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Differential Regulation of Cathepsin S and Cathepsin L in Interferon γ–treated Macrophages

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Abstract

Cathepsin S (catS) and cathepsin L (catL) mediate late stages of invariant chain (Ii) degradation in discrete antigen-presenting cell types. Macrophages (Mϕs) are unique in that they express both proteases and here we sought to determine the relative contribution of each enzyme. We observe that catL plays no significant role in Ii cleavage in interferon (IFN)-γ–stimulated Mϕs. In addition, our studies show that the level of catL activity is significantly decreased in Mϕs cultured in the presence of IFN-γ whereas catS activity increases. The decrease in catL activity upon cytokine treatment occurs despite the persistence of high levels of mature catL protein, suggesting that a specific inhibitor of the enzyme is up-regulated in IFN-γ–stimulated peritoneal Mϕs. Similar inhibition of activity is observed in dendritic cells engineered to overexpress catL. Such enzymatic inhibition in Mϕs exhibits only partial dependence upon Ii and therefore, other mechanisms of catL inhibition are regulated by IFN-γ. Thus, during a T helper cell type 1 immune response catL inhibition in Mϕs results in preferential usage of catS, such that major histocompatibility complex class II presentation by all bone marrow–derived antigen-presenting cell is regulated by catS.

Key words: cathepsin • macrophage • Ii processing • IFN-γ • p41

Introduction

MHC class II molecules expressed on the surface of APCs present proteolytic fragments of self- and foreign protein antigens to CD4$^+$ T cells (1). MHC class II molecules assemble in the endoplasmic reticulum with the help of the chaperone molecule invariant chain (Ii),* a type II glycoprotein that promotes the proper folding and assembly of the MHC class II α/β heterodimer (2, 3). The Ii cytoplasmic tail contains a sequence targeting this complex to the lysosomal/endosomal pathway (4, 5) where it is exposed to the activity of proteases including cathepsins, which mediate cleavage of Ii in a step-wise manner, leaving only the class II–associated Ii peptide associated with the MHC class II peptide binding groove (6–8). Before MHC class II trafficking to the cell surface, the MHC-like molecule HLA-DM (H-2M in mice) mediates removal of class II–associated Ii peptide in exchange for the diverse array of self- and nonself-peptides generated in the endosomes and lysosomes (9, 10).

The cathepsins are a large family of aspartyl (D and E) and cysteinal (B, S, and L) endosomal proteinases. Recent studies have indicated that cathepsin S (catS) and cathepsin L (catL) are differentially expressed in APCs and are the only identified enzymes from this family of proteinases to play a key role in regulating MHC class II presentation (11–14). CatS is expressed in B cells and dendritic cells (DCs) and catL activity is observed in cortical thymic epithelial cells whereas in macrophages (Mϕs) both catL and catS activity can be detected (11–14). In B cells and DCs catS mediates the late stages of Ii degradation, specifically cleavage of the p22 and p12 Ii fragments and therefore regulates presentation of exogenous peptides in the context of MHC class II (12–14). CatL-deficient mice, however, exhibit a profound defect in CD4$^+$ T cell selection as a result of the role played by catL in cleavage of these Ii fragments and in generation of the MHC class II bound peptides in cortical thymic epithelial cells (11, 15).

The observation that Mϕs express both of the cysteinal proteinases that mediate the late stages of Ii degradation has

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**Abbreviations used in this paper:* B6, C57BL/6; BMMϕ, bone marrow–derived macrophage; catB, cathepsin B; catF, cathepsin F; catL, cathepsin L; catS, cathepsin S; DC, dendritic cell; Ii, invariant chain; Mϕ, macrophage; pMϕ, peritoneal Mϕ; tg, transgenic.

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lead several groups to investigate the relative contribution of each enzyme to regulation of MHC class II presentation by these cells. One previous study reported that in alveolar and peritoneal Mφs (pMφs) lacking both catS and catL, degradation of Ii occurs as a result of activity of additional cysteine proteinases, potentially cathepsin F (catF; reference 16). However, we have previously observed a role for catS in regulating MHC II presentation of some exogenous antigens by Mφs (13). Thus, we sought to further investigate the roles of catS and catL in regulating MHC class II presentation by Mφs and determine the extent to which the functions of these two enzymes overlap. We demonstrate that catS mediates degradation of the p12 fragment of Ii in IFN-γ–stimulated pMφs whereas catL plays no significant role in this process. In addition, we show this is a result of down-regulation of intracellular catL activity and increased catS activity in pMφs after stimulation with this cytokine. This decrease in catL activity correlates with a decrease in mRNA levels, although IFN-γ–treated pMφs maintain high levels of mature catL protein. We also found catL activity secreted upon IFN-γ treatment of pMφ, which correlates with a small increase in the amount of secreted mature catL protein. However, as the total amount of secreted catL protein (pro-form plus mature form) and the amount of intracellular mature catL protein is not altered upon IFN-γ treatment of pMφs, we believe secretion of catL does not account for the down-modulation of catL activity. These data suggest that a specific inhibitor of catL activity is up-regulated upon IFN-γ stimulation of pMφs. Furthermore, we also report complete inhibition of catL activity when catL protein is overexpressed in DCs, suggesting that bone marrow–derived APCs i.e., DCs and Mφs, may express a catL-specific inhibitor. We found no significant evidence to suggest the p41 isoform of Ii effects such inhibition of catL activity in pMφs. These results lead us to hypothesize that during a Th1 type immune response, catS activity in pMφs is critical for Ii degradation and hence MHC class II antigen presentation.

Materials and Methods

Mice. C57BL/6 (B6) mice were purchased from Charles River Laboratories and maintained under specific pathogen-free conditions at the University of Washington. CatS$^{-/-}$, catL$^{-/-}$, catS$^{-/-}$ × catL$^{-/-}$, and Ii$^{-/-}$ animals were bred and maintained under these same conditions. CD11c-catL mice were generated using the CD11c-E$_{\alpha}{}^{d}$ construct (provided by K. Karjalainen, The Basel Institute for Immunology, Basel, Switzerland; reference 17). E$_{\alpha}{}^{d}$ was removed and catL cDNA was inserted into the construct by blunt end ligation using BamH1. CatL was flanked on either side by rabbit β globin gene fragments and provides the transgene with an intron and a polyadenylation signal. To generate CD11c-catL transgenic (tg) mice, purified DNA was injected into BDF1 × B6 fertilized embryos. Offspring were backcrossed with catS$^{-/-}$ B6 mice. CD4^{+} T cell development occurred normally in the CD11c-catL tg mice and their CD4/CD8 T cell ratio was comparable to that observed in non-tg littermate control animals. All animals were used at 2–8-mo of age. All procedures and care of the animals were in accordance with University of Washington guidelines.

Antibodies. The polyclonal rabbit antisera to mouse catL (provided by A. Erickson, University of North Carolina, Chapel Hill, NC) and the monoclonal antibodies M5/114 (anti-I-A$^{b,\ d,\ q}$ and anti-E$^{d,\ k}$) and IN-1 (anti-Ii) have been described previously (18–20). Streptavidin horseradish peroxidase was purchased from Vector Laboratories.

