

Fcε Receptor I on Dendritic Cells Delivers IgE-Bound Multivalent Antigens into a Cathepsin S-Dependent Pathway of MHC Class II Presentation¹

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In this study, we elucidate the FcεRI-mediated Ag uptake and presentation mechanisms of dendritic cells (DC). We found that FcεRI-bound IgE, after polyvalent but not after monovalent ligation, is efficiently internalized into acidic, proteolytic compartments, degraded, and delivered into organelles containing MHC class II, HLA-DM, and lysosomal proteins. To follow the fate of the fragmented ligand, we sought to interfere with invariant chain (Ii) degradation, a process critical for peptide loading of nascent MHC class II molecules. We found DC to express cathepsin (Cat) S, a cysteine protease involved in Ii processing by B cells. Exposure of DC to a specific, active-site inhibitor of Cat S resulted in the loss of anti-Cat S immunoreactivity, led to the appearance of an N-terminal Ii remnant, and decreased the export of newly synthesized MHC class II to the DC surface. Furthermore, inactivation of Cat S as well as blockade of protein neosynthesis by cycloheximide strongly reduced IgE/FcεRI-mediated Ag presentation by DC. Thus, multimeric ligands of FcεRI, instead of being delivered into a recycling MHC class II pathway, are channeled efficiently into MIIC (MHC class II compartment)-like organelles of DC, in which Cat S-dependent Ii processing and peptide loading of newly synthesized MHC class II molecules occur. This IgE/FcεRI-dependent signaling pathway in DC may be a particularly effective route for immunization and a promising target for interfering with the early steps of allergen presentation. *The Journal of Immunology*, 1998, 161: 2731–2739.

Dendritic cells (DC)³ are specialized APC that can acquire exogenous proteins/pathogens at peripheral sites and migrate/recirculate to lymphatic organs, where they display Ag-derived peptides for T cell recognition (1). DC surface receptors for enhanced Ag uptake have been identified and, ideally, may promote the capacity of these cells to discriminate between dangerous non-self and harmless self (2). The mannose receptor- and FcγRII-mediated Ag uptake by cytokine-stimulated human monocyte-derived DC are the interiorization pathways best investigated to date (3, 4). The nature and functionality of Ag uptake receptors used by virgin human DC (i.e., DC that have not been exposed to robust activation stimuli) are less well characterized. Freshly isolated, virgin tissue DC are clearly inferior to monocyte-derived DC

in their nonselective fluid-phase (macro)pinocytotic capacity (5–7). They may, therefore, rely on the expression of selective Ag binding and uptake receptors to present the relevant determinants in quantities needed to ensure vigorous T cell responses. Among others, the high affinity IgE receptor (FcεRI) is a candidate pattern recognition and Ag uptake moiety expressed by DC of the skin (i.e., epidermal Langerhans cells (8, 9) and dermal DC (10)) and DC circulating in the peripheral blood (PB-DC) (11). In fact, we have found previously that targeting of allergen-IgE complexes to FcεRI-bearing PB-DC results in a much stronger allergen-specific T cell response than that elicited following DC exposure to allergen in the absence of IgE (11). To unravel the biologic role of this Ag presentation mechanism and, perhaps, to define the targets for possible interference strategies, we investigated the cellular and molecular mechanisms that allow FcεRI-bearing DC to specifically recognize, internalize, and present IgE-defined Ags.

Materials and Methods

Abs and reagents

Chimeric (human Fc/mouse Fab) IgE anti-4-hydroxy-3-nitrophenacetyl (cIgE, IgE anti-NP) was from Serotec (Oxford, U.K.). Biotinylation and PE labeling of cIgE (cIgE biot, cIgE PE) were performed according to standard protocols. Purified, monoclonal human myeloma IgG1, IgG2, IgG3, IgG4, IgA2, and polyclonal human serum IgG were from Chemicon (Temecula, CA). The mouse anti-FcεRIα mAb 15-1 has been described (8). mAbs used for immunodepletion experiments included anti-CD3, anti-CD11b, anti-CD16, anti-CD19, anti-CD34, and anti-CD56 (all IgG1; Immunotech, Marseille, France). Fab fragments of the anti-IgE mAb (IgG1, clone B3102E8; Southern Biotechnology, Birmingham, AL) were prepared using a kit (ImmunoPure IgG1 Fab and F(ab')₂ Preparation Kit; Pierce, Rockford, IL) following the manufacturer's recommendations. FITC-conjugated mouse mAbs used were clone CR3/43 recognizing the β-chain of HLA-DR, HLA-DP, and HLA-DQ (Boehringer Mannheim, Mannheim, Germany); clone L243 recognizing HLA-DRA/β, and anti-CD3 (Leu4; both from Becton Dickinson, Mountain View, CA), anti-CD11b (Immunotech), anti-CD20

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³ Abbreviations used in this paper: DC, dendritic cell; aa, amino acid; BCR, B cell receptor; biot, biotin; Cat, cathepsin; CHX, cycloheximide; cIgE, chimeric human Fc/mouse Fab immunoglobulin E anti-4-hydroxy-3-nitrophenacetyl; CLIP, class II-associated invariant peptide; Ii, invariant chain; LAMP, lysosome-associated membrane protein; LHVS, *N*-morpholinurea-leucine-homophenylalanine-vinylsulfone-phenyl; Ly⁻, lymphocyte-depleted; MFC, mean fluorescence channel; MIIC, major histocompatibility complex class II compartment; NP, 4-hydroxy-3-nitrophenacetyl; PB-DC, dendritic cells circulating in peripheral blood; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SA, streptavidin; TCC, T cell clone; TRITC, tetramethylrhodamine isothiocyanate.

(Leu16; Becton Dickinson), anti-CD54 (PharMingen, Hamburg, Germany), mAb VICY1 recognizing the N terminus of invariant chain (Ii, CD74; kindly provided by Dr. O Majdic, University of Vienna Medical School, Austria), anti-LAMP-1 (CD107a), and anti-LAMP-2 (CD107b; both from PharMingen). PE-conjugated mAbs included mAb LN-2 (directed against the Ii C terminus) and IT2.2 (anti-CD86; both from PharMingen). Streptavidin PE (SA-PE) and anti-HLA-DR PerCP were from Becton Dickinson. Affinity-purified rabbit F(ab')₂ anti-human IgE was purchased from Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (CLB; Amsterdam, The Netherlands). The generation of affinity-purified rabbit anti-cathepsin (Cat) S Abs has been previously reported (12). The rabbit anti-HLA-DM β (R.DMB/c) Abs were kindly provided by Dr. P. Cresswell (Yale University School of Medicine, New Haven, CT). Affinity-purified rhodamine (TRITC)-conjugated and unlabeled mouse F(ab')₂-specific goat F(ab')₂, goat IgG (H+L)-specific donkey F(ab')₂ TRITC, and FITC-labeled goat F(ab')₂ anti-rabbit IgG (H+L) were from Jackson ImmunoResearch (West Grove, PA). Control mAbs were FITC- or PE-labeled mouse IgG1, IgG2a, and IgG2b (Becton Dickinson). The Cat S inhibitor *N*-morpholinurea-leucine-homophenylalanine-vinylsulfone-phenyl (LHVS; generous gift of Dr. J. T. Palmer, Arris Pharmaceuticals, South San Francisco, CA) was dissolved at 10 mM in DMSO. Bafilomycin A1 and cycloheximide (CHX) were from Calbiochem (La Jolla, CA).

