# Antigen Capture and Major Histocompatibility Class II Compartments of Freshly Isolated and Cultured Human Blood Dendritic Cells

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## Summary

Dendritic cells (DC) represent potent antigen-presenting cells for the induction of T cell–dependent immune responses. Previous work on antigen uptake and presentation by human DC is based largely on studies of blood DC that have been cultured for various periods of time before analysis. These cultured cells may therefore have undergone a maturation process from precursors that have different capacities for antigen capture and presentation. We have now used immunoelectron microscopy and antigen presentation assays to compare freshly isolated DC (f-DC) and cultured DC (c-DC). f-DC display a round appearance, whereas c-DC display characteristic long processes. c-DC express much more cell surface major histocompatibility complex (MHC) class II than f-DC. The uptake of colloidal gold–labeled bovine serum albumin (BSA), however, is greater in f-DC, as is the presentation of 65-kD heat shock protein to T cell clones. The most striking discovery is that the majority of MHC class II molecules in both f-DC and c-DC occur in intracellular vacuoles with a complex shape (multivesicular and multilaminar). These MHC class II enriched compartments (MIIC) represent the site to which BSA is transported within 30 min. Although MIIC appear as more dense structures with less MHC class II molecules in f-DC than c-DC, the marker characteristics are very similar. The MIIC in both types of DC are acidic, contain invariant chain, and express the recently described HLA-DM molecule that can contribute to antigen presentation. $CD19^+$ peripheral blood B cells have fewer MIIC and surface MHC class II expression than DCs, while monocytes had low levels of MIIC and surface MHC class II. These results demonstrate in dendritic cells the elaborate development of MIIC expressing several of the components that are required for efficient antigen presentation.

The classical cells expressing MHC class II molecules are B cells, macrophages, and dendritic cells (DC)[1]. DC are much more potent initiators of T cell responses than other APC types (1–9), and they have the capacity to overcome MHC-linked T cell immune response defects (10, 11). In addition, DC retain and present antigen for a relatively long period of time (12–14). Two stages of maturation of DC have been described. They exist as immature cells in nonlymphoid organs, exemplified by Langerhans cells (LC) in the skin (15–17), and by DC in the lung (18), where they ingest and process exogenous antigens. Subsequently, after migration to the T cell areas of lymphoid organs, they present antigenic peptides in the context of MHC class II molecules as activated mature cells (2, 15–17, 19). DC originate from the bone marrow and are distributed to various organs via the blood. Immature DC are therefore likely to be present in the blood. In this study, we have addressed the kinetics of antigen ingestion into freshly isolated DC (f-DC) and cultured DC

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[1] *Abbreviations used in this paper:* BSA-gold, BSA conjugated to 5 nm colloidal gold; c-DC, cultured dendritic cell; DAMP, 3-(2,4-dinitroanilino)-3-amino-*N* methyldipropylamine; f-DC, freshly isolated dendritic cell; I-chain, invariant chain; $Er^-$, erythrocyte rosetting negative; LAMP, lysosome-associated membrane protein; LC, Langerhans cell; MIIC, MHC class II compartment; RER, rough endoplasmic reticulum; TGR, *trans*-Golgi reticulum.


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(c-DC) by immunoelectron microscopy with special focus on colocalization of antigen and MHC class II in subcellular late endosomal/prelysosomal compartments (MIIC) and by a functional antigen processing and presentation assay. Compartments highly enriched for MHC class II molecules were first described in human B cells (20). MIIC have been isolated from macrophages (21), and recently endosomal compartments enriched in MHC class II were isolated from B lymphocytes and melanoma cells (22–25). Together, these studies strongly indicate that MHC class II–enriched compartments play an important role in loading MHC class II molecules with antigenic peptide.

We found that f-DC display immature DC-like characteristics such as minimal cytoplasmic processes and lower cell surface MHC class II expression; on the other hand, c-DC resemble mature DC displaying many long characteristic processes and high MHC class II cell surface expression. In agreement with a greater endocytic capacity, measured by the uptake of BSA conjugated to 5 nm colloidal gold (BSA-gold) particles, f-DC demonstrated superior antigen processing and presentation ability of intact protein when compared to c-DC. f-DC and c-DC contained MIIC, and the MIIC were found to be the site to which antigen is transported within 30 min after antigen exposure. Importantly, MIIC in f-DC and c-DC had similar characteristics, except for differences in morphology and I chain labeling.

## Materials and Methods

### *Isolation of DC*

*f-DC.* PBMC of healthy donors were isolated by harvesting the interphase of a buffy coat layered onto Ficoll-Paque (Pharmacia, Uppsala, Sweden). Every purification step was done on ice to prevent activation of the f-DC. T cells were separated by rosetting at 4°C with AET-treated SRBC. If SRBC persisted in the erythrocyte rosetting negative ($Er^-$) fraction $NH_4CL$ was used to lyse the SRBC. The $Er^-$ cells were left overnight on ice. CD14-, CD15-, CD16-, CD19-, and CD56-coated goat anti-mouse beads (Dynal, Inc., Great Neck, NY) were used to deplete the $Er^-$ cells for monocytes, B cells, NK cells, and granulocytes. The remaining cells were antibody labeled to be purified on a FACStar® cell sorter (Becton Dickinson and Co., Mountain View, CA). The cells were incubated with anti-HLA-DP (1:6,000) for 20 min at 4°C for the first step and GAM-F(ab)' FITC (Tago Inc., Burlingame, CA) as a second step. In the third step, the cells were incubated with anti-CD16-PE for 20 min. To prevent aspecific binding of antibodies, we blocked the Fc receptor by incubation of the cells with rabbit serum (20× diluted in RPMI, 20 min at 4°C). Then the cells were stained with CD3-PE, CD14-PE, CD19-PE, and CD56-PE. PE-negative and $FITC^+$ cells were sorted on a FACScan® cell sorter (Becton Dickinson and Co.). If necessary, 5 μg/ml DNAse (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to disrupt aggregates.

*c-DC.* A slightly modified version of the isolation method of Freudenthal et al. (26) was used. The $Er^-$ fraction was obtained as described above. The $Er^-$ cells were cultured for 36 h in 10-cm tissue culture dishes (Costar, Badhoevedorp, The Netherlands) at 37°C in humidified air at a concentration of $2 \times 10^6$ cells per ml in RPMI (Gibco Laboratories, Paisley, Scotland) supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml kanamycin and 10% FCS (Hyclone Laboratories, Logan, UT). After a 36-h culture period, cells were incubated twice on new tissue culture dishes for 40 min to deplete the adherent cells (the majority of these were monocytes). The T cell– and monocyte-depleted cells were layered onto a 14.5% metrizamide (dissolved in RPMI with 10% FCS) column to remove residual B and NK cells. The DC-enriched interface was harvested and washed before incubation on a tissue culture dish. The remaining nonadherent cells were antibody-labeled as described above for purification on a FACStar® cell sorter.