Mφ Isolation. To elicit pMφs, mice were injected intraperitoneally with 1 ml 4% thioglycollate (Becton Dickinson) and peritoneal exudate cells were collected 4–5 d later. Mφs were cultured in Hydron-treated (Hydro Med Sciences) plates with RPMI-S (RPMI 1640 containing 100 u penicillin-streptomycin, 2 mM L-Glutamine [Invitrogen Corporation], and 10% FBS [GIBCO BRL]). Bone marrow–derived Mφs (BMMφs) were generated by culturing bone marrow cells on bacterial plastic plates in RPMI-S with 10^{6} units/ml M-CSF (21). Mouse alveolar Mφs were harvested from lavage fluids as previously described (22). In brief, mouse lung was washed repeatedly with 1 ml of 6 mM EDTA PBS. Mφs were pelleted and cultured in RPMI-S. For cytokine treatment, Mφ populations were cultured for 48 h in the presence or absence of 0.3 ng/ml recombinant mouse IFN-γ (R&D Systems) or 200 U/ml recombinant mouse IL-4 (23).

DC Isolation. Spleens were enriched for DCs as previously described (14, 24). In brief, mice were injected subcutaneously with 5 × 10^{6} Flt3 ligand secreting B16 melanoma cells (provided by G. Dranoff, Dana-Farber Cancer Institute, Boston, MA) and spleens were harvested after the tumors reached 2 cm in diameter. Splenocytes were incubated with magnetic CD11c microbeads (Miltenyi Biotec), positively selected on an AutoMACS, and the purity of the positive fraction was assessed by flow cytometric analysis. All positive fractions were >96% CD11c^{hi}.

Cysteine Protease Active Site Labeling. 10^{6} cells were incubated for 2 h at 37°C with 0.25 μM of the iodinated cysteine protease inhibitor CBZ-^{125}I-Tyr-Ala-CN$_2$ (25). This inhibitor irreversibly binds to the active site of cysteine proteases via a thiol–ester bond. Cells were washed, lysed in cell lysis buffer (0.5% NP-40, 0.15 M NaCl, 50 mM Tris-HCL, pH 7.2), supplemented with a cocktail of protease inhibitors (Roche Molecular Biochemical), and run on a 12% wt/vol SDS-PAGE gel. ^{125}I-labeled proteins were visualized by autoradiography on Kodak BioMax M_r film. The results were quantified using the Bio-Rad GS-700 Imaging Densitometer and analyzed by Multi-Analyzer software Version 1.0.2. Alternatively, 10^{6} cells were incubated for 2 h at 37°C with 0.25 μM of the biotinylated cysteine proteinase inhibitor biotin-Tyr-Ala-FMK, as described above. Intracellular biotin-labeled proteases were detected by immunoblotting cell lysates (see below). Extracellular cathepsin activity was analyzed by Western blot after concentrating supernatant by ultrafiltration on YM10 Centricons (Amicon) and dialysis against 20 mM Tris, pH 7.5.

Immunoblotting. Mφs and fibroblasts were washed in PBS and lysed as described above. Debris was removed by centrifugation at 8,000 rpm for 10 min and the lysates and supernatant were analyzed for protein content using Coomassie® Plus Protein Assay Reagent (Pierce Chemical Co.). Samples containing the indicated amount of total protein or number of cell equivalents were boiled for 5 min in SDS-reducing buffer and separated by 12% SDS-PAGE. The proteins were electrophoretically transferred onto nitrocellulose membrane and this was probed using the indicated primary Ab. Binding was detected using the appropriate horseradish peroxidase–conjugated secondary Ab diluted 1:1,000 and visualized by chemiluminescence (Amersham Biosciences).

Pulse Chase Biosynthetic Radiolabeling and Immunoprecipitation. pMφs were preincubated at 37°C for 2 h in methionine/cysteine-free RPMI 1640 supplemented with 200 mM L-glutamine, 10 mM Hepes, 100 μg/ml penicillin-streptomycin, and 5% dialyzed FBS. Cells were pulsed (4–8 × 10^6 cells/ml) for 40 min with 1 mCi/ml ^{35}S-methionine/cysteine (Trans ^{35}S-label; ICN Biomedicals) and chased for 0, 1, 3, and 6 h in the presence of 30× unlabeled methionine (3 mM) and cysteine (16 mM). Pulse-labeled cells were washed in PBS and lysed in 1% NP-40, 0.01 M Tris, pH 7.4, and 0.15 M NaCl supplemented with a cocktail of protease inhibitors (Roche Molecular Biochemicals). Before precipitation with the M5/114 Ab, lysates were precleared with protein G Sepharose (Amersham Biosciences) and normal rat IgG (Caltag Laboratories Inc.). Precipitated proteins were boiled in SDS-reducing buffer and separated by 7.5–20% gradient SDS-PAGE. Gels were fixed in 50% methanol, 10% acetic acid, treated with Amplify™ (Amersham Biosciences), dried, and the labeled proteins were visualized by autoradiography.

RNA Extraction and cDNA Synthesis. pMϕs were purified by adherence to bacterial plastic plates and were lysed in RNA STAT-60 (3 ml RNA STAT-60 per 10 × 10^6 cells; TEL-TEST "B" INC.) directly on the plates 30 min after plating (ex vivo) and after 48 h of incubation in the presence or absence of IFN-γ. RNA was extracted according to the manufacturer's protocol and contaminating DNA was removed by treating 2 μg of sample RNA with amplification grade DNase I (Life Technologies). First strand cDNA was prepared by reverse transcription using the Life Technologies SUPERSCRIPT™ First-Strand Synthesis System for RT-PCR, as directed by the manufacturer.

Real-time PCR. Primer and probe sequences were selected with the assistance of Primer Express software (Applied Biosystems) using nucleotide sequences available on the GenBank Database (available from GenBank/EMBL/DDBJ under accession nos. NM_013556, X06086, and AJ002386 for HPRT, catL, and catS, respectively). Primers (Life Technologies) are as follows: HPRT-F 5′-3′ (5′-TGGAAAGAATGTCTTGATTGTTGAA-3′); HPRT-R 5′-3′ (5′-AGCTTGCAACCTTAACCATTTTG-3′); CatL-F 5′-3′ (5′-GACCGGGACAACCACTGTG-3′); CatL-R 5′-3′ (5′-CTACCCATCAATTCACGAC-3′); CatS-F 5′-3′ (5′-GCCATTCCTCCTTCTTCTTCTACA-3′); and CatS-R 5′-3′ (5′-CAAGAACACCATGATTCACATTGC-3′). The following probes were synthesized with a 5′ FAM reporter and 3′ TAMRA quencher (Biosearch Technologies Inc.): HPRT (5′-6-FAM-CAAACTTTGCTTTCCCTGGTTAAGCAGTACAGC-TAMRA-3′); CatL (5′-6-FAM-CTCAGGTGTTTGAACCCATGAATCTTTTACTC-TAMRA-3′); and CatS (5′-6-FAM-AAGCGGTGTCTATGATGACCCCTCCTGTATAMRA-3′).

