LYSOSOMAL CYSTEINE PROTEASES REGULATE ANTIGEN PRESENTATION

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Antigen presentation by both classical MHC class II molecules and the non-classical MHC class I-like molecule CD1D requires their entry into the endosomal/lysosomal compartment. Lysosomal cysteine proteases constitute an important subset of the enzymes that are present in this compartment and, here, we discuss the role of these proteases in regulating antigen presentation by both MHC class II and CD1D molecules.

T-cell function, maintenance and development are controlled largely by the interaction of the T-cell receptor (TCR) with ligand-loaded MHC molecules. Most T cells expressing an αβ TCR can be divided into two groups, depending on their expression of the co-receptor molecules CD4 and CD8. These co-receptors ligate the MHC molecule together with the TCR, providing the interaction with MHC specificity, as well as increasing its avidity. $CD8^+$ T cells recognize peptides that are derived from cytosolic proteins in the context of MHC class I molecules, whereas $CD4^+$ T cells interact with MHC class II molecules that present fragments of proteins that have been degraded in endocytic and phagocytic vesicles[1,2] (FIG. 1).

MHC class II molecules are αβ heterodimers that are assembled in the endoplasmic reticulum (ER) with the assistance of the invariant chain (Ii) chaperone molecule[3,4] (FIG. 1). A portion of Ii, known as the class II-associated invariant-chain peptide (CLIP), occupies the MHC class II peptide-binding groove, preventing premature loading of peptides in the ER and as the Ii–MHC class II complex traffics to the endosomal pathway[5–7]. This trafficking follows one of two pathways, the relative contributions of which seem to vary depending on the cell type. Newly synthesized Ii–MHC class II complexes can be exported directly to the plasma membrane, where they are present only transiently, as they are rapidly internalized and sorted into the endosomal compartment[8–10]. This internalization is mediated by a targeting motif in the cytoplasmic tail of Ii[11,12]. Alternatively, this motif can facilitate direct sorting of the Ii–MHC class II complexes from the trans-Golgi network to a relatively early aspect of the endocytic compartment, which is positioned between early recycling endosomes and late endosomes[13,14]. After entry into endosomes, Ii undergoes stepwise degradation by lysosomal proteases, leaving CLIP associated with the MHC peptide-binding groove[15,16]. Lysosomal proteases also degrade internalized and endogenous antigenic proteins that are present in the endosomal pathway[1]. Degradation of endosomal protein antigens that contain disulphide bonds is dependent on the cleavage of these bonds by lysosomal reductases, such as the recently described γ-interferon-inducible lysosomal thiol reductase (GILT)[17,18]. Exchange of CLIP for the proteolytic products that are generated by these lysosomal enzymes is mediated by the MHC-like molecule HLA-DM (H–2M in mice), and is followed by the trafficking of these peptide–MHC class II complexes to the cell surface[19,20]. Such trafficking has recently been shown to involve tubulation of the late endosomes that contain peptide–MHC class II complexes; small vesicles then bud off these tubules and fuse with the cell membrane[21–24].

Constitutive expression of MHC class II molecules in mice is restricted to professional antigen-presenting cells (APCs) — B cells, dendritic cells (DCs) and macrophages — as well as both cortical and medullary thymic epithelial cells (TECs). In addition to these cell types, in humans and rats, MHC class II molecules are also expressed by activated T cells. Expression of MHC class II molecules can also be transiently induced by inflammatory signals in other cell types, such as endothelial cells, fibroblasts and epithelial cells in organs other than the thymus. Expression of peptide–MHC class II

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doi:10.1038/nri1110

complexes in the thymus regulates CD4+ T-cell development. T cells expressing TCRs that have low avidity for peptide–MHC class II complexes expressed by cortical TECs undergo differentiation (positive selection), whereas potentially autoreactive T cells that express TCRs with high avidity for peptide–MHC class II complexes are eliminated after interaction with medullary TECs and bone-marrow-derived cells in the thymus such as DCs (negative selection). In all these cells, MHC class-II-restricted antigen presentation is regulated by lysosomal proteases at two stages — peptide generation and Ii degradation[1,25,26]. Many investigators have sought to characterize the enzymes that are involved in these processes and an important role in antigen presentation *in vivo* has been confirmed, so far, for only one group of proteases, the lysosomal cysteine proteases. A synopsis of the early findings that implicate lysosomal proteases in MHC class II antigen presentation is given in BOX 1.

It was originally predicted that lysosomal cysteine and aspartic proteases would have substantial redundancy in specificity and function, and initial investigations into the role of these enzymes in MHC class-II-restricted antigen presentation using chemical inhibitors of broad specificity were complicated by these expectations. However, recent analyses of mice with targeted deletions of lysosomal cysteine proteases and the lysosomal aspartic protease cathepsin D have indicated that the lysosomal proteases are likely to have unique functions. Furthermore, these studies indicate that specific lysosomal proteases are required for the presentation of the appropriate peptide–MHC class II repertoire by distinct cell types, which raises an intriguing question as to the biological role of such tissue specificity. These data also indicate that targeting of specific lysosomal cysteine proteases might be a viable clinical approach to controlling antigen presentation of defined peptide–MHC class II complexes. In addition, an increasing number of other crucial cellular functions for lysosomal cysteine proteases, both within and beyond the immune system, are being elucidated — including apoptosis, regulation of cytotoxic natural killer (NK)-cell and CD8+ T-cell function, epidermal homeostasis and antigen presentation by the MHC class-I-like molecule CD1d[27–30]. Here, we confine our discussion to studies that provide insight into the role of lysosomal cysteine proteases in regulating both the MHC class II and CD1d antigen-presentation pathways.

