

Dendritic Cells Use Macropinocytosis and the Mannose Receptor to Concentrate Macromolecules in the Major Histocompatibility Complex Class II Compartment: Downregulation by Cytokines and Bacterial Products

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Summary

We have previously demonstrated that human peripheral blood low density mononuclear cells cultured in granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 develop into dendritic cells (DCs) that are extremely efficient in presenting soluble antigens to T cells. To identify the mechanisms responsible for efficient antigen capture, we studied the endocytic capacity of DCs using fluorescein isothiocyanate-dextran, horseradish peroxidase, and lucifer yellow. We found that DCs use two distinct mechanisms for antigen capture. The first is a high level of fluid phase uptake via macropinocytosis. In contrast to what has been found with other cell types, macropinocytosis in DCs is constitutive and allows continuous internalization of large volumes of fluid. The second mechanism of capture is mediated via the mannose receptor (MR), which is expressed at high levels on DCs. At low ligand concentrations, the MR can deliver a large number of ligands to the cell in successive rounds. Thus, while macropinocytosis endows DCs with a high capacity, nonsaturable mechanism for capture of any soluble antigen, the MR gives an extra capacity for antigen capture with some degree of selectivity for non-self molecules. In addition to their high endocytic capacity, DCs from GM-CSF+IL-4-dependent cultures are characterized by the presence of a large intracellular compartment that contains high levels of class II molecules, cathepsin D, and lysosomal-associated membrane protein-1, and is rapidly accessible to endocytic markers. We investigated whether the capacity of DCs to capture and process antigen could be modulated by exogenous stimuli. We found that DCs respond to tumor necrosis factor α , CD40 ligand, IL-1, and lipopolysaccharide with a coordinate series of changes that include downregulation of macropinocytosis and Fc receptors, disappearance of the class II compartment, and upregulation of adhesion and costimulatory molecules. These changes occur within 1–2 d and are irreversible, since neither pinocytosis nor the class II compartment are recovered when the maturation-inducing stimulus is removed. The specificity of the MR and the capacity to respond to inflammatory stimuli maximize the capacity of DCs to present infectious non-self antigens to T cells.

Different mechanisms of antigen uptake are used by different cell types and determine their relative efficiency as APCs for MHC class II-restricted T cells. Capture of antigens by surface receptors such as mIg or FcR allows efficient delivery of the antigen to the processing compartment via receptor-mediated endocytosis (1). Antigens that fail to bind to cell surface receptors can still be taken up by fluid phase pinocytosis and presented by APCs, but with a much lower efficiency. Fluid phase uptake can occur via distinct mechanisms (2): (a) micropinocytosis, i.e., uptake of small vesicles ($\sim 0.1 \mu\text{m}$) via clathrin-coated pits; and (b) macropinocytosis, i.e., uptake of large vesicles ($0.5\text{--}3 \mu\text{m}$) mediated by membrane ruffling driven by the actin cytoskeleton. While micro-

pinocytosis occurs constitutively in all cells, macropinocytosis is limited to few cell types, such as macrophages and epithelial cells stimulated by growth factors (3–5).

Dendritic cells (DCs)¹ play a critical role in antigen presentation in vivo since they are the specialized APCs that prime virgin T cells (6). A large body of evidence from ex vivo experiments indicates that the capacity to interiorize and process antigen is a constitutive property of immature DCs present

¹ Abbreviations used in this paper: AML, amiloride; CCD, cytochalasin D; CD40L, CD40 ligand; DC, dendritic cell; DMA, 5-(N,N-dimethyl)-amiloride; DX, dextran; HRP, horseradish peroxidase; Lamp-1, lysosomal-associated membrane protein; LY, lucifer yellow; MR, mannose receptor.

in non-lymphoid organs. This property is rapidly lost when DCs, after in vivo antigenic challenge, move to the T cell-dependent areas of secondary lymphoid organs (6). It is also lost spontaneously when immature DCs such as Langerhans cells are cultured in vitro (7, 8). For these reasons, it has been difficult to characterize the mechanisms of antigen uptake by DCs. In particular, it is not clear whether DCs have high endocytic capacity and whether they may have specialized receptors for the capture of foreign antigens.

We have recently described an in vitro culture system using GM-CSF and IL-4 that permits DCs to be maintained in a state where they are competent for antigen capture and processing (9). Such cultured DCs present a soluble antigen taken up by fluid phase as efficiently as antigen-specific B cells that can use mIg for its capture via receptor-mediated endocytosis. Strikingly, stimulation with TNF- α results in an inhibition of the capacity to present a soluble antigen and, at the same time, an increase in T cell stimulatory capacity (9).

To understand the basis of the extraordinarily efficient antigen presentation, we have analyzed the mechanism of antigen capture by these cultured DCs. Here we report that DCs express the MR and use it for efficient capture of a variety of antigens. In addition, DCs are characterized by a very high level of macropinocytosis that allows them to take up large amounts of fluid and concentrate the macromolecules in a compartment that contains MHC class II molecules and proteases. TNF- α , as well as inflammatory stimuli, induce coordinate and irreversible changes that result in loss of the capturing machinery and a simultaneous increase in T cell stimulatory capacity.

Materials and Methods

Media and Reagents. The medium used throughout was RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 50 μ g/ml kanamycin (Gibco, Grand Island, NY), 5×10^{-5} M 2-ME (Merck, Darmstadt, Germany), and 10% FCS (Hyclone Laboratories, Logan, Utah). Human rIL-2, rIL-4, and rGM-CSF were produced in our laboratory by PCR cloning and expression in the myeloma expression system (10). IL-4 and GM-CSF concentrations were determined using commercial ELISA assays. Human recombinant TNF- α , TNF- α with point mutations at residues 32 and 86 (Trp32-Thr86-TNF- α , specific for TNF-R55), and TNF- α with point mutations at residues 143 and 145 (Asn143-Arg145-TNF- α , specific for TNF-R75) (11) were a generous gift from Dr. W. Lesslauer (Hoffmann-La Roche, Basel, Switzerland). Human rIL-1 α and rIL-1 β were gifts from Dr. L. Melli (IRIS, Siena, Italy). IL-6 was a kind gift from Dr. T. Rolink (Basel Institute for Immunology, Basel, Switzerland). Lucifer yellow CH, potassium salt, FITC-transferrin, and lysine-fixable FITC-dextran, $M_r = 40,000$, were purchased from Molecular Probes, Inc. (Eugene, OR) as lyophilized powder, reconstituted in PBS, and spun in a microfuge before use to eliminate aggregates. Horseradish peroxidase (HRP), o-phenylenediamine dihydrochloride, diaminobenzidine, FITC-mannosylated BSA, mannan from *Saccharomyces cerevisiae*, amylose (AML), 5-(N,N-dimethyl)-amyloride (DMA), cytochalasin D (CCD), and LPS from *Escherichia coli* were purchased from Sigma Immunochemicals (St. Louis, MO). J558 myeloma cells transfected with CD40L were kindly provided by Dr. P. Lane (Basel Institute for Immunology).

