The Expression and Function of Cathepsin E in Dendritic Cells

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The Expression and Function of Cathepsin E in Dendritic Cells

Benjamin M. Chain, Paul Free, Patrick Medd, Claire Swetman, Alethea B. Tabor, and Nadia Terrazzini

Cathepsin E is an aspartic proteinase that has been implicated in Ag processing within the class II MHC pathway. In this study, we document the presence of cathepsin E message and protein in human myeloid dendritic cells, the preeminent APCs of the immune system. Cathepsin E is found in a perinuclear compartment, which is likely to form part of the endoplasmic reticulum, and also a peripheral compartment just beneath the cell membrane, with a similar distribution to that of Texas Red-dextran within 2 min of endocytosis. To investigate the function of cathepsin E in processing, a new soluble targeted inhibitor was synthesized by linking the microbial aspartic proteinase inhibitor pepstatin to mannosylated BSA via a cleavable disulfide linker. This inhibitor was shown to block cathepsin D/E activity in cell-free assays and within dendritic cells. The inhibitor blocked the ability of dendritic cells from wild-type as well as cathepsin D-deficient mice to present intact OVA, but not an OVA-derived peptide, to cognate T cells. The data therefore support the hypothesis that cathepsin E has an important nonredundant role in the class II MHC Ag processing pathway within dendritic cells. The Journal of Immunology, 2005, 174: 1791–1800.

In this study, we have investigated the expression of cathepsin E in dendritic cells (DC), which are generally believed to be the key APCs in stimulating primary T cell immune responses. The observation that cathepsin E is expressed in both human and mice myeloid DC complements functional studies using a novel, targeted derivative of the aspartic proteinase inhibitor pepstatin and provides further evidence linking cathepsin E to Ag processing.

Materials and Methods

Dendritic cells

Human myeloid DC were generated from PBMC taken from healthy volunteers after informed consent (project approved by University College London (UCL) Ethics Board). Adherent monocytes were cultured for 7–8 days in GM-CSF and IL-4. Residual monocytes/lymphocytes were removed by magnetic bead depletion after 4 days as described in detail elsewhere (10). Resulting DC were >90% CD1a+ *CD133 *CD14 **HLA-DR **HLA-DR-DRβ1 *HLA-DR-DRβ1 DRβ1. In some experiments, DC were cultured for another 24 h in the presence of LPS (100 ng/ml; Sigma-Aldrich), which resulted in up-regulation of HLA and costimulatory molecules and down-regulation of endocytosis. Monocytes were isolated by adherence. PBMC were allowed to adhere for 2 h on tissue culture plastic at 37°C. Nonadherent cells were removed by several washings. The adherent cells were then incubated in 3 mM EDTA in HBSS for 30 min at 37°C, followed by vigorous resuspension. Residual B and T cells were removed by magnetic bead depletion as for the DC. The remaining monocyte population was >90% CD14 *.

Mouse myeloid DC were obtained by culture of bone marrow cells (5 × 10^7/ml) in Iscove’s medium (Invitrogen Life Technologies/Life Sciences), 10% FCS (Invitrogen Life Technologies) in GM-CSF (20 ng/ml; Pepro-Tech). Preliminary experiments showed that the standard culture methods had to be adapted to generate DC from bone marrow of young (20–23 days) mice as required for the experiments using cathepsin D-deficient mice and their littermates. Fresh medium and cytokine were added on day 4, and then again on day 8, and DC were harvested on days 11/12. In some experiments, DC were further purified by fluorescence-activated cell sorting of CD11c-expressing cells using a Beckman Coulter Epics Elite ESP cell sorter. The sorted population was 85–95% CD11c *.

Abbreviations used in this paper: DC, dendritic cell; Hb, hemoglobin; LAMP-1, lysosome-associated membrane protein 1; ER, endoplasmic reticulum.
**Quantitative PCR**

RNA was isolated from 1 to 3 × 10^6 monocytes or DC obtained either from human peripheral blood or from mouse bone marrow. As positive control for human cathepsin E expression, RNA was isolated from the EBV-transformed B cell line FC7, activated by the addition of 20 ng/ml PMA for 48 h. Total RNA extraction was performed using the RNeasy purification kit (Qiagen). Reverse transcription of 2 µg of RNA was performed by adding 400 U of Moloney murine leukemia virus-reverse transcriptase plus a reaction mix that consisted of 50 µM oligo(dT) primers, 1 mM dNTPs, 1x reverse transcriptase buffer (BRL), and 20 U of recombinant RNasin (Promega), all contained in a final reaction volume of 30 µl and incubated at 37°C for 1 h. PCRs were performed using a LightCycler (PE Biosystem). Amplification reactions were optimized for MgCl2 concentration and variable annealing temperatures of oligonucleotide primers. Each amplification reaction consisted of 1 µl of cDNA template, 0.5 µM forward and reverse oligonucleotide primers, 3 mM MgCl2, 1/30,000 dilution of SYBR Green dye (10X stock) and 1X LC master mix (BioGene) in a final volume of 10 µl. The primers and reaction conditions are summarized in Table I. Product formation was monitored at the end of each extension step within each cycle by measuring the fluorescence emitted by SYBR Green dye. At the completion of the 40th cycle of amplification, the temperature was increased steadily up to 94°C. With the increase of temperature and the melting of the DNA products, the fluorescent signal from DNA/SYBR Green was lost. Each PCR amplicon was subjected to analysis by agarose gel electrophoresis to confirm the expected size of the product.

