# Extracellular antigen processing and presentation by immature dendritic cells

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**In antigen presentation to CD4+ T cells, proteins are degraded to peptide fragments and loaded onto class II MHC molecules in a process involving the peptide exchange factors H-2M (murine) or HLA-DM (human). In many antigen-presenting cells these processes occur in intracellular endosomal compartments, where peptides are generated and loaded onto class II MHC proteins for subsequent transport to the surface and presentation to T cells. Here, we provide evidence for an additional antigen-processing pathway in immature dendritic cells (DC). Immature DC express at the cell surface empty or peptide-receptive class II MHC molecules, as well as H-2M or HLA-DM. Secreted DC proteases act extracellularly to process intact proteins into antigenic peptides. Peptides produced by such activity are efficiently loaded onto cell surface class II MHC molecules. Together these elements comprise an unusual extracellular presentation pathway in which antigen processing and peptide loading can occur entirely outside of the cell.**

class II MHC | antigen presentation | HLA-DM | H-2M | invariant chain

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) with unique capacities to activate naive T cells and overcome T cell nonresponsiveness *in vivo* (1–4). Immature DC are found in peripheral tissues where they very efficiently capture and process antigens. After contact with protein antigen, DC migrate to secondary lymphoid organs and present antigen to T cells. The migration completes the DC maturation process, and mature DC have decreased endocytic activity and increased surface expression of class II MHC and costimulatory molecules (5–7). Because of the importance of DC in the *de novo* immune response to antigens and the potential of DC to serve as antigen carriers in vaccines, the mechanisms of antigen processing and presentation in DC are of considerable interest. DC are able to take up antigen through fluid-phase endocytosis (2), a pathway that is prominent in macrophages but inefficient in B cells (8). DC also use receptor-mediated uptake for internalization of antigens (2), a pathway active in both B cells and macrophages (8). DC endocytic receptors include the mannose receptor (9) and DEC-205 (10) for glycosylated proteins and Ig Fc receptors for antigen/antibody complexes (11). Both fluid-phase and receptor-mediated endocytic pathways rely on intracellular processing followed by loading onto class II MHC proteins subsequent to antigen internalization (8).

Recently, unique aspects of the class II MHC antigen presentation pathway in DC have been described. First, the majority of class II MHC-Ii complexes in DC traffic to the cell surface before internalization into endosomal loading compartments, as compared with a much smaller fraction in B cells (12). Second, murine Langerhans cells have been reported to express the peptide exchange protein H-2M at the cell surface (13). Finally, immature DC express abundant empty class II MHC molecules at the cell surface (39). In principle, these elements together could function to reconstitute a peptide loading compartment on the cell membrane, similar to those previously described within the cell as the MIIC or CIIV endosomal antigen generation and loading compartments (14, 15). In this report, we also demonstrate that DC secrete a proteolytic activity able to process intact proteins into peptide antigens. This activity, together with cell-surface H-2M/HLA-DM and empty class II MHC proteins, functions as an unusual extracellular antigen-processing pathway active in immature and intermediate DC, in which antigen processing and loading events can occur completely outside of the cell.

## Methods

**Cell Culture.** Bone marrow DC were established by *ex vivo* differentiation of Thy1.2-, B220-, and GR1-negative precursor cells from SJL/J (I-$A^s$) or B.10.Br (I-$E^k$, I-$A^k$) mice, as described (16). For immature DC, cells were used after 4 days in culture with 10 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF) in DMEM supplemented with 5% FBS, 2 mM glutamine, nonessential amino acids, 1 mM sodium pyruvate, and 20 mM Hepes buffer (5% complete DMEM). In some experiments DC were further matured by subculture in media without GM-CSF for 24–48 hr or by differentiation with 1 $\mu$g/ml lipolysaccharide (LPS) (Sigma) for 48 hr. Splenic B cells were prepared from splenocytes after adherence of macrophages to plastic by complement lysis of T cells with Thy 1.2 (clone 30-H12, PharMingen) plus rabbit complement (Cederlane Laboratories) and were used either freshly prepared or after treatment for 48 hr with 1 $\mu$g/ml LPS. Human DC were obtained by Ficoll gradient separation of peripheral blood followed by selective adherence to plastic of monocytes, which then were cultured for 5 days with 2.5 units/ml of human recombinant IL-4 (Boehringer Mannheim) and 50 units/ml of human recombinant GM-CSF (Boehringer Mannheim). Cells were used either after 5-day culture with IL-4 and GM-CSF or after further treatment with 1 $\mu$g/ml LPS for 48 hr. Human B cells were obtained by Ficoll gradient separation of peripheral blood mononuclear cells with negative selection of macrophages by adherence and negative selection of T cells by using a mouse mAb to CD3 (clone HIT3a, PharMingen). Casein-specific, I-$A^s$ restricted T cell lines were derived from lymph nodes of SJL/J mice immunized 12 days earlier with 200 $\mu$g of $\beta$-casein (Sigma) in complete Freund's adjuvant (Sigma) and were maintained in hybridoma serum-free media (GIBCO/BRL). T cell lines were stimulated with $\beta$-casein (50 $\mu$g/ml) and syngeneic-irradiated splenocytes every 2 weeks. The 3A9 hybridoma was a gift from E. Unanue (Washington University, St. Louis) and was cultured in 10% complete DMEM.