Cells. The method for the in vitro culture of human DCs has been described (9). Briefly, PBMC obtained by the standard Ficoll-Paque method (Organon Teknica, Durham, NC) were separated on multistep Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and the light density fraction from the 42.5–50% interface was recovered and depleted of CD19⁺ and CD2⁺ cells using magnetic beads coated with specific antibodies (Dyna, Oslo, Norway). The remaining cells were cultured at 3×10^5 /ml in RPMI-10% FCS supplemented with 50 ng/ml GM-CSF and 1,000 U/ml IL-4 at 37°C at 5% CO₂ in air. DCs were used routinely from 8 to 15 d of culture. EBV-transformed B cell lines and T cell clones were obtained and maintained as previously described (12). To obtain human macrophages, monocytes were separated from PBMC using a single-step (43%) Percoll gradient and were cultured for 10 d in Teflon-coated petri dishes (Heraeus, Zürich, Switzerland) in Hepes-buffered RPMI 1640 supplemented with 2 mM L-glutamine and 50% heat-inactivated human serum. Mouse macrophages were obtained by washing the peritoneal cavity with cold PBS. Human fibroblast MRC-5 was obtained from American Type Culture Collection (Rockville, MD). The MonoMac cell line was kindly provided by Dr. G. Riethmuller (Munich University, Munich, Germany). Veiled cells from the afferent lymph of sheep were provided by Dr. Wayne Hein (Basel Institute for Immunology).

Staining with mAbs. Cell staining was performed using mouse mAbs followed by FITC- or PE-conjugated, affinity-purified, isotype-specific goat anti-mouse antibodies (Southern Biotechnology, Birmingham, AL). The following mAbs were used: L243 (IgG2a, anti-DR), W6/32 (IgG2a, anti-MHC class I), IV.3 (IgG2b, anti-CD32), SPVL3 (IgG2a, anti-DQ) (all from American Type Culture Collection), B7.24 (IgG2a, anti-CD80 was provided by Dr. Mark Deboer, Immunogenetics, Ghent, Belgium), R/R1.1 (IgG1, anti-CD54), TS2/9 (IgG1, anti-CD58) (both provided by Dr. T. Springer, Harvard Medical School, Boston, MA), LeuM3 (IgG2b, anti-CD14; Becton Dickinson Immunocytometry Systems, Mountain View, CA), G28.5 (IgG1, anti-CD40, provided by Dr. A. E. Clark, University of Washington, Seattle, WA), Tu1 (IgG3, anti-CD23, provided by Dr. A. Ziegler, University of Berlin, Berlin, Germany), and 3.29 (IgG1, anti-MR; Cella, M., unpublished data). The samples were analyzed on a FACScan[®] (Becton Dickinson) using propidium iodide to exclude dead cells.

Quantitation of Endocytosis in Single Cells by FACS[®] Analysis. Cells were resuspended in 10% FCS medium buffered with 25 mM Hepes at 37°C. FITC-DX or LY were added at the final concentration of 1 mg/ml if not otherwise indicated for different times. The cells were washed four times with cold PBS containing 1% FCS and 0.01% NaN₃ and were analyzed on a FACScan[®] (Becton Dickinson) using propidium iodide to exclude dead cells. The background (cells pulsed at 0°C) was always subtracted. In some experiments, the cells were pulsed at 37°C, washed four times with cold medium, and cultured at 37°C for different times in marker-free medium. CCD, AML, and DMA were added to the cell suspension at 37°C 15 min before the addition of the marker. The amount of fluorescent probe accumulated was calculated from the FITC to probe ratio with reference to a standard curve of beads labeled with known amount of fluorescein (Flow Cytometry Standard Corp., Research Triangle Park, NC).

Quantitation of Endocytosis in Bulk Populations. DCs were incubated at 37°C in the presence of different concentration of HRP. At various times, the cells were collected, washed four times in PBS containing 1% FCS and four times in PBS alone with one tube change, lysed with 0.05% Triton X-100 in 10 mM Tris buffer, pH 7.4, for 30 min, and the enzyme activity of the lysate was measured using o-phenylenediamine and H₂O₂ as substrates with ref-

erence to a standard curve. In other experiments, DCs were incubated with different concentrations of FITC-DX or LY, treated as described above, and the fluorescence of the lysate was measured with an automated fluorimeter (Titertek Fluoroskan II; Flow Laboratories, UK) with reference to a standard curve. The cell lysate did not interfere with the quantitation of the fluorochrome.

Identification of Macropinosomes by HRP. To visualize HRP uptake, the cells were incubated with 1 mg/ml HRP, washed with cold medium, and cytospun onto coverslips in a cytocentrifuge. The cells were fixed in 1% paraformaldehyde + 1.7% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4, for 12 min, washed four times in Na-cacodylate buffer, and the enzymatic reaction was developed using 1 mg/ml diaminobenzidine and 0.01% H₂O₂ in Na-cacodylate buffer.

Confocal Microscopy. Cells were adhered to glass slides (Polyscience; Miles Inc., Kanakee, IL) coated with 50 µg/ml poly-L-lysine (Sigma), fixed in 2% paraformaldehyde in Ca⁺⁺/Mg⁺⁺-free PBS, and quenched by washing with 50 mM ammonium chloride. Cells were permeabilized in PBS/saponin (0.01%)/gelatin (0.25%)/NP-40 (0.1%) and were sequentially incubated with different primary antibodies followed by isotype- or species-specific Texas red- or FITC-labeled secondary antibodies (Southern Biotechnology Associates, Birmingham, AL). The following primary antibodies were used: L243 (anti-DR, mouse IgG2a; American Type Culture Collection), anti-human cathepsin D (rabbit antisera, a generous gift from Dr. S. Kornfeld, Washington University, St. Louis, MO), anti-human Lamp-1 (rabbit antisera, a generous gift from Dr. S. Carlsson, Umea University, Sweden). Slides were mounted in 90% glycerol/PBS containing 2.5% DABCO (1-4-diazabicyclo[2.2.2]octane; Fluka Chemie AG, Buchs, Switzerland) to minimize photobleaching and were analyzed with a confocal laser scanning microscope system (MRC-600; Bio Rad Laboratories, Hercules, CA) attached to a microscope (Axiovert 35M; Carl Zeiss, Inc., Thornwood, NY). Appropriate controls were always included and bleedthrough corrections were done according to the manufacturer's recommendations and were tested in a single-labeling experiments. To visualize the relative positional distribution of the two fluorochromes, the images collected in the two channels were merged. To combine a three-dimensional z series into a two-dimensional image, 26 sections spanning the entire cell were projected and superimposed.

Results

Dissection of Fluid Phase and Receptor-mediated Endocytosis in DCs. We have studied the endocytic capacity of DCs using three classical markers: LY, FITC-DX, and HRP. While HRP could be used only to quantitate uptake in bulk populations, FITC-DX and LY also allow quantitation at the single-cell level by FACS[®] analysis. A typical experiment (Fig. 1 A) shows that DCs incubated at 37°C with FITC-DX accumulate this marker in a time-dependent fashion. The narrow distribution of the fluorescence profile indicates that all cells take up comparable amounts of the probe. On the contrary, EBV-B cells were dishomogeneous since only a small percent of the cells take up FITC-DX, and only in a much lower amount (Fig. 1 B). In addition, EBV-B cells do not accumulate the marker. Similar results were obtained with LY (see below).