Cell preparations and lines

E⁻ mononuclear cells (E⁻ PBMC; 2–4 $\times 10^8$ cells per donor) from healthy individuals and birch pollen-allergic patients (as defined by clinical criteria, skin prick test, and radioallergosorbent test) were prepared by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density-gradient centrifugation of heparinized venous blood, followed by depletion of SRBC-binding (E⁺) T lymphocytes. Thereafter, residual T cells, monocytes/basophils, NK cells, B, and progenitor cells were labeled with anti-CD3, anti-CD11b, anti-CD16/CD56, anti-CD19, and anti-CD34 mAbs (2 μ g/ml each), respectively, allowed to bind anti-mouse IgG1-coated paramagnetic particles (MACS; Milteny Biotec, Bergisch-Gladbach, Germany) and, finally, were depleted using a magnet (MiniMACS; Milteny Biotec). Non-bead-bound cells were recovered by two sequential washing steps. This procedure typically yielded 2 to 4 $\times 10^6$ cells, 90 to 95% of which displayed an HLA-DR^{high}, CD3⁻, CD20⁻, and CD14^{-low} immunophenotype and, thus, fulfill the phenotypic criteria of PB-DC (11). The great majority of these cells bound monomeric cIgE and reacted with the anti-Fc ϵ RI α mAb 15-1. The remaining non-DC population consisted of T cells, B cells, Fc ϵ RI⁺ basophils (together <2% of the total cell population), and some as yet unidentified cells. In certain experiments, the anti-CD11b mAb was omitted from the mAb mixture to obtain a lymphocyte-depleted cell population (Ly⁻ PBMC) that contains not only HLA-DR^{high}CD11b⁻ DC, but also HLA-DR⁺CD11b⁺ monocytes and Fc ϵ RI⁺HLA-DR⁻CD11b⁺ basophils. T cells were procured from the E⁺ PBMC fraction by NH₄Cl-mediated lysis of SRBC and were purified by anti-HLA-DR-, anti-CD16-, anti-CD19-, anti-CD20-, anti-CD56-based immunomagnetic depletion.

Flow-cytometry analysis

To release in vivo bound IgE molecules, isolated cells were subjected to lactic acid treatment (13). Ly⁻ PBMC (5 $\times 10^5$ in 50 μ l) were stained at 4°C with biotinylated cIgE, IgG1–4, or IgA (each 10 μ g/ml), and exposed to anti-CD11b FITC and anti-HLA-DR PerCP (5 μ g/ml each). Cell surface-bound biotin groups were visualized by SA-PE (1 μ g/ml). Aliquots of cell samples were preincubated for 30 min with purified mAb 15-1 (50 μ g/ml) or human IgG (200 μ g/ml) before staining. In all other experiments, isolated acid-stripped cells were allowed to rest for 30 min at 37°C before their further processing. In one experimental protocol, cIgE biot-loaded Ly⁻ PBMC, either left untreated or exposed to anti-IgE Fab (10 μ g/ml, 30 min at 4°C), were dispensed in Ca²⁺/Mg²⁺-containing HBSS and cultured at 37°C in the presence of anti-IgE Fab or goat F(ab')₂ anti-mouse F(ab')₂ (10 μ g/ml each). After the indicated 37°C culture periods, samples were placed on ice until the final time point of each experiment was reached and, thereafter, exposed to anti-CD11b FITC, anti-HLA-DR PerCP, and SA-PE. For internalization studies, cIgE-PE-loaded Ly⁻ PBMC were cultured at 37°C in the presence or absence of IgE-specific rabbit F(ab')₂ (30 μ g/ml) and, after the indicated culture periods, were placed on ice until the last sample was harvested. An aliquot of each sample was subjected to acid elution of cell surface Fc ϵ RI-bound cIgE PE. Thereafter, all samples were exposed to anti-CD11b FITC and anti-HLA-DR PerCP mAbs and analyzed by flow cytometry. Purified PB-DC were either kept on ice or cultured at 37°C for 4 to 12 h in granulocyte-macrophage CSF (100 U/ml; Novartis, Basel, Switzerland)-supplemented, serum-free UltraCulture medium (BioWhittaker, Walkersville, MD), and then analyzed by immunostaining or Western blotting (see below). Intact and/or permeabilized

PB-DC were stained with anti-HLA-DR FITC, VICY1 FITC, LN-2 PE, anti-ICAM-1 FITC, and anti-B7-2 PE. Permeabilization of PB-DC was performed using a kit (Fix & Perm; An der Grub, Kaumberg, Austria), according to the manufacturer's recommendations. Cellular fluorescence and light scatter parameters were analyzed on a FACScan (Becton Dickinson). Within the Ly⁻ PBMC population, PB-DC were gated as HLA-DR^{high}CD11b⁻ cells, monocytes as HLA-DR⁺CD11b^{high}, and basophils as HLA-DR⁻CD11b⁺, as previously described (11). The specific reactivity of biotinylated cIgE or human Ig and of FITC- or PE-labeled mAbs with a given cell type was expressed as the Δ -mean fluorescence channel number (Δ -MFC; biotinylated cIgE/human Ig, MFC of the SA-PE reactivity in the presence of the biotinylated Ab minus the MFC in its absence; FITC/PE-labeled mAbs, MFC of the reactivity of the FITC/PE-labeled mAb minus MFC of the reactivity of an isotype- and fluorochrome-matched control mAb). The percentage of cIgE-PE internalized by HLA-DR^{high}CD11b⁻ PB-DC was calculated using the formula: specific PE reactivity of DC upon acid stripping divided by the specific PE reactivity of non-acid-exposed DC multiplied by 100. Statistical significances were determined using the paired Student's *t* test, and *p* values of <0.05 were considered to be statistically significant.

Confocal laser microscopy

A total of 1 to 2 $\times 10^5$ purified PB-DC was mounted onto individual fields of adhesion slides (Bio-Rad, Richmond, CA) and exposed sequentially to cIgE, mouse anti-IgE Fab, TRITC-conjugated goat F(ab')₂, anti-mouse F(ab')₂, and donkey F(ab')₂ anti-goat IgG (each 10 μ g/ml, 30 min at 4°C). Slides were then overlaid with Ca²⁺/Mg²⁺-containing HBSS and cultured in a humidified atmosphere at 37°C, 5% CO₂. After the indicated culture periods, cells were fixed in 3% paraformaldehyde (Fluka Chemie AG, Buchs, Switzerland)/Ca²⁺/Mg²⁺-free PBS, washed, and quenched by washing with 50 mM NH₄Cl/PBS. For immunofluorescence-based staining of cytoplasmic Ags, cells were permeabilized with 0.3% saponin (Sigma, St. Louis, MO)/PBS, washed, and preincubated with 10% mouse serum/10% goat serum for 30 min at 4°C. Thereafter, cells were exposed to FITC-labeled anti-HLA-DR (clone CR3/43, diluted 1/10), anti-LAMP-1/2 (diluted 1/5), or isotype-matched control mAbs (2 μ g/ml), or were incubated with rabbit anti-HLA-DM β (diluted 1/1000) or control rabbit serum for 30 min at 4°C. All Ab dilutions and the washing buffer were supplemented with 0.1% saponin. After two washings, the binding of rabbit Abs was detected by an incubation step with FITC-labeled goat F(ab')₂ anti-rabbit IgG (H+L) (10 μ g/ml). After rinsing with PBS/saponin and embedding in Fluoprep medium (BioMerieux, Marcy L'Etoile, France), slides were examined using a confocal laser-scanning microscope system (LSM 410; Zeiss, Oberkochen, Germany) fitted with lasers emitting light at 488 and 543 nm for the excitation of FITC and TRITC, respectively. Images corresponding to fluorescence signals originating from 0.5- μ m sections of individual cells were acquired. To demonstrate the relative positional distribution of FITC- and TRITC-labeled Ags, the images collected in the two channels were merged.

