Monocyte-Derived Dendritic Cells Exhibit Increased Levels of Lysosomal Proteolysis as Compared to Other Human Dendritic Cell Populations

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Abstract

Background: Fine control of lysosomal degradation for limited processing of internalized antigens is a hallmark of professional antigen presenting cells. Previous work in mice has shown that dendritic cells (DCs) contain lysosomes with remarkably low protease content. Combined with the ability to modulate lysosomal pH during phagocytosis and maturation, murine DCs enhance their production of class II MHC-peptide complexes for presentation to T cells.

Methodology/Principal Findings: In this study we extend these findings to human DCs and distinguish between different subtypes of DCs based on their ability to preserve internalized antigen. Whereas DCs derived in vitro from CD34+ hematopoietic progenitor cells or isolated from peripheral blood of healthy donors are protease poor, DCs derived in vitro from monocytes (MDDCs) are more similar to macrophages (MΦs) in protease content. Unlike other DCs, MDDCs also fail to reduce their intralysosomal pH in response to maturation stimuli. Indeed, functional characterization of lysosomal proteolysis indicates that MDDCs are comparable to MΦs in the rapid degradation of antigen while other human DC subtypes are attenuated in this capacity.

Conclusions/Significance: Human DCs are comparable to murine DCs in exhibiting a markedly reduced level of lysosomal proteolysis. However, as an important exception to this, human MDDCs stand apart from all other DCs by a heightened capacity for proteolysis that resembles that of MΦs. Thus, caution should be exercised when using human MDDCs as a model for DC function and cell biology.

Introduction

The role of macrophages (MΦs) in the acquisition and degradation of exogenous material is well established throughout the phylogeny of metazoans [1]. Yet in vertebrates such complete degradation is inconsistent with the production of peptides of sufficient length (13–17 amino acids) to bind class II MHC molecules for presentation to T cells [2,3]. Antigen processing requires limited degradation of proteins and preservation of cognate T cell epitopes [4]. It was recently demonstrated in mice that the most efficient antigen presenting cells, dendritic cells (DCs) and B cells, are distinguished from MΦ in their ability to greatly attenuate lysosomal degradation of internalized antigen [5,6]. This is mechanistically mediated through a fine control of lysosomal proteolytic activity that was previously unappreciated. Both DCs and B cells, in vitro and in vivo, exhibit a remarkably low level of lysosomal protease expression. DCs furthermore control degradation by modulation of lysosomal pH that attenuates proteolysis in the immature state and moderately increases the level of proteolysis with maturation [7]. Additionally, in the case of phagocytosed antigens it has been demonstrated that NOX2 contributes to an increase in the alkalinity of the phagolysosome, further limiting proteolysis [8,9].

Both mouse and human DCs found in vivo have been categorized into a number of subsets based on phenotypic and functional differences [10,11]. Moreover, several methods have been developed for deriving subsets of human DCs in vitro from precursor cells, most commonly from CD34+ hematopoietic precursors (CD34DCs) and monocytes (MDDCs). CD34DCs have the advantage of being derived from an early hematopoietic precursor (analogous to bone marrow-derived DCs (BMDCs) in mice), though the number of starting cells can be limiting. On the other hand, monocytes are an abundant cell type from which large numbers of MDDCs can be cultured, though they are more...
derived precursors which are already committed to the monocyte/MΦ lineage. In the study that follows we extend the initial investigations of lysosomal function in mouse DCs to both in vivo- and in vitro-derived DCs of human origin.

Results

MDDCs are distinguished from other DC subsets in having high lysosomal protease content

We first investigated the relative abundance of representative lysosomal proteases in human monocyte-derived MΦs, MDDCs, and CD34DCs. These cells were cultured as previously described [12,13] and cell-free extracts were prepared for immunoblot analysis of the proteases and γ-interferon-inducible lysosomal thiol reductase (GILT). Surprisingly we found that cathepsins (cat) B, D, L and S, asparaginyl endopeptidase (AEP), and GILT were in near equal abundance in MΦs and immature MDDCs, slightly less abundant in populations of mature MDDCs (produced by overnight treatment with LPS), with only trace amounts present in CD34DCs (Fig. 1A). Overexposure of the blots revealed that these enzymes were present in CD34DCs, though in markedly lower abundance (Fig. 1B).