As shown in Table 1, the efficient accumulation of FITC-DX and LY is a peculiar property of cultured DCs and veiled cells freshly isolated from sheep afferent lymph, which is not

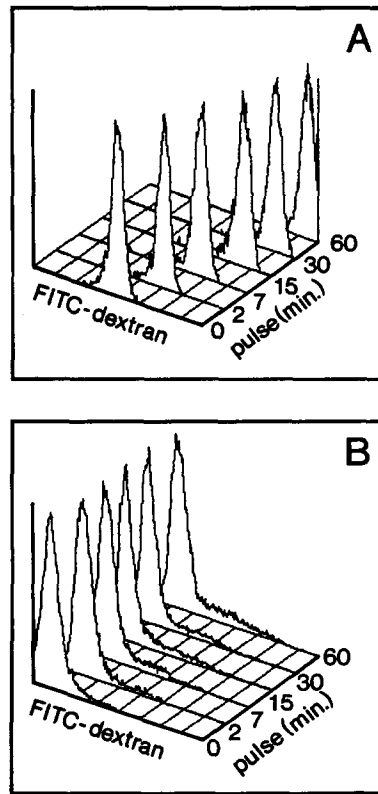


Figure 1. Accumulation of FITC-DX in DCs. DCs grown in GM-CSF + IL-4 (A) or EBV-B cells (B) were incubated in medium containing 1 mg/ml FITC-DX for various times, washed, and analyzed on a FACSscan[®]. The fluorescent stainings cannot be compared since much lower amplification was used for DCs than for EBV-B cells.

shared by other cell types analyzed, such as macrophages, monocytes, fibroblasts, lymphocytes, and various cell lines. This property is not lost when DCs are cultured in medium without GM-CSF and IL-4 for up to 5 d, making it very unlikely that the pinocytosis is dependent on stimulation by exogenous factors. Taken together, these results suggest that the high rate of endocytosis in DCs is constitutive.

A quantitative analysis of uptake of LY, FITC-DX, and HRP by DCs is shown in Fig. 2. All markers accumulate in a linear fashion with time. Upon chase in marker-free medium, FITC-DX is completely retained for long periods of time (up to 10 h, data not shown), while HRP and LY are lost to a variable extent (~50% in 1 h), possibly because they are degraded or released and thus will underestimate the amount taken up during prolonged incubations.

While fluid phase uptake is not saturable, uptake via specific receptors is saturable with increasing concentrations of the ligand. To get information about the mechanisms of uptake of the different markers, we measured the amount of marker taken up as a function of the concentration in solution. While the amount of LY accumulated by DCs was proportional to the concentration of LY in the medium, the amounts of FITC-DX and HRP showed a clear saturation at increasing doses of the marker. Thus, while LY behaves as a bona fide fluid

Table 1. Fresh and Cultured DCs Have a High and Constitutive Capacity to Accumulate Fluid Phase Markers

	Median fluorescence intensity:			
	LY*		FITC-DX*	
	7 min	30 min	7 min	30 min
DC (GM-CSF + IL-4)	408	1494	780	3290
DC no cytokines†	—	—	501	2229
Sheep veiled cells	60	150	456	1905
Fresh monocytes	—	—	32	19
Cultured macrophages	—	—	67	71
Mouse macrophages	—	—	16	35
MonoMac	17	58	11	12
EBV-B	19	50	19	11
T cell clone	13	31	0	0

* Cells were incubated with 1 mg/ml FITC-DX or LY for 7 or 30 min at 37°C, washed four times, and analyzed using the same amplification conditions. The mean fluorescence intensity is shown. The background fluorescence (cells incubated at 0°C for 30 min) was subtracted.

† DCs from GM-CSF + IL-4 cultures were washed and recultured in the absence of GM-CSF and IL-4 for 5 d before the assay.

phase marker, FITC-DX and HRP appear to be taken up by a saturable mechanism, implying the existence of a specific receptor.

Identification of the Mannose Receptor (MR) as the Major Receptor for Endocytosis of FITC-DX and HRP in DCs. We have recently produced an mAb to the human mannose receptor by immunizing mice with cultured DCs. This antibody stains both cultured DCs and DCs in tissue sections of both human and sheep. It also stains hepatic sinusoidal cells and scattered cells in the thymus. It also precipitates a 175-kD molecule from DC and inhibits uptake of mannosylated BSA (Cella, M., et al., manuscript in preparation). To test whether the putative receptor for FITC-DX is the MR, we measured FITC-DX uptake in the presence or absence of mannan, a bacterial polysaccharide that binds with high affinity to the MR. As shown in Fig. 3 A, mannan inhibits uptake of FITC-DX in a dose-dependent fashion. Maximum inhibition is reached at 0.3 mg/ml and does not further increase at higher concentrations of mannan. The inhibition of FITC-DX uptake is not complete and the noninhibitable component shows a linear increase with increasing concentrations of the marker, indicating that, in the presence of mannan, the uptake of the FITC-DX takes place by the fluid phase (Fig. 3 B).

To further investigate the role of MR in DC endocytosis, we tested the inhibitory effect of EDTA and specific anti-MR antibody on uptake of the three markers. As shown in Fig. 4, the uptake of FITC-DX and HRP was inhibited to a comparable extent by EDTA, mannan, and anti-MR antibody, while the same treatments did not affect the uptake

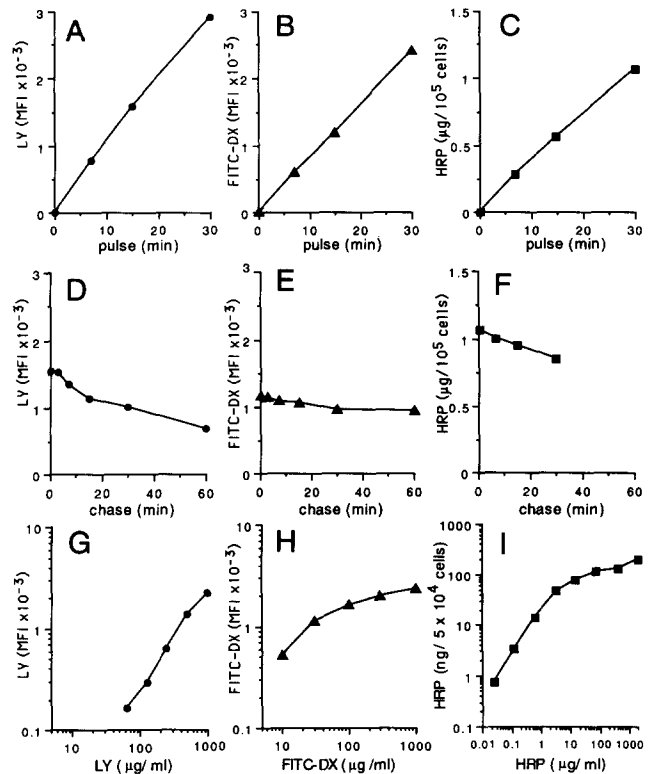


Figure 2. Endocytosis of different markers in DCs reveals saturable and nonsaturable mechanisms of uptake. (A–C) DCs were incubated with 1 mg/ml LY (●), FITC-DX (▲), or HRP (■) at 37°C. At various time points the cells were washed in cold medium and the amount of ligand accumulated was measured. (D–F) DCs were pulsed for 15 min with LY or FITC-DX or for 30 min with HRP, washed with cold medium, and analyzed immediately or after culture for various times in marker-free medium. (G–I) DCs were incubated at 37°C in the presence of different concentration of LY, FITC-DX, or HRP, and the amount accumulated was measured after 30 min (G–H) or 1 h (I). When the markers were used at 1 mg/ml, the uptake after incubation for 30 min at 0°C was <1% of the uptake at 37°C for LY and HRP and <2% for FITC-DX.