Western blot analysis

Native or cIgE/anti-IgE Fab-bound PB-DC were either kept on ice or cultured at 37°C in the presence of the indicated agents before their solubilization in Tris lysis buffer (20 mM, pH 8.2) containing 1% Nonidet P-40 (Sigma), 140 mM NaCl, 2 mM EDTA, 1 mM iodoacetamide, 1 mM PMSF, aprotinin, and leupeptin (both 10 μ g/ml; all from Sigma). Lysates were kept on ice for 30 min, reacted with SDS sample buffer, and analyzed by immunoblotting, as previously described (14). Briefly, aliquots of lysates were submitted to 7 to 15% SDS-PAGE and blotted onto Hybond-P nitrocellulose membranes (Amersham Life Science, Buckinghamshire, U.K.). Membranes were blocked with 10% dry milk/0.1% Tween-20 (Sigma)/PBS for at least 6 h. Thereafter, membranes were exposed to mAbs against the Ii N terminus (VICY1), CD45 (mAb MEM-28; kindly provided by Dr. V. Horejsi, Academy of Natural Sciences, Prague, Czech Republic), Cat D (Transduction Laboratories, Lexington, KY), or rabbit anti-Cat S Abs. The binding of peroxidase-conjugated rabbit anti-human IgE (Accurate Chemicals & Scientific, Westbury, NY), goat anti-mouse Ig (1:40,000), or goat anti-rabbit Ig (1:50,000; both from Bio-Rad) was detected using ECLplus developing solution (Amersham). Films were digitalized by high resolution scanning (S-12; UMAX Technologies, Fremont, CA), and integrated band densities were expressed as relative OD values using the VistaScan software package (UMAX Technologies).

Ag-specific T cell proliferation

Lactic acid-exposed, purified PB-DC from atopic individuals were used as stimulator cells and dispensed into 96-well round-bottom microtiter plates (1–2 $\times 10^4$ /well; Costar, Cambridge, MA). Autologous *Bet v* 1-specific T

cell clones (TCC) were established and characterized by techniques described previously (15). TCCs used in this study included GZ11 M (peptide specificity *Bet* v 1, amino acids (aa) 82–93) and GZ15R (aa 119–125); HR1217, HR2131, and HR2149 (aa 142–156); RR10/5 (aa 34–48), RR161/III (aa 76–90), and RR4R (aa 142–156); and HPR 17/II, HPR31/II, HPR 49/II, and HPR 77 (aa 142–156). Before the DC-TCC (5×10^4 cells/well) coculture, PB-DC were subjected to either of two experimental protocols. In the first experimental group, PB-DC were exposed for 2 h at 37°C to 5 nM LHVS or medium before their exposure to 1 μ g/ml NP-specific IgE and/or the indicated concentrations of NP-coupled recombinant birch pollen allergen *rBet* v 1 (Mayerhofer, Linz, Austria) (16). LHVS remained in the culture medium throughout the subsequent DC-TCC coculture period. In the second experimental group, DC were cultured in solvent- or CHX (5 μ g/ml)-conditioned medium for a total of 5 h at 37°C. After the initial 2-h incubation period, cultures were substituted with NP-specific IgE (1 μ g/ml final concentration). DC were allowed to bind IgE for 30 min and then exposed to *rBet* v 1-NP at the indicated final concentrations. After a 2.5-h Ag pulse, non-DC-bound culture additives were removed by several washings. DC-TCC cocultures and the appropriate controls were supplemented with stimulatory anti-CD28 mAbs (Leu28, 0.5 μ g/ml; Becton Dickinson) to correct for the CHX-induced inhibition of costimulatory molecule expression of DC. All proliferation assays were performed in granulocyte-macrophage CSF (100 U/ml)-supplemented serum-free UltraCulture medium. DC-TCC cocultures were incubated for 48 h before pulsing with 0.5 μ Ci [^{3} H]thymidine (Amersham) per well for additional 16 h. Incorporation of the radionucleotide was measured by liquid scintillation spectroscopy. Background counts of controls (DC, DC plus Ag, TCC, DC plus TCC, TCC plus Ag, or anti-CD28 mAbs) were always <500 cpm. The results are expressed as the mean cpm ($\pm$ SD) obtained in duplicate cultures.

Results

IgE is the isotype preferentially bound and ligation-dependently internalized by blood DC

In a first series of experiments, we investigated the Ig isotype- and the IgG subtype-binding capacity of PB-DC (HLA-DR^{high}CD11b⁺Ly⁺PBMC (11); referred to as DC throughout this work) and monocytes. As shown in Figure 1A, DC avidly bind monomeric IgE, but display no or only negligible affinity to the other isotypes/subtypes. The Fc ϵ RI dependency of IgE binding by DC was confirmed by the ability of a mouse anti-Fc ϵ RI α mAb (mAb 15-1) to prevent the IgE loading of DC. In contrast, monocytes bound IgG less efficiently than the other monomeric Ig isotypes/IgG subtypes tested (IgG3, IgG1 > IgG4 >> IgA, IgG2 > IgE, Fig. 1B). To study the fate of Fc ϵ RI-bound IgE, DC were monitored for surface-bound IgE upon exposure to monomeric (mouse anti-IgE Fab) and multimeric (anti-IgE Fab, followed by mouse F(ab')₂-specific goat F(ab')₂ ligands. Figure 1C shows that, at 37°C, cross-linking led to a rapid and almost complete disappearance of DC surface-bound IgE, whereas monomeric ligands of IgE hardly affected the amount of IgE at the plasma membrane. Basophils of the same donor and in the same experiment bound IgE 10-fold more efficiently than DC, but showed only modest down-regulation of this molecule upon polyvalent ligation (Fig. 1D). To determine whether the disappearance of surface-bound IgE moieties was due to preferential internalization or shedding of receptor-ligand complexes, DC were acid stripped and labeled with defined concentrations of IgE-PE. Following culture at 37°C in medium alone or in the presence of a multimeric ligand, they were again exposed to lactic acid to dissociate IgE-PE from surface-bound Fc ϵ RI. Figure 1E shows that in the presence, but not in the absence, of a multimeric ligand, the fluorescent moieties become rapidly resistant to acid elution, indicating that the receptor-ligand complexes had moved to an intracellular location. Multimerization of IgE had no effect on the amount of the total cell-associated PE fluorescence, reflecting the detectability of this dye irrespective of its membraneous or intracellular localization (Fig. 1E). Based on the ratio of lactic acid-resistant and total cell-associated PE fluorescence, we calculated the internalization efficacy