**Flow cytometry**

Phenotypic analysis of DC was conducted using standard methods as described previously (10). For intracellular staining, DC were fixed in 4% paraformaldehyde on ice for 10 min and then permeabilized in 0.1% Triton X-100 on ice for 10 min. Permeabilized cells were blocked and stained in the normal way.

**Confocal microscopy and endocytosis**

Briefly, 10^6 DC were incubated in the presence of Texas Red-labeled dextrans and endocytosed as described above. In some experiments, DC were first seeded onto fibronectin-coated coverslips, incubated at 37°C for 60–180 min to allow time for dendrite formation (12), and then allowed to endocytose as described above. After fixation, cells were permeabilized with 0.1% Triton X-100 for 5 min and then incubated with Ab to cathepsin E (supernatant of a monoclonal mouse Ab CE11.1 or a 1/100 dilution of polyclonal rabbit Ab (BMC11) (13) for 45 min, followed by a 45-min incubation in the dark with the appropriate secondary layer (DakoCytomation). In some experiments, cells were double stained with polyclonal rabbit antiserum raised against the endoplasmic reticulum (ER) retention sig-

**Table I. Quantitative PCR: primers and cycling times**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Cycling Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin E (human)</td>
<td>Forward, 5′-GGACATGTACCAATGTCACGCCA-3′</td>
<td>Denaturation: 94°C, 30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse, 5′-GGTTGAGGACACATCCACAGCA-3′</td>
<td>Annealing: 65°C, 3 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extension: 72°C, 18 s</td>
</tr>
<tr>
<td>Cathepsin E (mouse)</td>
<td>Forward, 5′-GGACATGTACCAATGTCACGCCA-3′</td>
<td>Denaturation: 94°C, 30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse, 5′-GGTTGAGGACACATCCACAGCA-3′</td>
<td>Annealing: 65°C, 3 s</td>
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<tr>
<td></td>
<td></td>
<td>Extension: 72°C, 18 s</td>
</tr>
<tr>
<td>Cathepsin D (mouse)</td>
<td>Forward, 5′-GGACATGTACCAATGTCACGCCA-3′</td>
<td>Denaturation: 94°C, 30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse, 5′-GGTTGAGGACACATCCACAGCA-3′</td>
<td>Annealing: 65°C, 3 s</td>
</tr>
<tr>
<td>β-actin (mouse)</td>
<td>Forward, 5′-CCCTTCCCTGCGGCTACGCTCCT-3′</td>
<td>Denaturation: 94°C, 30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse, 5′-GGACATGTACCAATGTCACGCCA-3′</td>
<td>Annealing: 65°C, 3 s</td>
</tr>
</tbody>
</table>

Mice

The colony of cathepsin D-deficient mice (11) were a gift from Prof. C. Peters (Institut für Molekulare Medizin und Zellforschung, Medizinische Universität Freiburg, Freiburg, Germany) and Prof. J. Kay (School of Biosciences, Cardiff University, Cardiff, U.K.). Since homozygous dele-

**MPC6 synthesis**

A detailed description of the synthesis of MPC6 will be published elsewhere. Briefly, asparagine derivatives were coupled to BSA (as carrier) via the ε amino group of lysine using an isothiocyanate linkage, to give a mannose-BSA neoglycoconjugate (mannose-BSA, Fig. 1). MALDI-TOF mass spectrometry showed that −24 mannosyl units were attached to each BSA. Biochemical experiments confirmed this: analysis of free trini-
trobenzene sulfonic acid-reactive amino groups (14) showed an approxi-
mate conjugation of 23 sugar units, and analysis of attached sugar units (15, 16) showed an approximate conjugation of 22.5 sugar units. Mannose-