Both f-DC and c-DC were stained with CD14-PE, CD16-PE, CD19-PE, and HLA-DR–PE to check the purity of the sorted cell suspensions. The cells were analyzed on a FACScan® flow cytometer (Becton Dickinson and Co.).

For the antigen presentation assay, we used HLA-DR15–type healthy blood donors. PBMC ($5 \times 10^6$) were stored at 4°C overnight (f-DC procedure) or in liquid nitrogen (c-DC procedure) to be used in the antigen processing and presentation assays.

### *mAbs for DC Isolation*

Anti-CD14, -CD15, -CD16, -CD19, -CD56, –CD3-PE, –CD14-PE, –CD16-PE, –CD56-PE, –CD19-PE, and –HLA-DR–PE antibodies were commercially obtained from Becton Dickinson and Co. The anti–HLA-DP antibody was provided by Dr. A. Mulder (AZL, Leiden, The Netherlands).

### *Immunoelectron Microscopy*

After isolation, cells were prepared for ultrathin cryosectioning and immunogold labeling according to Tokuyasu as adapted by Slot et al. (27). Briefly, cells were fixed in 2% paraformaldehyde and 1% acrolein for 3–4 d, washed twice in PBS, twice in PBS 0.15 M glycine, and finally embedded in 10% gelatin, which was solidified on ice. Small gelatin blocks were infiltrated with 2.3 M sucrose for 3 h at 4°C and then frozen in liquid nitrogen. Ultrathin cryosections were embedded in a mixture of sucrose and methyl cellulose according to a novel procedure that allows better visualization of membranes (28). Immunogold labeling was performed as described (29). Ultrathin cryosections were single labeled with 10-nm gold particles or double immunolabeled with 5- and 10- or 10- and 15-nm gold particles. The following antibodies were used: anti–MHC class II (polyclonal antibody recognizing the α chain; 30), DNP (3-[2,4-dinitroanilino]-3-amino-*N*-methyldipropylamine [DAMP] antibody; 31), CD20 (B cell marker), FS2 (DMα chain antibody; 32), ICN (I chain cytoplasmic tail, aa 1–20) kindly provided by Dr. Ph. Morton (Monsanto Co., St. Louis, MO), ICC (I chain luminal epitope, aa 191–210; 33) kindly provided by Dr. J. Pieters (Dept. of Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, The Netherlands), mouse mAb directed against the lysosomal protein CD63 (34), and rabbit anti–human lysosome-associated membrane protein (LAMP)-1 (35).

### *Internalization of Endocytic Tracer*

A pulse-chase experiment with BSA-gold (36) was used to visualize the endocytic route. Cells were pulsed for 10 min at 37°C in serum-free medium containing BSA-gold, washed, and chased for 0, 20, or 50 min at 37°C. Endocytosis was stopped in ice-cold medium and the cells were washed in cold medium to remove the surface-adhered gold. Cells were then fixed and processed as described above. To quantitate the number of MIIC containing BSA-gold in cryosections immunolabeled with MHC class II antibodies, 20 cell profiles were selected for each time of incubation. Quantita-

tion was performed directly in the electron microscope at an instrumental magnification of 15,000.

### *Detection of Acidic Compartments*

The weak base DAMP was used to visualize compartments with acidic contents. f-DC and c-DC were incubated for 30 min at 37°C in 50 μM DAMP in RPMI medium, washed with an excess of ice-cold medium, and then fixed in 2% paraformaldehyde + 0.2% glutaraldehyde. DAMP is able to diffuse through cell membranes and penetrate into cell organelles, where it accumulates in acidic compartments. Nonbound DAMP can be removed by washing. Ultrathin cryosections were double immunolabeled with antiDNP antibody, which recognizes DAMP and MHC class II antibody, visualized by 5- and 10-nm gold particles, respectively.

### *Cell Lines*

The EBV-transformed human B cell lines, GN (HLA-DR15$^+$), and JY (HLA-DR15$^-$) were cultured in RPMI medium containing 10% FCS.

The CD4$^+$ T cell clone R2F10 (gift from Dr. T. Ottenhoff, AZL) recognizes a peptide (aa 418–427) of the 65-kD heat shock protein (hsp65) of *Mycobacterium leprae* in the context of HLA-DR15 (37–39). The T cell clone was cultured in IMDM (Gibco Laboratories) supplemented with 100 IU/ml penicillin, 100 μg/ml kanamycin, 10% pooled human serum (HPS), 20 U/ml rIL-2 (Eurocetus, Amsterdam, The Netherlands), and $4 \times 10^{-8}$ M hsp65 protein (40). The hsp65 protein was a gift from Dr. J. D. A. van Embden (RIVM, Bilthoven, The Netherlands) and Dr. M. Singh (GBF, Braunschweig, Germany). Both institutes received financial support from the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases to purify and distribute recombinant proteins.