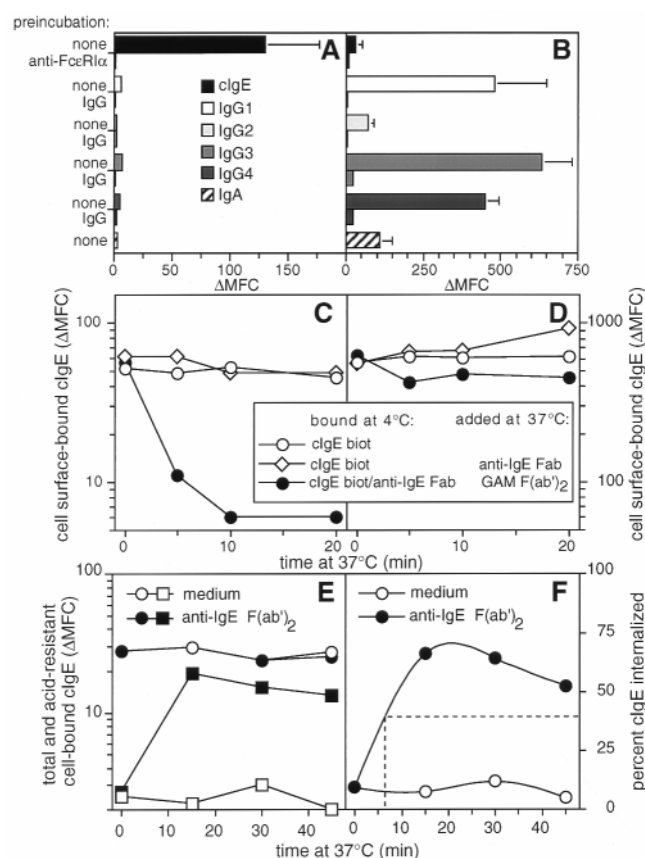
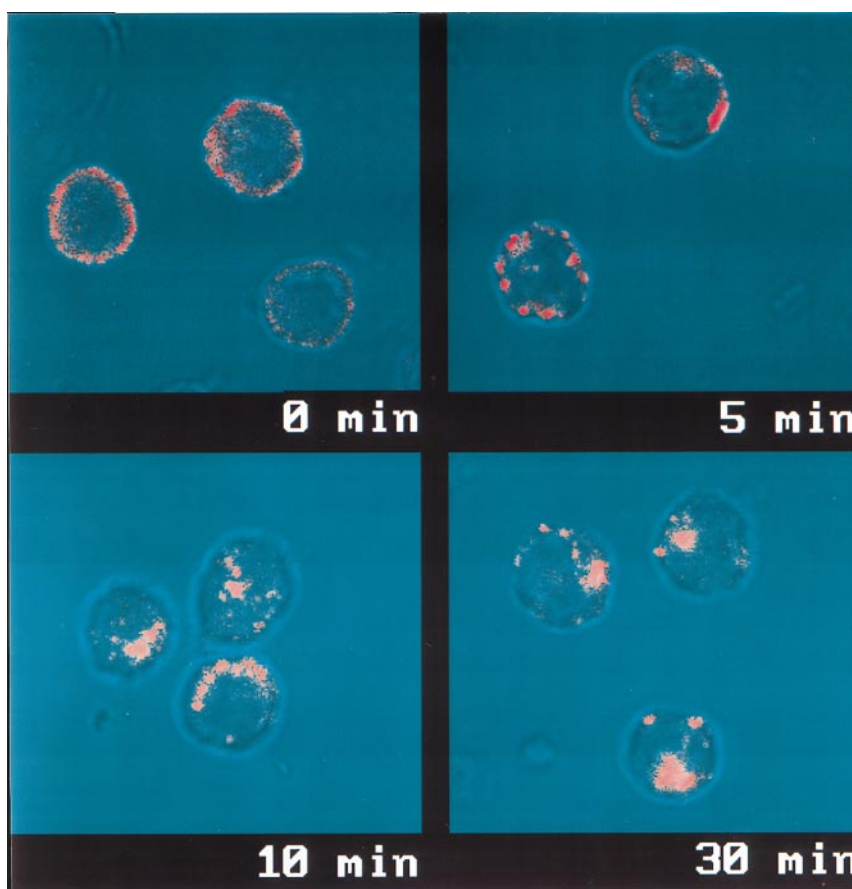


FIGURE 1. IgE is the isotype preferentially bound and ligation-dependently internalized by blood DC. A and B, IgE, but not other Ig isotypes or IgG subtypes bind to PB-DC. Ly⁺PBMC were exposed to biotinylated monomeric cIgE (solid bars), IgG1–4 (open and shaded bars), or IgA (hatched bars) with or without preexposure to an anti-Fc ϵ RI α mAb or purified human IgG. The specific SA-PE fluorescence of PB-DC (HLA-DR^{high}CD11b⁺; A) and monocytes (HLA-DR^{high}CD11b⁺; B) is given on the x-axis. C and D, Cross-linking-induced disappearance of surface Fc ϵ RI-bound IgE on DC. Ly⁺PBMC were exposed at 4°C to cIgE biot (open symbols) or to cIgE biot, followed by anti-IgE Fab (closed circles). Cells were then cultured for the indicated time periods at 37°C (x-axis) in the presence of medium alone (open circles), anti-IgE Fab (open diamonds), or goat F(ab')₂ anti-mouse F(ab')₂ (closed circles). DC (C) were gated as described above, and basophils (D) were identified as HLA-DR^{high}CD11b⁺ cells. The specific cell surface-bound SA-PE fluorescence is given on the y-axis. E and F, Multimerization of Fc ϵ RI-bound IgE results in rapid and efficient internalization of receptor-ligand complexes. Ly⁺PBMC were exposed to cIgE-PE and cultured for the indicated time periods at 37°C (x-axis) in the presence of medium alone (open squares and circles) or affinity-purified rabbit F(ab')₂ anti-human IgE (closed squares and circles). Aliquots of individual samples were subjected to lactic acid elution of surface-bound cIgE-PE (squares). The y-axes give the specific cIgE-PE reactivity (E) or the calculated percentage of internalized cIgE-PE (F) as a function of the elapsed time (x-axis).

and kinetics of Fc ϵ RI-bound IgE. As demonstrated in Figure 1F, multimerization of DC-bound IgE-IgE receptor complexes results in their effective (>70%) and rapid (6- to 7-min $t_{1/2}$ on the cell surface) internalization. Virtually no uptake was seen in the absence of the cross-linking agent (Fig. 1F).

In a next set of experiments, we used TRITC-labeled secondary Abs to follow the cytoplasmic route of IgE-IgE receptor complexes by confocal laser-scanning microscopy. These experiments revealed that IgE-Fc ϵ RI complexes on purified DC, while restricted to the cell surface when the cells were kept on ice, progressively accumulated in

FIGURE 2. IgE-Fc ϵ R1 complexes are efficiently internalized and progressively accumulate in distinctive cytosolic DC compartments. Purified DC were mounted onto slides and subjected to the TRITC-based IgE-labeling, Fc ϵ R1 cross-linking regimen, as described in *Materials and Methods*. Cells were either fixed immediately after the labeling procedure or incubated at 37°C for the indicated periods before fixation and analysis by confocal laser-scanning microscopy. Overlay exposures of TRITC fluorescence (red) and transmission light images are shown. No TRITC fluorescence could be measured when the IgE-loading step and/or the anti-IgE Fab incubation was/were omitted or when Fab fragments of an irrelevant mAb rather than of the anti-IgE reagent were used (not shown).



vesicular intracellular compartments after the DC had been shifted to 37°C (Fig. 2). We observed pronounced patching, capping, and partial internalization at 5 min and, thereafter, the continuous translocation of the complexes to certain distinctive cytosolic DC organelles that occur in a perinuclear location as well as adjacent to the cell membrane. Little or no fluorescent material remained on the cell surface.