**Antibodies.** For characterization of murine DC by flow cytometry, the following mAbs were used: mouse mAbs Y3P (anti-$IA^{s,u,f}$), and KL-304 (anti-$IA^{s,k,u,f}$ $\beta$ 57–68), hamster mAb CD11c, and rat mAb DEC-205, all purified from supernatant of

Abbreviations: APC, antigen-presenting cells; DC, dendritic cells; GM-CSF, granulocyte–macrophage colony-stimulating factor; LPS, lipolysaccharide; HEL, hen egg lysozyme.

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hybridomas (obtained from American Type Culture Collection) by ammonium sulfate precipitation and protein A or protein G chromatography; hamster mAb anti-CD80, rat mAb anti-CD86 and anti-CD74, purchased from PharMingen; and rabbit serum raised against recombinant extracellular domains of H-2M, a gift from P. Reay (Oxford University). mAB Aw3.1, specific for the I-$A^k$ HEL48–62 complex (17) was a gift from E. Unanue and was prepared from hybridoma supernatant as above. In some experiments Fc$\gamma$ receptor binding was blocked by preincubation with 1 $\mu$g of rat mAb CD16/CD32 (PharMingen). For characterization of human DC, the following additional mAbs were used: L243 (anti-HLA-DR, from American Type Culture Collection), and G2 (anti HLA-DM extracellular domain, a gift from P. Cresswell, Yale, New Haven, CT), both purified from hybridoma supernatant as above, and anti-Ii (anti-CD74, PharMingen). Species and isotype-matched control mAbs were purchased from PharMingen.

**Flow Cytometry.** For flow cytometry, DC or B cells were incubated with saturating amounts of primary mAb on ice for 30 min in staining buffer (PBS containing 1 mg/ml BSA and 12 mM $NaN_3$, pH 7.2), washed, and then incubated with fluorescein- or phycoerythrin-conjugated $(Fab')_2$ secondary antibody (Jackson Immunoresearch), washed again, and analyzed immediately by using a FACScalibur flow cytometer (Becton Dickinson). For sorting with KL-304, cells were stained as described above and sorted by using a Vantage FACScalibur flow cytometer (Becton Dickinson).

**Confocal Microscopy.** DC derived from a 5-day bone marrow culture in GM-CSF were sorted by flow cytometry using the CD11c marker (PharMingen). Positively sorted cells were layered in 5% complete DMEM with 10 ng/ml GM-CSF over a polylysine-coated coverslip for 8–12 hr. After adherence the coverslips were washed twice in staining buffer, and the cells were fixed for 15 min in 1% paraformaldehyde, washed three times with PBS containing 10 mM glycine, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$, and were permeabilized with 0.5% saponin in staining buffer. Fixed cells on coverslips were incubated with 10 $\mu$g/ml of biotinylated Y3P for 30 min at room temperature. After three washes in staining buffer, streptavidin-labeled Texas red (Molecular Probes) was added at 1:1,000 dilution for 20 min at room temperature. FITC-labeled KL-304 then was added at 10 $\mu$g/ml for 30 min at room temperature. After three additional washes the coverslips were mounted on a glass microscope slide with mounting medium (Biomedia, Foster City, CA). Isotype matching control antibodies (IgG2b FITC and IgG2a biotin) were used under the same conditions.

**Proteolysis of Intact Antigens.** Cell-free supernatants were prepared from 4-hr cultures of washed splenic cells ($10^6$ cells/ml) in complete DMEM, with or without 5% FCS as indicated, by centrifugation at 4,000 $\times$ *g* for 12 min. Sodium azide (0.1%) was added to the cell-free supernatants, which were stored at 4°C. In some experiments, splenic DC supernatants were further fractionated by centrifugation at 100,000 $\times$ *g* for 60 min at 4°C. The high-speed pellet contained numerous 50- to 100-nm vesicular exosomes (18), and the high-speed supernatant contained soluble proteins. Protease activity was measured by using generic proteolytic substrates in a fluorescence assay (19). Bodipy-labeled casein, ovalbumin, and BSA were purchased from Molecular Probes, and Bodipy-labeled myelin basic protein was prepared as described (19). These reagents are heavily labeled with a fluorescent dye (3–10 Bodipy-FL per molecule), resulting in almost total quenching of the conjugate fluorescence. On proteolysis, Bodipy-labeled fluorescent peptides are released. Labeled proteins (5 $\mu$g/ml) were incubated at 22°C in 100 mM Tris·Cl, pH 8.0, with $10^6$ DC, B cells, or macrophages, or with 10 $\mu$l of the corresponding 4,000 $\times$ *g* supernatants prepared as described. The net increase in fluorescence was measured by using 485-nm excitation and 535-nm emission wavelengths in a spectrofluorimeter.