### *Antigen Processing and Presentation Assay*

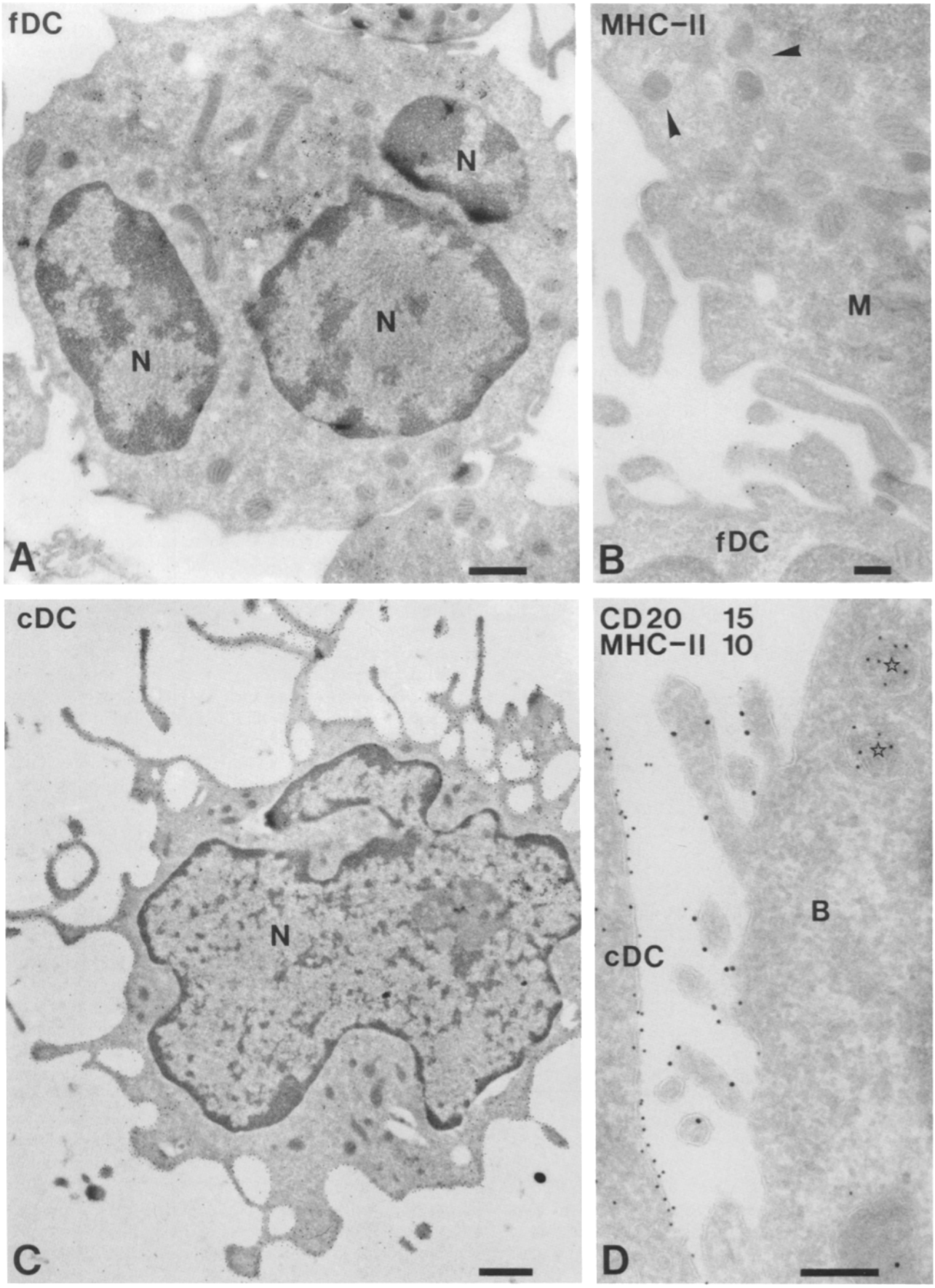
The processing and presentation efficiency of hsp65 protein as a model antigen was measured by culturing APC with the T cell clone R2F10 in the presence or absence of soluble whole protein. As APC we used f-PBMC (PBMC of the f-DC donor), c-PBMC (PBMC of the c-DC donor), f-DC, c-DC, HLA-DR15$^+$ (GN), and HLA-DR15$^-$ (JY) EBV-transformed B cells. PBMC and DC were irradiated with 3,000 rad and the EBV-transformed B cells with 6,000 rad. The cells were cultured in 150 μl 10% HPS containing IMDM in 96-well flat-bottom microtiter plates (Costar) for 72 h at 37°C, 5% $CO_2$ in humidified air, the last 8 h in the presence of 1 μCi of [$^3$H]thymidine. [$^3$H]Thymidine incorporation into DNA was determined by liquid scintillation counting. Each measurement was performed in triplicate. In the first set of experiments, the number of APC was titrated using a fixed amount of purified hsp65 protein ($8 \times 10^{-8}$ M). In the second set of experiments, fixed amounts of APC were used (2,500 DC and 10,000 PBMC), and the amount of hsp65 protein was titrated. The experiments shown were representative of results obtained in three independent experiments. The SEM was always <2%.

## Results

*Identification of Freshly Isolated and Cultured DC.* DC travel from bone marrow via the blood to the periphery to ingest antigen and, subsequently, from the site of antigen to a site of presentation to T cells. We compared f-DC and c-DC (36 h of culture) derived from blood to examine whether they represent immature and mature DC phenotypes, respectively. In both types, we monitored the subcellular localization of MHC class II, the endocytic capacity, as well as antigen processing and presentation capacity. f-DC and c-DC were obtained from human peripheral blood of healthy donors (HLA-DR15$^+$ if DC were used in antigen presentation assays). DC suspensions of 85–90% purity (f-DC) and 90–95% purity (c-DC) were generated by FACS® sorting (Becton Dickinson and Co.; data not shown). Contamination with B cells and monocytes was <2.5%. First, we identified and characterized DC in the sorted fractions using immunoelectron microscopy. f-DC showed only a few short cytoplasmic processes (Fig. 1 *A*) as compared to c-DC, which displayed many long processes (Fig. 1 *C*). Both f-DC and c-DC had a lobulated nucleus with a distinctive pattern of euchromatin and heterochromatin (Fig. 1, *A* and *C*). The B cell marker CD20 was used to identify B cells (Fig. 1 *D*), whereas monocytes were identified by the presence of characteristic azurophilic granules in their cytoplasm and low MHC class II expression (Fig. 1 *B*). The amount of MHC class II cell surface expression on f-DC and especially c-DC compared to B cells and monocytes was strikingly high (Fig. 1). As expected, B cells contained the classical multilaminar MIIC (Fig. 1 *D*), as described for B-lymphoid cell lines (20). Monocytes hardly showed distinguishable MIIC (Fig. 1 *B*). In most of the electromicroscopy studies, we used the starting material for cell sorting (see Materials and Methods) to obtain a high number of cells for sectioning.