To assess whether the differences in lysosomal protease expression could be accounted for at the transcriptional level, we performed quantitative RT-PCR on RNA samples from MΦs, MDDCs, and CD34DCs using primers for catB, catD, catL, catS, AEP, and GILT. The transcriptional profiles mostly segregated into two distinct groups: the MΦs and immature MDDCs with a high relative level of protease transcription and the immature and mature CD34DCs with a low level of transcription (Fig. 1C). Indeed, a general correlation between the abundance of protease transcripts and protein for these two groups was evident. The transcriptional profile for the mature MDDCs, however, was not proportional to the protein profile, as the level of transcription was closer to that of the CD34DCs, while the amount of protein present more closely matches the MΦs and immature MDDCs. The relative abundance of protease expression at the protein level in mature MDDCs likely reflects the fact that transcription of many genes is reduced following DC maturation but that lysosomal proteases are relatively long-lived.

Given the dramatic differences in protease expression between DCs derived in vivo from monocytes and from CD34+ hematopoietic progenitor cells, we assessed the protease expression profile of dendritic cells taken ex vivo from human blood. Cell-free extracts were prepared from myeloid DCs (MDCs) and plasmacytoid DCs (PDCs) that were purified from the blood of healthy donors as previously described [14]. Both MDCs and PDCs exhibited levels of protease expression that were very low, comparable to CD34DCs, and in marked contrast to MΦs (Fig. 1D).

Figure 1. MDDCs are abundant in lysosomal proteases compared to other DCs. (A) Immature (i-) and mature (m-) MDDCs are comparable to MΦs in protease protein abundance as assessed by immunoblot of cell lysates. By contrast, immature and mature CD34DCs exhibit remarkably lower expression levels of protease protein than either MΦs or MDDCs. (B) Overexposure of the blots from (A) reveals that the enzymes are present in CD34DCs, though in strikingly diminished amounts. (C) Quantitative RT-PCR shows that MΦs and MDDCs are also distinct in having a high quantity of transcripts for the enzymes compared to CD34DCs. Data are displayed as “fold-greater” than immature CD34DCs. (D) PDCs and MDCs taken ex vivo from healthy donors also display markedly low levels of lysosomal protease expression. γ-tubulin was used as loading control.

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Taken together these data confirm that DCs most commonly, but not always, contain a low level of lysosomal proteases. While human CD34DCs, PDCs and MDCs share the protease expression characteristics of murine BMDCs and DCs from mouse secondary lymphoid organs, human DCs derived from monocytes are distinguished by a protease expression profile similar to that of MΦs with whom they share a direct precursor (i.e., the monocyte).

MDDCs exhibit high levels of lysosomal proteolysis in vitro compared to CD34DCs

We next determined whether the observed differences in protease expression were reflected in the proteolytic capacity of MDDCs and CD34DCs. Initial results from in vitro degradation assays of OVA protein suggested that MDDCs hydrolyzed proteins at a level matching that of MΦs, while CD34DCs were attenuated in this capacity (Fig. 2A). Lysosomal proteolysis by these cells was quantitatively assessed using an in vitro kinetic degradation assay which demonstrated that immature MDDCs degraded the protein substrate at a rate equivalent to that of MΦs, while immature and mature CD34DCs exhibited a 17-fold and 28-fold lower level of degradation than MΦs, respectively (Fig. 2B). The mature MDDCs displayed an intermediate rate of degradation that was 2-fold less than MΦs. Thus the high level of protease expression in MDDCs was reflected in vitro by greater degradative capacity.

As described below, developmental upregulation of protease expression was evident in both MDDCs and MΦs derived in vitro from monocytes. Cell-free extracts were prepared from monocyte cultures at defined intervals as the cells differentiated into either MDDCs or MΦs. Using catB as a surrogate for the proteases, immunoblotting of these samples revealed that at an early time point of differentiation (day 2) the level of lysosomal protease expression was fairly low (Fig. 2C). CatB expression cumulatively increased in the MΦs on days 4 and 6. In MDDC cultures the level of protease expression on day 4 was roughly equivalent to day 6 MΦs. Maturation of the MDDCs and analysis of the cell extracts on day 6 demonstrated a decrease in protease expression. Again, the level of protease expression correlated with degradative capacity as measured by OVA degradation in vitro (Fig. 2D).