Real-time PCR amplification of HPRT, catL, and catS was performed as previously described (18). Triplicates of each cDNA sample were amplified alongside appropriate controls and each assay was performed on at least three independent occasions. Relative quantitation of catL and catS expression was determined using the comparative C_T method (user bulletin no. 2; Applied Biosystems). This method was used to calculate the fold increase of mRNA in pMϕs after 48 h of incubation in the presence or absence of IFN-γ compared with those cells isolated ex vivo.

Results

Ii Degradation in IFN-γ–stimulated Mϕs Is Predominately Mediated by CatS. The cysteinal lysosomal proteinases catS and catL play a role in regulating the MHC class II presentation pathway by virtue of their role as mediators of the late stages of Ii degradation (8, 11–14). These enzymes are differentially expressed in vivo in all APCs except Mϕs, in which both catS and catL activity can be detected. Thus, we wished to determine the relative contribution of each of these enzymes to Ii degradation and regulation of MHC class II presentation by Mϕs.

To elucidate the effects of catS and catL on the kinetics of Ii degradation, thioglycollate-elicited pMϕs isolated from B6, catL$^{-/-}$, catS$^{-/-}$, and catS$^{-/-}$ xcatL$^{-/-}$ mice were cultured for 48 h in the presence of IFN-γ to induce up-regulation of MHC class II (26). IFN-γ–stimulated pMϕs were metabolically labeled and chased for 0, 1, 3, and 6 h. Protein lysates were immunoprecipitated with an MHC class II–specific Ab and analyzed by SDS-PAGE (Fig. 1 A). B6 pMϕs showed minimal accumulation of the p12 fragment of Ii associated with MHC class II whereas in catS$^{-/-}$ cells marked accumulation of p12 was observed after 1 h of chase. This fragment was slowly degraded over the 6 h of chase, suggesting that in the absence of catS another proteinase is capable of Ii processing, albeit with substantially slower kinetics. The extent of p12 accumulation in catL$^{-/-}$ pMϕs was not significant compared with that observed in catS$^{-/-}$ pMϕs and therefore the delayed cleavage of Ii in the absence of catS is unlikely to be mediated by catL. In addition, in the absence of both catS and catL accumulation of p12 occurred with the same kinetics as observed in pMϕs lacking catS alone.

To test whether the defect in Ii processing in the absence of catS was limited to pMϕ, we examined different Mϕ populations and analyzed the steady state levels of Ii. We isolated alveolar, peritoneal, and BMMϕs from B6 and catS$^{-/-}$ mice (Fig. 2, B, C, and D). This experiment revealed accumulation of p12 Ii fragments in all Mϕ types in the absence of catS. These results clearly implicate catS as the key enzyme regulating the late stages of Ii degradation in Mϕs. In the absence of catS, another enzyme, likely catF, might be able to degrade Ii, albeit with significantly less efficiency.

IFN-γ Stimulation of Mϕs Modulates Cathepsin Activity. The observation that catL played only a minor role in Ii degradation in IFN-γ–treated pMϕs and previous reports indicating that IFN-γ modulates the mRNA level of cathepsins (27–29) lead us to investigate the effect of IFN-γ on the level of catS and catL of activity in pMϕs and BMMϕs. IL-4 has also been shown to up-regulate expression of MHC class II on the surface of Mϕs (26) and it has been shown that in DCs protease activity can be modulated by both pro- and antiinflammatory cytokines (30). Therefore, we sought to examine the effects of both IFN-γ and IL-4 on catS and catL activity in distinct Mϕ populations.

Thioglycollate-elicited pMϕs and BMMϕs were isolated from B6-, catL$^{-/-}$-, and catS$^{-/-}$-deficient mice and the level of intracellular cathepsin activity was analyzed immediately upon isolation (ex vivo) and after 48 h of culture in the presence or absence of IFN-γ or IL-4 (Fig. 2, A, B, and C). pMϕs analyzed directly ex vivo had barely detectable levels of catS and a very low level of catL activity. Upon culture, in the absence of cytokines, both pMϕs and BMMϕs showed a marked increase in catL activity whereas

