MHC class II and invariant chain biosynthesis and transport during maturation of human precursor dendritic cells

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Abstract

Dendritic cells (DC) are highly potent activators of the immune response. The precise mechanisms that give rise to the DC phenotype are not known. To investigate the mechanisms that contribute to the generation of the DC phenotype, precursor DC were freshly isolated from human blood and allowed to mature in vitro. These matured DC showed the phenotypical and functional characteristics of DC. Analysis of the MHC class II and invariant chain (Ii) biosynthesis revealed that upon maturation, class II synthesis was induced whereas Ii synthesis was significantly up-regulated. In mature DC, despite the presence of large amounts of Ii, export of MHC class II molecules from the endoplasmic reticulum was incomplete, up to 4 h after biosynthesis. Thus, MHC class II–Ii synthesis and transport in DC is highly regulated during maturation of DC. Analysis of the regulatory mechanisms may contribute to a better understanding of antigen-presenting capacities during the differentiation of DC.

Introduction

MHC class II molecules present peptides derived from exogenous antigens at the surface of antigen-presenting cells (APC) to CD4+ T cells (reviewed in 1). MHC class II molecules consist of an α and β chain; upon biosynthesis, αβ dimers become associated in the endoplasmic reticulum (ER) with trimers of the invariant chain (Ii) (2). The association of αβ dimers with Ii has several functions that contribute to enhancement of antigen presentation (3). First, Ii prevents premature binding of antigenic peptides to MHC class II molecules in the ER (4,5). Second, Ii assists in the folding of the class II complexes and their export from the ER (6). After exit from the ER and transport through the Golgi complex, αβ–Ii complexes are targeted to endocytic compartments due to the presence of endosomal/lysosomal targeting signals in the Ii cytoplasmic tail (7–9), illustrating a third function for Ii. Specialized compartments have been identified in various APC, highly enriched in MHC class II molecules, in which Ii is degraded and peptides are loaded onto MHC class II molecules (10,11). From these MHC class II compartments, MHC class II–peptide complexes are transported to the plasma membrane for presentation to T cells (12–15).

In human cells, the Ii gene encodes four forms: a 33 kDa isoform (IiP33) and a less abundant 41 kDa isoform (IiP41) that arises through alternative splicing (16,17). An alternative initiation site gives rise to two other forms, IiP35 and IiP43, that contain an additional 15 amino acid residues (16). IiP41 contains an additional exon (exon 6B) that has been suggested to play a role in intracellular transport as well as in antigen degradation (17). Although it has been suggested that enhanced antigen presentation is restricted to IiP41 (18), when reconstituted in Ii−/− mice, both IiP33 and IiP41 were shown to assist in class II transport and maturation to a similar
degree (19–22). The precise functions of the Ii isoforms IiP33 and IiP41 within the same APC, as is the case in dendritic cells (DC), are not known; Recently, the alternatively spliced exon (exon 6B) present in IiP41 was shown to be a potent inhibitor of cathepsin L (23,24). By virtue of its protease inhibitory effect, IiP41 might alter class II–li trafficking as well as modulate the types of epitopes loaded on class II molecules.

Many types of APC are capable of generating MHC class II–peptide complexes and presenting these to primed T cells, but the activation of naive T cells seems to be restricted to DC (25,26). DC were identified in lymphoid organs of mice as a cell type distinct from mononuclear phagocytes and lymphocytes (27). DC originate from bone marrow and migrate via the blood towards various tissues (26). Freshly-isolated human blood DC can develop into mature DC that show an increase in surface expression of MHC class II and accessory molecules, and an increased stimulatory capacity (26,28–33).

Some of the mechanisms possibly contributing to the potent immunostimulatory capacity of DC have recently been identified. These include a high surface expression of costimulatory and adhesion molecules, several highly active endocytic mechanisms, and the existence of MHC class II compartments (33,34). However, these cannot be the sole contributors to the potent antigen-presenting function of DC, since these characteristics are not unique to DC. An additional level of regulation might be provided through synthesis of MHC class II–peptide complexes and presenting these to primed T cells, since these characteristics are not unique to DC. An additional level of regulation might be provided through synthesis of MHC class II–peptide complexes and presenting these to primed T cells, since these characteristics are not unique to DC.

Methods

Cells and antibodies

Precursor DC were isolated as described below. To obtain DC, pre-DC were cultured for 40 h in 50% RPMI/FCS [RPMI 1640 medium supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamycin, 2 mM L-glutamine, 10% heat-inactivated FCS (Gibco, Paisley, UK) and 100 µM β-mercaptoethanol (Sigma, St Louis, MO)] and 50% conditioned medium (filtered supernatant of an overnight culture of T cell-depleted mononuclear cells).