**Proteolysis of Labeled Peptides.** Using a variety of peptide substrates carrying C-terminal fluorescent reporter groups, we determined that the secreted DC protease(s) efficiently cleaved the 7-amino-4-methylcoumarin (AMC)-labeled peptide Z-Gly-Pro-Arg-AMC (Bachem). Enzyme assays were performed at 37°C in 200 mM Tris·Cl with 0.1 mM peptide and 25 $\mu$l of cell-free supernatant. Fluorescence measurements were made by using excitation and emission wavelengths of 380 and 460 nm, respectively. The fluorescence was measured in increments of 10 sec for 5 or more min, and the initial velocity was calculated for each sample. For evaluation of protease inhibitors, cell-free supernatants were preincubated before protease analysis for 30 min at 25°C with 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, 1 mM), loxastatin (20 $\mu$g/ml), E64 [trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, 5 $\mu$M], EDTA (5 mM), iodoacetamide (10 mM), leupeptin (50 $\mu$M), pepstatin (1 $\mu$M), PMSF (1 mM), *N*-tosyllysinechloromethylketone (100 $\mu$M), *N*-tosylphenylalaninechloromethylketone (100 $\mu$M), or $\alpha$2-macroglobulin, or by boiling the supernatant for 5 min before assay, or by changing the pH of the assay from 8.0 to 5.1 followed by assay as above.

**Generation of Immunodominant Antigens.** Cell-free conditioned culture supernatants from 4-hr cultures of splenic B cells or DC from SJL (I-$A^s$) or B10.BR (I-$A^k$) mice, or culture medium alone as control, were incubated overnight with 1 mg/ml intact $\beta$-casein or hen egg lysozyme (HEL), respectively. Various amounts of these mixtures then were applied to 1% paraformaldehyde-fixed APC ($5 \times 10^5$): either cells from a B cell lymphoma line (LS35.2), splenic B10.BR B cells, or bone marrow-derived B10.BR DC, for detection of HEL48–62; or splenic SJL/J B cells, for detection of casein antigens. Peptide binding was allowed to proceed for 2 hr at 37°C before T cell assay as described below. As a positive control, 10 $\mu$g of synthetic HEL48–62 peptide was applied instead of the digestion mixtures. In addition to the T cell assay, bound peptide also was detected by flow cytometry using the I-$A^k$-restricted, HEL48–62-specific mAb Aw3.1 (17).

**T Cell Proliferation.** T cells ($10^5$) from I-$A^k$-restricted, HEL48–62-specific T cell hybridoma 3A9 (20), or from an I-$A^s$-restricted, casein-specific T cell line (see above), were incubated with APC. T cell activation was measured by using either the IL-2-dependent CTLL line for 3A9 or a T cell proliferation assay for the $\beta$-casein T cell line. For cloroquine dependence, I-$A^s$ DC or B cells ($5 \times 10^5$) from SJL/J mice were incubated with various concentrations of intact $\beta$-casein for 6 hr in the presence or absence of 0.1 mM chloroquine, extensively washed, fixed with 1% paraformaldehyde, washed with PBS containing 10 mM glycine, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$, and incubated with $1 \times 10^5$ $\beta$-casein-specific T cells. After incubation at 37°C 5% $CO_2$ for 48 hr, [$^3$H]thymidine was added (1 $\mu$Ci per well), and incorporated radioactivity was determined.

## Results

**Characterization of Immature DC Sorted for Expression of Empty Class II MHC Molecule.** Bone marrow-derived murine DC can be prepared in different maturational states depending on the time of culture *in vitro* and the cytokines present (6, 7). DC from a 4-day culture with GM-CSF are in an immature state with high capacity to phagocytose antigens but with low antigen presentation function (5). Bone marrow cells from a 4-day culture with GM-CSF were sorted with the mAb KL-304 (21) (Fig. 1*A*). This