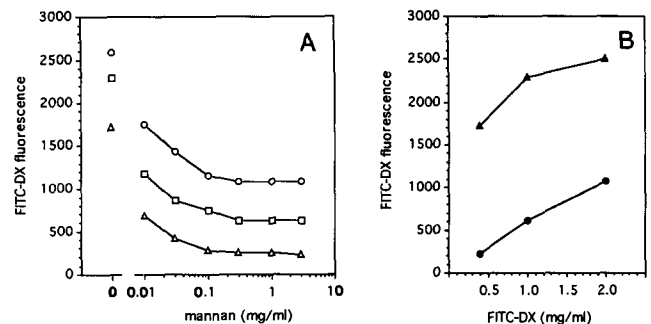


Figure 3. The inhibitory effect of mannan reveals saturable and nonsaturable components in the uptake of FITC-DX by DCs. (A) DCs were incubated at 37°C in the presence or absence of different concentrations of mannan, and after 10 min, FITC-DX was added at the final concentration of 2 (○), 1 (□), or 0.4 (△) mg/ml. The data show the fluorescence of the cells after 30 min. (B) Amount of FITC-DX taken up in the absence (▲) or in the presence (●) of 3 mg/ml mannan as a function of the FITC-DX concentration in the medium.

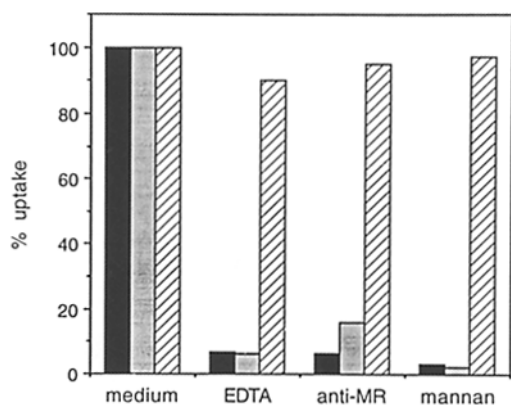


Figure 4. The MR is the major receptor for endocytosis of FITC-DX and HRP. DCs were incubated at 37°C in normal medium or in medium containing anti-MR antibody or 3 mg/ml mannan, or in PBS-10% dialyzed FCS containing 0.5 mM EDTA. After 10 min, FITC-DX (black bars), HRP (grey bars), or LY (stippled bars), all at 1 mg/ml, were added. The uptake was measured after 15 min and expressed as percent of the uptake in medium alone.

of LY. Thus ligand specificity, calcium dependency, and a specific antibody identify MR as the most important if not the exclusive receptor involved in endocytosis of FITC-DX and HRP by DCs.

Quantitation of Marker Taken up via MR and Fluid Phase. Table 2 shows an estimate of the amount of marker taken up by DCs by fluid phase or MR-mediated uptake. Fluid phase uptake was measured using LY or FITC-DX in the presence

of inhibitory concentrations of mannan. We estimate that a single DC can take up every hour an amount of marker corresponding to a volume of 1,000–1,500 μm^3 . These volumes, which are probably underestimated because of losses, are close to the volume of the cell itself. The fact that the markers are present in a discrete cellular compartment indicates that DCs must be able to concentrate the markers.

Uptake via the MR was quantitated using ligands that bind to the MR (FITC-DX, FITC-mannosylated BSA, and HRP). As shown in Table 2, the MR allows DCs to accumulate a large number of molecules per cell, even at low concentrations of the ligand. The number of molecules taken up via the MR exceeds the number of MR per cell, indicating that the receptor can be used in successive rounds of binding, internalization, and delivery.

Evidence for Macropinocytosis by DCs In Vitro and In Vivo. The very high level of fluid phase uptake in DCs prompted us to search for the responsible mechanism. Morphological examination of DCs pulsed for very short times with various markers revealed the formation of large numbers of structures resembling macropinosomes. Macropinosomes could be detected as early as 30 s after addition of HRP (Fig. 5 A), their number increased with time at 37°C for the first 2–3 min. The size of the macropinosomes varied from 0.5 to 3 μm in diameter (mean = 1 μm) in different cells, but was rather homogeneous in each individual cell. Similar results were obtained using HRP (Fig. 5, A and B), FITC-DX (Fig. 5, C and D), and LY (Fig. 5 E). Interestingly, similar structures were observed to form when freshly isolated veiled cells from sheep afferent lymph were incubated with FITC-DX (Fig.

Table 2. Amount of Molecules Accumulated per Hour by Fluid Phase or MR-mediated Uptake in a Single DC

Marker concentration	Molecules/cell per hour	Equivalent volume (μm^3)
LY (2×10^{-3} M)*	$1.1 - 1.3 \times 10^9$	920–1100
FITC-DX (2.5×10^{-5} M + mannan)*	$14 - 22 \times 10^6$	930–1500
FITC-DX (2.5×10^{-8} M) [†]	300,000	20,000
FITC-BSA-Mann. (10^{-8} M) [‡]	2,200,000	366,000
FITC-BSA-Mann. (10^{-9} M)	440,000	733,000
HRP (4×10^{-7} M) [§]	24,000,000	100,000
HRP (8×10^{-8} M)	14,200,000	296,000
HRP (1.6×10^{-8} M)	4,600,000	478,000
HRP (3.2×10^{-9} M)	1,050,000	547,000
HRP (6.4×10^{-10} M)	240,000	625,000
HRP (1.3×10^{-10} M)	66,000	860,000
HRP (2.6×10^{-11} M)	13,500	877,000

* The amount of marker was measured in cell lysates using a fluorimeter by reference to a standard curve. The cell lysate did not interfere with the quantitation of the marker. Data are from two separate experiments.

† The amount of marker was measured by cytofluorimetric analysis on the basis of the FITC content of the marker by reference to a standard curve of beads containing known amounts of FITC.

§ The amount of marker was measured in cell lysates by reference to a standard curve of HRP.

|| Equivalent volume corresponds to the volume of fluid that contains the amount of marker accumulated in the cell. The volume of a DC was $\sim 2,500 \mu\text{m}^3$, as determined by hematocrit.