MDDCs and CD34DCs are comparable in lysosomal degradation of non-protein substrates

The initial investigation of lysosomal degradation in DCs of mice demonstrated that, in contrast to proteolysis, DCs were comparable to MΦs in lysosomal degradation of non-protein substrates [5]. Indeed, this finding is consistent with the observation that post-translational modifications of proteins only rarely contribute to the cognate T cell epitopes bound to class II MHC [15,16,17], perhaps because these modifications are removed in lysosomes. We therefore investigated whether the attenuated proteolytic capacity of human CD34DCs was due to an overall decrease in lysosomal hydrolytic activity or whether it was protease-specific. Cell-free extracts were prepared from MΦs, MDDCs, and CD34DCs and were tested against substrates specific for the activity of lysosomal acid phosphatase, β-glucuronidase, and γ-mannosidase. In contrast to the marked difference in protease activity between MDDCs and CD34DCs, these other lysosomal hydrolases were comparable in activity between the two DC subsets (Fig. 3A). Though the greatest difference in hydrolytic activity was seen between the mature CD34DCs and the immature MDDCs when assaying for β-glucuronidase activity, this difference was at most 5-fold, substantially less then the 28-fold difference in protease activity between these two DC types (Fig. 3B). The difference in lysosomal hydrolytic capacity between MDDCs and CD34DCs was therefore predominantly limited to proteolysis, analogous to our previous findings using bone marrow-derived mouse DCs vs. mouse macrophages [3].
Exogenous antigen is rapidly degraded by MDDCs and preserved by CD34DCs

To determine whether antigen was degraded in intact MDDCs and CD34DCs as well as in cell-free preparations, we developed an assay for assessing protein degradation in live DCs. MDDCs and CD34DCs were pulsed for 2 hrs with immune complexes of HRP and polyclonal anti-HRP antibodies, washed, and then returned to culture for 24–48 hr in the presence or absence of a maturation stimulus. After these incubations, the cell-associated HRP activity was determined using a kinetic assay. Intriguingly, while the immature MDDCs displayed an expected loss of HRP activity due to lysosomal degradation, the MDDCs that were matured showed only a modest level of HRP degradation (Fig. 4A and 4B). This was likely due to the decrease in lysosomal protease content as well as an increase in lysosomal pH (see below) that occurred during MDDC maturation. Conversely, even 48 hours after loading the immature CD34DCs, the internalized HRP displayed a minimal amount of degradation. Maturation of the CD34DCs resulted in an increase in lysosomal degradation, presumably reflecting a decrease in lysosomal pH that accompanies maturation (see below).

In an independent set of experiments we measured lysosomal pH of MDDCs and CD34DCs. These studies revealed that lysosomal acidification in CD34DCs was regulated in response to maturation stimuli, as found previously for mouse bone marrow-derived DCs (Fig. 4C). In the immature state, the lysosomes of human CD34DCs exhibited an elevated pH (~5.6). Given the strict acid requirement for lysosomal proteolytic activity, such an elevated lysosomal pH would result in a significant reduction in the activity of the proteases present. Following LPS-induced maturation of these cells, lysosomal pH dropped closer to the pH optimum of most lysosomal hydrolases (~4.5) therefore providing an environment more conducive to proteolysis. MDDCs exhibited a low lysosomal pH in the immature state (~4.5), similar to that found in macrophages [pH 4.7–4.8 [18]] and most other cells.

Two general conclusions can be inferred from this set of data. First, as in the case of murine DCs, human DCs also exhibit a markedly reduced capacity for antigen proteolysis. Second, there is an important exception to this conclusion. MDDCs, the widely used model for DCs derived directly from monocytes, are indeed far more reminiscent of macrophages with respect to their capacity for lysosomal proteolysis than they are similar to DCs, either human or mouse, conventional or plasmacytoid.

Discussion

DCs were originally identified by their remarkable capacity to stimulate antigen-specific T cell proliferation [19,20,21]. Investigation into the mechanisms underlying this capacity revealed that these cells utilize a number of cell biological specializations to achieve this end [4,22]. In addition to the phenotypic changes that occur with DC maturation and the tight regulation of MHC expression and distribution, recent work has shown that these cells are acutely distinguished from other myeloid leukocytes by specializations in antigen handling and processing within lysosomes [23,24].