Lysosomal proteases in lymphoid tissue

Proteases mediate the hydrolysis of peptide bonds and can be classified, depending on the mechanism by which they elicit proteolysis, as aspartic, cysteine, serine or

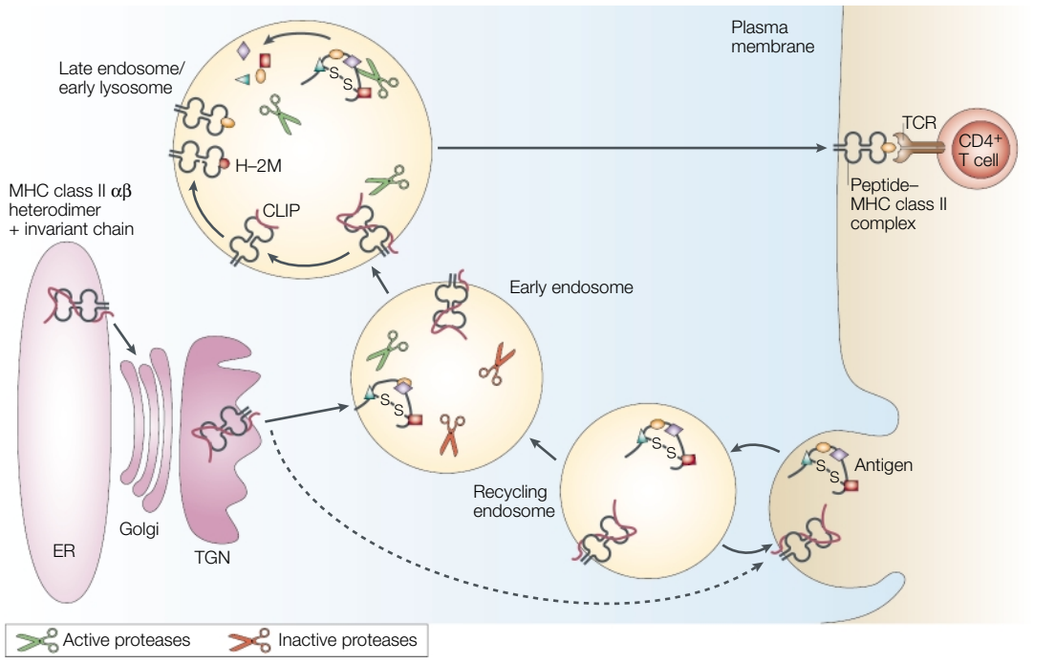

Figure 1 | **The MHC class II antigen-presentation pathway.** MHC class II αβ heterodimers assemble in the endoplasmic reticulum (ER) with the assistance of invariant chain (Ii). The cytoplasmic tail of Ii contains a motif that targets the Ii–MHC class II complex to the endosomal pathway, either directly from the trans-Golgi network (TGN) to early endosomes or via the plasma membrane. Ii–MHC class II complexes at the cell surface are rapidly internalized into recycling endosomes and then traffic to the early endosomes. Maturation of the early endosome leads to activation of lysosomal enzymes, including cysteine proteases, which degrade endogenous endosomal proteins, internalized proteins and Ii. In addition to these proteases, lysosomal reductases that cleave disulphide bonds, such as γ-interferon-inducible lysosomal thiol reductase (GILT), are required for the processing of protein antigens that contain disulphide bonds. Following Ii cleavage, the MHC class II peptide-binding groove remains occupied by the class-II-associated invariant chain peptide (CLIP), which prevents premature peptide loading. Removal of CLIP and loading of peptides is mediated by the MHC-like molecule H–2M. These peptide–MHC class II complexes then traffic to the plasma membrane. TCR, T-cell receptor.

Box 1 | **MHC class II molecules and lysosomal proteases: early findings**

- **1982** Treatment of macrophages with ammonia or chloroquine inhibited MHC class-II-restricted antigen presentation by macrophages, as a result of an increased lysosomal pH[104].
- **1983** MHC class-II-restricted antigen presentation was shown to require intracellular fragmentation of the antigen[105].
- **1985** Disruption of lysosomal acidification in the presence of lysosomotropic reagents resulted in the accumulation of invariant chain (Ii) fragments that were associated with MHC class II molecules in B cells[106].
- **1988** Treatment of a human B-cell line with the protease inhibitor leupeptin led to the accumulation of Ii fragments associated with MHC class II molecules[37].
- **1991** Degradation of antigen for MHC class II presentation was shown to occur in the late endosomal/lysosomal compartment[107].
- **1994** Treatment of a human B-cell line with chemical inhibitors of aspartic proteases blocked the initiation of Ii degradation[15].
- **1996** Inhibition of lysosomal cysteine proteases expressed by a human B-cell line with the chemical inhibitor morpholinurea-leucine-homophenylalanine-vinylsulphone-phenyl (LHVS) indicated that cathepsin S mediates the late stages of Ii degradation[16].

metallo proteases. Members of the aspartic and cysteine groups make up most of the proteases that are present in the endosomal/lysosomal compartment. These proteases originally evolved to catabolize both internalized and cellular proteins, and at different stages of evolution they became crucially involved in many processes — such as cellular homeostasis, autophagy, apoptosis and antigen presentation[25–28]. Most mammalian lysosomal cysteine proteases are known as cathepsins, although not all cathepsins are cysteine proteases[31] (TABLE 1). In addition, asparagine endopeptidase is a recently identified mammalian lysosomal cysteine protease that is homologous to the legumain family of proteases, which were originally isolated from plants and parasites[32]. Similar to other proteases, these enzymes are synthesized as zymogens, requiring removal of the amino-terminal pro-domain to become active. Excision of this pro-domain can be facilitated by other proteases or by an autocatalytic mechanism after exposure to an acidic pH, as is the case for cathepsin L[33]. Although an acidic pH is optimal for the activity of lysosomal cysteine proteases, individual enzymes are active over different pH ranges[25,26].

The enzymatically active forms of lysosomal cysteine proteases can be detected using SUBSTRATE-ANALOGUE INHIBITORS, which become covalently linked to the active site of the enzyme. These irreversible inhibitors can be labelled with radioactive (^{125}iodine) or affinity (biotin) tags, which allow the enzymes to be visualized. Several different irreversible inhibitors[16,34,35] have been used to show that although expression of some lysosomal proteases, for example, cathepsin B, is ubiquitous, other cathepsins are expressed in a more tissue-specific manner[26,36] (TABLE 1). Cathepsin-S activity can be detected in the thyroid and the spleen, but not in thymic-tissue extracts, whereas cathepsin L is active in the kidney, liver and thymus, but not the spleen[25,26,36]. This discrete expression of active cathepsins S and L by lymphoid tissues led several laboratories to investigate further the cellular distribution and function of lysosomal cysteine proteases. These analyses indicated that one crucial function of these enzymes is the regulation of the MHC class II and CD1D antigen-presentation pathways.

SUBSTRATE-ANALOGUE INHIBITORS
Small molecules that mimic the natural enzyme substrate and covalently bind to the cysteine residue present in the active site of the cysteine protease.