B cells were isolated from peripheral blood mononuclear cells (PBMC). After removal of monocytes by adherence (2 h at 37°C), non-adherent cells were incubated with anti-CD19–coupled magnetic beads (Dynal, Oslo, Norway) for 40 min on ice. CD19+ B cells were separated using a strong magnet and cultured overnight to allow the magnetic beads to detach. Purity of CD19+ B cells after magnetic cell sorting was ≥90%. The following antibodies were used: Leu4 (anti-CD3; Becton Dickinson, Mountain View, CA), OKM1 (anti-CD11b; ATCC, Rockville, MD), 3G84 (anti-CD16; ATCC), THB5 (anti-CD21; ATCC), anti-CD19–FITC (Dako, Copenhagen, Denmark), anti-HLA-DR–phycoerythrin (PE) (Becton Dickinson), TIB228 (anti-CD14 (34)), 6E10 [anti-transferrin receptor (TfR) (35)], a polyclonal anti-MHC class II antiserum (kind gift from Dr H. L. Ploegh), and the following antibodies against Ii: MDDQ, a rabbit antisera generated against a synthetic peptide consisting of amino acids 1–29 of li, B7U-43 and B7U-45 (36,37; kind gifts from Drs D. L. Hardy and I. C. M. MacLennan), and VIC-Y1 (38; kind gift from Dr W. Knapp).

Isolation of pre-DC

A modified version of the isolation method of O’Doherty et al. (31) was used to isolate pre-DC. Buffy coats were obtained from the Central Laboratory of Blood Transfusion (Amsterdam, The Netherlands) or from Kanton Spital Bietschpande (Basel, Switzerland). Mononuclear cells were isolated by flotation on Lymphoprep (Nycomed, Oslo, Norway); 2–7×10^8 PBMC were obtained from each buffy coat. T cells were removed by adherence to sheep erythrocytes treated with neuraminidase (Calbiochem, La Jolla, CA). The remaining cells were incubated with antibodies against T cells (anti-CD3), monocytes (anti-CD11b), NK cells (anti-CD16) and B cells (anti-CD21). Cells were washed to remove unbound antibodies using HBSS without Ca^2+ and Mg^2+ (Gibco), supplemented with 0.5% w/v BSA fraction V (Sigma) and 1 mM Na-EDTA (Sigma). The cells were panned by adherence to Petri dishes coated with goat anti-mouse IgG (Cappel, West Chester, PA). Non-adherent cells were incubated with goat anti-mouse FITC to stain labeled cells that had not been removed, washed in HBSS with 1% w/v mouse serum and subsequently incubated with anti-CD19–FITC to stain remaining B cells and with anti-HLA-DR–PE to stain remaining B cells. Cells were purified using a FACStar Plus cell sorter equipped with Consort 30 software (Becton Dickinson). The instrument was set up using single-labeled samples; cellular debris and dead cells were excluded by setting a forward scatter threshold. Pre-DC (2–7×10^5) were obtained from each buffy coat.

Electron microscopy

Cells were fixed in 1.5% v/v glutaraldehyde (Merck, Darmstadt, Germany) in Na-cacodylate (0.1 M, pH 7.4) at 4°C. After fixation in 1% w/v OsO_4 (Merck) in Na-cacodylate for 1 h at 4°C, the cells were pelleted in 1% agar (Sigma), dehydrated using increasing percentages of ethanol (70, 90, and 100% v/v) and finally propylene oxide, and embedded in a mixture of araldite 502 (Sigma) and epon (Merck) (1:1). Ultra-thin sections were stained with 7% w/v uranyl acetate and lead (1.7% w/v) citrate (1.7% w/v), and examined using a Philips EM 301 electron microscope at 60 kV.

Cell staining

For surface-staining, 10^4 cells were incubated with HLA-DR–PE and washed 3 times in 0.1% w/v BSA in PBS (PBS/BSA). To reveal both intracellular and surface staining, 10^5 cells were fixed in 3% w/v paraformaldehyde (Merck) for 20 min at 4°C, treated with 0.5% w/v saponin in PBS/BSA, blocked with 0.1% w/v glycine and incubated with HLA-DR–PE. Washing was performed in 0.5% w/v saponin in PBS/BSA. Samples
Fig. 1. Purification of pre-DC. Pre-DC were isolated from human blood. Method of isolation (A). Characterization of isolated cell populations during purification (B). Dot-plots of 4×10^3 events after two color staining (CD3, CD11b, CD16, CD19 and CD21–FITC versus HLA-DR–PE) on aliquots of each cell fraction: (a) PBMC, (b) T cell-depleted PBMC, (c) non-adherent cells after panning and (d) pre-DC. The percentages of the boxed dots are given in each panel and represent the percentage of pre-DC.

were analyzed using a FACScan flow cytometer (Becton Dickinson). The instrument was set up as described for the sorting.

For confocal analysis, cells were adhered to glass slides coated with 50 μg/ml poly-L-lysine (Sigma), fixed in 0.1% glutaraldehyde and 3.7% formaldehyde in PBS, and permeabilized in 0.2% Triton X-100. After quenching by washing in 0.5 mg/ml NaBH₄, the cells were incubated with a control or polyclonal anti-MHC class II antiserum, followed by FITC-labeled secondary antibodies (Zymed, South San Francisco, CA). Slides were analyzed using a confocal laser scanning microscope system (MRC-600; BioRad, Hercules, CA) attached to an Axiovert 35M microscope (Carl Zeiss, Thornwood, NY).