Consistent with the discovery of restricted lysosomal proteolysis in DCs of mice, lysosomes of human DCs taken from blood or derived from hematopoietic progenitors harbor a protease poor, antigen-preserving environment. The combination of low protease content and attenuated lysosomal pH in immature CD34DCs leads to limited degradation of internalized antigen. Concomitant with maturation, lysosomal pH drops and degradation increases. Thus, in addition to well-established mechanisms for antigen acquisition and T cell stimulation, human DCs also utilize mechanisms for antigen preservation that are similar to those of mice.

DCs derived in vitro from human monocytes are set apart from other DCs by resembling MΦs in lysosomal degradative capacity.
The details of DC ontogeny are under active investigation and the current data indicate that in steady state conditions dendritic cells and monocytes arise from a common precursor cell, while under inflammatory conditions dendritic cells differentiate directly from newly immigrated monocytes [25,26,27]. Our data suggest that monocytes have already engaged a developmental program that...
gives rise to cells with high protease content and that as monocytes differentiate into DCs they acquire many of the characteristic phenotypic traits of DCs while also developing MΦ-like lysosomes. One can distinguish between different subsets of DCs based on functional and phenotypic variation [25] and the presence of highly degradative lysosomes in MDDCs points to a cell biological specialization that separates this subset from other DCs. Regardless of subset, DCs are collectively set apart from other cell types by an exquisite capacity for antigen acquisition and T cell stimulation. In this regard the tremendous rate of macrophagocytosis by MDDCs coupled with very high expression of class II MHC may partly account for their ability to rescue some peptides for presentation to T cells despite the very proteolytic nature of their lysosomes [12]. Additionally, in vivo these cells are found predominantly at active inflammatory sites and may be particularly well suited for the acquisition, processing, and presentation of bulky particulate and microbial antigens more so than soluble proteinaceous antigens. This contrasts with other DC subsets which, while exhibiting a similar capacity for degradation of non-proteinaceous material, would easily preserve T cell epitopes from either a particulate or soluble source. Indeed, our previous studies using murine DCs demonstrate that they have a reduced capacity for the degradation of yeast as compared to murine macrophages [7].

As highly degradative cells, MΦs have a clear function in innate immunity, in wound healing, and in the effector arm of adaptive immunity where they participate in antigen clearance and in microbial killing and digestion. Native immunologically relevant antigens consist of biological macromolecules that must be degraded prior to presentation to T cells. *Prima facie* it is counterintuitive that the antigen presenting cells best equipped to stimulate T cells are poorly degradative, yet this underscores that *partial* degradation of antigens is an unequivocal requirement for the production of cognate T cell epitopes [23]. Indeed, though degradative cells have an ancient role in wound healing and innate immunity, the onset of adaptive immunity drove the need for a specialized cell type capable of preserving small peptides in the context of otherwise degradative lysosomes [28,29].

**Materials and Methods**

**Antibodies**

The following antibodies were used for western blotting: mouse anti-human CatB (Serotec), rabbit anti-human CatD (Dako), goat anti-human CatL (Santa Cruz Biotechnology), rabbit anti-human CatS (CalBioChem), sheep anti-human AEP (R&D Systems), rabbit anti-human GILT (a kind gift of P. Cresswell, Yale University), and mouse anti-human γ-tubulin (Sigma). The anti-human monoclonal antibodies used for flow cytometry were as follows: anti-CD1a, -CD11c, -CD86, -CD123, -HLA-DR, and Lin1 (Lineage cocktail 1, a cocktail of antibodies directed against CD3, CD14, CD16, CD19, CD20, and CD56) (BD Biosciences).

**Cell Isolation and Culture**

Human monocytes were isolated from buffy coats of healthy donors (New York Blood Bank) using RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies) according to the manufacturer’s protocol. For MDDC cultures, monocytes were grown at 1 × 10^6 cells/mL in 10 cm bacteriological-grade petri dishes (BD Biosciences) in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 20 μg/mL gentamicin (Gibco/Invitrogen), and 25 ng/mL GM-CSF (Peprotech) at 37 °C. Immature MDDCs were harvested at day 5. For mature cells, maturation was induced on day 5 by adding 100 ng/mL lipopolysaccharide (LPS, Sigma) or DH5α bacteria (Stratagene) and allowing maturation to proceed for 24 hours.