Lysosomal proteases and MHC class II molecules

Lysosomal cysteine proteases and Ii degradation. Initial studies to identify the proteolytic enzymes that are involved in Ii degradation used protease inhibitors of broad specificity, and implicated both aspartic and cysteine proteases[15,37]. These analyses indicated that Ii cleavage, which is characterized by a series of clearly defined Ii intermediates, was initiated by an aspartic protease with subsequent steps being mediated by cysteine proteases (FIG. 2). The most prevalent lysosomal aspartic and cysteine proteases, cathepsin D and cathepsin B, respectively, were therefore suggested to be the crucial enzymes that regulate Ii degradation. However, in both cathepsin-B- and cathepsin-D-deficient mice, Ii degradation and antigen presentation to several MHC class-II-restricted T-cell hybridomas were shown to occur normally[38,39]. Furthermore, studies using protease inhibitors indicated that cathepsin S, but not cathepsin B, could mediate Ii cleavage, and that inhibition of cathepsin S impaired MHC class-II-restricted antigen presentation *in vitro* and *in vivo*[16,40]. So, these data excluded cathepsin B and cathepsin D as the non-redundant proteases that elicit Ii degradation and regulate MHC class II antigen presentation *in vivo*.

Despite the use of many protease inhibitors of varying specificity and the generation of knockout mice for several candidate proteases, the identity of the enzyme involved in the initial cleavage of Ii remains unknown. It is conceivable that its original identification as an aspartic protease was somewhat misleading and, indeed, recent evidence indicates that asparagine endopeptidase might have an important role in this process in B cells and DCs[41]. However, the results of Manoury *et al.*[41] further indicate that, in addition to asparagine endopeptidase, other unknown proteases can also mediate this initiating step of Ii degradation. Future analyses of knockout mice should indicate more clearly the role of asparagine endopeptidase and other proteases in the initiation of Ii degradation.

The data obtained from analysis of cathepsin-deficient mice have, however, elucidated a clear, non-redundant role for the lysosomal cysteine proteases, cathepsins S and L, in the late stages of Ii degradation[42–44]. Consistent with the discrete tissue distribution of cathepsins S and L, cathepsin S activity was detected in B cells, DCs and macrophages, whereas cathepsin L was expressed by cortical TECs and macrophages[42–45] (TABLE 1). Interestingly, despite the lack of activity of cathepsins S and L in thymic and splenic tissues, respectively, cathepsin-S expression was detected in DCs isolated from the thymus[42] and, more recently, expression of mature cathepsin L protein by both B cells and DCs was observed, although no enzyme activity could be detected[46].

Analysis of thymic epithelium and purified cortical TECs — the cells that mediate positive selection of T cells — from cathepsin-L-deficient mice indicated that cathepsin L was responsible for mediating the late stages of Ii degradation in these cells[42,47]. Impaired Ii degradation by cortical TECs affects MHC class-II-restricted presentation by these cells, and as a result, cathepsin-L-deficient mice have markedly diminished numbers of $CD4^+$ T cells — only 25–30% of those detected in littermate control animals. The absence of cathepsin L does not alter the level of MHC class II molecules that are expressed on the cell surface of cortical TECs. Therefore, the cathepsin-L deficiency must affect the expression of peptide–MHC class II complexes qualitatively, in part, as a result of cell-surface expression of MHC class II molecules bound to the Ii degradation intermediates p12 and p18 (REFS 42,47). Unsurprisingly, concomitant analysis of peripheral APCs, B cells and DCs indicated that in the absence of cathepsin L, these cells have normal Ii processing and presentation of a range of MHC class-II-restricted antigens. Interestingly, in humans, cathepsin V, also known as cathepsin L2, is a recently described lysosomal cysteine protease that has high homology with cathepsin L[48,49]. Cathepsin V has no mouse orthologue, and in humans, expression of this enzyme is restricted to the testis and thymus. Furthermore, the protein sequence of cathepsin V has greater homology with mouse cathepsin L than human cathepsin L does, suggesting that in humans cathepsin V might have a role that is analogous to the function of cathepsin L in mice[49].

In studies similar to those carried out in cathepsin-L-deficient mice, analysis of B cells and DCs isolated from cathepsin-S-deficient mice showed the accumulation of degradation intermediates associated with MHC class II molecules in these cells[43,44]. This inefficient Ii degradation reduced the presentation of exogenous antigens by these APCs to most of the MHC class-II-restricted T-cell hybridomas that were tested. Further evidence of deficient MHC class-II-restricted antigen presentation was provided by the observation that $CD4^+$ T-cell-dependent formation of germinal centres and immunoglobulin-isotype class switching are impaired in cathepsin-S-deficient mice[44]. These studies, as well as those described for cathepsin-L-deficient mice, used mice of the $H\text{–}2^b$ haplotype that express the MHC class II molecule $I\text{–}A^b$, a molecule that has a high affinity for Ii and its degradation intermediates. However, it should be noted that the accumulation of MHC class II-bound Ii fragments that is observed in cathepsin-L- or -S-deficient APCs has haplotype variability. In animals that express MHC class II alleles with a low affinity for Ii fragments, these degradation intermediates do not accumulate in association with MHC class II molecules, and as a result, MHC class II antigen presentation is less markedly impaired by the absence of cathepsins S or L[26,29,50].

In contrast to bone-marrow-derived APCs, Ii degradation occurred normally in cortical TECs that were isolated from cathepsin-S-deficient mice, and these animals have normal numbers of $CD4^+$ T cells, despite the fact that negatively selecting thymic DCs express cathepsin S and not cathepsin L[42]. This indicates that presentation of many endogenous peptide–MHC class II complexes by thymic DCs is not markedly affected by a deficiency in cathepsin S, as has previously been reported for splenic DCs[43]. Alternatively, other cells in the thymus, such as medullary TECs, might be able to compensate for any defect in MHC class II antigen presentation by thymic DCs that lack expression of cathepsin S and mediate negative selection.

Table 1 | **Lysosomal proteases implicated in antigen presentation**

Enzyme	Protease type	Expression	Knockout phenotype	References
Cathepsin B	Cysteine	B cells, DCs and macrophages	No marked immune-system phenotype reported Decreased trypsin activation and onset of pancreatitis Decreased susceptibility to TNF-induced hepatocyte apoptosis	38 108 109
Cathepsin D	Aspartic	B cells, DCs and macrophages	No immune-system phenotype reported Die at ~21 days as a result of atrophy of the ileal mucosa Lysosomal storage disorder in the central nervous system neurons	38,39 110 111
Cathepsin F	Cysteine	Macrophages and epithelial cells	N.D.	112,113
Cathepsin K	Cysteine	Macrophages and osteoclasts	No immune-system phenotype reported Have osteopetrotic phenotype	113,114
Cathepsin L	Cysteine	Activity in cortical TECs, macrophages and thymocytes; protein expression by B cells and DCs	Decreased $CD4^+$ T-cell selection due to impaired Ii degradation and generation of MHC class-II-bound peptides in cortical TECs Decreased $V\alpha14^+J\alpha18^+$ NKT-cell selection as a result of impaired thymocyte presentation of endogenous CD1d-bound ligands Epidermal hyperplasia and hair-follicle deficiencies Dilated cardiomyopathy	28,42–56 30 115 116
Cathepsin S	Cysteine	B cells, DCs, macrophages and epithelial cells	Decreased MHC class II presentation of exogenous antigens by B cells and DCs, and decreased numbers of $V\alpha14^+J\alpha18^+$ NKT cells as a result of accumulation of MHC class-II-bound Ii intermediates Deficient germinal-centre formation and impaired class switching to IgG2a and IgG3 isotypes Diminished susceptibility to collagen-induced arthritis	29,43 44
AEP	Cysteine	B cells and DCs	N.D.	41,58–60

AEP, asparagine endopeptidase; DCs, dendritic cells; Ii, invariant chain; N.D., not determined; NKT, natural killer T; TECs, thymic epithelial cells; TNF, tumour-necrosis factor.