**Protease assay**

Protease activity was measured using a modification of the 14C-hemo-
globin (Hb) degradation assay (8, 17). Hb (50 mg in sodium borate buffer, pH 7.4) was acetylated by reaction with [14C]acetic anhydride (American Radiolabeled Chemicals) and purified by chromatography over G-50 Sephadex, followed by precipitation in 3% TCA and dialysis against PBS. To measure protease activity, enzyme sample (total volume 25 µl) was added to 50 µl of sodium acetate buffer (1 M, pH 4.0). 14C-Hb (∼2.5 µCi, 100 µg µCi), unlabeled Hb (125 µg, 50 µl), and inhibitor or water as required (25 µl). The mixture was incubated for 60 min at 37°C and the reaction was terminated by addition of 500 µl of cold 3% TCA. Undigested Hb was removed by centrifugation (12,000 g, 15 min), and the TCA-soluble radioactive material was counted by liquid scintillation in Optiscint Supermix scintillant (Wallace). Each reaction was conducted in duplicate or triplicate, but variation between replicates was very small (<5%). Under these reaction conditions, assay of cellular lysates measured predominantly aspartic protein activity, as shown by the fact that >95% of the activity was blocked in the presence of pepstatin.
Functional assay of Ag presentation

Spleen cells from the BALB/c DO11.10 OVA-specific TCR-transgenic mouse (gift from GlaxoSmithKline) were cultured in RPMI 1640 medium and 5% FCS in the presence of OVA peptide 323–339. The cultures were split on day 2 and 10 ng/ml IL-2 was added. The cultures were split again on day 4, with the addition of fresh IL-2, and harvested on day 7 or 8. Dead cells were removed by centrifugation over Ficoll-Hypaque, and the remaining cells (95% TCR-transgenic T cells) were harvested and aliquots (10^6) were frozen until required. For assay of Ag presentation, variable numbers of DC were cocultured with OVA-specific T cells (5% of contaminating B cells, T cells, and monocytes) were generated from peripheral blood adherent cells by a modification of the standard GM-CSF/IL-4 cultures, with a negative depletion step on day 4 of culture.

Quantitative real-time RT-PCR on these cells was used to detect message for cathepsin E (Fig. 2a). The melting curve (derived as \(-\frac{dF}{dT}\), the rate of change of fluorescence, over the rate of change of temperature) shows peak characteristics of each specific product and differentiate between nonspecifically and specifically amplified products. To confirm that the product was derived from cathepsin E message, the PCR products were analyzed by gel electrophoresis and found to contain a band of the expected size (Fig. 2a, inset). The identity of the band in the DC lane was further confirmed by sequencing. In contrast, monocytes contained no cathepsin E message, as judged either by melting curves of products or by gel electrophoresis. Analysis of cDNA from the EBV B cell line Fc7, both stimulated and unstimulated by PMA, was included as a positive control on the basis of previous studies (9). The PCR products from this cell line demonstrated two bands whose sizes corresponded with the presence of the splice variants of cathepsin E described previously (18). No evidence of such multiple transcripts was seen in the DC however, and this observation was not pursued further.

Statistics

Wherever appropriate, the results were analyzed by Student’s t test or one-way ANOVA with post hoc Dunnett’s modification (conducted using SPSS software) as indicated in the figure legends.

Results

Expression of cathepsin E message and protein in human DC

Human DC (containing <5% of contaminating B cells, T cells, and monocytes) were generated from peripheral blood adherent cells by a modification of the standard GM-CSF/IL-4 cultures, with a negative depletion step on day 4 of culture.

Functional assay of Ag presentation

Spleen cells from the BALB/c DO11.10 OVA-specific TCR-transgenic mouse (gift from GlaxoSmithKline) were cultured in RPMI 1640 medium and 5% FCS in the presence of OVA peptide 323–339. The cultures were split on day 2 and 10 ng/ml IL-2 was added. The cultures were split again on day 4, with the addition of fresh IL-2, and harvested on day 7 or 8. Dead cells were removed by centrifugation over Ficoll-Hypaque, and the remaining cells (95% TCR-transgenic T cells) were harvested and aliquots (10^6) were frozen until required. For assay of Ag presentation, variable numbers of DC were cocultured with OVA-specific T cells (5% of contaminating B cells, T cells, and monocytes) were generated from peripheral blood adherent cells by a modification of the standard GM-CSF/IL-4 cultures, with a negative depletion step on day 4 of culture.

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Statistics

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Intracellular distribution of cathepsin E

The distribution of cathepsin E within DC and its relationship to the endocytic pathway was studied by confocal microscopy (Fig. 3). The distribution of cathepsin E was reticular as observed previously (13, 19) and completely different from the vesicular (lysosomal) distribution of cathepsin D (Fig. 3a) or LAMP-1 (Fig. 3b). Cathepsin E was also absent from the class II MHC containing “Ag-loading” compartment of the DC (Fig. 3c), but there was just a suggestion of colocalization with a class II-containing compartment at the periphery of the cell (Fig. 3c, see arrows). In support of this observation, peripheral cathepsin E also showed some colocalization with the transferring-containing early endosomal reticulum (Fig. 3d). Because the cells were very thin and because of the diffuseness of the compartment, it was difficult to show conclusively that this represented true colocalization, rather than adjacent overlapping/superimposed compartments. In contrast to peripheral cathepsin E which forms part of the endosomal system, perinuclear cathepsin E is predominantly in the ER, as shown by colocalization with the ER marker KDDDKDEL (Fig. 3e).