Multimeric proteinaceous ligands of Fc ϵ R1 are subject to acidification-dependent degradation and selective targeting into lysosomal, MIIC-like compartments of DC

We then studied, by Western blotting, the fate of Fc ϵ R1 ligands upon internalization. Lysates of IgE-loaded DC that had been cultured in the presence or absence of an anti-IgE-directed cross-linking agent were blotted, and membranes were probed for anti-IgE reactivity. As shown in Figure 3A, the lysates of DC that had been consecutively reacted with cIgE/anti-IgE and, then, were exposed to goat anti-mouse F(ab')₂ progressively lost the anti-IgE-reactive 180-kDa band (representative experiment, $n = 5$). No decrease in the amount of intact IgE was seen in the absence of the cross-linking stimulus (Fig. 3A), and the 180-kDa anti-IgE reactivity was still discernible even after a 12-h culture period (not shown). When the amount of DC-bound IgE was adjusted to the total cellular content of CD45 at any given time point, it appeared that intact IgE (180 kDa) had a ~ 20 -min $t_{1/2}$ upon cross-linking (Fig. 3B). In two of five experiments, the fast disappearance kinetics was followed by a slower phase, during which anti-IgE-reactive moieties with m.w. characteristics of free IgE heavy chains emerged (Fig. 3, A and B). To investigate whether internalized IgE is subject to acidification-dependent proteolysis, DC were exposed to bafilomycin A1, a drug that affects lysosomal function by increasing the lysosomal pH (17). While this com-

pound did not interfere with the rate of Fc ϵ R1-mediated internalization of IgE (two independent experiments, data not shown), it did inhibit the loss of intact IgE and the appearance of IgE fragments (Fig. 3A). Altogether, these results demonstrate that Fc ϵ R1 on DC can efficiently and rapidly direct multimeric ligands into subcellular acidic compartments containing the machinery for protein degradation.

To identify the nature of the vesicles in which protein Ags or fragments thereof accumulate after their internalization via Fc ϵ R1, DC were subjected to immunofluorescence double-labeling procedures and analyzed by confocal laser microscopy. IgE/Fc ϵ R1 complexes on purified DC were cross-linked with secondary Abs conjugated to a pH-stable fluorescent dye (TRITC), washed, and cultured for 40 min at 37°C. Cells were then permeabilized and stained with Abs recognizing HLA-DR, HLA-DM β , or LAMP-1 (CD107a) and LAMP-2 (CD107b). As shown in Figure 4, the intracellular vesicles that are accessible for IgE/Fc ϵ R1 complexes in DC stained prominently for HLA-DR and HLA-DM β , and were, at least partly, positive for LAMP-2. These data demonstrate that Ags taken up via Fc ϵ R1 on DC are preferentially targeted to compartments that display the same antigenic profile as the lysosomal/late endosomal MIIC previously identified in immature DC and various other APC (18–21). In fact, li degradation and the loading of antigenic peptides onto class II molecules have been shown to take place in these organelles (21).

Expression and function of Cat S in dendritic cells

In view of the critical role of Cat S in li processing by EBV-transformed human B cells (22) and murine splenocytes (23), we searched for the presence of this cysteine protease in DC. Using affinity-purified rabbit anti-Cat S Abs and Western blotting, we

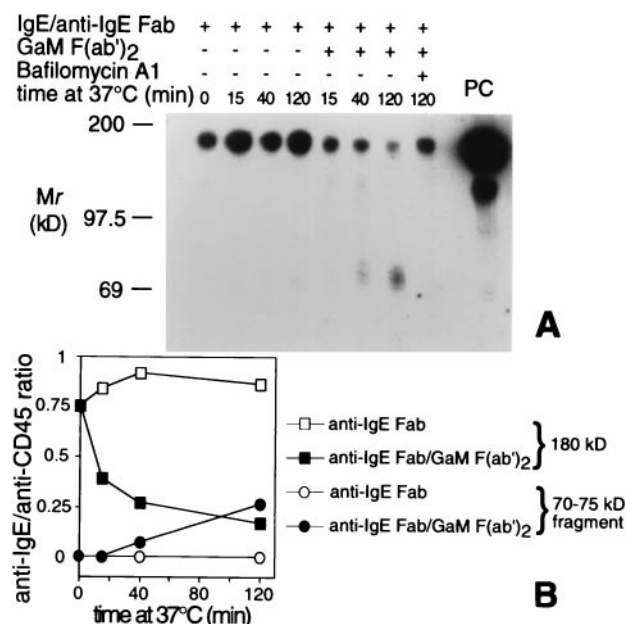


FIGURE 3. Acidification-dependent degradation of FcεRI-bound ligands upon internalization. *A*, Purified DC from three donors were pooled, loaded with cIgE/anti-IgE Fab, and cultured at 37°C for the indicated periods in the presence or absence of Bafilomycin A1 (1 μM) and/or goat F(ab')₂ anti-mouse F(ab')₂. Lysates corresponding to 0.5 × 10⁶ purified DC were subjected to anti-IgE (*A*) and anti-CD45 Western blotting (not shown). cIgE (250 ng) was applied as a positive control (PC). *B*, Ratios of integrated densities of the 180-kDa (squares) and the 70- to 75-kDa (circles) anti-IgE-reactive bands over the anti-CD45 reactivity were determined for any given time point. The ratios calculated for DC cultured in the presence (closed symbols) or absence (open symbols) of the IgE-directed cross-linking agent are given on the y-axis as a function of the elapsed time (x-axis). The positions of m.w. markers (in kDa) are indicated at the left margins of *A*.

detected anti-Cat S-reactive, 28-kDa moieties in lysates prepared from freshly isolated DC (Fig. 5A). Bands of identical and also different m.w. were identified in EBV-transformed human B cells (Fig. 5A). Short-term culture of DC in the presence of 5 nM LHVS, a specific and irreversible active-site inhibitor of Cat S (22–24), but not in solvent-conditioned medium only, resulted in a complete loss of the DC- and in a reduction of the B cell-expressed 28-kDa anti-Cat S reactivity (Fig. 5A). (Control experiments revealed a 43-kDa anti-Cat D immunoreactivity that was not sensitive to LHVS in either of the two cell types (data not shown).) The other anti-Cat S immunoreactive moieties of B cells were not reduced by LHVS treatment. The inhibitor-induced loss of Cat S is not due to toxic effects since culture in the presence of this compound affected neither DC viability (data not shown) nor the level of CD45 expression by these cells (Fig. 5A). The further observation that the exposure of DC lysates to even high concentrations of LHVS did not alter the level of anti-Cat S immunoreactivity (not shown) demonstrated that this inhibitor does not interfere with the immunodetection of Cat S. One may therefore assume that LHVS not only blocks the function, but also leads to the elimination of Cat S in viable DC.