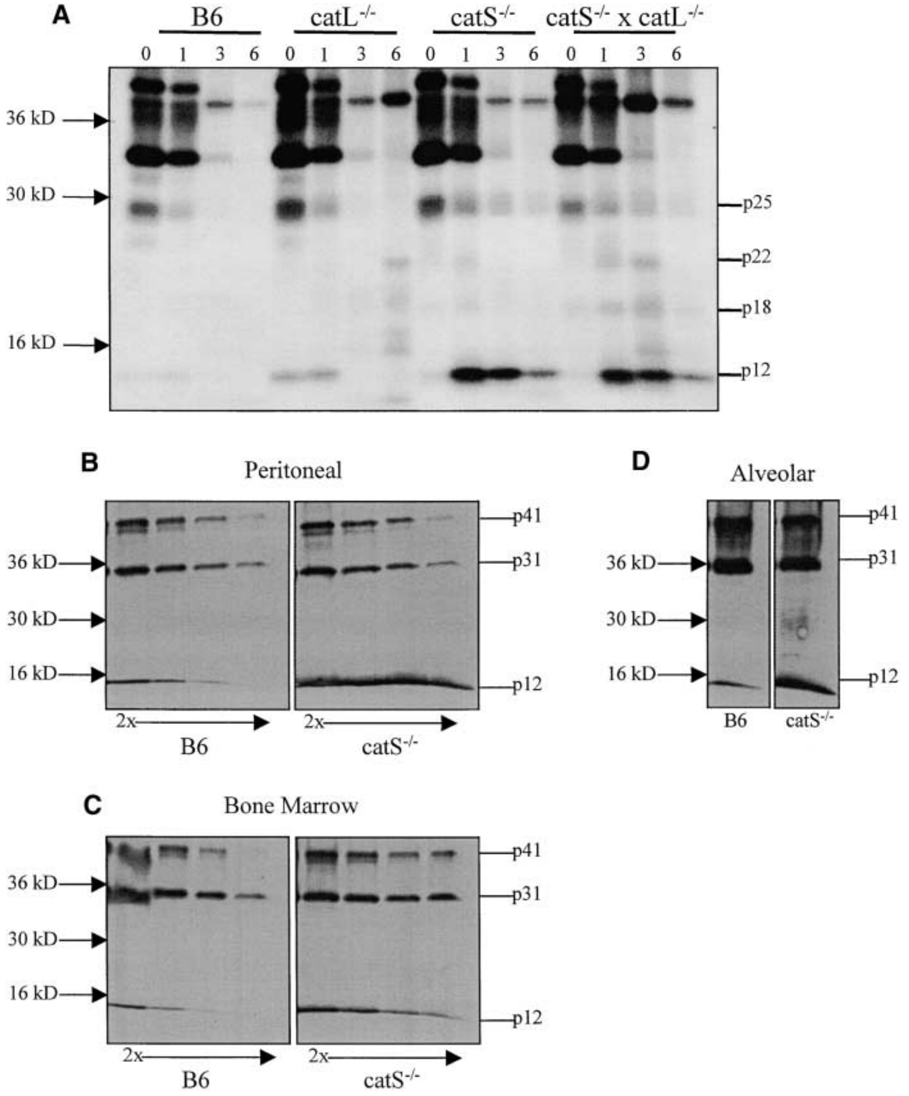

Figure 1. Ii degradation in cathepsin-deficient Mϕs. (A) Thioglycollate-induced Mϕs from B6, catS$^{-/-}$, catL$^{-/-}$, and catS$^{-/-}$ xcatL$^{-/-}$ mice were pulse labeled and chased for 0, 1, 3, and 6 h. Cell lysates were immunoprecipitated with the I-A^{b}-specific Ab M5/114 and immunoprecipitates were analyzed by 7.5–20% gradient SDS-PAGE under denaturing conditions. (B) Thioglycollate-induced pMϕs, (C) BMMϕs, and (D) alveolar Mϕs from B6 and catS$^{-/-}$ mice were cultured for 48 h in the presence of IFN-γ and immunoblotted with the monoclonal Ab IN-1. Cells were solubilized in lysis buffer (1% NP-40/TBS in the presence of protease inhibitors) and lysates were then twofold serially diluted starting at 5 μg of total protein (alveolar Mϕs are shown at 5 μg only). The position of Ii fragments p41, p31, and p12 are indicated.

the activity of catS increased only slightly. Interestingly, the addition of IFN-γ resulted in an increase in catS activity and a dramatic decrease in catL activity. We quantified the changes in catL activity after IFN-γ stimulation by densitometry and found catL activity decreased between 50–100-fold (Fig. 2 C). IL-4, however, had no affect on cathepsin activity as compared with Mϕs cultured in the absence of cytokines (unpublished data; see Figs. 4 and 5). These data suggest our previous observation that catS but not catL was critical for regulating the late stages of Ii cleavage in IFN-γ–stimulated Mϕs can be explained by the effect of this cytokine on down-modulating catL activity.

We have previously observed that the level of mature catL protein in B cells is regulated by the presence or absence of catS (18). Thus, we wished to determine whether such regulation of the cysteinal proteinases was involved in the changes in cathepsin activity observed in both pMϕs and BMMϕs cultured in the presence of cytokines. Modulation of catL activity after IFN-γ stimulation of catS-deficient Mϕs was comparable to that observed in B6-derived cells (Fig. 2, A, B, and C), implying that in Mϕs the activities of catS and catL are regulated independently upon IFN-γ stimulation.

CatL mRNA Levels Are Decreased upon IFN-γ Stimulation of pMϕs. Our finding that catL activity was decreased upon IFN-γ stimulation of pMϕs lead us to investigate the mechanisms by which this down-modulation was regulated. In view of several studies in which it has been reported that in a variety of cell types cathepsin mRNA levels are altered by IFN-γ stimulation (27–29), we sought to determine whether transcription of catL mRNA was decreased in pMϕs upon IFN-γ stimulation.

pMϕs were purified by adherence to tissue culture plates and mRNA was isolated from cells directly ex vivo and after culturing for 48 h in the presence or absence of IFN-γ. Real time PCR amplification and subsequent quantitative analysis indicated that the level of catL mRNA in IFN-γ–stimulated pMϕs was decreased approximately eightfold compared with cells analyzed directly ex vivo (Fig. 3). Furthermore, we were unable to detect any significant change

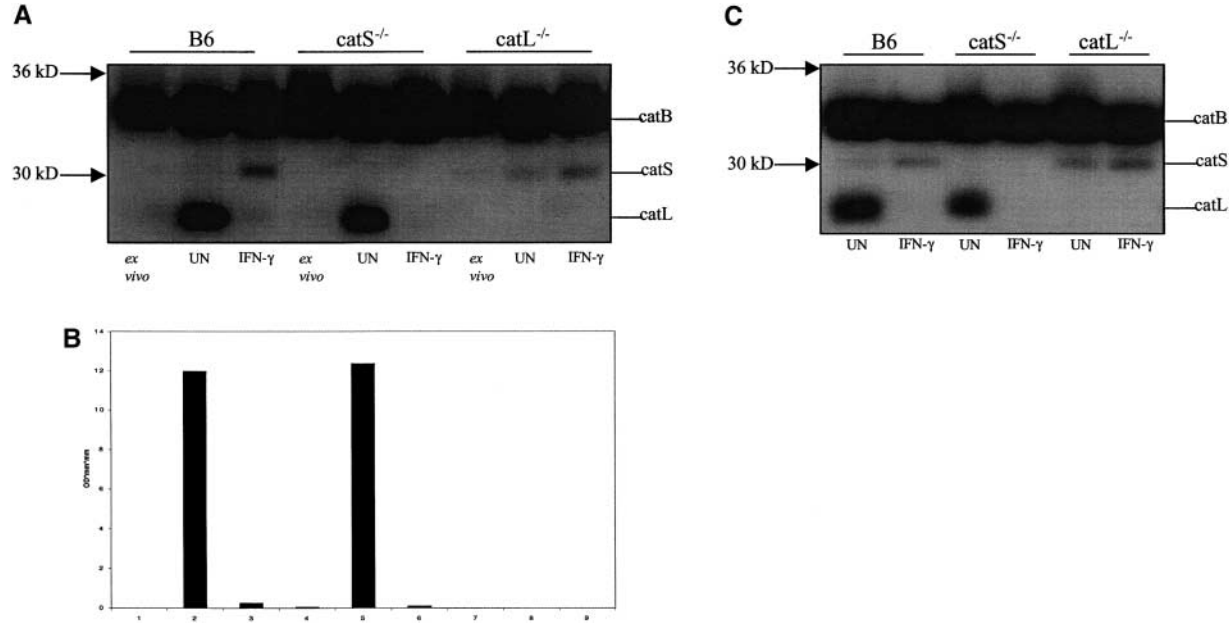

Figure 2. Active site labeling of cytokine-treated Mϕ. (A) Thioglycollate-induced pMϕs and (B) BMMϕs from B6, catS$^{-/-}$, and catL$^{-/-}$ mice were either taken directly ex vivo or activated for 48 h with or without IFN-γ. Cells were incubated for 2 h with the irreversible cysteine protease inhibitor Cbz- [^{125}I]-Tyr-Ala-CN$_2$. Cells were lysed and radiolabeled enzymes were analyzed on a 12% SDS-PAGE gel. Levels of active cathepsin in cells taken directly ex vivo (ex vivo) and after 48 h of plating with IFN-γ (IFN-γ) or without (UN) are shown. The position of cathepsin B (catB), cathepsin S (catS), and cathepsin L (catL) are indicated on the gel. C) The intensity of the active site labeling was quantified by the BioRad GS-700 Imaging Densitometer and analyzed by Multi-Analyzer software Version 1.0.2. The numbers correspond to the following: 1, B6 ex vivo; 2, B6 UN; 3, B6 IFN-γ; 4, catS$^{-/-}$ ex vivo; 5, catS$^{-/-}$ IFN-γ; 6, catS$^{-/-}$ UN; 7, catL$^{-/-}$ ex vivo; 8, catL$^{-/-}$ UN; 9, catL$^{-/-}$ IFN-γ.

in the level of catL mRNA in cells cultured in the absence of cytokine. In addition, we analyzed catS mRNA levels in pMϕs and observed no significant changes with or without cytokine treatment. These results are in agreement with previously published data indicating that catL mRNA is decreased in microglial cells stimulated with IFN-γ (27) and suggests that the IFN-γ–induced decrease in catL activity might in part be a result of decreased transcription of catL mRNA.