For monocyte-derived MΦ cultures, monocytes were grown at 1 × 10^6 cells/mL in 10 cm bacteriological-grade petri dishes (BD Biosciences) in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 20 μg/mL gentamicin (Gibco/Invitrogen), and 25 ng/mL GM-CSF (Peprotech) at 37°C. MΦs were harvested on day 7.

CD34DCs were derived from CD34+ hematopoietic progenitor cells as previously described [13]. Briefly, purified CD34+ cells (generously provided by D. Krause, Yale University) were cultured at a density of 4 × 10^4 cells/mL in X-VIVO 10 medium (Cambrex) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco/Invitrogen), 100 ng/mL GM-CSF (Leukine (sargramostim), Bayer HealthCare Pharmaceuticals), 20 ng/mL SCF, 2.5 ng/mL TNFα, 0.5 ng/mL TGFβ1, and 100 ng/mL Flt3L (Peprotech) at 37°C. After 7–10 days of culture the clustered cells were purified over a 7.5% BSA density cushion at 1xg for 30 minutes on ice. The pellets were retrieved and washed with cold PBS. The cells were then either taken as immature CD34DCs or were matured by culturing them in the growth medium supplemented with either 100 ng/mL LPS (Sigma) or DH5α bacteria (Stratagene) and allowing maturation to proceed for 48 hours.

Blood MDCs and PDCs were isolated as previously described [14]. Briefly, mononuclear cells were first isolated from buffy coats (New York Blood Bank) on a Ficoll-Paque gradient (GE Healthcare). The samples were enriched for DCs using a negative selection enrichment cocktail (EasySep Human Pan-DC Pre-enrichment Kit, StemCell Technologies) according to the manufacturer’s protocol. The cells were labeled with Lin1, anti-CD123, anti-HLA-DR, and anti-CD11c and were sorted by FACS where the DCs were separated according to their phenotype. MDCs were Lin1+, HLA-DR+, CD11c+, CD125+. PDCs were Lin1+, HLA-DR+, CD11c+, CD123+.

**Preparation of Cell Lysates and Immunoblotting**

As many lysosomal proteases are inactivated by alkaline or neutral conditions [30,31,32], the preparation of cell lysates was consistently performed in a slightly acidic buffer. The cell-free extracts were prepared in sucrose buffer (0.25 M sucrose, 20 mM HEPES, 2 mM EDTA (Sigma), pH 6.5) with 1% Triton X-100 (Sigma).

Gel electrophoresis and coomassie staining were performed according to standard protocols. Immunoblotting was performed with the indicated antibodies following SDS-PAGE and transfer to nitrocellulose filters (Schleicher and Schuell). All secondary antibodies used for western blotting were conjugated to HRP and the membranes were developed using an enzymatic chemiluminescence system (Pierce Biotechnology).

**Quantitative RT-PCR**

Total RNA was isolated using the RNeasy Kit (Qiagen) according to the manufacturer’s recommendations. Quantitative real-time RT-PCR was performed using the Quantitect SYBR Green One-Step RT-PCR Kit (Qiagen) and detected with the Mx3000P QPCR system (Stratagene). The data were normalized to the level of GAPDH expression in each individual sample. The ratio of transcript abundance was calculated using the immature CD34DC values as a base unit equal to one, thus allowing for the display of data as “fold-greater” than the immature CD34DCs.
**Lysosomal pH Measurements**