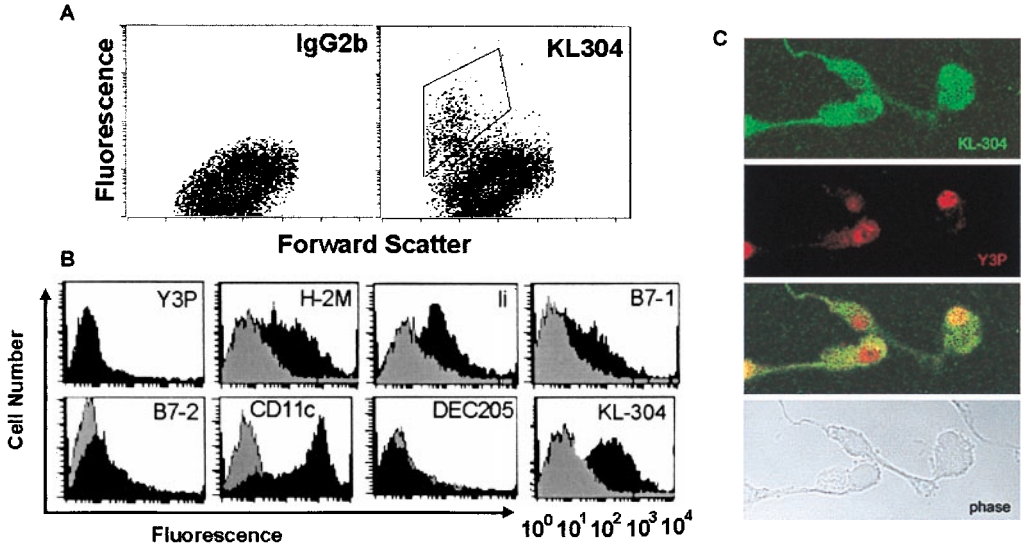


**Fig. 1.** KL-304-positive DC express surface markers consistent with immature DC. Bone marrow DC from SJL mice express peptide-receptive class II MHC molecules at the cell surface. (*A*) Staining with KL-304, specific for empty or peptide-receptive I-A$^s$, relative to an isotype-matched control antibody. (*B*) The KL-304-positive population sorted and analyzed for DC surface markers. (*C*) Confocal microscopy of DC doubly stained with FITC-labeled KL-304 and biotinylated Y3P/Texas red-labeled streptavidin. Shown are fluorescence images (optical magnification × 100) for KL-304, Y3P, costaining, and a phase-contrast image of the same field (top to bottom).

antibody recognizes the class II MHC protein I-A in its empty or peptide-receptive conformation (22) in four different stains of mice (H-2$^{s,k,f,u}$) (39). KL304$^+$ bone marrow cells presented cell surface markers typical of immature myeloid-derived DC, with very low expression of class II MHC/peptide complexes detected by Y3P (23), low expression of the costimulatory molecule B7–1, no detectable B7–2, high levels of CD11c, and no detectable DEC-205 (Fig. 1*B*). The same population expressed at the surface relatively high levels of KL-304 reactive empty class II MHC molecules, the class II-associated invariant chain (Ii, CD74), and the peptide exchange factor H-2M (Fig. 1*B*). In a separate experiment, DC sorted for CD11c also stained positively for KL-304 (Fig. 1*B*, last panel). Confocal microscopy with fixed and permeabilized DC revealed empty IA$^s$, as detected by KL-304, uniformly distributed at the cell surface, whereas Y3P-reactive, I-A$^s$/peptide complexes were found intracellularly, as reported (25), with localization clearly distinct from the KL-304-reactive material (Fig. 1*C*).

**Developmental Regulation of Ii, H2-M, and Empty Class II MHC Expression.** DC undergo several phenotypic changes as part of their developmental program, including changes in the relative amounts of surface and internal class II MHC/peptide complexes (25). Splenic DC were tested for developmental regulation of empty and peptide-loaded class II MHC and H-2M, by using mAbs recognizing the relevant extracellular domains. Murine splenic DC cultured in GM-CSF, which are in an immature/intermediate developmental state, expressed moderate levels of empty class II MHC and H-2M, and higher levels of class II MHC peptide complexes (Fig. 2*A*, open profiles). After treatment with LPS to induce DC maturation, essentially all of the cell- surface class II MHC molecules appear to be complexed to peptide, with essentially no detectable empty protein, and significantly reduced surface expression of H-2M (Fig. 2*A*, filled profiles). Thus, the components necessary for loading antigen onto class II MHC molecules, including peptide-receptive class II MHC molecules and H2-M, are expressed together at the DC surface in a developmentally regulated fashion. For B cells derived from the same initial population of splenocytes, no empty class II MHC protein and low levels of H-2M were detected. LPS treatment induced a doubling in B cell expression of class II MHC/peptide complex and a decrease of H2-M (Fig. 2).

Human DC also can be cultured *ex vivo* in various developmental states, by using human GM-CSF to induce differentiation of peripheral blood monocytes into immature DC and LPS to induce further DC maturation. Human DC were tested for cell-surface expression of HLA-DR, a human class II MHC molecule, and HLA-DM, the human homologue of H-2M.