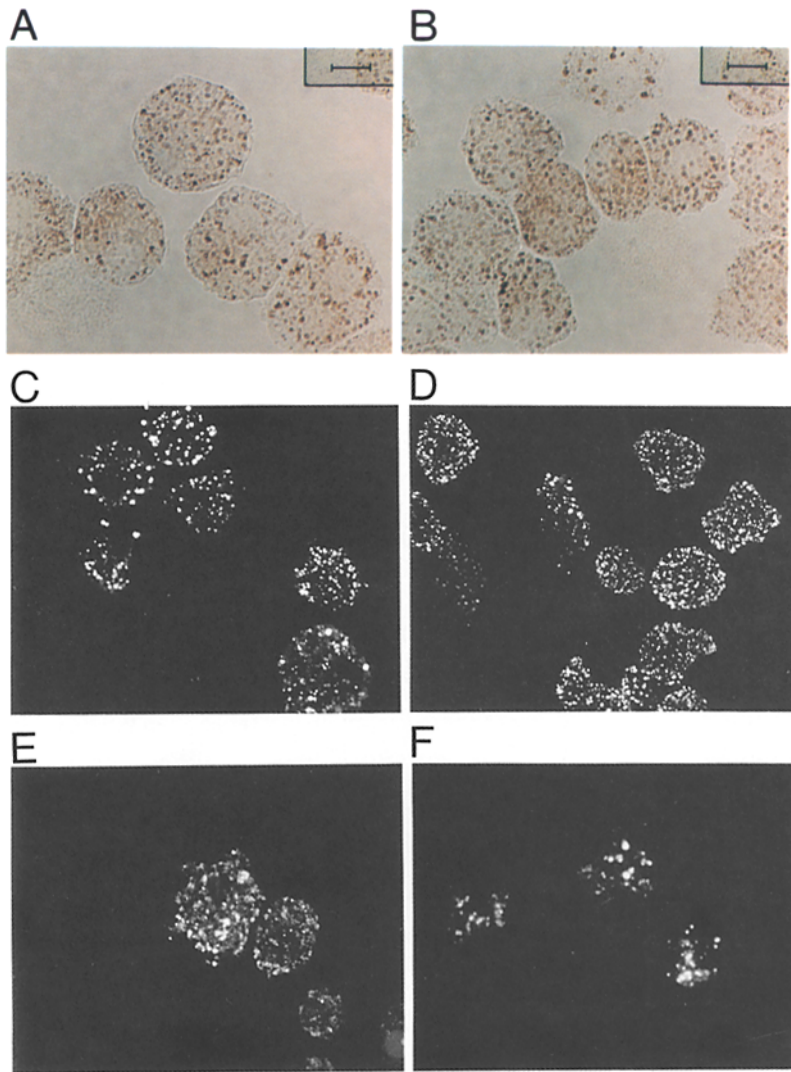


Figure 5. Morphological evidence for macropinosomes in DCs in vitro and in vivo. DCs were incubated for 30 s (A) or 1 min (B) with 1 mg/ml HRP, washed in cold medium, fixed, and the enzymatic reaction was developed. Bar, 20 μ m. Unpulsed cells or cells incubated for the same times at 0°C were completely negative (not shown). DCs were incubated with 1 mg/ml FITC-DX for 1 min (C) or 3 min (D) at 37°C, washed in cold medium, fixed, and analyzed by taking the z series using a confocal microscope. Each image results from projection of 26 sections. DCs were incubated with 1 mg/ml LY at 37°C for 2 min (E), washed in cold medium, and analyzed as above. Cells from sheep afferent lymph were pulsed for 5 min with 1 mg/ml FITC-DX, sorted according to physical parameters, and analyzed as described above (F).

5 F). Macropinosomes were difficult to visualize under phase contrast because the cells are not adherent.

Macropinocytosis is dependent on membrane ruffling and has been reported to be inhibitable by CCD (4), AML (13), and DMA (Watts, C., personal communication). As shown in Fig. 6, uptake of LY and FITC-DX is inhibited by these three drugs while the same drugs have a much lower effect on uptake of FITC-transferrin by EBV-B cells, which occurs only by endocytosis mediated by the transferrin receptor. In all cases, inhibition by DMA was completely reversed 5 min after removal of the drug (data not shown).

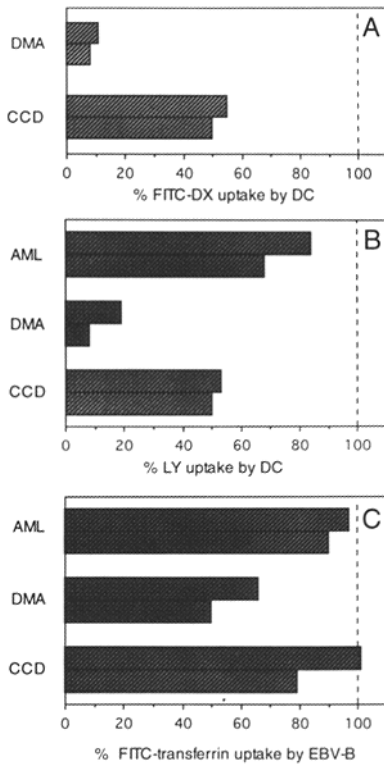
Taken together, these results indicate that macropinocytosis is a constitutive function of both in vitro-cultured and fresh DCs.

Macropinosomes Deliver their Content to a Large Compartment that Contains MHC Class II, Cathepsin D, and Lysosomal-associated Membrane Protein-1 (Lamp-1). DCs grown in GM-CSF + IL-4 were fixed, permeabilized, and stained with antibodies to mature class II molecules and cathepsin D or Lamp-1. As shown in Fig. 7, mature class II molecules were present on

the cell surface and in an extensive intracellular compartment. This compartment contains also cathepsin D and Lamp-1 (yellow structures in Fig. 7, A and B).

To establish whether this class II compartment might be reached by fluid phase markers, DCs were pulsed with FITC-DX for 2 min at 37°C and examined either immediately or after a chase at 37°C for 15 min. 2 min after pulsing, FITC-DX was present in a large number of macropinosomes as a ring at the periphery of the cell (Fig. 8 A, green structures), which did not colocalize with intracellular class II molecules (Fig. 8 A, red structures) or cathepsin D (data not shown). When DCs pulsed with FITC-DX were chased in marker-free medium, the green fluorescence became redistributed throughout the cell in what appeared as a large tubular and vesicular network. After 10–15 min of chase, we observed an extensive colocalization of FITC-dextran with class II molecules (Fig. 8 B), cathepsin D, and Lamp-1 (not shown).

Downregulation of Macropinocytosis and Disappearance of the MHC Class II Compartment Induced by TNF- α , CD40L, IL-1 and LPS. We have used GM-CSF + IL-4-dependent DCs



as a model of antigen presentation competent, “immature” DCs to identify the signals responsible for DC maturation. As likely candidates, we tested the effect of various cytokines that may be present at the site of inflammation and a bacterial product such as LPS.

The effects of these stimuli on the endocytic capacity of DCs is shown in Fig. 9. Incubation with TNF- α for 40 h resulted in a marked downregulation of the uptake of FITC-DX. Using mutant TNF- α molecules that interact selectively with the p55 or the p75 receptors, we found that this inhibitory effect is entirely mediated via the p55 receptor. The inhibitory activity of TNF- α was lost after boiling and was blocked by a specific antibody to TNF- α , ruling out the possibility that other contaminants may be responsible for this effect. In addition to TNF- α , preincubation with IL-1 β and cell-bound CD40L resulted in a similar inhibitory effect. LPS was also effective in downregulating pinocytosis. In this case,

Figure 6. Inhibition of LY and FITC-DX uptake by drugs that affect macropinocytosis. Uptake of FITC-DX (1 mg/ml) (A) or LY (1 mg/ml) (B) by DCs in the presence of various drugs. Uptake of FITC-transferrin (1 μ g/ml) by EBV-B cells in the presence of the same drugs (C). The drugs were added at 37°C 15 min before the addition of the marker. The results are expressed as percent of the uptake in the absence of inhibitors. The vehicle did not show any effect.