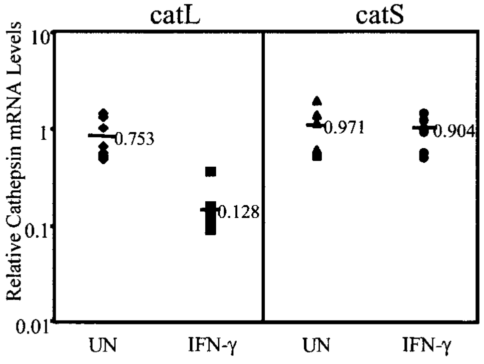

Figure 3. Regulation of catL activity by mRNA. pMϕs were purified by adherence to bacterial plastic plates. Cells were lysed directly on the plates 30 min after plating (ex vivo) and after 48 h of incubation in the presence or absence of IFN-γ. After RNA extraction and cDNA synthesis, quantitative PCR was performed in an ABI Prism 7700 sequence detector. Primers and probes for HPRT, catL and catS were designed using Primer Express software. The probes were labeled at the 5′ and 3′ ends with the fluorochromes FAM and TAMRA. mRNA levels were quantitated using the comparative C$_T$ method described in the ABI Prism 7700 sequence detector user bulletin number 2. The data is shown as the level of cathepsin mRNA in cells after 48 h of plating with IFN-γ (IFN-γ) or without (UN) compared with the level of mRNA ex vivo.

Mature CatL Protein Levels Are Not Reduced in IFN-γ–treated pMϕs. Having demonstrated that the decrease in catL activity in IFN-γ–stimulated pMϕs correlated with diminished transcription of catL mRNA, we wished to establish whether there was a corresponding decrease in intracellular catL protein levels. We performed a twofold serial dilution of cell lysate derived from B6 pMϕs isolated directly ex vivo or cultured for 48 h in the presence or absence of IFN-γ. Lysates were separated by SDS-PAGE and analyzed by immunoblotting for catL protein (Fig. 4 A). Unexpectedly, we observed that the amount of mature catL protein detected in pMϕs cultured in the presence or absence of IFN-γ was comparable and that this level of protein was substantially greater than that detected in ex vivo–isolated cells. A decrease in the amount of the 38 kD pro-form of catL was observed, however, in IFN-γ–stimulated pMϕs when compared with cells analyzed directly ex vivo. Thus, the reduction in catL mRNA upon IFN-γ stimulation of pMϕs resulted in a decrease in pro-form catL protein although there was no significant concomitant decrease in the level of mature catL protein, presumably as a result of the long half-life of this protein. Taken together, our results demonstrating that catL activity is substantially diminished upon IFN-γ stimulation of pMϕs whereas levels of mature catL pro-