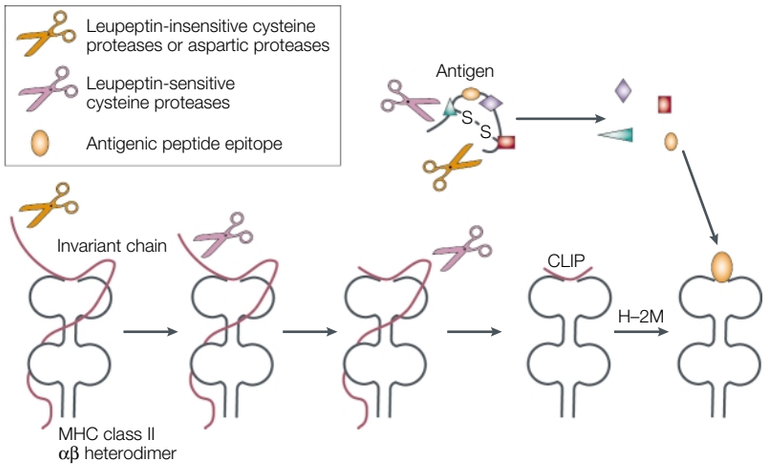

Figure 2 | **Degradation of invariant chain.** Invariant chain (Ii) is degraded in a stepwise manner in the endosomes. The initial cleavage is thought to be mediated by a leupeptin-insensitive cysteine protease or an aspartic protease, whereas subsequent steps are a result of the activity of leupeptin-sensitive cysteine proteases and leave the MHC class II peptide-binding groove occupied by the class-II-associated invariant chain peptide (CLIP). Aspartic and cysteine proteases also degrade internalized and endogenous proteins that are present in the endosomal compartment, and the MHC-like molecule H–2M then exchanges CLIP for the peptides that are generated by these enzymes. The size and relative abundance of the Ii fragments that accumulate in the presence of leupeptin vary depending on the species from which the antigen-presenting cell was derived and the MHC haplotype.

These data indicate that two distinct lysosomal cysteine proteases mediate Ii degradation in different APCs. This leaves us with the question as to why MHC class-II-restricted antigen presentation is regulated by two different enzymes in the cells that mediate positive selection, cortical TECs, and the cells that are responsible for the induction of immune responses in the periphery. One possibility is that cathepsins S and L not only cleave Ii, but are also intimately involved in the other proteolytic process that affects MHC class II antigen presentation — degradation of endosomal/lysosomal proteins to generate the peptide fragments that are loaded into the MHC class II peptide-binding groove.

Lysosomal cysteine proteases and peptide generation. Little is known about the identity of the specific enzymes that generate particular MHC class-II-bound peptides *in vivo*. *In vitro* analyses of purified proteases and substrates previously indicated that the aspartic proteases, cathepsins D[51,52] and E[53], might have a role in peptide generation. However, subsequent analysis of APCs derived from cathepsin-D-deficient mice indicated that this enzyme was not essential for MHC class II antigen presentation *in vivo*[38,39], whereas it remains unclear whether or not cathepsin E is present in endosomes and lysosomes[54]. In a more recent report, cysteine proteases were shown to be involved in the endosomal/lysosomal degradation of immunoglobulins that are taken up by bone-marrow-derived APCs through the Fc receptor[55]. More specifically, cathepsin B, and to a lesser extent cathepsin S, were shown to be the proteolytic effectors, although whether such degradation is important for the generation of T-cell epitopes remains to be determined.

The effect of cathepsins S and L on the generation of T-cell epitopes has been assessed by mass spectrometry of peptides eluted from MHC class II molecules that were purified from a fibroblast cell line engineered to express either cathepsin S or cathepsin L, or neither enzyme[56]. It was observed that, although neither enzyme was required for the generation of most of the abundantly expressed MHC class-II-bound peptides, these enzymes had an important role in the presentation of a subset of peptides. More specifically, the generation of some peptides required cathepsin S or L, or either of the enzymes, whereas other peptides were destroyed in their presence. So, cathepsins S and L can influence the endogenous peptide–MHC class II repertoire of a model APC. However, these studies did not address the physiological relevance of peptides that are generated differentially by these proteases. To address this, animals that are deficient in both an enzyme and Ii were analysed. This prevented any potential direct role of cathepsins in peptide generation from being obscured by the impaired MHC-class II-restricted antigen presentation that results from a defect in cathepsin-mediated Ii degradation. Mice deficient in both cathepsin L and Ii had a more marked defect in $CD4^+$ T-cell selection than single-knockout control mice[50], indicating that cathepsin L influences the repertoire of peptide–MHC class II complexes expressed by cortical TECs independently of its role in Ii degradation, and thereby implicating the enzyme in the generation of T-cell epitopes *in vivo* (FIG. 3). Analysis of DCs derived from mice that are deficient in both cathepsin S and Ii provided no evidence of a similar non-redundant role for cathepsin S in peptide generation (K.H. and A.Y.R., unpublished observations), although cathepsin S was shown to destroy T-cell epitopes derived from IgM[43,56]. However, in a separate study using mice that are engineered to express a B-cell receptor specific for hen egg lysozyme (HEL), cathepsin S was shown to be required for the generation of two specific HEL-derived T-cell epitopes, whereas a third T-cell epitope was cathepsin-S independent[57]. These data indicate that cathepsin L directly influences the generation of most of the cortical TEC peptide epitopes that are involved in $CD4^+$ T-cell selection, whereas in B cells and DCs, cathepsin S influences the generation of some of the MHC class-II-bound peptides, but not most of those presented at the cell surface. It is possible that this might be due to an increased diversity, and therefore greater redundancy, of lysosomal proteases that are involved in antigen processing in DCs and B cells compared with cortical TECs. So, the overlap of peptides that are generated by cathepsin S with the peptides that are generated by other enzymes might be far greater than for cathepsin L. Alternatively, it is possible that cathepsin-L-dependent peptides disproportionately contribute to the positive selection of $CD4^+$ T cells. To determine which of these scenarios occurs *in vivo*, it will be necessary to identify the cathepsin-S- and -L-dependent peptides.