The relationship between cathepsin E and the endocytic pathway is studied further in Fig. 3, f and g, using the uptake of FITC-dextran to identify endosomal structures. Some apparent colocalization of cathepsin E was seen with the very early endosomal compartment, as identified by the presence of dextran within 2–5 min after uptake (Fig. 3, f and g), although it was impossible to rule out that cathepsin E and dextran were in fact in very closely

FIGURE 1. A diagrammatic representation of the structure of MPC6 and the mannone-BSA precursor.
derived DC and analyzed for expression of cathepsin E and negative control (5), and Fc7 EBV cells (4), PMA-stimulated Fc7 B cells (3), cyto-\textgreek{m}tes (2), and negative control (5). The area under the major peak is proportional to the amount of product. Inset, Qualitative analysis of products for each group by agarose gel electrophoresis. b, Purified DC and monocytes were stained with test Ab (filled histogram) or isotype control (open histogram) and analyzed by flow cytometry as described in Materials and Methods. Staining for CD1a and CD14 was conducted before fixation. Staining for cathepsin E using the monoclonal mouse Ab CE1.1 or the polyclonal rabbit antiserum BMC11 was conducted on fixed permeabilized cells. No staining for cathepsin E was observed using intact (viable) cells. The monocytes and DC populations contained \textgreek{m}<5\% T or B cells (data not shown). c, Purified DC were cultured for 24 h in the presence or absence of LPS (100 ng/ml) and stained for CD86 and CE1.1. LPS-treated DC (mature) also showed increased HLA-DR, HLA-DQ, and CD40 expression (data not shown).

Expression of cathepsin E in wild-type and cathepsin D-deficient mice

The study of cathepsin E function has been hampered by the absence of cell-permeable selective inhibitors and by the absence of cathepsin E-deficient mice. To obtain functional data on cathepsin E in DC, we have synthesized a new cell-targeted derivative of pepstatin. Pepstatin is a microbial inhibitor with potent activity against a wide spectrum of aspartic proteinases. However, only two pepstatin-sensitive aspartic proteinases are present within hemopoietic cells, the lysosomal enzyme cathepsin D and cathepsin E. Pepstatin will therefore act as a specific inhibitor of cathepsin E in cells lacking cathepsin D, e.g., DC derived from cathepsin D-deficient mice.

We first confirmed that cathepsin E message was present in bone marrow-derived mouse DC from wild-type and cathepsin D-deficient null mice. Gel electrophoresis of RT-PCR products (Fig. 4a, left panels) confirmed that cathepsin E message could be detected in DC from both mice genotypes. As expected, DC from wild-type, but not from cathepsin D-deficient mice contained message for cathepsin D. Quantitative PCR (Fig. 4a, right panel) confirmed that no difference in cathepsin E levels could be detected between DC from the two mice strains, implying that cathepsin E message levels were independent of cathepsin D expression.

No Ab to murine cathepsin E has been described. The activity of cathepsin E in the bone marrow DC was therefore measured enzymatically. The Hb-degrading activity of cellular lysates at pH 4 is predominantly due to aspartic proteinase activity (8). The total Hb-degrading activity in DC derived from cathepsin D-expressing and nonexpressing mice is shown in Fig. 4b. Cathepsin D-deficient DC had \textgreek{m}sim\textgreek{u}12\% of the activity of wild-type derived DC. This residual activity was completely (>98\%) inhibited by pepstatin (data not shown), confirming that it was due to residual aspartic proteinase activity, presumed to be cathepsin E.

Despite this decrease of 88\% in acid proteinase activity and a total lack of cathepsin D, there was no consistent difference in either the yield or growth of bone marrow-derived DC from wild-type or cathepsin D-deficient mice. In addition, although some variation in phenotype was observed between individual cultures of DC from different animals, no consistent differences were seen between the surface phenotype of wild-type and cathepsin D-deficient DC (Fig. 4c). Approximately 40–60\% of the nonadherent bone marrow cultures were CD11c and MHC class II positive, with the remainder probably representing immature precursors, since they showed no expression of any other lineage markers tested. The slightly lower percentage of CD11c expression and the somewhat longer culture time required than that commonly reported from other laboratories (see Materials and Methods) may have reflected the fact that the bone marrow cells had to be collected from very young mice, because the cathepsin D mutation is lethal at 23 days.