Studies of lysosomal pH in intact cells were performed using an acidotropic probe that selectively partitions into the lysosomal compartments of living cells. The probe used (LysoSensor Yellow/Blue DND-160, Molecular Probes) consists of a dye with two distinct optimal pH sensitivities, which allows dual-emission measurements and ratiometric quantitation of lysosomal pH. The procedure used for lysosomal pH measurements was adapted from the Molecular Probes Handbook [33], previous work by Haugland and colleagues [34], and previous work by Poole and colleagues [19]. This approach required a minimum of $10^6$ cells in suspension. $2 \times 10^6$ cells were aliquoted out for use as a blank in later pH measurements. The staining medium containing 5 μM LysoSensor probe in 5 mL growth medium was allowed to equilibrate with the calibration samples, a standard curve was calculated by plotting the OD at all wavelengths. Using the data from the pH calibration and were each resuspended in Mes/HEPES buffer, pH 7.4. This variable pH buffer with one of the following levels of acidity: pH 4.0 to pH 7.4. Mes/HEPES buffer (50 mM HEPES, 50 mM NaCl, 30 mM Ammonium Acetate, 40 mM Sodium Azide (Sigma), pH 4.0) with 10−6 M to the aliquots used for pH calibration and were each resuspended in a Mes/HEPES buffer and facilitated the creation of a standard calibration. This allowed lysosomal pH to equilibrate with the sample above. All subsequent steps were done quickly and on ice.

A series of Mes/HEPES pH buffers were previously prepared by mixing Mes buffer (50 mM Mes, 50 mM NaCl, 30 mM Ammonium Acetate, 40 mM Sodium Azide (Sigma), pH 4.0) with HEPES buffer (50 mM HEPES, 50 mM NaCl, 30 mM Ammonium Acetate, 40 mM Sodium Azide (Sigma), pH 7.5) to achieve buffers of varying pH, ranging from pH 4.0 to pH 7.4.

Five of the LysoSensor-labeled aliquots were used for lysosomal pH calibration and were each resuspended in a Mes/HEPES buffer of the following levels of acidity: pH 4.0, pH 4.5, pH 5.0, pH 5.5, and pH 6.0. The remaining aliquots were resuspended in Mes/HEPES buffer, pH 7.4. This first resuspension in Mes/HEPES buffer was used as a wash and after centrifuging the aliquots, each was resuspended in 2 mL (1×10^6 cells/mL) of the corresponding Mes/HEPES buffer.

Two minutes prior to fluorescence measurements of the samples, nigericin and monensin (CalBioChem) were added to a final concentration of 10 μM to the aliquots used for pH calibration. This allowed lysosomal pH to equilibrate with the Mes/HEPES buffer and facilitated the creation of a standard curve correlating lysosomal pH with the magnitude of fluorescence emission.

Fluorescence intensity of all samples was measured with a fluorescence spectrophotometer (Perkin Elmer) at an excitation wavelength of 365 nm and emission wavelength of 450 nm.

**In vitro Protein Degradation Assays**

Protein degradation assays were developed in house to assess the proteolytic capacity of lysates from different cell types. Ovalbumin (OVA; CalBioChem) was used for degradation assays at a concentration of 0.5 mg/mL and cell lysates at a concentration of 1 mg/mL. Reactions were performed in degradation reaction buffer (0.1 M citrate, 1 mM EDTA, 2 mM DTT, 1% Triton X-100 (Sigma), pH 4.5) or control buffer (0.1 M Tris, 1 mM EDTA, 1% Triton X-100 (Sigma), pH 7.4) at 37°C for 60 minutes and were stopped with 0.4 M carbonate buffer (pH 9.0). Fluorescence intensity was measured on a fluorescence spectrophotometer with an excitation wavelength of 585 nm and emission wavelength of 617 nm.

**Other Acid Hydrolase Activity Assays**

The following hydrolysis substrates were used for acid phosphatase activity, 4-methylumbelliferyl-β-D-glucuronidase activity, and 4-methylumbelliferyl-α-D-mannopyranoside (Sigma) for α-mannosidase activity. Cell lysates were used at a concentration of 0.5 mg/mL in degradation reaction buffer (0.1 M citrate, 1 mM EDTA, 1% Triton X-100 (Sigma), pH 4.5) or control buffer (0.1 M Tris, 1 mM EDTA, 1% Triton X-100 (Sigma), pH 7.4). Reactions proceeded at 37°C for 60 minutes and were stopped with 0.4 M carbonate buffer (pH 9.0). Fluorescence intensity was measured on a fluorescence spectrophotometer (Molecular Devices) with an excitation wavelength of 365 nm and emission wavelength of 450 nm.

**Endocytosis of HRP Immune Complexes and HRP Activity Assays**

HRP immune complexes were formed by incubation of HRP or FITC-HRP (Roche Applied Science) with rabbit anti-HRP (Jackson ImmunoResearch Laboratories) at a mole:mole ratio of 1 mol HRP to 6.8 mol anti-HRP. DCs were pulsed with the 5 μg/mL HRP immune complexes for 2 hours, washed three times and chased for 30 minutes.