To search for the effect(s) of Cat S on the trafficking of MHC class II-related gene products at the cellular level, purified DC were exposed to either LHVS or solvent only and analyzed for cell surface and total cellular HLA-DR as well as cytoplasmic and surface Ii expression. As shown in Figure 5B, the elimination of Cat S activity resulted in a significantly decreased culture-induced HLA-DR surface expression by DC. This observation combined

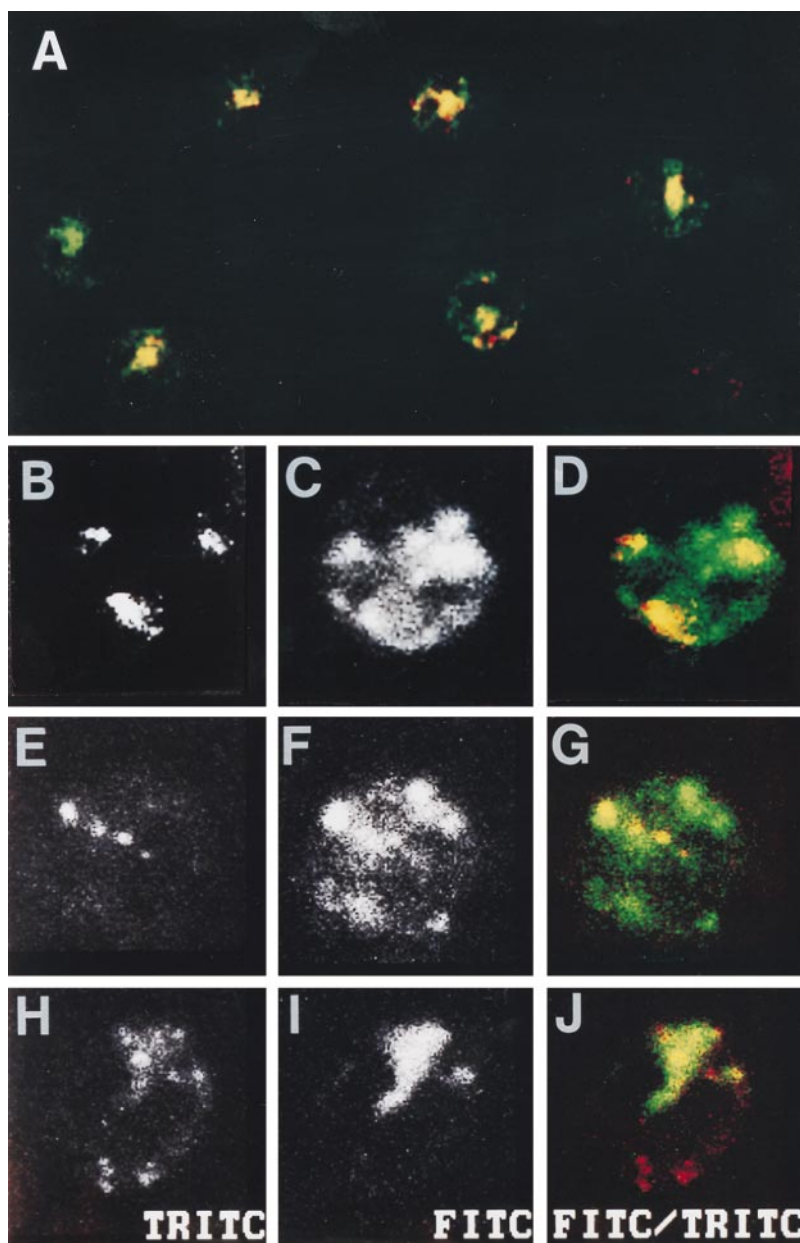
with the further finding that the total cellular content of HLA-DR was essentially unaffected by LHVS suggests that, in the absence of functionally intact Cat S, DC accumulate HLA class II molecules in an intracellular location. Since the inhibitory effect of CHX on HLA-DR synthesis could not be enhanced by the addition of LHVS, it appears that de novo synthesized rather than preexisting HLA class II molecules require Cat S-mediated, lysosomal Ii degradation for their efficient transport to the cell surface. In line with previous findings in human B cells (22), the functional inactivation of Cat S in DC led to the accumulation of intracellular, anti-N terminus-reactive Ii. These moieties apparently represent newly synthesized Ii, since the LHVS-induced effect was not observed in the presence of CHX. In contrast, the culture-induced surface expression of mAb LN-2 (anti-Ii C terminus)-reactive, intact Ii was not changed significantly by LHVS (Fig. 5B). It is not yet clear whether these LN-2-reactive Ii moieties are MHC class II αβ associated. In DC, such complexes have been shown to exist and to undergo Ii degradation after their internalization from the cell surface (25). In contrast to the profound effects on HLA-DR and Ii expression, the inhibition of Cat S activity did not impair the culture-induced up-regulation of the costimulatory molecules B7-2 and ICAM-1 (Fig. 5B).

The molecular nature of the LHVS-inducible anti-Ii-reactive moieties was investigated by Western blotting using a mAb directed against the N terminus of Ii. Purified PB-DC (and B cells) express the p33 (Fig. 5B) and, to a lesser extent, p41 Ii isoforms (visible after prolonged exposure time only; data not shown). DC culture in LHVS- but not in solvent-conditioned medium led to the appearance and pronounced accumulation of an N-terminal Ii breakdown intermediate of approximately 13 kDa both at early (4 h, data not shown) and later time points of the culture (12 h, Fig. 5C), while the steady state level of the Ii p33 was not influenced by LHVS. These findings demonstrate that Cat S is essential for complete Ii degradation by DC and, thus, perhaps for the proper Ii-dependent Ag presentation.

Essential role of Cat S-mediated Ii processing for conventional and receptor-mediated Ag presentation by DC

To explore the relative contribution of the Ii-dependent vs the Ii-independent pathway for FcεRI-mediated Ag presentation, we used NP-conjugated recombinant birch pollen allergen (rBet v 1-NP) as well as synthetic Bet v 1 peptides as model Ags, NP-specific IgE, and HLA-DR-restricted T cell clones (TCC) to assess peptide presentation by purified, autologous DC (11, 16). Bet v 1 peptides, which bind to cell surface-expressed HLA-DR and, conceivably, are presented in an Ii-independent manner, were added to DC-T cell cocultures with or without LHVS. As exemplified in Figure 6A, DC can present synthetic peptides in a largely Cat S-independent fashion (mean inhibition of peptide-induced TCC proliferation by LHVS: 4.1 ± 16.1%; seven independent experiments). Expectedly, IL-2-stimulated TCC proliferation was not affected by LHVS treatment. In striking contrast, functionally active Cat S was essential for the optimal presentation of epitopes generated from intact protein (Fig. 6B). When DC were exposed to high Ag concentrations (10 μg/ml), the blockade of Cat S activity resulted in proliferative TCC responses that were reduced drastically, albeit not entirely abrogated (mean inhibition: 63%, results obtained with six TCC from three donors recognizing five different Bet v 1 epitopes, i.e., aa 34–48, 76–90, 82–93 (Fig. 6B), 118–129, 142–156). This indicates that, in the presence of saturating Ag concentrations, DC can load MHC class II in a Cat S-dependent and, perhaps, also Cat S-independent fashion. Figure 6B also demonstrates that, at lower Ag concentrations (100 ng/ml), significant peptide-specific TCC responses occur selectively in the presence

FIGURE 4. FcεRI delivers ligands into MIIC-like vesicles of DC. Purified DC were mounted onto glass slides, and FcεRI-bound IgE was cross-linked with TRITC-labeled secondary Abs, as described in *Materials and Methods*. After 40 min at 37°C, DC were permeabilized and exposed to FITC-labeled mAbs recognizing HLA class II β -chain (A–D) or LAMP-2 (H–J), or to rabbit anti-HLA-DM β , followed by FITC-conjugated goat anti-rabbit IgG (E–G). The TRITC- and FITC-derived light emission of corresponding individual cells is depicted in B, E, H, and C, F, I, respectively. To demonstrate the relative positional distribution of the material internalized via FcεRI (TRITC, red) and the various FITC (green)-labeled Ags, the images collected in the two channels were merged (A, D, G, and J). The (sub-) cellular areas in which the TRITC and the FITC fluorescence are colocalized appear in yellow. As visualized, the intracellular vesicles that are accessible for IgE/FcεRI complexes stain for MHC class II Ags (A, D) and HLA-DM β (G), and are, in part, LAMP-2⁺ (J). Anti-LAMP-1 immunostaining revealed a similar reactivity and colocalization with TRITC-derived signals, as seen with anti-LAMP-2 immunostaining (not shown). No FITC signals were obtained when FITC-labeled, isotype-matched control mAbs were used instead of the anti-MHC class II or anti-LAMP-2 mAbs, or when the anti-HLA-DM β Abs were omitted or substituted for by control rabbit serum (not shown).