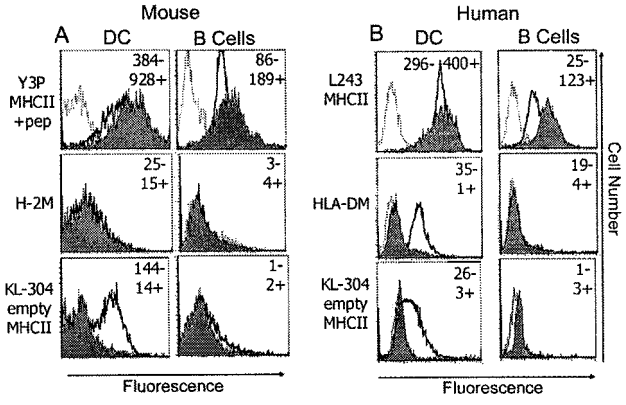


**Fig. 2.** Immature DC express molecules involved in antigen presentation. Distinct patterns of expression of molecules involved in antigen processing in DC and B cells. (*A*) Flow cytometry of immature DC (*Left*) and B cells (*Right*) derived from murine spleen, stained for expression of class II MHC/peptide complexes (Y3P), H2-M, and empty peptide-receptive class II MHC molecules (KL-304). Cells were cultured in the absence (open profiles) or presence of 1 $\mu$g/ml LPS (dark profiles) before staining. (*B*) Flow cytometry of immature DC derived from human peripheral blood monocytes (*Left*) and peripheral blood B cells (*Right*), stained for expression of total class II MHC HLA-DR (L243), HLA-DM, and peptide-receptive class II MHC molecules (KL-304), with LPS-induced maturation as above. The specificity of KL-304 for human HLA-DR alleles is not known, and staining was observed for one of five preparations tested. Staining intensity for isotype-matched control antibodies indicated by dotted profiles. Numbers indicate mean fluorescence intensity (−) before and (+) after LPS treatment.

KL-304 was used to detect empty class II MHC protein, making use of cross-reactivity of HLA-DR with I-A$^s$ because of similarity in the epitope region (21). mAb L243 was used to detect both peptide-bound and empty class II MHC protein. Like murine DC, immature human DC expressed empty class II MHC protein at the cell surface, along with HLA-DM (Fig. 2*B*, light profiles). On DC maturation, levels of empty class II MHC protein and HLA-DM decreased, and levels of HLA-DR increased (Fig. 2, dark profiles), as observed for murine DC. In B cells, no empty class II MHC protein was detected at the surface of immature or mature cells, with the level of total cell-surface class II MHC protein increasing on maturation, again as observed for murine B cells. Thus, human DC exhibited the same cell type and maturation state-dependent expression of cell surface empty class II MHC molecules and HLA-DM as observed for murine DC.

**DC-Associated Proteases.** Secreted as well as membrane-bound proteases from a variety of cells are essential for the host defense and are part of the responsiveness to the environment of many APC (2). DC themselves might express an extracellular proteolytic activity that could contribute to antigen processing, as suggested by the finding of proteolysis associated with DC-secreted exosomes that can contribute to MHC class I-mediated presentation (24). Extracellular proteolytic activity was tested in DC cultures by using casein, a generic proteolysis substrate, in a fluorescence assay that relies on relief of intermolecular quenching as fluorescent peptides are released from a highly labeled, internally quenched protein substrate (19). Both splenic and bone marrow-derived murine DC expressed substantial extracellular proteolytic activity, as compared with a very much lower level for resting macrophages, B cells, or T cells (Fig. 3*A*). The DC activity was associated with cell-free supernatants collected from 4-hr culture, as well as with intact cells treated with sodium azide (Fig. 3*B*). The soluble proteolytic activity was not caused by cell lysis, as similar levels were observed from untreated cultures that maintained full viability during the culture period, including freshly sorted viable CD11c$^+$ cells. The activity was not associated with the exosomal fraction (Fig. 3*B*), which as reported (18) consisted of numerous 50- to 100-nm unilamellar vesicles. In addition to casein, proteolysis of other protein substrates by conditioned cell-free DC supernatants was observed, including myelin basic protein, ovalbumin, and BSA (Fig. 3*C*). Using a conventional proteolysis assay based on hydrolysis of a short fluorescent peptide (CBZ-Pro-Gly-Arg-7-amino-4-methylcoumarin) that was efficiently cleaved by the supernatants (not shown), further characterizations of the proteolytic activity present in conditioned medium were performed, by using a variety of inhibitors. The activity was completely blocked by boiling, by low pH or by the serine protease inhibitors PMSF and 4-(2-aminoethyl)benzenesulfonyl fluoride, but not by inhibitors of cysteine proteases, metalloproteases, and acid proteases, or by other inhibitors of serine and cysteine proteases such as leupeptin, *N*-tosyllysinechloromethylketone, *N*-tosylphenylalaninechloromethylketone, or $\alpha$2-macroglobulin (Fig. 3*D*).