One lysosomal cysteine protease that has been shown to have a central role in the generation of specific T-cell epitopes from several defined antigens is asparagine endopeptidase[58–60]. Asparagine endopeptidase was shown

CLASS II TRANSACTIVATOR (CIITA). A non-DNA-binding transcriptional activator that functions as a master control factor for the expression of MHC class II molecules. It is believed that CIITA alone provides the tissue specificity for the expression of MHC class II molecules and the accessory molecules invariant chain and HLA-DM (H–2M in mice).

to initiate the degradation of tetanus toxin, but was insufficient to generate the MHC class-II-bound tetanus-toxin-derived peptides[58,59]. In these studies, the initiating proteolytic event mediated by asparagine endopeptidase was required for MHC class-II-restricted antigen presentation of several different tetanus-toxin T-cell epitopes, presumably eliciting unfolding of the antigen to generate substrates for other enzymes — for example, cathepsin D and cathepsin E, which have previously been shown to degrade denatured tetanus toxin[61]. Similarly, *in vitro* degradation of myelin basic protein has been shown to be initiated by asparagine endopeptidase and cathepsin S, with subsequent proteolysis being mediated by many enzymes, including cathepsins S, L and D[62]. These data imply that a series of defined enzymatic steps are likely to be involved in the cleavage of proteins to generate MHC class-II-bound peptides. In addition, these studies indicate that by identifying the enzyme that initiates proteolysis, it might be possible to manipulate the generation of individual, clinically important peptides — for example, auto-antigenic peptides — by designing viable therapeutic strategies to target these proteases. However, subsequent studies showed that such a strategy is potentially dangerous, as asparagine endopeptidase not only influences peptide generation positively, but was also shown to destroy a T-cell epitope of the putative human auto-antigen myelin basic protein, which potentially affects the induction of tolerance to this peptide[60]. Similar observations have been made for other lysosomal cysteine proteases[63]; therefore, inhibiting the generation of one peptide might lead to the generation of other potentially auto-antigenic T-cell epitopes. A second potential problem with therapeutic inhibition of lysosomal cysteine proteases is highlighted by the observation that these enzymes have many roles in the regulation of MHC class-II-restricted antigen presentation — for example, cathepsins S and L mediate epitope generation and Ii degradation, which are crucial steps in the MHC class II antigen-presentation pathway. Further characterization of the proteolytic mechanisms by which particular T-cell epitopes are generated is, therefore, required before these enzymes can be considered as clinical targets.

MHC class II antigen presentation by macrophages. Macrophages are unique APCs, in that they have easily detectable levels of activity of both cathepsin S and cathepsin L. However, the relative contribution of these two proteases to the regulation of MHC class-II-restricted antigen presentation by macrophages has only recently been defined. Macrophages express only a low level of cell-surface MHC class II molecules constitutively; however, after exposure to pro-inflammatory stimuli, such as interferon-γ (IFN-γ), expression of MHC class II molecules increases markedly. Therefore, the role of proteases in MHC class II antigen presentation by macrophages is studied in cells that are stimulated by IFN-γ. Work from our laboratory has indicated that cathepsin S has an important role in the late stages of Ii degradation in these cells, as other proteases that substitute for cathepsin S seem to cleave Ii with substantially slower kinetics[64]. Further analysis of macrophages that are deficient in cathepsin L, or in both cathepsin S and cathepsin L, indicated that the delayed Ii processing in cells isolated from cathepsin-S-deficient mice was not mediated by cathepsin L. This conclusion was in agreement with other reports implicating cathepsin F — a lysosomal cysteine protease that is expressed at high levels by macrophages, but not expressed by DCs or B cells — in Ii degradation in the absence of cathepsin S[63]. Generation and analysis of cathepsin-F-deficient mice should allow this hypothesis to be tested more rigorously.

Despite the expression of both cathepsins S and L by bone-marrow-derived macrophages and *ex vivo* macrophages that are isolated from the lung and peritoneum, no role has been defined for the latter enzyme in regulating the MHC class II antigen-presentation pathway of macrophages. This is surprising, considering that cathepsin L has a crucial role in Ii degradation in cortical TECs and can mediate efficient Ii proteolysis in fibroblast cell lines that express CLASS II TRANSACTIVATOR (CIITA)[42,56]. However, further analysis of the effect of cytokines on the expression of cathepsins by macrophages indicated that cathepsin-S activity increased in the presence of IFN-γ, whereas cathepsin-L activity was markedly downregulated to barely detectable levels[64]. So, as a result of this shift in the balance of cathepsin activity after cytokine treatment, cathepsin S becomes the main enzyme that can elicit Ii degradation and, therefore, regulate MHC class-II-restricted antigen presentation by macrophages.