The immune complex-loaded cells were split into four different samples. The first sample was assessed for maturation markers by FACs and the second was used for an HRP activity assay as detailed below. The third and fourth samples were placed back into culture with either DC growth medium alone or DC growth medium plus a maturation stimulus (LPS or DH5α bacteria). After 24 hours (MDDCs) or 48 hours (CD34DCs) of incubation the samples were assessed for maturation markers by FACs and used for HRP-activity assay.

For measurement of HRP activity, DCs loaded with HRP-ICs were washed three times with PBS and dispensed (in 100 μL PBS) into microtiter plates (BD Biosciences). To each well 100 μL TMB substrate (3, 3′, 5, 5′-tetramethylbenzidine, Pierce Biotechnology) was added and OD(650) absorbance readings were acquired for each well at 10 second intervals using a plate-reading UV/Vis spectrophotometer (Molecular Devices).

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**Author Contributions**

Conceived and designed the experiments: NM IM. Performed the experiments: NM. Analyzed the data: NM IM. Wrote the paper: NM IM.
References

1. Hartenstein V (2006) Blood Cells and Blood Cell Development in the Animal Kingdom. Annu Rev Cell Dev Biol 22: 677–712.

2. Rudensky A, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA Jr (1991) Sequence analysis of peptides bound to MHC class II molecules. Nature 353: 622–627.

3. Brown JH, Jardelezyk TS, Gorga JC, Stern LJ, Urban RG, et al. (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364: 35–39.

4. Trombeta ES, Mollman I (2005) Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol 23: 975–1028.

5. Delamarre L, Couture R, Mellman I, Trombeta ES (2006) Enhancing immunogenecity by limiting susceptibility to lysosomal proteolysis. J Exp Med 203: 2049–2055.

6. Delamarre L, Pack M, Chang H, Mellman I, Trombeta ES (2005) Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. Science 307: 1630–1634.

7. Delamarre L, Pack M, Chang H, Mellman I, Trombeta ES (2006) Enhancing immunogenecity by limiting susceptibility to lysosomal proteolysis. J Exp Med 203: 2049–2055.

8. Tumors ES, Wasmóe C, Tumors ES, Tumors L, Tumors J, et al. (2007) Rab27a regulates phagosomal pH and NAPDH oxidase recruitment to dendritic cell phagosomes. Nat Cell Biol 9: 367–378.

9. Savina A, Tumors C, Tumors S, Tumors M, Tumors M, et al. (2006) NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. Cell 126: 205–218.

10. Shortman K, Naik SH (2007) Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 7: 19–30.

11. Villadangos JA, Schnorrer P (2007) Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol 7: 541–553.

12. Sallusto F, Lanzavecchia A (1994) Efficient presentation of soluble antigen by dendritic cells. J Immunol 152: 257–263.

13. Gatti E, Velleca MA, Biedermann BC, Ma W, Unternaehrer J, et al. (2000) Lysosomal proteolysis in antigen-presenting cells determines antigen fate. Science 290: 1400–1403.

14. Chicz RM, Urban RG, Vignali DA, Vignali DA, Vignali DA, et al. (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364: 35–39.

15. Trombeta ES, Mollman I, Trombeta ES (2005) Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol 23: 975–1028.

16. Williams ES, Wasmóe C, Tumors ES, Tumors L, Tumors J, et al. (2007) Rab27a regulates phagosomal pH and NAPDH oxidase recruitment to dendritic cell phagosomes. Nat Cell Biol 9: 367–378.

17. Savina A, Tumors C, Tumors S, Tumors M, Tumors M, et al. (2006) NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. Cell 126: 205–218.

18. Shortman K, Naik SH (2007) Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 7: 19–30.

19. Villadangos JA, Schnorrer P (2007) Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol 7: 541–553.

20. Sallusto F, Lanzavecchia A (1994) Efficient presentation of soluble antigen by dendritic cells. J Immunol 152: 257–263.

21. Tumors ES, Wasmóe C, Tumors ES, Tumors L, Tumors J, et al. (2007) Rab27a regulates phagosomal pH and NAPDH oxidase recruitment to dendritic cell phagosomes. Nat Cell Biol 9: 367–378.