of NP-specific IgE. This IgE-dependent Ag presentation was almost entirely abolished (inhibition 80–90%) when the Cat S activity of DC was eliminated (Fig. 6B, one of three experiments with TCC recognizing *Bet* v 1 aa 82–93, 118–129, and 142–156). In Western blotting experiments, we addressed whether Cat S plays a major role in the proteolysis of r*Bet* v 1-NP taken up via FcεRI and found that the anti-*Bet* v 1-reactive material disappeared within a 4-h incubation period whether or not Cat S activity was inhibited (data not shown). This finding supports the notion that Cat S has no exclusive function during the earliest steps of exogenous protein degradation. However, the possibility cannot be ruled out that Cat S activity is of relevance for the liberation of certain peptides from partially digested Ags. Based on our observations that FcεRI-mediated Ag presentation depends on Cat S activity and that ongoing protein production was required for LHVS-induced Ii accumulation, we reasoned that FcεRI-dependent Ag delivery could result in the peptide loading of newly synthesized MHC class II. To explore this possibility, IgE-loaded and, for control purposes, nonloaded DC were Ag pulsed in the pres-

ence or absence of CHX. These experiments showed that FcεRI-dependent Ag presentation indeed critically depends on protein neosynthesis (Fig. 6C). Thus, uptake via FcεRI results in the sorting of Ags into DC compartments, where nascent class II molecules acquire peptides in a Cat S-dependent manner.

Discussion

Selective Ag targeting may profoundly affect the occurrence, the quantity, and, perhaps also, the quality of Ag-specific T cell responses. Ag uptake receptors known to date include lectin receptors, e.g., the macrophage mannose receptor (4, 26) and DEC-205 (27), as well as members of the Ig supergene family, i.e., the B cell receptor (BCR) (28, 29), FcγRI (30, 31), FcγRII (3, 31, 32), ILT3 (33), and FcεRI (11, 16). The possibility that some of these receptors can acquire a special function when expressed by DC is exemplified by our finding that DC- rather than basophil-expressed FcεRI can be efficiently internalized. This may be due to the fact

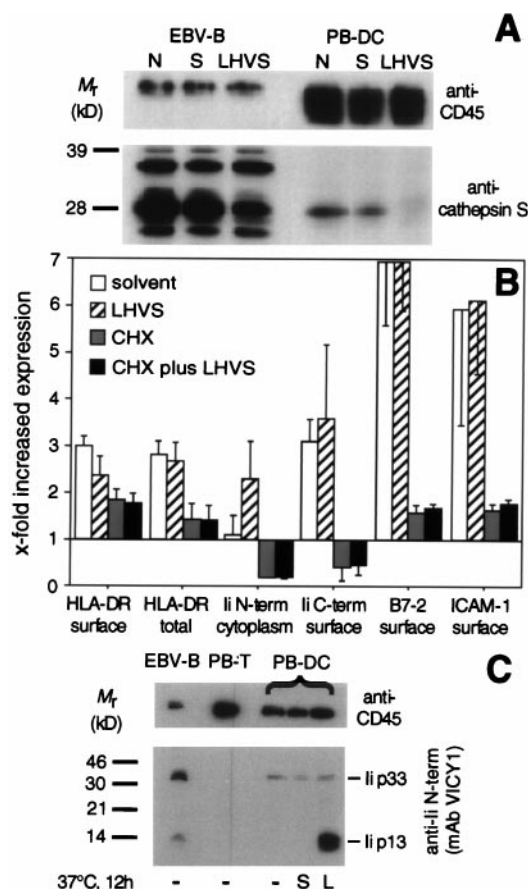


FIGURE 5. Expression and function of Cat S in DC. *A*, Expression of Cat S and LHVS-induced loss of anti-Cat S immunoreactivity in DC. Lysates of DC either noncultured (N) or cultured for 12 h in the presence of 5 nM LHVS (LHVS) or solvent (S) were subjected to anti-Cat S and anti-CD45 Western blotting. For control purposes, lysates of EBV-transformed B cells (EBV-B) that had been subjected to the same culture protocol were analyzed in parallel. Results are representative of four independent experiments. *B*, Inactivation of Cat S in DC impairs the export of HLA-DR complexes and leads to the accumulation of cytoplasmic Ii moieties. Purified DC were cultured for 12 h at 37°C in the presence of solvent (open bars), 5 nM LHVS (hatched bars), 10 μ g/ml CHX (grey bars), or CHX plus LHVS (closed bars). A fraction of the purified DC population was kept on ice for the determination of baseline Ag expression. Intact cells were analyzed for cell surface HLA-DR, Ii (C terminus), B7-2, and ICAM-1 expression. Permeabilized DC were used to measure the total cellular HLA-DR and Ii expression. Data are presented as the mean ($\pm$ SD) culture-induced change in Ag expression for each condition (LHVS vs solvent, $n = 4$; CHX vs LHVS plus CHX, $n = 3$) relative to the baseline value obtained with the DC, which were kept on ice. *C*, The blockade of Cat S activity results in the appearance and accumulation of a 13-kDa N-terminal Ii intermediate. Purified DC were either kept on ice (–) or cultured in the presence of LHVS (L) or solvent (S), lysed, and subjected to Western blotting using the mAb VICY1 recognizing the Ii N terminus or an anti-CD45 mAb. For control purposes, lysates of PB-T and EBV-B were analyzed in parallel. Results are representative of four independent experiments. The positions of m.w. markers (in kDa) are indicated at the left margins of *A* and *C*.

that DC express Fc ϵ RI complexes that differ in subunit composition from those encountered on basophils (11). We further show that DC display affinity for monomeric IgE, but are largely unable to bind monomeric IgA or IgG. Thus, Ags/pathogens against which IgE responses have been mounted may be preferentially retargeted to DC, whereas IgG-defined Ags may be predominantly trapped by monocytic cells and other cell types that display abun-

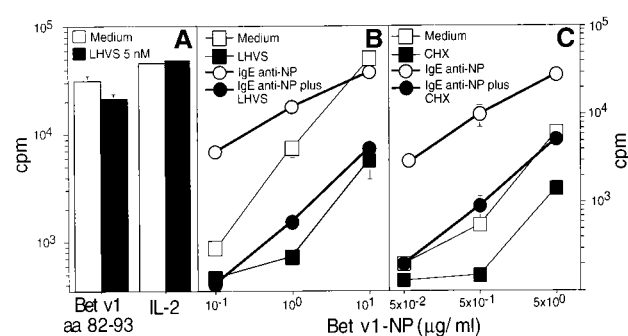


FIGURE 6. Fc ϵ RI/IgE-mediated Ag presentation by DC requires Cat S-dependent Ii processing and protein neosynthesis. *A* and *B*, Purified DC were precultured for 2 h in the presence (closed symbols) or absence (open symbols) of 5 nM LHVS (open symbols) before their exposure to synthetic peptides spanning aa 82–93 of Bet v 1 (0.2 μ g/ml), to IL-2 (20 U/ml) (*A*), or to the indicated concentrations of Bet v 1-NP (*B*) with (circles, solid lines) or without IgE anti-NP (squares, fine lines). Thereafter, autologous responder cells (TCC GZ11 M, specificity: aa 82–93 of Bet v 1) were added, and [³H]thymidine uptake was measured (y-axis: mean cpm $\pm$ SD). *C*, Purified DC were precultured in the presence (closed symbols) or absence (open symbols) of 5 μ g/ml CHX, exposed to IgE anti-NP (circles, solid lines) or medium only (squares, fine lines), and pulsed for 2.5 h with the indicated concentrations of Bet v 1-NP. After removal of non-DC-bound Ag, autologous Bet v 1 aa 142–156-specific TCC (clones HPR 77, HPR 49/2, and HPR31/2) cells were added and cultures were processed, as in *A*.

dant high and low affinity IgG receptors. Prolonged expression of Fc ϵ RI-bound monomeric IgE should enable DC to acquire and continuously display Ag-specific memory receptors and, thus, to efficiently interact with Ags/pathogens both within the circulation and, depending on their migratory properties, within the tissue.