Inhibition of Ag processing in wild-type and cathepsin D-deficient mice

Although previous studies have examined Ag processing in cathepsin D-deficient mice (3), these experiments did not specifically investigate DC from these mice. The ability of wild-type and cathepsin D-deficient DC to process and present OVA to DO11.10-(g) T cells was therefore addressed (Fig. 4d). No significant difference between presentation by the two populations of DC was observed, for either whole protein or peptide.

Pepstatin is a potent inhibitor of aspartic proteinases in cell-free studies. However, it is extremely poorly soluble in water and in initial experiments formed insoluble precipitates if added to DC
cultures. To increase the solubility of pepstatin and also target it to the endosomal uptake system of DC, a novel inhibitor (MPC6) was constructed (Fig. 1). As described in Materials and Methods, MPC6 consisted of a BSA molecule, to which mannose residues were first conjugated via lysine side chain amino groups. Pepstatin was then conjugated to the BSA backbone via a linker containing a cleavable disulfide bond.

The activity of MPC6 compared with pepstatin was first tested against purified human cathepsin D and human cathepsin E (Fig. 5a). MPC6, but not the mannose-BSA precursor, inhibited both enzymes with an IC50 of around 20 nM (cathepsin E) and 0.7 nM (cathepsin D). These values were at least an order of magnitude less potent than pepstatin (Fig. 5a), suggesting the coupling to BSA might cause some steric hindrance. The true Ki values could not easily be ascertained using the Hb assay, because they depend on the number of bonds within Hb targeted by the two enzymes and the average Km for cleaving these bonds. The relative potency of pepstatin and MPC6 agree closely, however, with the Ki values obtained using a peptide fluorogenic substrate. One hundred nanomolar MPC6 and pepstatin both completely abrogated Hb-degrading activity in DC lysates, confirming that the protease activity measured in this assay is due to aspartic proteinases only (Fig. 5b). The ability of MPC6 and pepstatin to target aspartic proteinase activity in intact DC is shown in Fig. 5, c and d. DC were incubated for 180 min in the presence of various concentrations of MPC6 or pepstatin. The DC were washed extensively and then lysed, and the lysates were tested for Hb-degrading activity. MPC6 inhibited 90% proteinase activity at concentrations above 1 μM. In contrast, pepstatin in this whole cell culture system was much less effective, showing only partial inhibition even using 100 μM concentrations (Fig. 5c). Mannose-BSA (Fig. 5d) had no significant effect on proteinase activity.

Having established that MPC6 was water soluble, active, and could inhibit aspartic proteinase within viable DC much more effectively than pepstatin, the ability of the inhibitor to affect Ag processing was investigated (Fig. 6). Since much smaller numbers of cells were required for these functional studies, the DC were further enriched by cell sorting before assay, providing a population that was >85% positive for CD11c (Fig. 6a). Preliminary experiments showed that the sorted population retained the same phenotype, but were more potent on a cell per cell basis, reflecting the increased frequency of DC.

4 P. Free, The function of cathepsin E in antigen processing. Submitted for publication.
Initial experiments were done pulsing DC with Ag and inhibitor, followed by fixation (20). However, even very light fixation reduced the ability of DC to activate cognate T cells by 10–100 times, making it difficult to interpret these experiments. The experiments were therefore conducted by adding inhibitor directly to the DC/T cell cocultures. To rule out that MPC6 was affecting the responding T cells, rather than the DC, MPC6 was added directly to T cells stimulated with IL-2. No significant inhibition was observed at any of the concentrations tested (Fig. 6c, right panel). The effects of MPC6 (Fig. 6c, left panel) and pepstatin (Fig. 6c, right panel) on processing of OVA and 323–339 peptide were first incubated for 1 h in medium and then either MPC6 (2 μM) or mannos-BSA conjugate (2 μM). Excess inhibitor was removed by repeated centrifugation, and the cells were then lysed and assayed for Hb-degrading activity as above. Background cpm is 192. cpm in the presence of cell extract but no inhibitor is 1469. One experiment of three is represented.