*Subcellular Localization of MHC Class II Molecules.* f-DC and c-DC were distinguished not only morphologically but also by the amount of detectable MHC class II (Figs. 1 and 2). c-DC displayed higher MHC class II expression than f-DC, both at the plasma membrane and intracellularly (Fig. 2, *A* and *B*). After quantitation of MHC class II labeling, we found an average of 89 gold particles on the plasma membrane and 160 intracellularly per f-DC cell profile. For c-DC, the numbers for plasma membrane and intracellular MHC class II labeling were 590 and 262, respectively. The total plasma membrane length of c-DC compared to f-DC was approximately twice that of f-DC. Thus, the density of MHC class II molecules on the plasma membrane of c-DC was about three times higher than on f-DC. Intracellularly, in both f-DC and c-DC, the MHC class II labeling was concentrated in typical MIIC present in the Golgi region (see below), with either internal vesicles and/or membrane sheets (Fig. 2). The number of MIIC in f-DC (9 per cell profile) and c-DC (11 per cell profile) was about twice as much compared to B cells (5 per cell profile), and monocytes almost did not express the MIIC at all. In comparison to c-DC, the MIIC in f-DC were more electron dense and had less MHC class II labeling per compartment (Fig. 2 *A*). Neither type of DC showed significant MHC class II labeling in the rough endoplasmic reticulum (RER) or Golgi complex (Fig. 2).

*Characterization of MIIC.* In B cells (20) and macrophages (41), MIIC represent late endocytic prelysosomal compartments. To investigate the functional presence of MIIC in f-DC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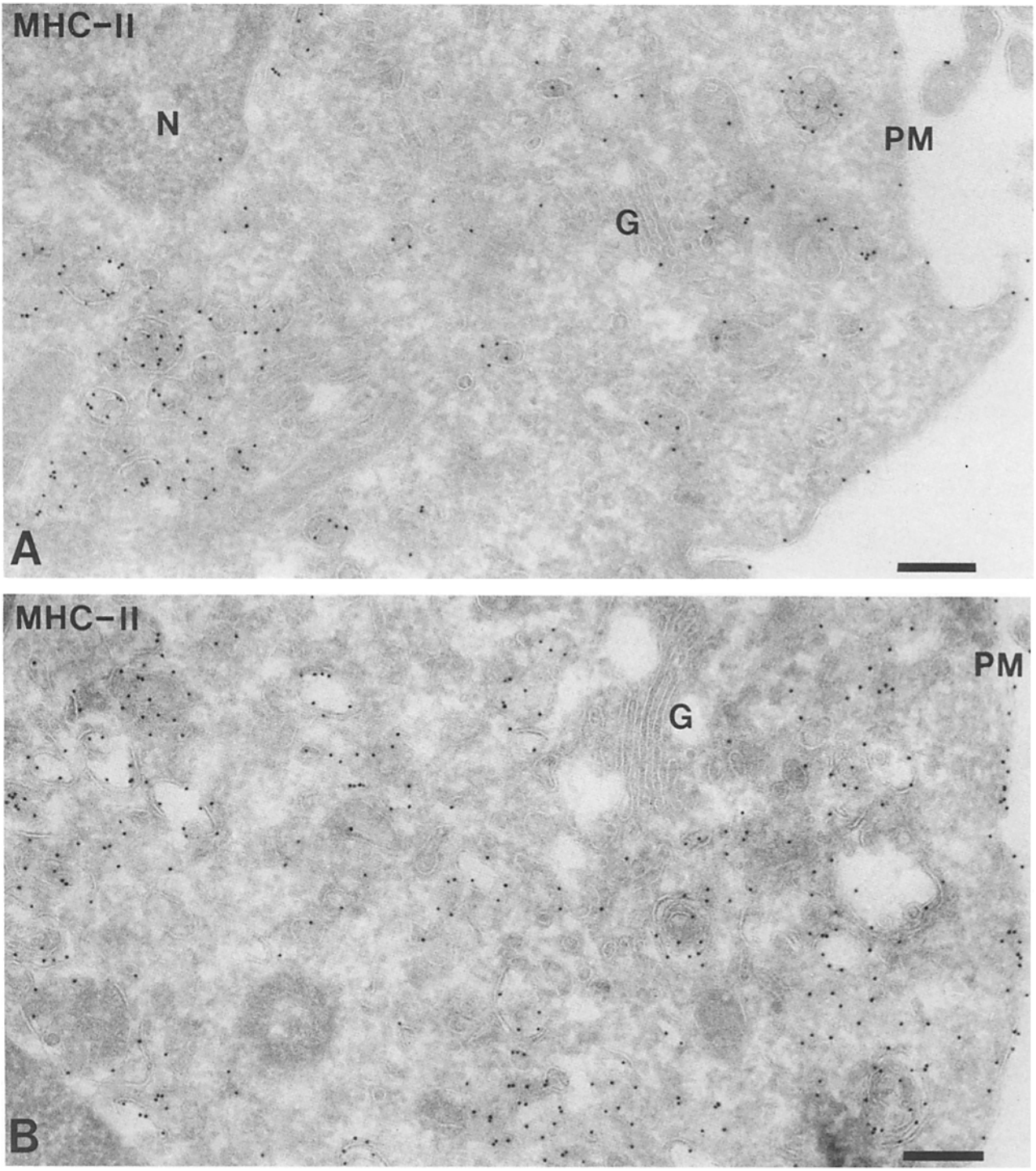


**Figure 2.** Intracellular distribution of MHC class II. (*A*) f-DC showing some MHC class II labeling at the plasma membrane (*PM*) and in MIIC, and little labeling is present in the Golgi complex (*G*). *N*, nucleus. (*B*) c-DC showing higher expression of MHC class II at the plasma membrane (*PM*) as compared to *A*, whereas labeling for MHC class II in the Golgi region (G) is similar to f-DC. Many MIIC are present in the cytoplasm, some with a multilaminar appearance, or with an electron-lucent content with internal vesicles. Bars, 200 nm.

**Figure 1.** Identification of DC in ultrathin cryosections. (*A*) f-DC, showing a lobulated nucleus, short cytoplasmic processes, and low surface labeling of MHC class II compared to c-DC. *N*, nucleus. Bar, 1 μm. (*B*) A monocyte (*M*) is identified by the presence of dense azurophilic granules (*arrowheads*) in the cytoplasm and no detectable MHC class II labeling at the cell surface in contrast to the f-DC. Bar, 200 nm. (*C*) c-DC showing long cytoplasmic processes and a lobulated nucleus. Note the abundant labeling of MHC class II on the plasma membrane. Bar, 1 μm. (*D*) The B cell (*B*) is labeled for the B cell marker CD20 and for MHC class II. Note the presence of two MIIC (*asterisks*) in the cytoplasm of the B cell. There is a striking difference in the density of MHC class II gold labeling between the c-DC and the B cell. Bar, 200 nm.