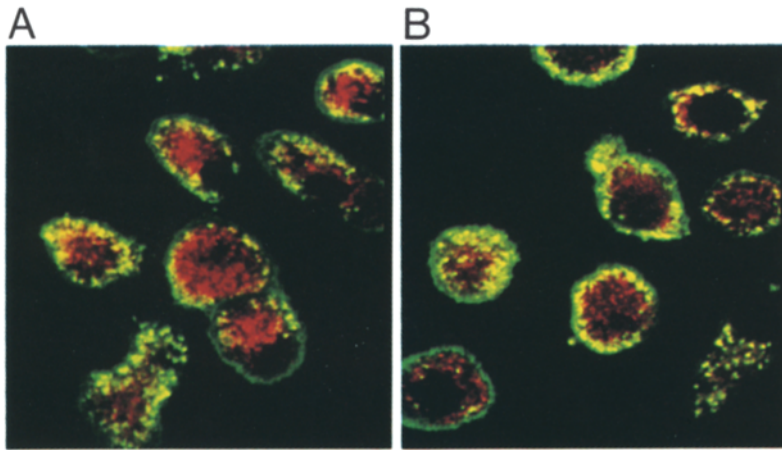


Figure 7. DCs have a large class II compartment that colocalizes with cathepsin D and Lamp-1. DCs were fixed, permeabilized, and stained by indirect immunofluorescence. (A) DR (green), cathepsin D (red); (B) DR (green) Lamp-1 (red). When red and green staining overlap, the compartment appears yellow.

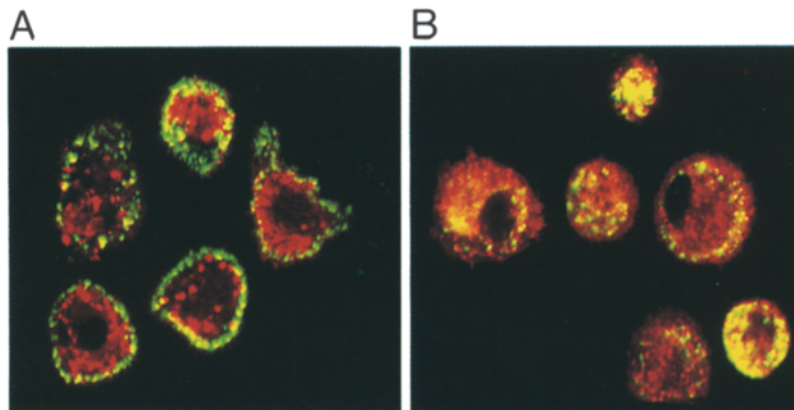


Figure 8. The content of macropinosomes is delivered to the MHC class II compartment. DCs were pulsed with FITC-DX for 2 min and analyzed immediately (A) or after a 15-min chase in marker-free medium (B). The cells were fixed, permeabilized, and stained with anti-DR (L243) followed by Texas red-labeled second antibody.

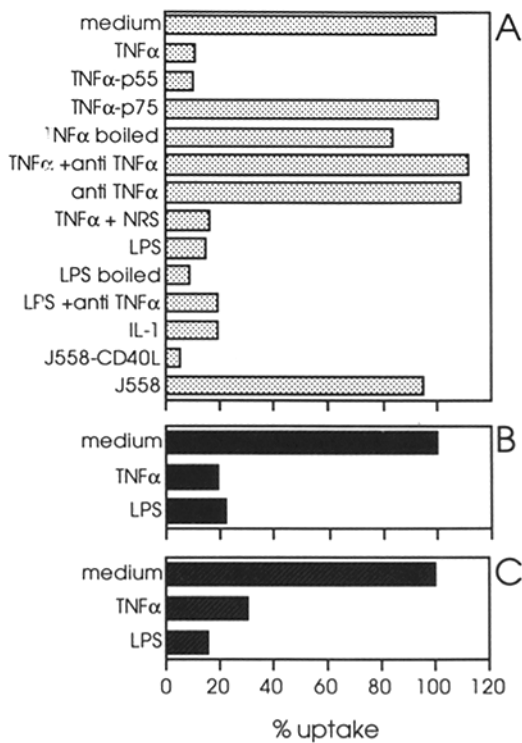


Figure 9. In DCs pinocytosis is downregulated by TNF- α , CD40L, IL-1 β , and LPS. DCs from GM-CSF + IL-4-dependent cultures were incubated for 40 h with various stimuli and the uptake of FITC-DX (A), HRP (B), and LY (C), all at 1 mg/ml, were measured after 30 min of pulse. TNF- α was added at 20 ng/ml, TNF- α mutants that interact only with the p55 or with the p75 TNF receptors at 200 ng/ml, IL-1 β at 10⁴ U/ml, and LPS at 20 ng/ml. Irradiated J558 cells transfected with CD40L were added at a 1:1 ratio and were gated out during the acquisition using an anti-DR antibody to identify DCs. Rabbit anti-TNF- α antiserum or normal rabbit serum were added together with the inducing stimuli.

the effect was not inhibited by boiling nor by an anti-TNF- α antibody, indicating that LPS does not act indirectly via induction of TNF- α production (Fig. 9 A). A similar inhibition of uptake was observed using HRP (Fig. 9 B), as well as LY (Fig. 9 C), suggesting that these stimuli decrease both fluid phase- and receptor-mediated uptake.

In addition to decreased uptake, DCs treated with TNF- α did not show formation of macropinosomes even after prolonged incubation (Fig. 10 A). The same treatment induced a disappearance of the class II compartment, as well as of the cathepsin-D-positive organelles (Fig. 10 B). Surprisingly, the loss of the class II compartment was not accompanied by a reduction in class II synthesis, which was actually transiently increased after treatment with TNF- α (Sallusto, F., unpublished results).

When compared to untreated cells, cells treated with TNF- α or LPS showed an increased expression of class II and class I molecules, B7.1 (CD80), LFA3 (CD58), and intracellular adhesion molecule 1 (CD54), as well as a decreased expression of FcR (CD32 and CD23) (Fig. 11). Interestingly, the expression of MR was reduced by less than 50%. These coordinate changes were observed in all the 10 different cell lines tested. The most striking induction was observed with TNF- α and LPS, while a lower effect was seen with IL-1 β . The maturation-inducing effects of TNF- α and LPS are irreversible since neither pinocytosis nor the class II compartment were recovered, even several days after removal of the inducing stimuli.

Discussion

We have previously demonstrated that human DCs from GM-CSF + IL-4-dependent cultures are highly efficient in presenting soluble antigens and that they lose this capacity when treated with TNF- α (9). Here we provide an explanation for both findings. First, we show that DCs have two specialized mechanisms for antigen capture: the MR and macropinocytosis that allow them to take up and concentrate macromolecules in the MHC class II compartment. Second, we show that inflammatory cytokines and bacterial products induce a coordinate and irreversible change in DCs with loss of macropinocytosis and disappearance of the class II compartment and upregulation of adhesion and costimulatory molecules. We discuss these results in the context of the model of DC maturation (6).