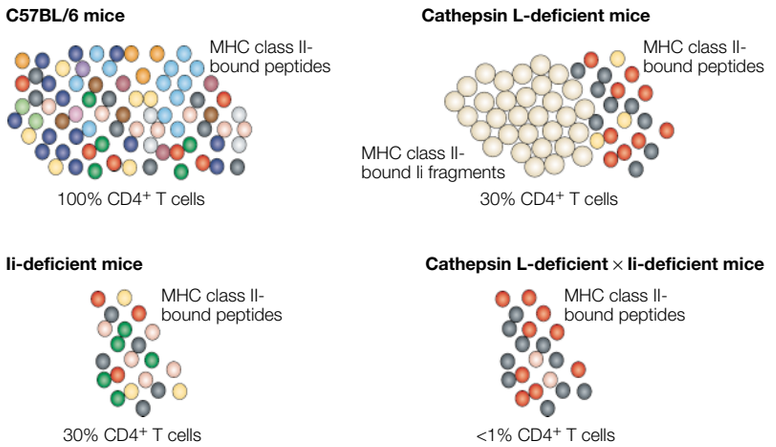

Figure 3 | **The role of cathepsin L in determining the peptide–MHC class II repertoire.** In wild-type mice (C57BL/6), the level of expression of MHC class II molecules by cortical thymic epithelial cells (TECs) and the diversity of the peptides that they present elicit the selection of a normal number of $CD4^+$ T cells (100%). In cathepsin-L-deficient mice, many MHC class II molecules are associated with Ii fragments and the remaining molecules present a markedly decreased peptide repertoire, reducing $CD4^+$ T-cell selection by 70%. In the absence of invariant chain (Ii), the level of MHC class II expression is decreased and there is a concomitant reduction in the peptide diversity, which results in the selection of only 30% of $CD4^+$ T cells, compared with C57BL/6 animals. In cathepsin-L-deficient × Ii-deficient mice, the level of expression of MHC class II molecules is comparable to that of Ii-deficient animals, but $CD4^+$ T-cell selection is reduced to background levels. As $CD4^+$ T-cell numbers correlate with the diversity of MHC class-II-bound peptides that are presented by cortical TECs, this indicates that cathepsin L influences the peptide–MHC class II repertoire that is expressed by cortical TECs by regulating the late stages of Ii degradation and by an alternative mechanism that is independent of Ii cleavage, presumably involving the generation of MHC class-II-bound peptides.

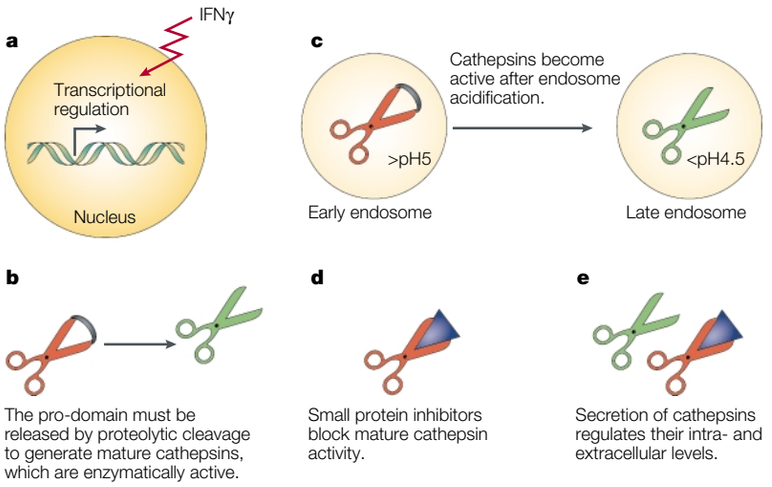

Figure 4 | **Regulation of lysosomal cysteine protease activity.** Lysosomal cysteine protease activity is regulated at many different levels, some of which are shown in this schematic. **a** | The level of cathepsin messenger RNA has been observed to change after stimulation of cells by the pro-inflammatory cytokine interferon-γ (IFN-γ), indicating that transcription of cathepsins is tightly regulated. **b** | Lysosomal cysteine proteases are synthesized as inactive precursors that become enzymatically active after proteolytic removal of the amino-terminal pro-domain. **c** | The pH of the endosomal compartment affects the enzyme activity of lysosomal cysteine proteases. Endosome acidification decreases the intravesicular pH, which initiates pro-domain removal and establishes an optimal pH for proteolysis. **d** | Lysosomal cysteine protease activity is also regulated by the presence or absence of small-molecule inhibitors, such as cystatins or the unique region of the p41 isoform of invariant chain, which has been shown to inhibit the activity of cathepsin L. **e** | Secretion of lysosomal cysteine proteases allows enzymatic activity to be regulated intracellularly and extracellularly.

Selective use of cathepsin S by peripheral APCs. The observation that IFN-γ-treated macrophages specifically downregulate cathepsin-L activity and use cathepsin S for efficient Ii degradation has led us to hypothesize that APCs in the periphery require this process to be regulated by cathepsin S and not cathepsin L[64]. In all peripheral APCs, B cells, DCs and macrophages, cathepsin-S activity is observed at high levels, whereas cathepsin-L activity is barely detectable, if at all, despite the presence of mature protein[46,64]. By contrast, cathepsin L has a crucial role in both Ii degradation and peptide generation in cortical TECs, cells in which cathepsin-S activity cannot be detected[42,50]. The biological relevance of such divergent expression remains to be determined. However, as a direct role in the generation of MHC class-II-bound peptides *in vivo* has been indicated only for cathepsin L, it is interesting to speculate that restricting the expression of cathepsin L to cortical TECs might be crucial in preventing the peripheral expression of cathepsin-L-dependent T-cell epitopes that might induce T-cell activation and elicit autoimmunity. One flaw to this hypothesis is that the aforementioned mass-spectrometry analysis of MHC class-II-bound peptides generated in the presence of cathepsins S or L indicated that the overlap between the peptide repertoires generated by these two enzymes was substantial, although by no means complete[56]. One approach to address the question of whether divergent protease expression is important in generating disparate repertoires of MHC class-II-bound peptides in the thymus and periphery, and whether this has a role in the maintenance of T-cell tolerance, is to generate mice in which cathepsin S is expressed by cortical TECs and cathepsin L is expressed by peripheral APCs.

In our initial attempt to generate cathepsin-S-deficient mice with cathepsin L expressed by DCs, we used the DC-specific CD11c promoter to drive the expression of cathepsin L. However, no cathepsin L activity could be detected in DCs, despite the presence of extremely high levels of expression of mature cathepsin-L protein[64]. So, this approach to understanding the reason for divergent expression of cathepsins S and L has, so far, been uninformative. However, this lack of cathepsin-L activity, despite the presence of high levels of expression of mature protein by both transgenic DCs and macrophages[64], suggests that cathepsin-L activity is specifically downregulated in peripheral APCs, presumably by an as-yet-unidentified inhibitor. These data reinforce our hypothesis that cathepsin S has a leading, non-redundant role in Ii processing in the periphery and indicate that cathepsin activity is tightly regulated in a highly specific manner.

Regulation of lysosomal protease activity

The activity of lysosomal cysteine proteases can be regulated at many different levels, including transcription, trafficking, environmental pH, activation of the pro-enzyme and by the presence or absence of inhibitors (FIG. 4). However, despite these numerous mechanisms of control, there is little consensus as to how this occurs *in vivo*. Several groups studying various cell types have observed regulation of cysteine protease transcription after exposure to an inflammatory stimulus. In one report, the pro-inflammatory cytokine IFN-γ was shown to downregulate levels of cathepsin S and cathepsin B messenger RNA in microglia and macrophage cell lines, as well as primary alveolar macrophages[65]. However, other groups have observed that stimulation with IFN-γ increases the transcription of cathepsin S by an epithelial cell line, bone marrow cells and lung tissue[66,67]. In another study, there was no change in the level of cathepsin S mRNA, but a marked decrease in the transcription of cathepsin L, in peritoneal macrophages that were treated with IFN-γ[64]. The disparity between these data indicates that regulation of cathepsin mRNA is highly sensitive to environmental changes, such as the cytokine milieu, cell adhesion to substrates, cell–cell contact and perhaps even the handling of cells. So, it is probable that transcriptional regulation of cathepsin activity is of some importance in the control of expression of cathepsins, although its precise role *in vivo* remains poorly defined.