**Generation of Immunodominant Peptides.** To investigate whether the protease(s) secreted by DC could be used to generate antigenic peptides for presentation to T cells, conditioned cell-free medium from 4-hr cultures of murine DC or B cells was incubated with intact HEL or $\beta$-casein proteins. After digestion, the mixtures were applied to fixed I-A$^k$ or I-A$^s$ APC. Immunodominant antigenic peptide fragments were detected by proliferation of either a T cell hybridoma specific for HEL48–62 in the context of I-A$^k$ or of a T cell line raised against $\beta$-casein in the context of I-A$^s$. Incubation of intact HEL with cell-free supernatant resulted in the generation of an antigenic peptide containing the immunodominant HEL48–62 peptide in the culture

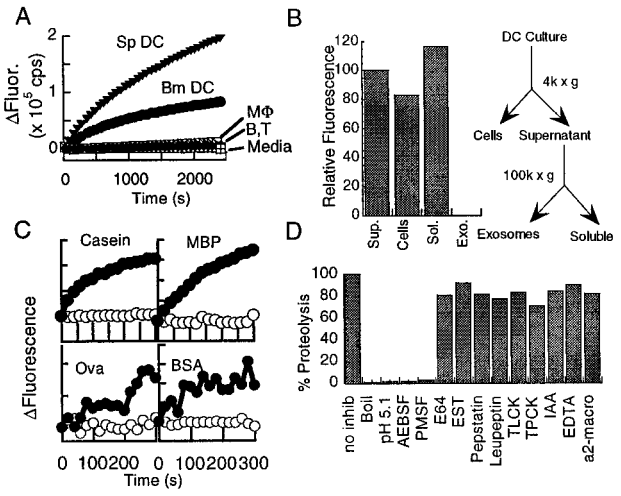


**Fig. 3.** DC produce soluble proteases. (*A*) Proteolytic activity of splenic (Sp) DC, bone-marrow derived (Bm) DC, splenic macrophages (MΦ), or a mixture of splenic B and T cells (B, T), measured by using Bodipy-derivitized casein in fluorescence assay (28). (*B*) Subcellular fractionation. Proteolytic activity for cells, culture supernatant, exosomes, and high-speed soluble fraction, prepared as shown. (*C*). Conditioned medium from splenic DC has proteolytic activity against Bodipy-casein, myelin basic protein (MBP), ovalbumin (OVA), and serum albumin (BSA). ●, conditioned medium from DC cultures; ○, medium alone. (*D*) Protease inhibitors. Conditioned serum-free medium can hydrolyze an aminocoumarin-labeled peptide substrate. Hydrolyis is blocked by boiling, low pH, and incubation with the general serine protease inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and PMSF, but not inhibitors of other protease families or more specific serine protease inhibitors. TLCK, *N*-tosyllysinechloromethylketone; TPCK, *N*-tosylphenylalaninechloromethylketone.

supernatant from immature DC but not B cells, as detected by the T cell hybridoma 3A9 (Fig. 4*A*). Both fixed B cells and fixed DC were able to present the digested mixture, with the DC inducing at least 20-fold higher levels of T cell activation, as expected from their more potent costimulatory capacity (Fig. 4*B*). Comparison of the level of T cell activation induced by fixed B cells presenting HEL fragments generated in the DC cell-free supernatant, with that induced by unfixed B cells incubated with intact HEL antigen, suggested that the efficiency of the extracellular processing pathway approached half of that for the conventional, intracellular B cell pathway (data not shown). Generation of a peptide containing the HEL48–62 antigen and presentation by I-A$^k$ molecules was confirmed by flow cytometry using the antibody Aw3.1 (17), which specifically binds the I-A$^k$/HEL48–62 complex. I-A$^k$/HEL complexes were generated by incubation with HEL digested by the DC supernatant, whereas essentially no peptide was generated by B cell supernatant (Fig. 4*C*). The amount of I-A$^k$/HEL complex produced by 100 $\mu$l of DC supernatant was comparable to that generated by direct addition of 1 $\mu$g HEL48–62 peptide, suggesting a processing efficiency of 10%. Intact $\beta$-casein also was digested into immunodominant peptide fragments, as detected by an IA$^s$-restricted, casein-specific T cell line (Fig. 4*D*). Thus, the proteolytic activity secreted by DC can act to produce antigenic peptides from intact proteins. However, the size(s) of the peptide(s) containing the antigenic fragments remains to be determined.