Antigen Capture via MR. The MR is a surface 175-kD C-type lectin containing eight carbohydrate recognition do-

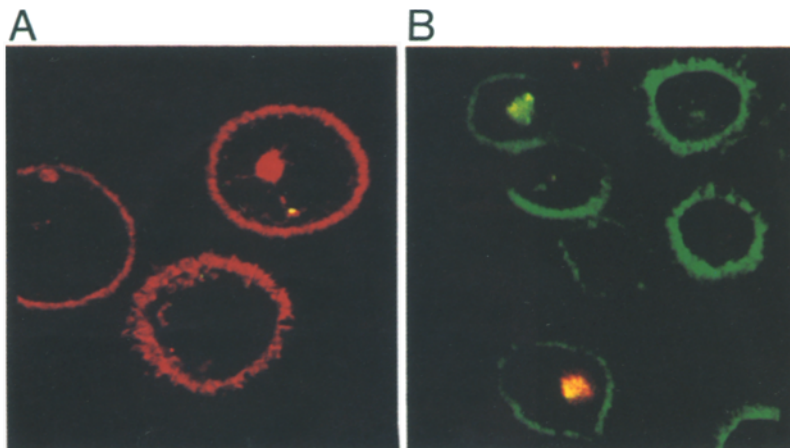


Figure 10. Macropinosomes and the class II compartment disappear after TNF- α treatment. DC maturation was induced by 20 ng/ml TNF- α for 40 h. (A) The cells were incubated with 1 mg/ml FITC-DX (green) for 30 min and subsequently stained with anti-DR antibody (red) as in Fig. 8. (B) The cells were stained with anti-DR (green) and anti-cathepsin D (red) as in Fig. 7 A.

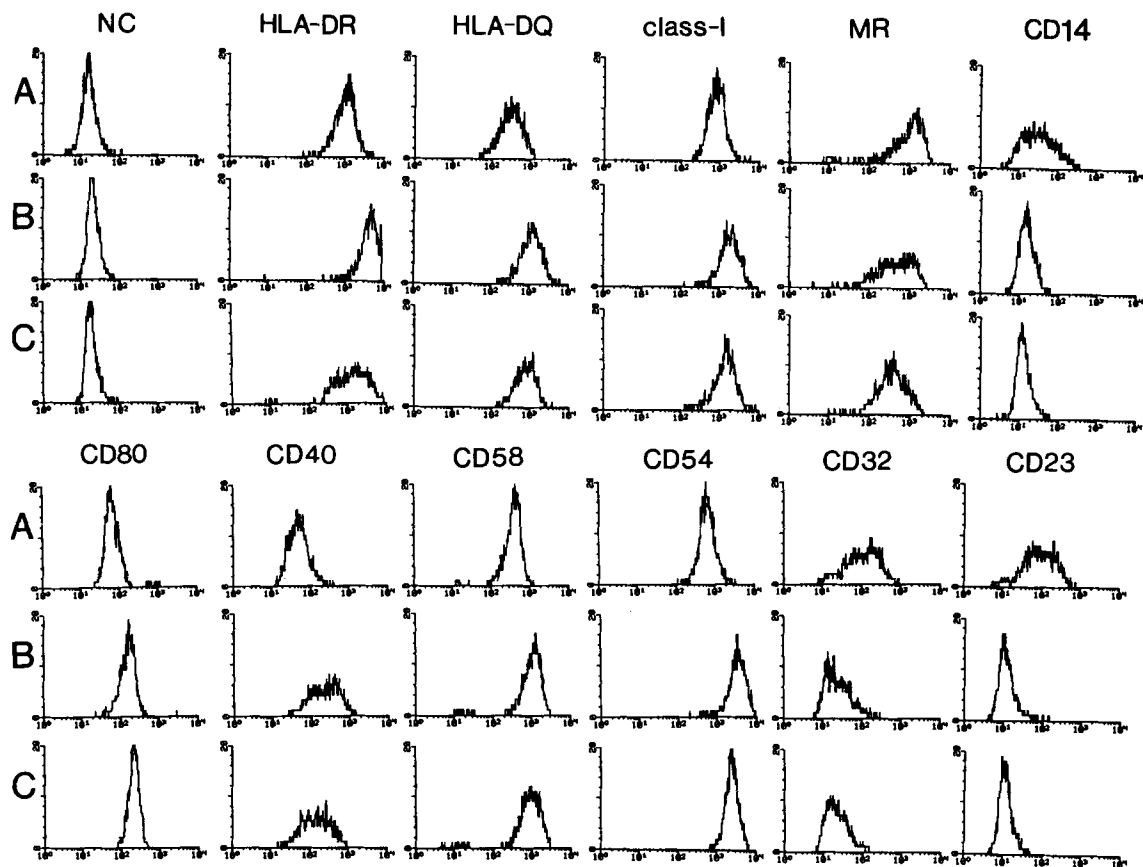


Figure 11. Changes in cell surface phenotype induced by treatment of DCs with TNF- α or LPS. DCs untreated (A) or treated with 20 ng/ml TNF- α (B) or 20 ng/ml LPS (C) for 40 h were stained with different mAbs. The first panel (NC) shows the profile obtained using an isotype-matched control antibody.

mains with broad specificity for sugars (14). It mediates phagocytosis of mannose-coated particles such as yeasts and endocytosis of mannosylated glycoproteins in macrophages (15). So far, MR has been demonstrated only on scavenger macrophages. Its presence on DCs has been suggested on the basis of their capacity to phagocytize yeast particles (16).

We have identified the MR as the major receptor responsible for endocytosis of HRP and FITC-DX in DCs. This conclusion is based on the observation that uptake of these markers is saturable and can be inhibited by mannan, EDTA, and by an antibody to the human MR. Using this antibody, we have demonstrated that the MR is expressed in cultured DCs, and we have evidence that it is also expressed on DCs *in vivo*, as detected by staining of tissue sections (Cella, M., et al., manuscript in preparation).

There are two features of the MR that are crucial for its function in antigen presentation: (a) the broad ligand specificity; and (b) the capacity to release the ligand at low pH and recycle after delivery of the ligand.

MR is known to bind various types of sugars such as mannose, fucose, and *N*-acetyl-glucosamine, as well as hydrophobic molecules (14). It is possible that even very low affinity interactions may be sufficient to allow adsorptive endocytosis via the MR. Thus, MR can be viewed as a pattern recogni-

tion molecule that can provide DCs with some capacity of self non-self discrimination at the level of antigen uptake on the basis of glycosylation and perhaps hydrophobicity. It will be interesting to see whether self proteins released by lysed cells in the high mannose form may be captured and presented with high efficiency by DCs.

The second important feature of the MR is the capacity to release its ligand at low pH and recycle to the cell surface (17). In this way, the MR can deliver ligands in consecutive rounds and thus in amounts that far exceed the number of receptors (see Table 2). Indeed, the amount of FITC-DX and HRP bound to DC at 0°C is only ~1% of the amount taken up in 30 min at 37°C. This is at variance with mIg or FcR, which do not dissociate antigen at low pH and are thus degraded together with their cargo (18, 19). Thus, while mIg and FcR offer a limited capacity for antigen capture, the MR offers an unlimited capacity to accumulate antigen.

The presence of the MR on DCs suggests that mannosylated antigens or toxins can be targeted *in vivo* to DCs to enhance immunogenicity or to deplete DCs from tissue grafts.

Macropinocytosis. Macropinocytosis has been characterized in epithelial cells and macrophages as a cytoskeleton-dependent mechanism of fluid uptake induced by stimulation with

growth factors or PMA, which is distinct from micropinocytosis, which occurs via clathrin-coated pits (3–5). While the importance of micropinocytosis in the capture of membrane-bound ligands has been well recognized, the functional significance of macropinocytosis has been an unresolved issue. Our results point to a specialized function for macropinocytosis in DCs, i.e., volume capture and solute concentration in the class II compartment.