The most obvious mechanism by which cathepsin activity is regulated is by the removal of the amino-terminal pro-domain that is required for these proteases to become enzymatically active. The pro-domain occupies the active site of the enzyme, thereby preventing inappropriate protease activity in the ER and Golgi apparatus. The mechanisms of pro-domain removal have been extensively studied, and seem to vary for the different cysteine proteases. In the case of

both cathepsins L and B, it has been shown that exposure to the acidic environment of the late endosomal compartment initiates autocatalytic activation of the protease[68,69]. However, whether this is mediated by an intramolecular[70,71] or intermolecular[69,72] reaction remains unclear. Other cathepsins are incapable of autocatalytic activation and require other proteases for pro-domain removal — for example, cathepsin L has been shown to activate cathepsin C[73]. However, as all these studies involve *in vitro* analysis of purified cathepsins, the precise *in vivo* mechanisms of pro-enzyme activation have yet to be determined.

After pro-domain removal, inhibitory molecules and numerous factors, such as pH and a reducing environment, further regulate the activity of cathepsins. Cystatins are a superfamily of small proteins that have been shown, in many *in vitro* studies, to act as potent inhibitors of cysteine proteases[74–76]. However, the role of cystatins in the regulation of MHC class-II-restricted antigen presentation is poorly understood. For example, cystatin C, a class II cystatin, has been implicated in regulating the expression of cathepsin S and the trafficking of MHC class II molecules in DCs[77], although in subsequent reports, no correlation between cathepsin-S activity and cystatin-C expression was observed in either DCs or macrophages, indicating that cathepsin-S activity must be regulated in other ways[78,79] (A.Y.R., unpublished observations).

Given the role of cathepsin L in mediating Ii degradation, one intriguing observation is that the unique region of the p41 isoform of Ii was found to be non-covalently bound to cathepsin L[80]. Furthermore, this fragment of p41 was subsequently shown to bind to the active site of cathepsin L and inhibit its enzyme activity[81–83], raising the possibility that a negative-feedback mechanism could regulate cathepsin-L activity and, thereby, MHC class-II-restricted antigen presentation. Many groups have sought to define such a regulatory loop *in vivo*, but p41-deficient mice have no defect in MHC class II antigen presentation or $CD4^+$ T-cell development[84,85], and we have found no role for Ii in regulating cathepsin-L activity in bone-marrow-derived and thioglycollate-induced macrophages[64]. Furthermore, in a recent study, Ploegh and colleagues[86] made the surprising observation that cathepsin-L activity was barely detectable in bone-marrow-derived macrophages that were generated from mice lacking only the p41 Ii isoform. This study implicated the p41 isoform of Ii not as an inhibitor of cathepsin L, but as a chaperone molecule that stabilizes the mature form of the protease. Therefore, the physiological relevance of cathepsin-L inhibition by p41 requires further investigation. Subsequent analysis of the function of p41-mediated stabilization of the mature cathepsin L protein indicated that this interaction might allow the extracellular accumulation of active cathepsin L[87]. These data provide a mechanism by which p41 can regulate cathepsin-L activity both intracellularly and extracellularly, thereby influencing the effect of cathepsin L on MHC class-II-restricted antigen presentation and the extracellular-matrix remodelling that is associated with angiogenesis and cell migration.

The discussion, so far, has largely focused on the mechanisms by which the activity of the proteases is regulated directly. However, recent data have indicated that the lysosomal proteolytic environment might also be regulated in a spatial and temporal manner[79,88,89]. In one report, it was observed that internalized proteins are not exposed to all enzymes at the same time, but that lysosomal cysteine proteases are recruited to the phagosome sequentially[88]. In other studies, protease activity was shown to be regulated temporally, with increased cathepsin activity in late endosomes detected after stimulation of DCs with lipopolysaccharide (LPS)[79,89]. This increase in protease activity after DC maturation was shown to be a result of enhanced endosome acidification[89]. As mentioned previously, the decrease in pH initiates autocatalytic activation of proteases[68,69] and establishes an optimum pH for the activity of lysosomal cysteine proteases. Furthermore, the enhanced activity of lysosomal cysteine proteases that occurs after endosome acidification might account, at least in part, for the observation that phagocytosed particles do not encounter distinct protease activities synchronously[88]. These studies indicate that the cellular environment modulates enzyme