**Extra-Endosomal Antigen Processing.** The presence of the key components of antigen processing, i.e., peptide-receptive MHC molecules, H-2M or HLA-DM, and proteases, all together at the surface of immature DC suggests that these cells might be able to present antigen by direct binding at the cell surface, without

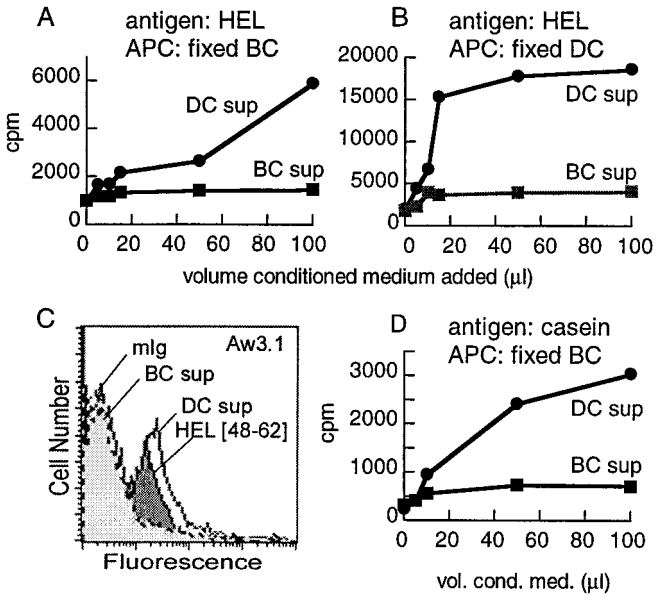


**Fig. 4.** DC proteases can generate immunodominant peptide fragment. (*A* and *B*) Generation of HEL48–62 peptide antigen from intact HEL protein by conditioned medium from splenic DC (●) but not from B cells (BC) (■). Cell-free supernatants from 4-hr DC or B cell cultures were incubated with intact HEL protein before addition to (*A*) fixed B cells or (*B*) DC for presentation to an $IA^k$-restricted and HEL48–62-specific T cell hybridoma. T cell activation was measured by using an IL-2 secretion assay (21). (*C*) The I-$A^k$-HEL complex detected by using the complex specific antibody Aw3.1 instead of the T-cell hybridoma. (*D*) Same as for *A* but using intact β-casein antigen and an I-$A^s$-restricted, casein-specific T cell line, with T cell activation determined by using a proliferation assay (21).

a requirement for internalization and endosomal processing. Recombinant expression of HLA-DR and HLA-DM at the cell surface has been shown to be sufficient for efficient surface binding and presentation of peptides in transfected COS-7 cells (25). To investigate whether such a pathway for antigen processing and presentation was active, DC and B cells were treated with chloroquine to block endosomal proteolysis and peptide loading. The treated cells then were incubated with intact β-casein. After a 6-hr incubation at 37°C, the cells were fixed with paraformaldehyde to prevent further internalization or processing and were used as APC in a T cell activation assay. DC, but not B cells, could process and present antigen in the presence of chloroquine (Fig. 5). The level of T cell activation induced by antigen-pulsed, chloroquine-treated DC was about two-thirds of the level induced by untreated, unfixed DC (not shown), indicating that the chloroquine-insensitive pathway represents a substantial fraction of the overall processing capacity.

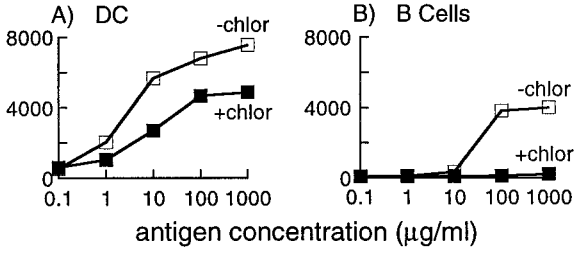


**Fig. 5.** Extra-endosomal antigen processing and presentation. Splenic DC (*A*) or B cells (*B*) were incubated with various amounts of β-casein in the absence (□) or presence (■) of 0.1 mM chloroquine, washed, fixed, and tested for antigen presentation in a standard T cell proliferation assay.

## Discussion

DC act as the sentinels of the immune system. Immature DC in peripheral tissue constantly sample the environment for antigens, which are presented to T cells after DC maturation and migration to lymph notes. Mature DC are particularly efficient APC, in part because of high levels of the costimulatory molecules B7–1 and B7–2, and they have an important role in stimulation of naïve T cells. In current models proposed for DC maturation, immature DC express most or all of their class II MHC molecules in endosomal/lysosomal compartments (5, 6) caused at least in part by inhibition cathepsin S necessary for cleavage and release of invariant chain (26, 27). Only on DC maturation do endosomal class II MHC molecules become released from Ii and able to bind peptide fragments of endocytosed antigens; the resultant peptide complexes then are transported to the cell surface for presentation to T cells. This mechanism is believed to provide for efficient surveillance of foreign antigens in peripheral tissue with subsequent presentation in lymphoid tissue.