We further demonstrate that the critical signal to internalize antigenic moieties in an IgE-dependent manner is conferred by the Ag's ability to cross-link Fc ϵ RI. Thus, the decision to take up and, consequently, to present Ags in an IgE-dependent fashion is made on the basis of whether or not Ags/pathogens carry repetitive and/or multiple different IgE epitopes. We have also found that Fc ϵ RI-mediated internalization results in the proteolysis of the ligand and the sorting of the resulting fragments into lysosome-related vesicles that display the same antigenic profile (HLA-DR⁺, HLA-DM⁺, LAMP⁺) as the MIICs previously identified in various types of APC (18–21). Fc ϵ RI expressed by DC is composed of an Fc ϵ RI α -chain linked to a pair of immunotyrosine activation motif-bearing Fc ϵ RI γ -chains (11). It, thus, has a structural design similar to that of the BCR complex containing the immunotyrosine activation motif-bearing molecules Ig α and Ig β . As opposed to the BCR, which apparently requires Ig α for being channeled to organelles, where peptide loading of nascent MHC class II occurs (34), the molecules directing Fc ϵ RI to these compartments have yet to be clarified. Conceivably, Fc ϵ RI γ and Ig α , but not Ig β , will associate with common but yet unknown adaptor molecules that allow their trafficking to MIICs and/or prevent their entry into recycling endosomes.

The cysteine protease Cat S critically contributes to Ii degradation to class II-associated invariant peptide (CLIP) and, thus, to the formation of peptide-bound MHC class II dimers in B cells (22). We have now demonstrated that human DC express Cat S and require this enzyme for optimal presentation of protein allergens taken up by fluid-phase and receptor-mediated endocytosis. Exposure of DC to the active-site Cat S inhibitor LHVS significantly decreased the surface export of newly synthesized HLA-DR and led to the intracellular accumulation of N-terminal Ii fragments with migratory properties similar to those of the LHVS-inducible

Ii remnants previously detected in human B cells (22). As a predicted Cat S cleavage site in Ii is located in closest proximity to the N terminus of CLIP, the 13-kDa Ii intermediate in DC most likely contains the abluminal localization/retention signal as well as the peptide competitor CLIP (22, 35–37). This LHSV-mediated effect in DC occurs at inhibitor concentrations that do not interfere with the expression and function of other cathepsins (22–24; data not shown). Another observation was that LHSV not merely inhibited Cat S function, but further led to a disappearance of DC-expressed anti-Cat S immunoreactivity. We have evidence that LHSV does not interfere with the detection of Cat S by polyclonal Abs and, therefore, assume that enhanced lysosomal degradation of and/or decreased autocatalytic activation of procathepsin S by LHSV-bound Cat S is/are responsible for the observed phenomenon.

In preliminary experiments, we have comparatively analyzed the effects of the multiple cysteine/serine protease inhibitor leupeptin and of the Cat S-specific compound LHSV, and found both substances to display a similar inhibitory potency for Ag presentation by PB-DC (data not shown). Together with the observations of nonaltered Ii degradation/Ag presentation by bone marrow-derived APC of Cat D^{-/-} (23) and Cat L^{-/-} mice (38), this finding suggests that Cat S, rather than other leupeptin-sensitive proteases, appears to be the most potent catalyst of complete Ii degradation in DC. Thus, the observation that protein-pulsed DC require Cat S activity for the elicitation of robust peptide-specific T cell responses is apparently attributable to a unique role of Cat S for Ii degradation to CLIP. However, it should be taken into account that, at least in murine B cells, delayed Ii degradation by leupeptin leads to the mistargeting of Ii remnant-associated MHC class II complexes to high density lysosomes, from where compact dimers move only slowly to the cell surface (39). Our demonstration of decreased MHC class II surface transport in PB-DC together with the observation of delayed, rather than prevented, appearance of newly synthesized MHC class II on monocyte-derived DC in the presence of LHSV (manuscript in preparation), suggests that Cat S inhibition also leads to an altered trafficking of MHC class II molecules in DC. Although to be tested, it is possible that part of the inhibitory activity of LHSV on Ag presentation by DC may be related to the preferential loading of nascent MHC class II with peptides that are not derived from exogenous proteins.

The requirement of Cat S is particularly pronounced under conditions when DC depend on FcεRI-mediated Ag uptake to mount a proliferative T cell response but, less so, when high Ag concentrations are applied. In this context, it is conceivable that DC express proteases other than Cat S, which allow for sufficient MHC class II loading only if peptides are generated in high copy numbers or, alternatively, that Cat S-independent Ag presentation via mature, recycling MHC class II αβ dimers operates particularly efficiently at high Ag concentrations (40–42). Another candidate Cat S-independent presentation mechanism relates to surface-targeted class II αβ_{li}. In DC, HLA-DR αβ_{li} can directly reach the plasma membrane, internalize into early endosomes, and rapidly recycle to the cell surface as compact dimer (25). While the specific protease requirement of this early endosomal pathway remains to be established, the observation of a stringent Cat S dependency for the presentation of FcεRI-targeted Ags strongly suggests that Cat S plays a major role for the maturation and peptide loading of, at least, those MHC class II molecules that access MIIC-like organelles of DC.

Peripheral blood DC, based on the differential anti-CD11c reactivity, contain two major subpopulations, both of which express and internalize FcεRI-bound IgE complexes (Refs. 11 and 43; unpublished observations). Counterparts of the CD11c⁻ and CD11c⁺ DC have been identified in T and B cell-rich areas of

lymphoid tissues, respectively (6, 7). It is feasible that PB-DC as well as epidermal Langerhans cells at peripheral sites bind and internalize Ag/pathogen in an FcεRI/IgE-dependent manner and, upon homing to lymphoid tissues, fulfill their T cell-priming capacity. The harsh proteolytic conditions operative in the FcεRI-dependent internalization pathway may allow the efficient liberation not only of previously recognized, but also of cryptic peptides of IgE-defined Ags, thus widening the repertoire of allergen/pathogen-specific T cells. This could be of particular importance for atopy since multiple Ags of the house dust mite, a major source of allergen in atopic dermatitis and asthma, have revealed to be proteases and, thus, are presumably resistant to rapid endosomal degradation (44). Similarly, a common biochemical feature of food allergens is their remarkable resistance to protease destruction (45). In a scenario of limited IL-12 production by APC, as has been demonstrated in atopy (46), FcεRI-dependent allergen degradation and presentation may amplify and widen allergen-specific Th2 cell responses (47) and even induce IgE-specific Th responses. These T cells may be able to provide help for any B cell that internalizes and presents IgE in a CD23-dependent manner as well as for IgE-specific B cells, resulting in the occurrence of anti-IgE-directed immune responses frequently observed in atopy (48).

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