and c-DC, we allowed the cells to take up 5-nm BSA-gold particles and we determined the time for the tracer to reach the MIIC. After a pulse of 10 min uptake and a chase for 0, 20, and 50 min, cells were fixed and ultrathin cryosections were immunogold labeled with an MHC class II antibody and 10-nm gold particles. We quantitated the number of MIIC that contained BSA-gold at each time point in 20 cell profiles of f-DC and c-DC. Fig. 3 shows that after 10 min of uptake, only occasional BSA-gold was detectable in MIIC of both DC types. After 20 min of chase 2.2 MIIC per cell profile in c-DC and 3.5 MIIC in f-DC contained BSA-gold. A further increase in the number of MIIC positive for BSA-gold was found after 50 min of chase for both types of DC, 4.2 MIIC per c-DC and 7.7 MIIC per f-DC contained BSA-gold. This corresponded to 45% of the MIIC in c-DC and 85% of the MIIC in f-DC. Note that in the f-DC, about twice as many MIIC had acquired BSA-gold, compared to the c-DC (Fig. 3). These data thus demonstrate that significant numbers of MIIC acquire internalized tracer particles only after 30 min and therefore the MIIC in both DC types are typical of late endocytic compartments. The higher endocytic capacity of f-DC compared to c-DC is in agreement with data on total BSA-gold uptake after 10 min pulse.

We next determined whether MIIC are acidic organelles, as is expected for prelysosomal compartments. For this purpose, DC were incubated in the presence of the weak base DAMP, which accumulates in acidic structures, e.g., endosomes, lysosomes (31). Ultrathin cryosections were double immunolabeled with DNP antibody, which recognizes DAMP, and with MHC class II antibody. Since the MIIC in f-DC and c-DC displayed similar characteristics, only micrographs of MIIC in c-DC are shown. MIIC in DC showed abundant DNP labeling (Fig. 4 *A*). To determine whether MIIC in DC showed late endosomal/lysosomal marker proteins, we performed double immunolabeling experiments for MHC class II molecules and the lysosomal membrane proteins CD63 or LAMP-1. MIIC in DC showed labeling for both CD63 (Fig. 4 *B*) and LAMP-1 (not shown).

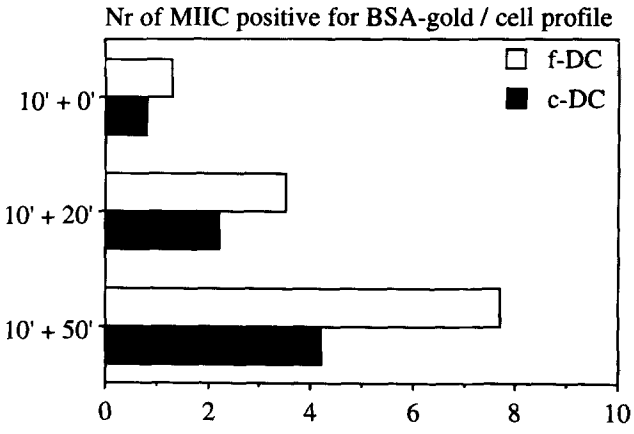


**Figure 3.** Quantitive analysis of the appearance of BSA-gold in MIIC after incubation of f-DC and c-DC in the presence of 5-nm BSA-gold for 10 min and further incubation in the absence of the tracer for 20 and 50 min. Shown are the number of MIIC containing BSA-gold. The figure shown is representative for experiments done in duplicate.

*I Chain and HLA-DM Localization and Distribution.* To further explore the differences between f-DC and c-DC, we compared their I chain localizations and distributions. Since the I-chain is associated with MHC class II molecules during the early part of the biosynthetic route, and is degraded, probably starting from the luminal part, shortly after passage of the Golgi complex (42), the presence of the I chain allowed us to distinguish between different stages of MIIC. For the immunolocalization of the I chain, we used antibodies against its $NH_2$-terminal cytoplasmic tail (ICN, aa 1–20) and its COOH terminus luminal part (ICC, aa 191–210). Both f-DC and c-DC (not shown) showed ICN and ICC labeling in the RER, Golgi region, and MIIC (Table 1). The luminal part of the I chain was found in MIIC with a distinct morphology with electron lucent content and few internal vesicles (Fig. 5 *A*). These MIIC probably represent early stages in their formation and were located in the Golgi region (Glickman, J. N., P. A. Morton, J. W. Slot, S. Kornfeld, and H. J. Geuze, manuscript in preparation). The cytoplasmic tail of the I chain could also be detected in dense MIIC (Fig. 5 *A*).

To obtain a semiquantitative impression of the distribution of ICN and ICC, gold particles on 15 cell profiles were counted of both f-DC and c-DC (Table 1). The ICN and ICC antibodies were mainly detected in the RER, except for ICN in f-DC (57% in MIIC). The percentage of MIIC labeling decreased for ICN and for ICC in c-DC (Table 1). Interestingly, both ICN and ICC can be detected in small amounts on the cell surfaces of f-DC and c-DC (Table 1).

The novel MHC product HLA-DM has been shown to be essential for proper binding of antigenic peptide to MHC class II (43). It has recently been shown that HLA-DM is enriched in MIIC of B cells, while it was undetectable at the cell surface. It was therefore of interest to study the localization of this molecule in DC. Using a DM$\alpha$ chain-specific rabbit antiserum (32), the MIIC of both f-DC (Fig. 5 *B*) and c-DC (not shown) were HLA-DM$^+$, while the cell surface was negative. Most of the HLA-DM labeling was seen in MIIC that contained BSA-gold particles that were internalized during 60 min (Fig. 5 *B*).

*Endocytic Capacity and Antigen Presentation Assay.* To investigate whether differences in MHC class II expression between f-DC and c-DC are related to their capacity to take up and present antigen, we studied the endocytic capacity by allowing the cells to internalize BSA-gold particles as an endocytic tracer. We quantitated the total number of BSA-gold particles taken up after 10 min at 37°C in 20 random cell profiles. f-DC had taken up 269 gold particles per cell profile and c-DC 139 gold particles per cell profile, indicating that f-DC were more efficient in endocytosis. To extend these findings, we studied the f-DC, c-DC, and PBMC of HLA-DR15$^+$ healthy donors for their capacity to present the *M. leprae* hsp65 to an HLA-DR15-restricted CD4$^+$ hsp65-specific T cell clone. We used either a fixed amount of *M. leprae* hsp65 ($8 \times 10^{-8}$ M) during a 72-h culture period and we titrated the number of APC (Fig. 6 *A*), or we alternatively used a fixed number of APC and titrated the amount of