FIGURE 5. A mannose-pepstatin-BSA conjugate (MPC6) inhibits acid proteinase activity in mouse bone marrow-derived DC. a, Purified cathepsin D (40 nM) or cathepsin E (10 nM) was incubated with varying concentrations of MPC6, pepstatin, or mannos-BSA conjugate (without conjugated pepstatin) in the presence of 14C-Hb as described in Materials and Methods. Background cpm is 231. Counts per minute in the presence of enzyme but no inhibitor is 2477 for cathepsin E and 1985 for cathepsin D. The dotted line shows the IC50 values. One experiment of three is represented. b, DC (2 × 105) from bone marrow of wild-type mice were resuspended in lysis buffer (1 M sodium acetate (pH 4) and 0.1% Nonidet P-40) and the lysate was assayed for Hb-degrading activity in the presence or absence of MPC6 or pepstatin as described. One experiment of three is represented. c, DC (2 × 105) were incubated for 180 min in medium and different concentrations of pepstatin (at 100, 30, or 10 μM) or MPC6 (at 10, 3, or 1 μM). Excess inhibitor was removed by repeated centrifugation, and the cells were then lysed and assayed for Hb-degrading activity as above. Background cpm is 192. cpm in the presence of cell extract but no inhibitor is 1469. One experiment of three is represented. d, DC (2 × 105) were incubated for 1 h in medium and either MPC6 (2 μM) or mannos-BSA conjugate (2 μM). Excess inhibitor was removed by repeated centrifugation, and the cells were then lysed and assayed for Hb-degrading activity as above. Background cpm is 72.
FIGURE 6. MPC6 inhibits Ag processing of OVA by DC from wild-type and cathepsin D-deficient mice. 

a, Bone marrow-derived DC from cathepsin D-deficient mice were sorted for the expression of CD11c. The expression of CD11c before and after sorting is shown. Similar results were obtained with DC from wild-type littermates. 

b, DO11.10 T cells (5 × 10^5) were incubated in the presence of different concentrations of MPC6 and IL-2 (10 ng/ml) but no DC. After 24 h, the wells were pulsed with 1 μCi of [^3]H]thymidine for 16 h and then frozen until harvested, and thymidine incorporation was measured. Incorporation by T cells cultured in the absence of IL-2 was <1000 cpm. The proliferation in the presence of each concentration of inhibitor was compared with that in the absence of inhibitor using a one-way ANOVA with post hoc Dunnett’s modification. No significant differences were observed. 

c, Bone marrow-derived DC from BALB/c mice were incubated with OVA-specific DO11.10 T cells (5 × 10^5) and OVA (2 μM) or OVA peptide 323–339 (0.02 μM) or medium (✘). Pepstatin or MPC6 at various concentrations was added to some cultures as shown. After 24 h, the wells were pulsed with 1 μCi of [^3]H]thymidine for 16 h and then frozen until harvested, and thymidine incorporation was measured. Incorporation by T cells cultured in the absence of DC both in the presence and absence of Ag was <3000 cpm. One experiment of two is represented. 

d, Bone marrow-derived DC from wild-type or cathepsin D-deficient DC were incubated with OVA-specific DO11.10 T cells (5 × 10^5) and OVA (2 μM) or OVA peptide 323–339 (0.02 μM) or medium (✘). MPC6 at various concentrations was added to some cultures as shown. After 24 h, the wells were pulsed with 1 μCi of [^3]H]thymidine for 16 h and then frozen until harvested, and thymidine incorporation was measured. Incorporation by T cells cultured in the absence of DC both in the presence and absence of Ag was <3000 cpm. The proliferation in the presence of each concentration of inhibitor was compared with that in the absence of inhibitor using a one-way ANOVA with post hoc Dunnett’s modification. Groups differing significantly from control (p < 0.01) are shown with an asterisk. One experiment of three is represented. 

e, Percent inhibition data at different inhibitor concentrations (DC number 2000–3000) combined from results of six experiments, combining data on wild-type and cathepsin D-deficient mice. The solid line shows best-fit trend. Two-way ANOVA was used to analyze the inhibition in terms of both concentration and whether the cells were derived from wild-type (WT, ▲) or cathepsin D-deficient mice (knockout (KO), ◆). The data were highly significant in terms of concentration (p < 0.003) but showed no difference between knockout or wild-type groups (p > 0.8). 

f, Bone marrow DC from cathepsin D-deficient DC were incubated with OVA-specific DO11.10 T cells (5 × 10^5) and OVA (2 μM) or medium (first column only). MPC6 or mannose-BSA at various concentrations was added to cultures as shown. After 24 h, the wells were pulsed with 1 μCi of [^3]H]thymidine for 16 h and then frozen until harvested, and thymidine incorporation was measured. Incorporation by T cells cultured in the absence of Ag was <2000 cpm. The proliferation in the presence of each concentration of inhibitor was compared with that in the absence of inhibitor using a one-way ANOVA with post hoc Dunnett’s modification. Groups differing significantly from control (p < 0.01) are shown with an asterisk. One experiment of two is represented.
The inhibition of OVA and peptide presentation in wild-type and cathepsin D-deficient mice is shown in Fig. 6d. Processing of OVA was inhibited in a dose-dependent manner by MPC6 using DC derived from either cathepsin D-deficient or wild-type mice. In contrast, MPC6 had little or no effect on presentation of the OVA peptide (a 20% inhibition by the highest concentration of MPC6 was observed in wild-type DC in the experiment shown in Fig. 6d, compared with a >90% inhibition of OVA presentation at the same concentration). The data from six independent experiments using wild-type and cathepsin D-deficient mice are shown in Fig. 6e. Two-factor ANOVA analysis of these data showed that the degree of inhibition was dependent on inhibitor concentration (p < 0.003) but that there was no significant difference (p = 0.85) between the degree of inhibition observed using wild-type or cathepsin D-deficient mice. In contrast to MPC6, mannose-BSA had no ability to inhibit processing of OVA (Fig. 6f).