22. Savina A, Tumors C, Tumors S, Tumors M, Tumors M, et al. (2006) NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. Cell 126: 205–218.

23. Shortman K, Naik SH (2007) Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 7: 19–30.

24. Williams ES, Wasmóe C, Tumors ES, Tumors L, Tumors J, et al. (2007) Rab27a regulates phagosomal pH and NAPDH oxidase recruitment to dendritic cell phagosomes. Nat Cell Biol 9: 367–378.

25. Shortman K, Naik SH (2007) Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 7: 19–30.

26. Auffray C, Sieweke M, Gennemann F, et al. (2006) Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol 26: 72–76.

27. Randolph G, Jakubzick C, Qu C (2008) Antigen presentation by monocytes and monocyte-derived cells. Curr Opin Immunol 20: 52–60.

28. Rumfelt LL, McKinney EC, Taylor E, Flajnik MF (2002) The development of primary and secondary lymphoid tissues in the nurse shark Ginglymostoma cirratum: B-cell zones precede dendritic cell immigration and T-cell zone formation during ontogeny of the spleen. Scand J Immunol 56: 130–148.

29. Lovy J, Wright GM, Speare DJ (2006) Morphological presentation of a dendritic-like cell within the gills of chinook salmon infected with Loma salmonae. Dev Comp Immunol 30: 259–263.

30. Turk B, Dolezal I, Turk V, Beeth JG (1993) Kinetics of the pH-induced inactivation of human cathepsin L. Biochemistry 32: 375–380.

31. Turk B, Dolezal I, Zervonik E, Turk D, Gubensek F (1994) Human cathepsin B is a metastable enzyme stabilized by specific ionic interactions associated with the active site. Biochemistry 33: 14800–14806.

32. Ali SY, Evans L, Stainthorpe E, Lack CH (1967) Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. J Exp Med 126: 307–316.

33. Haugland RP (2007) Handbook of Fluorescent Probes and Research Chemicals: Molecular Probes.

34. Di Pucchio T, Chatterjee B, Smed-Sorensen A, Clayton S, Palazzo A, et al. (2008) Lysosomal proteolysis in antigen-presenting cells determines antigen fate. Science 299: 203–208.

35. Rumfelt LL, McKinney EC, Taylor E, Flajnik MF (2002) The development of primary and secondary lymphoid tissues in the nurse shark Ginglymostoma cirratum: B-cell zones precede dendritic cell immigration and T-cell zone formation during ontogeny of the spleen. Scand J Immunol 56: 130–148.

36. Shortman K, Naik SH (2007) Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 7: 19–30.

37. Randolph G, Jakubzick C, Qu C (2008) Antigen presentation by monocytes and monocyte-derived cells. Curr Opin Immunol 20: 52–60.

38. Rumfelt LL, McKinney EC, Taylor E, Flajnik MF (2002) The development of primary and secondary lymphoid tissues in the nurse shark Ginglymostoma cirratum: B-cell zones precede dendritic cell immigration and T-cell zone formation during ontogeny of the spleen. Scand J Immunol 56: 130–148.

39. Lovy J, Wright GM, Speare DJ (2006) Morphological presentation of a dendritic-like cell within the gills of chinook salmon infected with Loma salmonae. Dev Comp Immunol 30: 259–263.

40. Turk B, Dolezal I, Turk V, Beeth JG (1993) Kinetics of the pH-induced inactivation of human cathepsin L. Biochemistry 32: 375–380.

41. Turk B, Dolezal I, Zervonik E, Turk D, Gubensek F (1994) Human cathepsin B is a metastable enzyme stabilized by specific ionic interactions associated with the active site. Biochemistry 33: 14800–14806.

42. Ali SY, Evans L, Stainthorpe E, Lack CH (1967) Characterization of cathepsins in cartilage. Biochem J 105: 549–557.

43. Haugland RP (2007) Handbook of Fluorescent Probes and Research Chemicals: Molecular Probes.

44. Diou Z, Chen CS, Zhang C, Klauberst DH, Haugland RP (1999) A novel acidotropic pH indicator and its potential application in labeling acidic organelles of live cells. Chem Biol 6: 411–418.

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