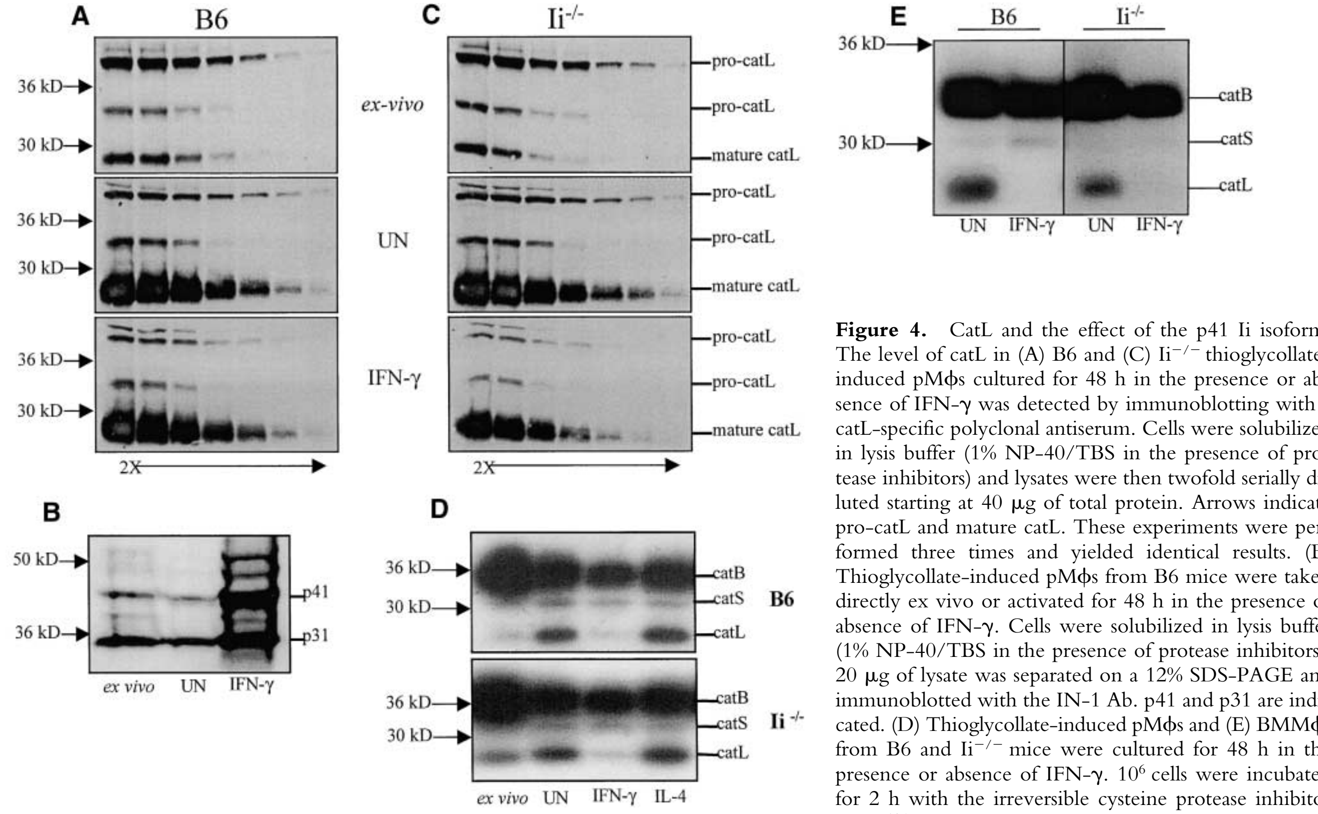

Figure 4. CatL and the effect of the p41 Ii isoform. The level of catL in (A) B6 and (C) $Ii^{-/-}$ thioglycollate-induced pMϕs cultured for 48 h in the presence or absence of IFN-γ was detected by immunoblotting with a catL-specific polyclonal antiserum. Cells were solubilized in lysis buffer (1% NP-40/TBS in the presence of protease inhibitors) and lysates were then twofold serially diluted starting at 40 μg of total protein. Arrows indicate pro-catL and mature catL. These experiments were performed three times and yielded identical results. (B) Thioglycollate-induced pMϕs from B6 mice were taken directly ex vivo or activated for 48 h in the presence or absence of IFN-γ. Cells were solubilized in lysis buffer (1% NP-40/TBS in the presence of protease inhibitors). 20 μg of lysate was separated on a 12% SDS-PAGE and immunoblotted with the IN-1 Ab. p41 and p31 are indicated. (D) Thioglycollate-induced pMϕs and (E) BMMϕs from B6 and $Ii^{-/-}$ mice were cultured for 48 h in the presence or absence of IFN-γ. 10^6 cells were incubated for 2 h with the irreversible cysteine protease inhibitor Cbz-[^{125}I]-Tyr-Ala-CN_2, lysed, and separated by 12% SDS-PAGE. Levels of active cathepsin in cells taken directly ex vivo (ex vivo) and after 48 h of plating with IFN-γ (IFN-γ) or without (UN) are shown. Arrows indicate the positions of cathepsin B (catB), cathepsin S (catS), and cathepsin L (catL).

tein are increased suggest an inhibitor of catL might be up-regulated in these cells.

Modulation of CatL Activity in IFN-γ–stimulated pMϕs Occurs in the Absence of Ii. Several molecules have been implicated as inhibitors of catL, including the p41 isoform of Ii (31–33). Previous studies have shown that Ii is up-regulated in response to IFN-γ (20), making p41 an attractive candidate inhibitor of catL in pMϕs cultured in the presence of this cytokine. Thus, we examined the regulation of catL in thioglycollate-elicited pMϕs isolated from $Ii^{-/-}$ mice.

To first test whether p41 could be an inhibitor of catL activity in IFN-γ–stimulated pMϕs, we analyzed by immunoblotting the level of Ii protein in B6 pMϕs isolated directly ex vivo or cultured in the presence or absence of IFN-γ for 48 h (Fig. 4 B). Upon IFN-γ stimulation the p41 isoform of Ii was substantially up-regulated whereas the increase in p31 was more moderate. No such change in the level of either p31 or p41 was observed when cells were cultured in the absence of cytokine. This increase in p41 upon IFN-γ stimulation of pMϕs provided us with evidence that p41 was a viable candidate inhibitor of catL.

It has previously been reported that the level of mature catL protein detected in BMMϕs is significantly decreased in the absence of the p41 isoform of Ii (33). Therefore, we sought to determine whether a similar defect could be observed in $Ii^{-/-}$ pMϕs. The level of catL protein in pMϕs isolated from $Ii^{-/-}$ mice was analyzed by serial dilution of cell lysates and immunoblotting as described above. The level of mature catL protein in $Ii^{-/-}$ pMϕs was increased upon culturing the cells in the presence or absence of IFN-γ (Fig. 4 C), as observed in B6 pMϕs (Fig. 4 A). The extent of up-regulation of mature catL in cells cultured in the absence of cytokine was the same for both $Ii^{-/-}$ and B6-derived cells. However, the amount of mature catL protein detected in $Ii^{-/-}$ cells cultured in the presence of IFN-γ was two- to threefold less than in cells cultured in the absence of this cytokine and also two- to threefold less than in B6 pMϕs cultured in IFN-γ (Fig. 4 A). Taken together, these data suggest that p41 may play some role in stabilizing the mature form of catL in IFN-γ–stimulated pMϕs as has been suggested for BMMϕs (33), however, we observed no role for p41 in maintaining the level of mature catL protein in pMϕs isolated directly ex vivo or cultured in the absence of cytokines.

Having observed that $Ii^{-/-}$ pMϕs stimulated with IFN-γ express noticeably lower levels of mature catL protein than B6 cells cultured under the same conditions, we aimed to establish whether Ii regulated the decrease in catL activity detected upon IFN-γ stimulation of B6 Mϕs. pMϕs and BMMϕs from B6 and $Ii^{-/-}$ mice were isolated directly ex vivo or cultured for 48 h in the presence or absence of IFN-γ or IL-4. Intracellular cathepsin activity was detected using the irreversible cysteine protease inhibitor Cbz-^{125}I-Tyr-Ala-CN_2 and the proteins were separated by SDS-

PAGE (Fig. 4, D and E). The modulation of cathepsin activity we observed was the same in both cell types. After culture of cells in the absence of cytokine catL activity was up-regulated and in the presence of IFN-γ this up-regulation was not observed. In addition, catL activity was up-regulated in cells cultured in the presence of IL-4. These results provide no evidence for a major role for the p41 isoform of Ii in regulating catL activity in pMφs and BMMφs although it may play a modest role in stabilizing the levels of mature catL protein in IFN-γ–stimulated Mφs.

CatL Is Secreted When Mφs Are Treated with IFN-γ. Cathepsins are soluble proteinases and specific secretion of catL may explain the decrease in intracellular catL activity we observed upon IFN-γ stimulation of pMφs. To address this possibility we compared intracellular and extracellular cathepsin activity using a new biotinylated active site labeling reagent, biotin-Tyr-Ala-FMK. pMφs were labeled for 2 h at 37°C with the biotinylated inhibitor and labeled enzymes in the culture supernatant and cell lysates were detected by Western blot analysis. As shown in Fig. 5, A and B, catL activity was markedly increased in the culture supernatant upon IFN-γ treatment whereas intracellular catL activity sharply decreased. CatL activity was barely detectable in supernatant of untreated or IL-4–treated pMφs. In contrast, both extracellular and intracellular catS activity increased upon treatment with IFN-γ. It is important to note that endocytic uptake can be a rate-limiting step, thus, the efficiency of the active site labeling of intracellular enzymes is significantly less efficient than that of extracellular enzymes. Therefore, we assessed the overall amount of catL protein secreted by IFN-γ–induced pMφs by Western blot analysis with an anti-catL Ab (Fig. 5 D). We observed a two- to fourfold increase in mature catL whereas pro-catL levels are decreased in the supernatant of IFN-γ–treated cells as compared with the cells cultured in the absence of cytokine. In these experiments analyzing catL levels in culture supernatants, we used 7–10-fold more cell equivalents per lane as compared with intracellular catL analysis. Taken together with intracellular mature catL protein levels not changing, these experiments indicate that secretion of catL protein cannot account for the significant loss in catL activity upon IFN-γ stimulation of pMφs.