Discussion

The aspartic proteinase cathepsin E was originally implicated in Ag processing by murine B cells (8), but the role of this enzyme in DC has not previously been investigated, either in vivo or in vitro. Both cathepsin E message and protein were found in human DC, but were absent in monocytes (Fig. 2). The PCR and enzymological data are also consistent with the presence of cathepsin E in murine bone marrow-derived DC, although the lack of Ab reagents means we cannot rule out that cathepsin E is actually present in a small, contaminating non-DC population in this model. Expression of this enzyme is therefore associated temporally with the development of the “professional Ag-presenting” phenotype. In contrast to other components of the Ag-processing machinery including some proteases (21), the level of cathepsin E was not further regulated during maturation induced by LPS, although more extensive studies will be required to determine whether other signals of DC maturation have any effect.

The intracellular distribution of cathepsin E has remained rather mysterious. In contrast to cathepsin D (22) and the proteases previously implicated in Ag processing (e.g., cathepsins L and S and asparagine endopeptidase) (5, 7), cathepsin E is nonlysosomal (8), an observation confirmed here in DC (Fig. 3). A proportion of the enzyme (perhaps inactive precursor, although this has not been addressed in this study) is found in the ER, as described previously in fibroblasts transfected with cathepsin E (23). However, a proportion is also found in a peripheral reticular structure, which may correspond to a very early component of the endosomal pathway. Cathepsin E in this compartment appears to colocalize with both transferrin and class II MHC, although immunoelectron microscopy will be necessary to conclusively demonstrate this. The concentration of cathepsin E at the site of early endocytosis is particularly noticeable in DC adhered to fibronectin, which extend long dendrites, the tips of which demonstrate extensive membrane ruffling and are the site of avid pinocytosis. The localization of cathepsin E at a very early stage of endocytosis is consistent with enzymatic studies showing that, in contrast to cathepsin D, this protease retains activity near neutral pH (24), albeit with a somewhat different peptide selectivity (25). Although cathepsin E could not be detected in the classical late endosomal class II-loading compartment (26), we cannot rule out the possibility that some enzyme is transported into this compartment along with Ag during active endocytosis.

Previous analysis of the role of aspartic proteinases in Ag processing has been confounded by two problems, namely, the lack of solubility and thus bioavailability of the most widely used inhibitor pepstatin and the absence of an inhibitor which discriminates clearly between the two major cellular aspartic proteinases cathepsin D and E. To overcome its low solubility in water, pepstatin is routinely dissolved in DMSO, a solvent that is highly toxic to DC even at very low concentrations (our unpublished observations), adding to the difficulties of interpretation. The question of selectivity has previously been approached using a peptide inhibitor derived from the round worm Ascaris (27), which was reported to have little or no activity against cathepsin D compared with cathepsin E. However, studies with the naturally occurring inhibitor have been limited by difficulties of purification, and recent analysis has suggested that the recombinant inhibitor may have a slightly different inhibitory profile (28). Follow-up of the initial studies of cathepsin E’s role in Ag processing has therefore been limited (29).

An alternative to small molecular mass inhibitors is the use of homologous recombination to produce cathepsin-deficient mice. Cathepsin D-deficient mice (but not so far cathepsin E, despite attempts in two laboratories) have been generated and analyzed fairly extensively in terms of Ag processing (3, 30). No major defects in Ag processing in these mice has been detected, although the analysis is complicated by the fact that mice die by day 25 because of atrophy of the gastrointestinal tract. No study on DC from these mice has been previously reported.