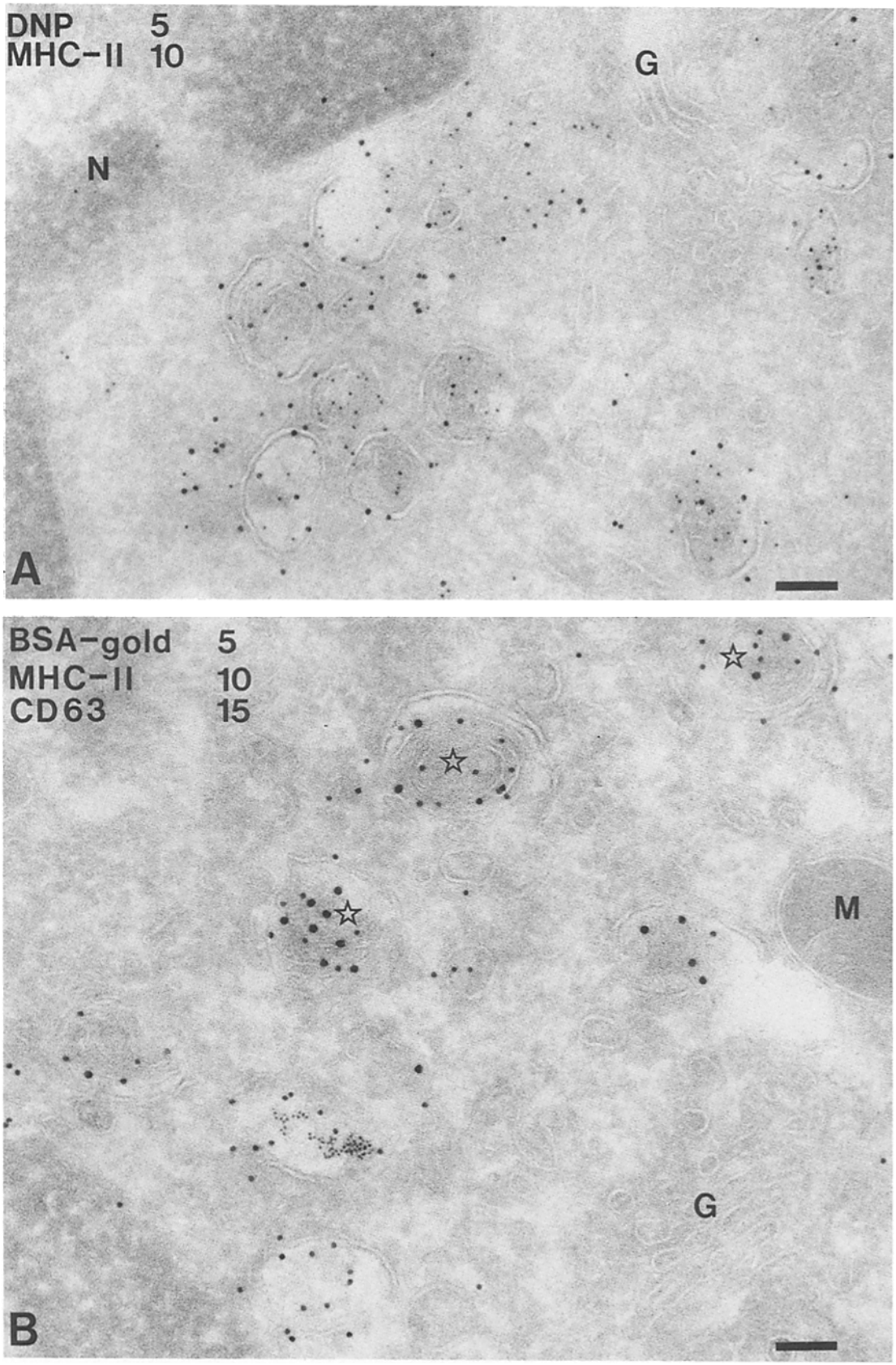


**Figure 4.** Characterization of MIIC. (*A*) The acidic content of MIIC in a c-DC is shown by the accumulation of the weak base DAMP immunolabeled with anti-DNP and 5-nm gold particles. *G*, Golgi complex; *N*, nucleus. (*B*) c-DC double immunolabeled for the lysosomal membrane protein CD63 and MHC class II as indicated on the figure. Especially those MIIC containing membrane sheets show CD63 (*asterisks*). 5-nm BSA-gold that is added for 10 min and chased for 50 min can also be detected in MIIC. *M*, mitochondrion. Bars, 100 nm.