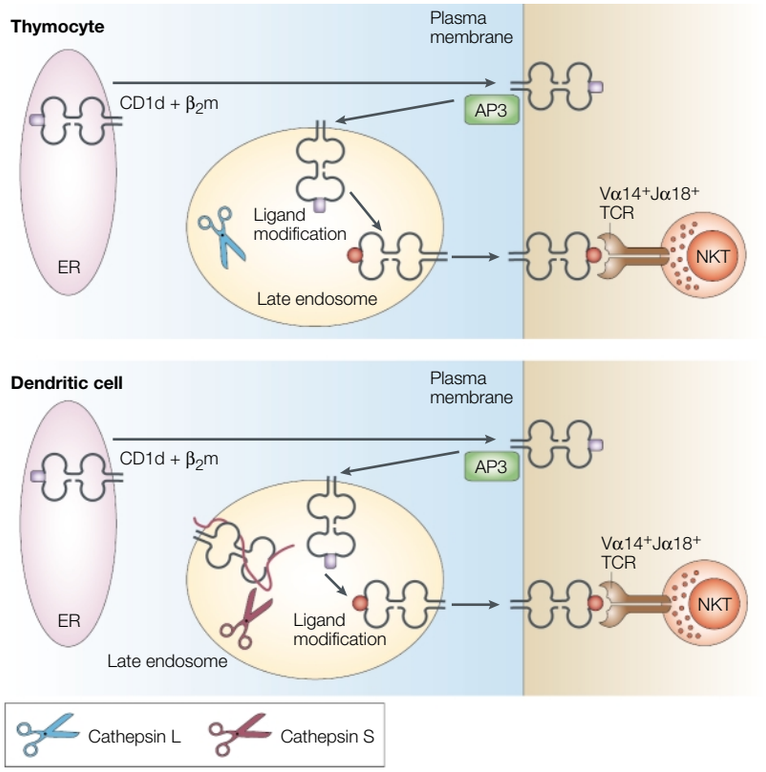

Figure 5 | **Lysosomal cysteine proteases and the CD1d antigen presentation pathway.** CD1d molecules associate with β_2-microglobulin (β_2m) in the endoplasmic reticulum (ER) before trafficking directly to the plasma membrane. The cytoplasmic tail of CD1d contains a tyrosine-based motif that binds the adaptor protein complex AP3 — an interaction that induces CD1d internalization and sorting to late endosomes. In the late endosomes, the CD1d complex is modified, such that after trafficking back to the plasma membrane, $V\alpha14^+J\alpha18^+$ natural killer T (NKT) cells are now able to recognize the CD1d-bound ligand. In the absence of cathepsin S, MHC class II molecules bound to Ii fragments accumulate and disrupt the endosomal compartments, resulting in the disruption of endosomal processes, including presentation of CD1d to $V\alpha14^+J\alpha18^+$ NKT cells. In thymocytes, however, cathepsin L specifically regulates the expression of the CD1d ligands that are involved in the selection of $V\alpha14^+J\alpha18^+$ NKT cells.

NATURAL KILLER T CELLS
NKT cells constitute a lymphocyte subset that is defined by co-expression of the NK-cell marker NK1.1 and an αβ T-cell receptor (TCR). As a result of the heterogeneity of this population, this T-cell subset is in the process of being more precisely defined; for example, in the mouse, most of these cells are CD1d restricted and express a semi-invariant TCR ($V\alpha14^+J\alpha18^+$) and the CD4 co-receptor.

activity, and provide another avenue of research to advance our understanding of how the lysosomal cysteine proteases that are involved in the MHC class II antigen-presentation pathway are regulated.

Lysosomal proteases and CD1D

The non-polymorphic MHC class-I-like molecule CD1d elicits selection and activation of a subset of T cells that express both an αβ TCR and NK-cell markers, such as NK1.1 and Ly49a[90–92]. This subset, known as NATURAL KILLER T CELLS, is selected by TCR interaction with CD1d molecules that are expressed by double-positive ($CD4^+CD8^+$) and single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) thymocytes[93,94]. However, the endogenous CD1d-bound ligands that initiate both this process and peripheral NKT-cell activation have not been identified. A substantial proportion of NKT cells express the semi-invariant TCR Vα14Jα18, and the natural CD1d ligands that are recognized by these cells can be mimicked by the marine-sponge-derived glycolipid α-galactosylceramide (α-GalCer)[91]. Such glycolipid presentation is compatible with the crystal structure of CD1d, which indicates that the binding groove is large and lined with hydrophobic amino acids[95]. Indeed, binding of glycophosphatidylinositol to CD1d has been reported[96].

Interestingly, despite being an MHC class-I-like molecule, CD1d presents endogenous ligands to $V\alpha14^+NK1.1^+$ T cells only after trafficking through endosomes[97–99] (FIG. 5). Recent work from several groups has begun to shed light on the mechanisms by which endosomal CD1 trafficking is regulated[99–101] (M. Kronenberg, personal communication), and the endosomal proteins that influence CD1d presentation to $V\alpha14^+NK1.1^+$ T cells[29,30,102,103]. The endosomal proteins involved in CD1d-restricted presentation include Ii and MHC class II molecules, both of which have been shown to associate with CD1d, and more surprisingly, the lysosomal cysteine proteases cathepsins S and L.

Cathepsin S was implicated in the CD1d presentation pathway by a report indicating that cathepsin-S-deficient mice had decreased numbers of $V\alpha14^+NK1.1^+$ T cells, and that DCs isolated from these mice induced inefficient *in vitro* stimulation of $V\alpha14^+NK1.1^+$ T-cell hybridomas[29]. However, these defects were not a specific effect of cathepsin S on the CD1d presentation pathway, but a result of accumulated MHC class II-bound Ii fragments that modify the endosomal compartment of cathepsin-S-deficient DCs. This was in contrast to the role of cathepsin L in regulating CD1d-restricted antigen presentation by thymocytes[30]. These latter cells do not express MHC class II molecules or Ii, and so the defect in CD1d presentation cannot be a consequence of the role of cathepsin L in Ii processing. In this report, it was shown that cathepsin-L-deficient thymocytes could not stimulate the production of interleukin-2 (IL-2) by $V\alpha14^+NK1.1^+$ T-cell hybridomas *in vitro*, or elicit $V\alpha14^+NK1.1^+$ T-cell selection *in vivo*, despite normal cell-surface expression of CD1d by thymocytes, endosome localization, and structure and number of lysosomes. These data further indicate that cathepsin L specifically regulates thymocyte presentation of endogenous CD1d ligands. Additional studies will, however, be required to elucidate whether cathepsin L generates the CD1d-bound ligands directly, for example, by mediating cleavage of glycolipoproteins, or whether cathepsin L regulates other proteins in the CD1d presentation pathway, for example, lipid hydrolases, enzymatic co-factors or as-yet-unidentified CD1d chaperone proteins. Data obtained from such analyses should provide us with new substrates for cathepsin L and, therefore, further our understanding of its role in regulating CD1d-restricted antigen presentation.

Concluding remarks

The lysosomal cysteine proteases, in particular cathepsin S and cathepsin L, have an important role in regulating antigen presentation by both MHC class II molecules and the MHC class-I-like molecule CD1D. As the function of cathepsins S and L seems to be non-redundant, and their proteolytic activity is tightly controlled, it is probable that these enzymes have discrete roles in antigen presentation that remain to be defined. This use of individual proteases to regulate an exact process is unlike the general turnover of proteins in lysosomes, which can be mediated in a redundant manner by several enzymes. Further understanding of both the precise role of lysosomal cysteine proteases in antigen presentation and the mechanisms by which these proteases are themselves regulated might provide us with an approach to modulating immune responses. For example, $V\alpha14^+NK1.1^+$ T cells are potent immunomodulatory cells that are implicated in resistance to autoimmunity, infection and cancer[90,91]. Regulating CD1D-restricted antigen presentation, therefore, is one potential mechanism for influencing the function of these cells, whereas adaptation of MHC class-II-restricted antigen presentation offers many possibilities for manipulating $CD4^+$ T-cell responses, both positively and negatively. It is, however, necessary to gain a deeper understanding of the complex proteolytic mechanisms that regulate antigen presentation before such therapeutic approaches can be developed.

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Acknowledgements
We would like to thank C. Beers and P. Gough for critical review of the manuscript. A. Y. R. is supported by the Howard Hughes Medical Institute and grants from the National Institutes of Health.

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