In this study, we have attempted to identify a role for cathepsin E in Ag processing using a combination of genetic deficiency and novel inhibitors. We initially confirmed that DC cathepsin D-deficient mice can be obtained normally from bone marrow of mice harvested around days 20–23 of life. The cathepsin D-deficient DC retained only 10% of the levels of total aspartic proteinase (pepstatin-sensitive Hb-degrading) activity of their wild-type litter mates, suggesting that cathepsin E makes a minor contribution to the total acid proteinase activity of these cells. Remarkably, however, these DC grew normally, showed a normal phenotype and showed no defect in Ag processing activity (a slight increase in OVA processing was occasionally observed in the cathepsin D−/− mice). Cathepsin D is therefore not required for growth, differentiation, or processing/presentation activity of these cells, confirming previous studies which used total splenocytes as a source of APCs (3). Cathepsin D and cathepsin E are the only pepstatin-sensitive aspartic proteinases described in hemopoietic cells (the more recently described BACE enzymes (β-amylloid β-secretases) being pepstatin resistant), and analysis of the human genome sequence has not identified any further homologues of these enzymes (J. Kay, unpublished data). Delivery of pepstatin to cathepsin D deficient mice should therefore selectively block only cathepsin E, and therefore provide a specific tool for analysis of Ag processing requirement.

To overcome the poor bioavailability of pepstatin, a novel inhibitor was designed in which pepstatin was coupled to mannosylated BSA via a cleavable linker (details of this synthesis are being published elsewhere). Not only would the BSA mannosyl carrier provide solubility in culture medium, but the complex should be efficiently taken up by DC by virtue of their expression of the mannos receptors DEC 205 and the macrophage mannos receptor (31). It should also be noted that disulfide reduction has been shown to take place in endosomes via a number of pathways (32). This would result in the cleavage of the disulfide linkage of MPC6, releasing pepstatin from the mannos-BSA neoglycoconjugate into the endosome. Freeing the pepstatin from the mannos-BSA neoglycoconjugate is important for the activity of MPC6 within the cell, since analogues with a noncleavable linkage were not effective in inhibiting Ag processing (data not shown).
MPC6 has been shown here to retain potent activity against both cathepsins D and E (suggesting the carrier per se does not inhibit accessibility of pepstatin to the enzyme active site), along with greatly enhanced water solubility (>40 µM). Critically, MPC6 was at least 100-fold more potent than pepstatin in inhibiting aspartic proteinase activity in DC, presumably as a result of increased solubility and uptake. The concentration of MPC6 required in these whole cell assays was in the order of 1 µM, consistent with the known affinity of mannoside receptors for their ligands (31), suggesting that uptake is related to activity in this model.

MPC6 was therefore used to block aspartic proteinases in Ag-processing DC. In contrast to pepstatin, MPC6 showed a dose-dependent inhibition of processing of OVA, but had no effect on presentation of peptide, consistent with inhibition of the Ag-processing pathway. Although previous studies have used pepstatin to block Ag processing (8, 33), those studies did not use DC. The very high levels of MHC on DC, coupled with a very high efficiency of Ag presentation, may mean that partial inhibition of enzyme may have little effect on the overall level of presentation. Alternatively, pepstatin may fail to reach the right processing compartment in DC.

The inhibitor should target both cathepsin D and E in mice expressing both of these enzymes, but only cathepsin E in cathepsin D-deficient DC. Although slightly higher inhibition of one or the other strain was sometimes seen in individual experiments (e.g., Fig. 6d), analysis of many experiments showed that MPC6 was in fact equally active in blocking processing in DC from both strains of mice (Fig. 6d).

The absence of cathepsin D had no effect on processing by DC and conversely processing by wild-type and cathepsin D-deficient mice was equally inhibited by MPC6. These two pieces of data taken together strongly imply a role for cathepsin D in overall Ag-processing pathway will require further elucidation. Cathepsin E could be involved in the initial cleavage of the invariant chain, in which an unidentified aspartic proteinase has been implicated (33, 34). Although cathepsin E is absent from the late endosomal vesicles containing cathepsin S and in which the invariant chain processing has been thought to take place, it is possible that the invariant chain traffic transits via an early endosomal compartment in route for the class II-loading compartment (35, 36). Alternatively, or additionally, cathepsin E may play a role in early cleavage of Ags en route to the class II-loading pathway. Cathepsin E cleave OVA in vitro, generating the DO11.10 epitope. Both cathepsin E cleavage and Ag processing in vitro are greatly enhanced by mild protein denaturation (our unpublished observations), a process which could occur extracellularly following in vivo immunization. There is strong evidence that asparagine endopeptidase plays a similar role in initial Ag cleavage (6, 7), and the relationship of these two enzymes in a variety of Ag systems clearly merits further investigation.

In conclusion, our study is the first to document both the presence and a functional role for cathepsin E in DC. The equal sensitivity of Ag processing to inhibition of aspartic proteinase activity in the presence or absence of cathepsin D, taken together with the lack of any effect of cathepsin D deficiency alone on Ag processing, provide convincing support for an important role for cathepsin E in generation of the CD4 repertoire in response to foreign and self-proteins. Targeted inhibition of this enzyme in APCs may therefore provide an alternative approach to regulation of autoimmune disease.

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References