A large fraction of the class II MHC molecules that are expressed on the surface of immature DC are present in the empty, peptide-receptive state. Thus, immature DC may use alternate pathways for trafficking and loading of class II MHC molecules, in addition to the endosomal retention pathway described above. Here, expression of the peptide exchange factors H2-M or HLA-DM, along with empty class II MHC molecules, on the surface of immature (but not mature) murine and human DC is reported. Also secretion by DC of protease(s), able to cleave a variety of protein antigens and to generate antigenic peptide fragments for presentation on class II MHC molecules, is demonstrated. Finally, DC can use a chloroquine-independent pathway for antigen processing and loading, which appears to act entirely at the cell surface. Such an extracellular mode of antigen presentation clearly would be distinct from current endosomal processing models. These data are consistent with some previously published data, including expression of HLA-DM on the surface of Langerhans cells (13) and secretion of proteases from DC (24).

Two mechanisms can be envisioned for the production of empty class II MHC molecules at the DC surface. One possibility is that they are derived from newly synthesized class II MHC/Ii complexes that traffic to the cell surface en route to endosomal compartments. This pathway is minor in B cells (28), but has been reported to be an important trafficking route in DC (12). Cell-surface class II MHC/Ii complexes could interact with cell-surface H-2M or HLA-DM to release Ii, perhaps after cleavage of Ii by extracellular proteases. Ii is particularly sensitive to protease digestion (29), and DC-conditioned medium has been observed to degrade Ii into small peptide fragments (G.J.C. and L.J.S., unpublished data). Cell-surface class II MHC molecules released from Ii targeting signals by peptide exchange or by Ii processing would remain at the surface, whereas unprocessed class II MHC/Ii complexes would traffic to endosomes for processing and loading in the conventional pathway. Another possibility is that the empty cell-surface population is derived directly from class II MHC molecules that have not associated with invariant chain in the ER. Some allotypes of class II MHC molecules, including I-$A^k$ (but not I-$A^b$), are able to fold correctly in the endoplasmic reticulum (ER) in the absence of invariant chain (30–32). In B cells such molecules are retained in the ER, but in DC they are expressed at the surface (33). Ii-deficient mice express high levels of KL-304 reactive molecules on immature DC (L.S. and L.J.S., unpublished observations), indicating that empty class II MHC molecules can traffic to the surface without Ii. Cathepsin S-deficient mice express normal levels of class II MHC protein on the surface of DC, suggesting that an Ii-independent pathway can be active in DC

(34). Whether this Ii-independent pathway occurs in DC of normal $Ii^+$ mice and whether it is accessible to class II MHC molecules other than $I\text{-}A^k$ remains unclear. In either case, cell-surface H-2M or HLA-DM would act to both stabilize the empty class II MHC molecules at the surface (35, 36) and to help load peptides at the cell surface (25).

The extracellular antigen presentation pathway described here could play an important role in the immunological function of immature DC. The ability of immature DC to sample their environment by producing and binding peptides directly in the extracellular space would serve to broaden the spectrum of antigens available for presentation to T cells. In another antigen-presenting system, different molecules of the CD1 family bind antigen in different cellular compartments (37), emphasizing that complete antigenic coverage may be enhanced by binding antigen at a variety of cellular sites. In DC, labile peptides could be protected from terminal degradation that might otherwise occur in the extremely proteolytic endosomal environment, by binding to empty class II MHC at the surface. Such class II MHC-mediated protection from proteolytic degradation has been observed for particular peptides in B cells (38). The problem of protecting labile peptides may be particularly acute for immature DC, as they do not appear to generate a continuous supply of class II MHC molecules in the endosomes until maturation, and potential antigens would need to survive in an internal compartment until generation of active class II MHC molecules. The empty class II MHC molecules also could act as antigen receptors, able to collect at the cell surface extracellular peptide antigens that might not be taken up efficiently by fluid phase uptake. Additionally, maintenance of class II MHC molecules at the surface in an empty, peptide-receptive state would allow binding and presentation of relatively low-affinity, weakly binding peptides that might not be able to compete efficiently for binding in the endosomes. This latter function may be more important in maintenance of peripheral T cell tolerance and to thymic T cell selection than in presentation of foreign antigens at the lymph node. We recently have observed KL-304-positive cells in murine thymic epithelium (L.S. and F. Fischer, unpublished results). Relevant antigens for these tolerance and selection pathways include proteins and peptides released from apoptotic and necrotic cells, which would be available at the DC surface.

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