Cathepsins have been implicated in inflammatory extracellular matrix remodeling, tumor metastasis, and angiogenesis (34) and catL has a potent elastinolytic activity (35). We wanted to determine if the secreted enzymes are active extracellularly by labeling supernatants of pMφ cultures with biotin-Tyr-Ala-FMK at neutral pH and found that both secreted catL and catS are active at physiological pH (Fig. 5 C). This data suggests that secreted catL may play a role in extracellular matrix remodeling at the sites of inflammation.

CatL Activity Is Inhibited When Overexpressed in DCs. B cells and DCs exhibit no catL activity and therefore utilize catS for late stage Ii chain processing whereas Mφs are the only APC in which active catS and catL can be detected concurrently. In light of our observation that catL plays no significant role in Ii degradation in Mφs, we sought to investigate the effect of catL expression in a second catS-dependent APC. tg mice were generated using the CD11c promoter to specifically overexpress catL in DCs. To rule out any effect of catS upon MHC class II maturation in these cells, these mice were crossed onto a catS$^{-/-}$ background.

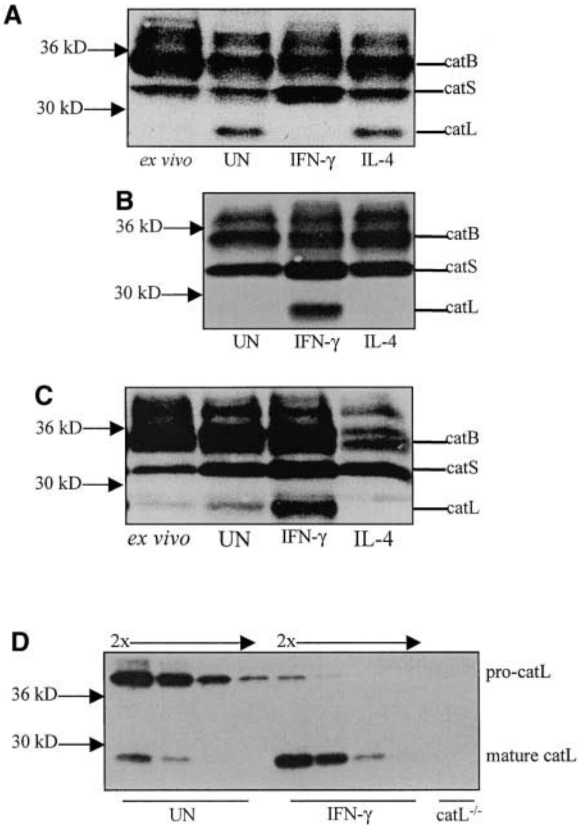

Figure 5. Secretion of catL upon IFN-γ treatment. B6 thioglycollate-induced pMφs taken directly ex vivo or cultured for 48 h in the presence or absence of IFN-γ or IL-4 were incubated serum free with the cysteine proteinase inhibitor biotin-Tyr-Ala-FMK for 2 h. Labeled cathepsin present (A) intracellularly and (B) in the supernatant were detected by immunoblotting. (C) Cells were incubated for 2 h in serum-free medium, the cells were removed, and the inhibitor biotin-Tyr-Ala-FMK was added for 20 min before analysis of labeled cathepsins by immunoblotting. (D) Thioglycollate-elicited pMφs cultured for 48 h in the presence or absence of IFN-γ were incubated for 2 h in serum-free medium. 5 μg of total supernatant protein were titrated and separated before catL immunoblotting.

We wished to determine the level of catL protein expressed in DCs isolated from tg mice and non-tg littermate control animals. Highly purified DCs were isolated and protein lysates from the indicated number of cells were analyzed for catL protein (Fig. 6). B6 DCs expressed significantly less catL protein than the equivalent number of CD11c-catL tg DCs, indicating that the CD11c promoter efficiently induces overexpression of catL protein in DCs. To our surprise, using the Cbz-^{125}I-Tyr-Ala-CN$_2$ irreversible inhibitor we were unable to detect catL activity in the CD11c-catL tg DCs in which we observed high levels of mature catL protein. We observed the same results using DCs isolated from progeny of a second founder CD11c-catL tg mouse (unpublished data).

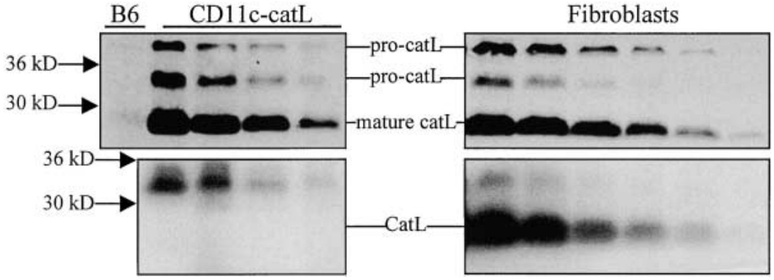

Figure 6. Inhibition of catL activity in CD11c-catL tg mice. DCs purified from Flt3 ligand–treated CD11c-catL tg mice were twofold serially diluted starting at 10^6 cells. Transformed murine embryonic fibroblasts were twofold serially diluted starting at 2.5×10^5 cells. For Western blot analysis, cells were solubilized in lysis buffer (1% NP-40/TBS in the presence of protease inhibitors) and lysates were separated by 12% SDS-PAGE and immunoblotted for catL. Pro-catL and mature catL are indicated. For active site labeling, cells were incubated for 2 h with the irreversible cysteine protease inhibitor Cbz-[^{125}I]-Tyr-Ala-CN_2. Cells were lysed and radiolabeled enzymes were analyzed on a 12% SDS-PAGE gel. Arrows indicate active catL.

To ensure that the level of mature catL protein expressed by the CD11c-catL tg DCs generates sufficient signal to be detected in our active site labeling assay, we compared catL protein and activity in tg DCs to fibroblasts, a cell type known to express high levels of catL activity (11). We were able to detect both mature catL protein and catL activity in as few as 8,000 fibroblast cells (Fig. 6). However, in DCs expressing comparable catL protein levels to 1.25×10^5 fibroblasts we were unable to detect catL activity. Thus, the quantity of mature catL protein expressed in the CD11c-catL tg DCs is not below the level of detection in our active site labeling assay, indicating that DCs express a specific inhibitor of catL activity.