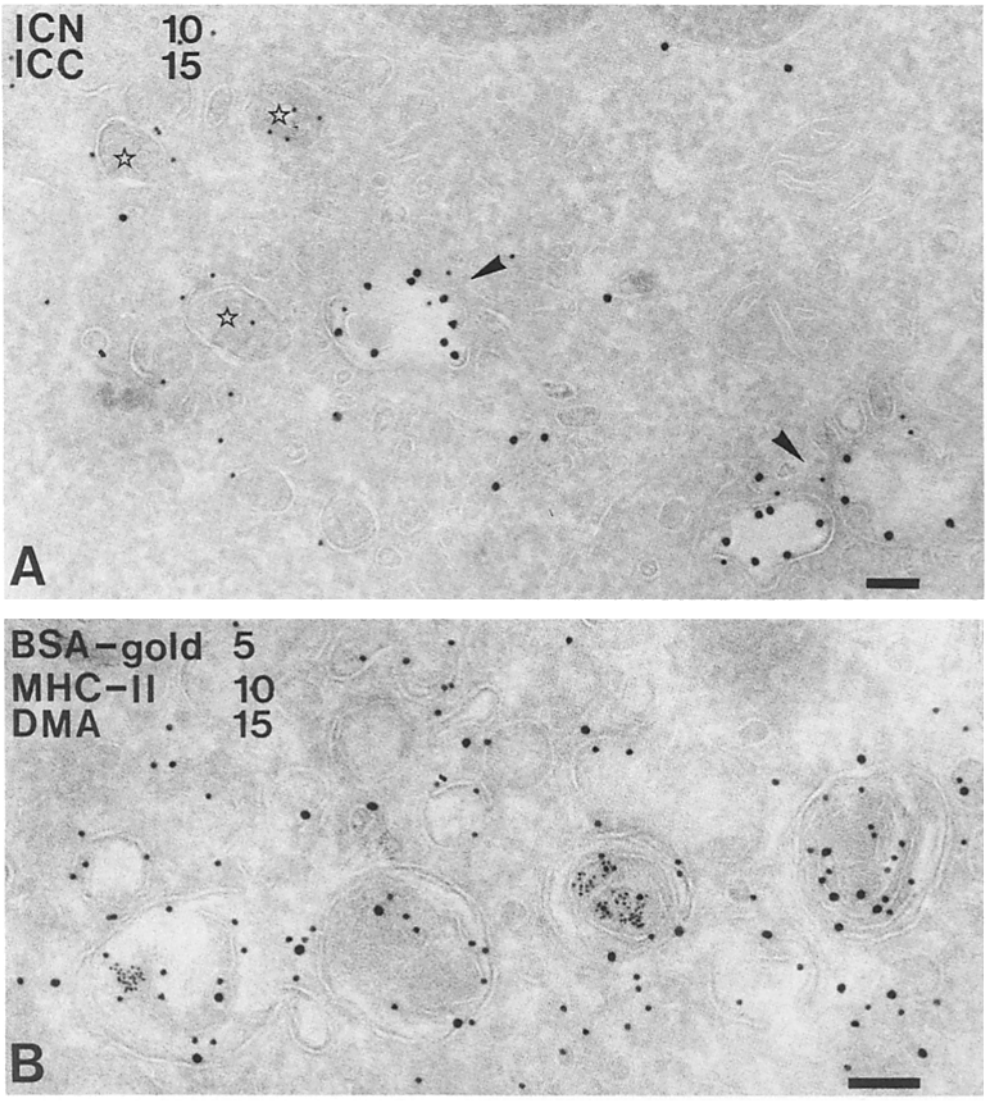


**Figure 5.** Expression of I chain and HLA-DMα in MIIC. (*A*) A double immunolabeling of an f-DC for ICN and ICC shows that multivesicular MIIC (*arrowheads*) contain both the luminal and cytoplasmic part of the I chain. The dense MIIC (*asterisks*) display primarily labeling for ICN. (*B*) MIIC are positive for the α chain of HLA-DM (*DMA*). These MIIC also show 5-nm BSA-gold after 10 min pulse and 50 min chase. Bars, 100 nm.

**Table 1.** *Semiquantitative Immunogold Distribution of I Chain in f-DC and c-DC Using Antibodies against the $NH_2$ Terminus (ICN) and COOH terminus (ICC)*

| | F-DC | | C-DC | |
|---|---|---|---|---|
| | ICN | ICC | ICN | ICC |
| RER | 27% | 51% | 40% | 54% |
| Golgi | 7% | 14% | 15% | 18% |
| TGR | 6% | 8% | 8% | 5% |
| MIIC | 57% | 24% | 32% | 14% |
| Cell surface | 2% | 2% | 3% | 8% |

Gold particles were counted on 15 cell profiles of both f-DC and c-DC in ultrathin cryosections. The numbers represent the percentages of ICN or ICC.

*M. leprae* hsp65 (Fig. 6 *B*). No differences in proliferation of the T cell clone R2F10 after stimulation with purified hsp65 pulsed HLA-DR15$^+$ EBV cell line GN were observed between independent experiments (data not shown), allowing us to compare all data obtained. An hsp65-pulsed HLA-DR15$^-$ EBV cell line used as a negative control did not stimulate the T cell clone (data not shown). GN cells fixed with 2% paraformaldehyde + 0.2% glutaraldehyde did not induce proliferation of the T cell clone in the presence of 5 μg/ml *M. leprae* hsp65 (data not shown), demonstrating that the hsp65 protein is not contaminated with peptide fragments capable of binding exogenously to the HLA-DR15 molecules at the cell surface. Proliferation of the T cell clone was observed after addition of peptide (aa 418–427, $5 \times 10^{-6}$ M) to fixed GN cells (data not shown).

Fig. 6 shows that f-DC are superior in processing and presentation of soluble protein antigen; c-DC are still potent, however. At a fixed antigen dose, f-DC can be diluted ap-

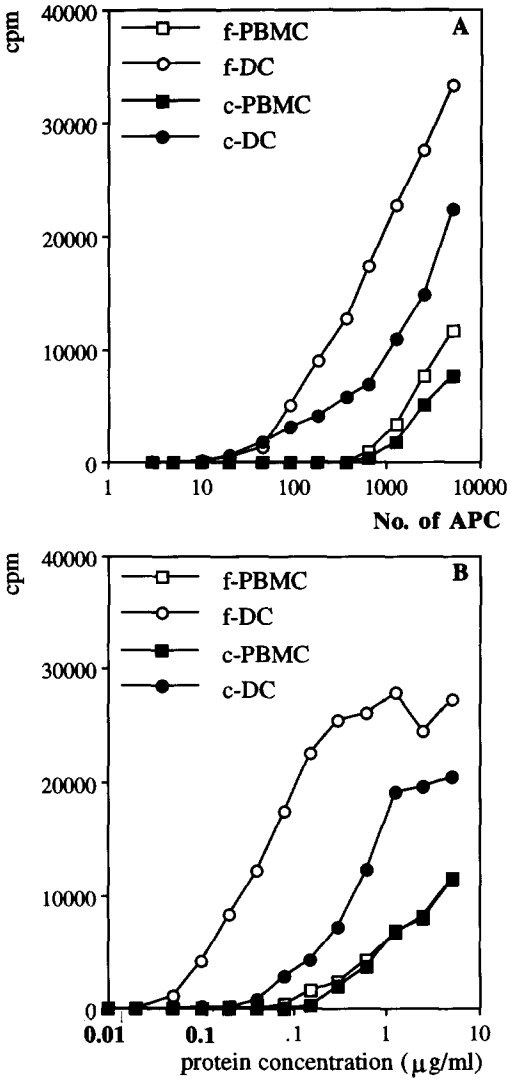


**Figure 6.** Comparison of f-DC, c-DC, f-PBMC, and c-PBMC for their capacity to present *M. leprae* hsp65 to an HLA-DR15-restricted CD4+ hsp65-specific T cell clone. We used a fixed amount of *M. leprae* hsp65 (5 μg/ml) during the 72-h culture period and titrated down the number of APC (*A*), or we used a fixed number of APC (2,500 DC and 10,000 PBMC) and titrated down the *M. leprae* hsp amount (*B*). The experiments shown were representative of results obtained in three independent experiments. The SEM was always <2%.

proximately 4 times more than c-DC and 10 times more than f-PBMC or c-PBMC to induce the same proliferation of the T cell clone (Fig. 6 *A*). Using different concentrations of hsp65 protein, f-DC (2,500 cells per well) induce comparable T cell proliferation at concentrations approximately 10 times lower than c-DC (2,500 cells per well) and 100 times lower than f-PBMC or c-PBMC (10,000 cells per well) (Fig. 6 *B*).