We have shown that DCs are characterized by a very high level of fluid endocytosis, as measured by the uptake of LY, which behaves as a pure fluid phase marker, or by the uptake of FITC-DX in the presence of concentrations of mannan that inhibit MR-mediated uptake. The fluid volume taken up per hour by a single DC has been estimated to be 1,000–1,500 μm^3 , a volume that is close to that of the cell itself. This clearly indicates that these cells are able to concentrate the marker in a discrete intracellular compartment.

The evidence for macropinocytosis in DCs is based on morphological criteria and sensitivity to inhibitory drugs. As early as 30 s after addition of the marker, several macropinosomes measuring 0.5–3 μm in diameter are formed in DCs and their number increases to ~ 100 at 1 min. The uptake of ~ 100 pinosomes of $\sim 1 \mu\text{m}$ diameter would account for a fluid uptake of 50 $\mu\text{m}^3/\text{min}$ and therefore $\sim 3,000 \mu\text{m}^3/\text{h}$, a figure that would be compatible with that estimated using fluid phase markers. This type of calculation would require a more careful stereological analysis (20).

In these cultured DCs, the level of macropinocytosis is much higher than in other cell types (4, 5), probably because of the very active membrane ruffling. In addition, while macropinocytosis in macrophages or epithelial cells is present only for a few minutes after growth factor stimulation, it is constitutive in DCs since it is present even days after removal of GM-CSF and IL-4 and can be detected in fresh veiled cells *ex vivo*. Our results are in apparent contrast with previous studies in which DCs have been shown to have a detectable but rather low level of endocytosis (21, 22). This discrepancy may be explained by the fact that different cell populations have been analyzed in these studies. It is well known that DC can change their properties after maturation and lose antigen capturing capacity (references 6, 8 and this study). The cells from GM-CSF + IL-4 cultures may be the equivalent of immature DCs, a stage that is difficult to preserve *ex vivo* or in different culture conditions.

How can macrosolutes be concentrated in DCs? The formation and fate of macropinosomes differs in different cell types. In epithelial cells stimulated by epidermal growth factor, the pinosomes do not fuse with intracellular vesicles, but only among themselves, and recycle back within 20–30 min (13). In contrast, in macrophages stimulated by macrophage-CSF (23) and in DCs, the macropinosomes mature and go on to fuse with a lysosomal compartment. In the latter case, the vesicles that recycle back from this compartment to the cell surface are much smaller than the macropinosomes and thus will recycle much more membrane than fluid (24). This imbalance causes shrinkage of the compartment, loss of water and permeable compounds, and concentration of the macrosolutes.

The fate of the molecules taken up by macropinocytosis depends on their physical and chemical properties. Membrane-permeable compounds will passively diffuse out, while impermeable compounds such as sucrose (25), LY, or FITC-DX will accumulate. In steady-state conditions protein antigens that are delivered to this compartment will be continuously eliminated by degradation into peptides and subsequently into permeable amino acids (26). Peptides carrying the appropriate motifs may be rescued by MHC class II molecules and transported to the cell surface, where they will effectively accumulate as long lived complexes (27). In this context, the very high level of class II synthesis (up to 10-fold that of EBV-B cells; Sallusto, F., unpublished data) is an important attribute of DCs as it endows them with powerful peptide sampling capacity.

The very high level of macropinocytosis may have important consequences for the traffic of surface molecules. Indeed, while in all cell types surface molecules are internalized via coated pits that act as molecular filters, in DCs all surface molecules must be internalized primarily via macropinocytosis, which is expected to be nonselective. Thus, this alternative pathway will allow molecules that are usually excluded from coated pits to be internalized at high rate, a fact that may have functional consequences in the case of MHC class I and II molecules as discussed below.

The MHC Class II Compartment in DCs. We have shown that DCs generated in culture with GM-CSF and IL-4 are characterized by the presence of a large intracellular compartment that contains class II molecules, cathepsin D, and Lamp-1, and is rapidly accessible to endocytic markers. It is not clear how this compartment relates to the MHC class II compartment described in EBV-B cells and other class II⁺ cells (28–32). Specifically, it will need to be established whether the class II molecules in this compartment are derived only from the biosynthetic pathway or also from mature cell surface molecules taken up via macropinocytosis. Thus, while in B cells mature surface class II molecules are internalized only to a very small extent and recycle through early endosomes and thus do not reenter the compartment where antigen processing occurs (33, 34), it is possible that in DCs mature class II molecules may be internalized at high rates via macropinocytosis into this compartment and recycle back to the cell surface. It will be important to establish whether empty cell surface class II molecules may be efficiently internalized and loaded with peptide from processed antigen in DCs. It is interesting that the kinetics of antigen processing in DCs is much faster than in antigen-specific B cells (35), suggesting that the trafficking of antigen and class II molecules may be different in these two cell types (Sallusto, F., unpublished data).

Induction of DC Maturation In Vitro. A breakthrough in understanding the function of DCs has been the identification of two stages of maturation (6). According to this scheme, immature DCs residing in nonlymphoid tissues take up and process antigen and migrate to the regional lymph nodes, where they arrive as mature DCs that have lost antigen capturing and processing capacity and that have gained increased T cell stimulatory capacity (7, 8). The nature of the signal

that induces maturation and migration of DCs in vivo is not clear, although there is some evidence that TNF- α may have an important role (36–39).

We have used DCs generated in culture with GM-CSF and IL-4 as a model of immature DCs to identify the signals that may induce their maturation. We have found that DCs respond not only to TNF- α , but also to CD40L, IL-1, and LPS with a coordinate series of changes that include a complete downregulation of macropinocytosis and a disappearance of the class II compartment, concomitant with an upregulation of adhesion and costimulatory molecules. These changes occur within 24–48 h and are irreversible, since the immature phenotype is not recovered when the maturation-inducing stimulus is removed. Interestingly, the uptake of HRP and FITC-DX is downregulated to a much higher extent than the surface MR, which is only partially downregulated. This discrepancy suggests that in immature DCs, macropinocytosis may be the major pathway of internalization of the MR.

The complex changes in DCs best serve their physiolog-

ical function. The upregulation of CD44V expression (9) and possibly downregulation of E-cadherin (40) may change their migratory properties. The reduction of pinocytosis, the downregulation of MR, and the loss of the class II compartment result in “freezing” the cell into the presentation of the antigen that was present at the time of contact with the maturation inducing signal. Finally, the increase in adhesion and costimulatory molecules will facilitate priming of naive T cells.

It is tempting to speculate that the capacity of DCs to respond with these changes to inflammatory cytokines or exogenous bacterial products might have developed to favor the earliest possible recognition of invading pathogens, since presentation of antigens from dangerous pathogens will be favored compared to presentation of nondangerous self antigens (41, 42). The identification of substances that induce DC maturation and modulate their function may help to understand the regulation of antigen presentation in physiological and pathological situations and may lead to the design of more effective adjuvants.

We thank Werner Lesslauer and Hansruedi Loetscher for providing GM-CSF and the TNF- α mutants, Wayne Hein for providing sheep afferent lymph, Mark Dessing for help with confocal analysis, Colin Watts and Joel Swanson for discussion and suggestions, Philip Stahl for help in characterizing the anti-MR antibody, and Marco Colonna, Gillian Griffiths, and Kristian Hannestad for critical reading of the manuscript.

The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche & Co. Ltd. (Basel, Switzerland).

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Received for publication 14 October 1994 and in revised form 27 March 1995.

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