Discussion

The lysosomal cysteinal proteinases catS and catL have previously been shown to play a critical role in the late stages of Ii degradation in DCs, B cells, and cortical thymic epithelial cells (36, 37). In these cells, activity of only one enzyme can be detected and thus the relative role of catS and catL in regulating Ii processing cannot be assessed. Mϕs provide an ideal APC in which to study whether catS and catL are redundant enzymes in the regulation of MHC class II presentation, as activity of both enzymes can be detected in these cells. Here, we report that in thioglycollate-elicited pMϕs stimulated with IFN-γ, catS is principally responsible for mediating Ii cleavage whereas catL plays little part in this process. This result is surprising given that efficient Ii proteolysis in cortical thymic epithelial cells requires catL and that in MHC class II–expressing fibroblast cell lines effective Ii cleavage can be mediated by catL (11, 38). However, an explanation for our results is provided by our subsequent observation that although intracellular activity of catL is profoundly down-regulated in pMϕs in response to the proinflammatory cytokine IFN-γ, intracellular catS activity increases. This modulation in catL activity in IFN-γ–stimulated pMϕs coincided with a decrease in catL mRNA levels whereas catS mRNA levels were not diminished. These data indicate that modulation of catL activity in pMϕs in response to IFN-γ is in part regulated at the level of transcription as has previously been reported for microglial cells (27). Down-regulation of catL mRNA was concomitant with a decrease in pro-catL, however, levels of the mature protein were not decreased in IFN-γ–stimulated pMϕs. One possible explanation for this maintenance of high levels of mature catL protein despite decreased mRNA is that the mature enzyme is long lived in endosomes. Additional studies showed that the decrease in catL activity upon IFN-γ stimulation was concomitant with an increase in secretion of mature catL protein that exhibited protease activity. However, this increase in secretion had little effect on intracellular catL protein levels and therefore, secretion of mature protein does not explain the specific inhibition of intracellular catL activity. Taken together with our observation that intracellular catL activity decreases in IFN-γ–stimulated pMϕs whereas levels of the mature protein are maintained, these results suggest that an inhibitor(s) of enzyme activity is increased in response to this proinflammatory cytokine and is critical for regulating catL activity in pMϕs and BMMϕs.

A number of lysosomal cysteinal proteinases inhibitors have been identified, including cystatin C (39, 40), the propeptide regions of the cathepsins (41–43), and the p41 isoform of Ii (31, 32). We found no difference in cystatin C expression or localization in pMϕs cultured in the presence or absence of IFN-γ (unpublished data), indicating that this cysteinal proteinase inhibitor plays no significant role in regulating catL activity upon IFN-γ stimulation of pMϕs. In addition, the propeptide regions of catS and cathepsin B (catB) do not affect the level of catL activity in Mϕs treated with IFN-γ. We examined catS- and catB-deficient pMϕs and BMMϕs and were unable to determine any differences in catL activity with or without treatment of IFN-γ when compared with B6 Mϕs (Fig. 2 and unpublished data). Recent studies have reported that p41 is not only an inhibitor of catL enzymatic activity but that it is also required for catL activity in BMMϕs where it acts as a chaperone, stabilizing the mature form of this enzyme (33). Because both MHC class II and Ii, including p41, are up-regulated in pMϕs stimulated with IFN-γ, we examined whether p41 and its fragments were responsible for the observed down-regulation of catL activity and preservation of high levels of mature protein in these cells. We observed that levels of mature catL protein in $Ii^{-/-}$ and B6 pMϕs and BMMϕs were identical, except upon IFN-γ stimulation when catL levels were two- to threefold lower in $Ii^{-/-}$ cells compared with B6 cells. However, we detected equivalent levels of catL activity in $Ii^{-/-}$ and B6 pMϕs and BMMϕs cultured in the presence or absence of IFN-γ. Furthermore, the extent to which catL activity was down-regulated upon IFN-γ stimulation was equivalent in $Ii^{-/-}$ and B6 cells. These data demonstrate that Ii does not play an important role in regulating catL activity in either thioglycollate-induced pMϕs or BMMϕs and are in agreement with our previous observations in thymic stromal cells (15). However, these results suggest that Ii does play a minor role in stabilizing the mature form of catL in pMϕs, as has been previously suggested by others for BMMϕs (33). Another possible inhibitor of catL activity is cystatin F, which is specifically expressed in hematopoietic cells and preferentially binds catL and papain in vitro (44, 45). We believe cystatin F could potentially be involved in the catL inhibition observed in Mϕs or DCs and would like to investigate this in the future. Thus, although it is possible that p41 may contribute modestly to the observed down-modulation of intracellular catL activity upon IFN-γ stimulation of pMϕs, we suggest that an unknown inhibitor is the pivotal regulator of catL enzymatic activity in pMϕs.

We have previously reported that catL activity cannot be detected in ex vivo–isolated DCs (11, 18), however, significant levels of catL activity can be detected in DCs derived from bone marrow in culture (unpublished data). In view of our observations implying that catL activity in pMϕs is regulated in a highly specific manner by an as yet unidentified inhibitor, we sought to examine whether such a mechanism was present in ex vivo DCs. Analysis of DCs isolated from the spleen of mice engineered to express catL under the control of the CD11c promoter revealed significant levels of mature catL protein but no detectable catL activity in these cells. Expression of this catL cDNA when driven by a retroviral promoter in fibroblasts generated high levels of catL activity (38), indicating that inhibition of enzymatic activity was specific to ex vivo–isolated DCs. Thus, we have shown that both DCs and pMϕs use a mechanism of catL inhibition although the nature of the catL inhibitor in DCs and pMϕs remains to be identified.

Our data indicate that catL activity is down-regulated upon IFN-γ stimulation of pMϕs and that catS is the predominant mediator of the late stages of Ii degradation, and hence MHC class II peptide presentation. In addition, catS mediates Ii degradation in other Mϕ populations. These observations appear to differ from a recent report in which it was shown that other cysteinal proteinases, perhaps catF, are able to compensate for the absence of catS and catL and elicit degradation of Ii in pMϕs and alveolar Mϕs (16). However, in this latter study, Ii processing was analyzed immediately after pulse and after an overnight chase. Thus, the pronounced accumulation of MHC class II–associated p12 Ii fragments we observe between 1 and 6 h of chase was not detected. The significantly delayed kinetics of Ii degradation and accumulation of MHC class II–bound Ii fragments in catS-deficient pMϕs affect the efficiency of generation of MHC class II–peptide complexes and thus account for the previously reported defect in MHC class II presentation of some but not other protein antigens (13). Therefore, we believe that while although enzymes can mediate Ii cleavage with delayed kinetics, catS plays a major role in regulating Ii degradation and MHC class II presentation in Mϕs.

In conclusion, we have shown that in pMϕs, in which both cysteinal proteinases catS and catL are expressed, catS is the predominant enzyme processing Ii. Furthermore, catL is shown to have no major role in this process as a result of substantial down-regulation of its enzymatic activity upon IFN-γ stimulation. This decline in catL activity correlates with a decrease in catL mRNA and an increase in secretion of mature catL protein but not a decrease in intracellular mature catL protein levels, suggesting that catL activity in IFN-γ–stimulated pMϕs is regulated by a specific inhibitor. In addition, we find the p41 isoform of Ii does not contribute significantly to this regulation of catL activity in pMϕs and that DCs also use a catL-specific inhibition mechanism. The results presented here indicate that upon IFN-γ activation of pMϕs, enzymatic activity of catL is specifically inhibited, such that catS mediates Ii degradation and regulates MHC class II maturation. Thus, we suggest that as a result of differential regulation of catL and catS the latter enzyme governs MHC class II presentation by APCs in secondary lymphoid organs.

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