## Discussion

The most striking result of our studies is that protein antigen ingested into human blood DC trafficks to abundant MHC class II+ endosomal prelysosomal compartments (MIIC), presumably the site of antigen degradation and peptide loading into MHC class II molecules. Thus DC and LC (44), the most professional of all APC, use similar subcellular compartments as B cells (20) and the extraordinary antigen processing and presenting qualities of DC and LC are not associated with the presence of unique subcellular organelles. Several phenotypic differences between f-DC and c-DC were found. First, c-DC showed characteristic long cytoplasmic processes, whereas f-DC had little or no such processes. Second, as expected, the MHC class II immunolabeling at the plasma membrane of c-DC was more dense when compared to that of f-DC. These data agree with those obtained from LC, studied either in situ, shortly after isolation, or after short-term in vitro culture (15, 44–46). Similarly, culture of mouse DC leads to an increase in MHC class II expression and remodeling from a rounded morphology to one in which long cytoplasmic processes are present (47, 48). Of note, MHC class II localization shifted from intracellular to the cell surface upon culturing. In addition, the overall MHC class II expression in c-DC was higher. LC studied in situ also contain most MHC class II labeling intracellularly (44). However, LC show a dense labeling of MHC class II in the RER, whereas the RER in f-DC contains little MHC class II labeling. Most of MHC class II in DC is found in the MIIC.

Both f-DC and c-DC contained typical MIIC, similar in morphology and marker profile to those described in other APC (20–25, 41, 44). Characteristics of the compartments isolated from B lymphocytes and melanoma cells (21–25) resemble the MIIC in EBV-transformed B cells and macrophages (20, 41), and they stress the importance of MIIC in antigen processing. The characteristics of the MIIC in DC are significant, especially since they represent the first physiological evaluation of MIIC in the most professional APC. f-DC contained fewer MIIC of a relatively denser structure than c-DC (Fig. 2). Different developmental stages of MIIC can be distinguished by the presence of I chain (in the MIIC with internal vesicles) or the absence of I chain (in MIIC with membrane sheets) (44; Glickman, J. N., P. A. Morton, J. W. Slot, S. Kornfeld, and H. J. Geuze, manuscript submitted for publication). The I chain has two main functions. Part of the COOH-terminal domain of I chain binds to MHC class II, preventing the premature binding of antigenic peptides present in the RER (49–52). The I chain also directs $\alpha/\beta$ dimers into a compartment, the MIIC, located at the intersection of the biosynthetic and the endocytic routes (53–56). After proteolytic cleavage of I chain, $\alpha/\beta$ dimers form a complex with the antigenic peptide, an event that most likely occurs in MIIC (21, 49, 56). The present observations are the first description of the intracellular distribution in DC derived from peripheral blood. In both f-DC and c-DC, pronounced ICN and ICC labeling was found in the RER and in MIIC. In agreement with the activation state of the c-DC, the amount of ICC and ICN is increased in the RER. LC studied in situ showed ICN to be localized mainly in the RER (69%; 44), whereas in f-DC, we detected a large portion of ICN in the RER (27%), but the majority

is present in MIIC. The f-DC contain most of their MHC class II intracellularly. It is therefore not surprising that ICN is so abundantly present in MIIC, keeping the MHC class II molecules in the right state to form a complex with antigen after activation. Irregularly shaped electron-lucent MIIC with internal vesicles were best labeled with antibody against the COOH-terminal part of I chain (ICC). Because the COOH terminus of I chain is degraded immediately after passage to the Golgi complex (41), these multivesicular MIIC most likely represent early MIIC.

Recently, an additional MHC class II product, HLA-DM, has been described. The predicted structure of HLA-DM is a heterodimer consisting of a DM$\alpha$ and $\beta$ chain (32, 57, 58). HLA-DM possibly assists in MHC class II–peptide binding. In the Burkitt lymphoma cell line Raji, HLA-DM was mainly localized in MIIC and was absent from the cell surface (32). Interestingly, HLA-DM was also localized in MIIC of the DC, a professional APC. The important role of HLA-DM in class II–restricted antigen processing has recently been demonstrated (43). Denzin et al. (43) have shown that the antigen processing–defective cell line T2 transfected with HLA-DR3 (T2.DR3) is fully restored in its ability of class II–restricted antigen processing after stable transfection of T2.DR3 with the HLA-DMA and HLA-DMB cDNA.

The superior processing and presenting ability of f-DC compared to c-DC correlates with their superior endocytic capacity. Although the processing and presenting ability of f-DC was superior, c-DC were still capable of processing and presenting soluble antigen, in agreement with other studies (6, 59–62). In this respect, c-DC differ from cultured mouse LC, which virtually lack antigen processing capacity (12, 16). It is likely that during the 72-h incubation period with soluble antigen, the f-DC mature to c-DC, expressing a large amount of MHC class II on the cell surface complexed with the *M. leprae* epitope.

Others have shown that progenitors of human DC are present both in bone marrow and blood, and even subpopulations of f-DC may exist (63–67). With lymphokines such as GM-CSF, IL-4, and TNF, DC precursors developed into mature DC and could be cultured for several weeks (63–65). When TNF was added to such DC cultured with GM-CSF and IL-4, a redistribution of intracellularly localized MHC class II to the cell surface was observed (65). Our c-DC were not kept in the presence of additional growth factors, but it cannot be excluded that factors are produced by other cell types present during culture (68).

Based on their morphology, MHC class II expression, endocytic capacity, and antigen processing and presentation capacity, we suggest that f-DC and c-DC represent immature and mature DC phenotypes, respectively. We show that DC contain MIIC with the same characteristics as in other APC, suggesting that this compartment plays a general role in antigen presentation. We have shown recently that multivesicular MIIC probably play a role in the transport of MHC class II molecules to the cell surface (Raposo, G., H. W. Nijman, W. Stoorvogel, R. Leyendekker, C. V. Harding, C. J. M. Melief, and H. J. Geuze, manuscript submitted for publication). The large amount of multivesicular MIIC in c-DC, together with high MHC class II cell surface expression, is in agreement with this observation.

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