Mixed lymphocyte reaction

T cells were isolated from buffy coat blood by adherence to sheep erythrocytes treated with neuraminidase (Calbiochem). After lysis of the erythrocytes with NH₄Cl in PBS, the cells were cultured at 37°C overnight in RPMI/FCS. The non-adherent cells were >95% T cells, as determined by CD3 staining on cytospin preparations. After irradiation with 2000 rad of 37Cs γ radiation, pre-DC and DC were co-cultured with 2×10^5 T cells from the same allogeneic donor for 6 days in 96-well plates in 200 μl RPMI/FCS. As a control, pre-DC, DC and T cells were cultured alone. Sixteen hours before cell harvesting, 1 μCi [3H]thymidine (Amersham, Amersham, UK) was added to each well. Incorporation of the isotope was measured in a liquid scintillation counter and expressed as the average c.p.m. per well of triplicate cultures. The HLA-DR–PE antibody used for sorting of pre-DC did not interfere with T cell stimulation.

Fluid-phase endocytosis

Pre-DC and DC were incubated with 0.1 mg/ml DNP-albumin (Sigma) in RPMI/FCS for 2 h at 37°C and 5% CO₂. Subsequently, cells were fixed using 2% w/v paraformaldehyde and 0.1% v/v glutaraldehyde and impregnated in Lowicryl HM20 as described before (39). Ultra-thin sections were labeled with anti-DNP antibodies (Dako) followed by 20 nm Protein A gold. The number of gold particles as well as background labeling on the nucleus was quantified in 20 cell profiles in a Philips EM 401 electron microscope at a magnification of ×3000.

Metabolic labeling

Metabolic labeling and pulse-chase was essentially performed as described before (11). Briefly, cells were cultured for 20 min in methionine and cysteine-free medium prior to labeling. This medium was replaced by methionine and cysteine-free medium containing 0.5 mCi/ml [35S]methionine/cysteine mix (Amersham). After 20 min, cells were washed in RPMI/FCS containing 2 mM methionine and 2 mM cysteine, and incubated in the same medium for the times indicated in the figure legend. At the times indicated, cells were lysed in 20 mM HEPES (pH 7.5) with 100 mM NaCl, 5 mM MgCl₂ and 1% Triton X-100 with protease inhibitors (10 μg/ml chymostatin, 10 μg/ml leupeptin, 10 μg/ml apronin, 10 μg/ml antipain, 10 μg/ml pepstatin and 1 mM PMSF).

Immunoprecipitation and electrophoresis

Cell lysates from 10⁶ pre-DC and DC or 2×10⁶ B cells were incubated with antibodies (polyclonal anti-MHC class II antiserum, a combination of MDDQ, BU-45 and VIC-Y1 or
66Ig10] at 4°C for 2 h, followed by the addition of 40 µl Protein A–Sepharose [Pharmacia, Uppsala, Sweden (1:1 slurry in low salt buffer, see below)]. After 1 h incubation at 4°C, the immune complexes were washed 3 times with 1 ml of low salt buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% NP-40 and 2 mM EDTA), 3 times with 1 ml of high salt buffer (10 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.2% NP-40 and 2 mM EDTA) and twice with 1 ml of 10 mM Tris–HCl, pH 7.5. Where indicated, samples were treated for 16 h at 37°C with 2 ml endoglycosidase H (Endo H; Boehringer Mannheim, Mannheim, Germany) in 100 mM Na-acetate, 150 mM NaCl and 9 mM CaCl2 or as a control incubated for 16 h at 37°C in the same buffer in the absence of Endo H. The immune complexes were eluted from the Protein A–Sepharose beads by incubation at 95°C for 7 min in Laemmli sample buffer (40), and subjected to 12% SDS–PAGE (40), fluorography and autoradiography.

Quantification of gels was performed using a Phospho-Imager (Fuji Bas 2000 TR) and attached PCBas Beta software (Raytest Isotopenmessgeräte, Staubenhardt, Germany).

Western blotting
Cell lysates from DC were incubated at 95°C for 7 min in Laemmli sample buffer (42) and subjected to 12% SDS–PAGE. Proteins were transferred to nitrocellulose by semi-dry blotting. After incubation of the membrane in 5% low-fat milk in PBS containing 0.2% Tween 20, the nitrocellulose was incubated with antibodies to Ii (BU-43 and VIC-Y1), washed and incubated with peroxidase-coupled goat anti-mouse antibodies (Chemicon, Temecula, CA) and developed by chemiluminescence (ECL; Amersham).

Results

Purification of pre-DC
DC originate from the bone marrow and are transported through the blood to various organs. There they are capable to capture antigens that are carried to the lymph nodes for priming T cells. To obtain early pre-DC from peripheral blood, a modified procedure originally described by O’Doherty et al. (29) was followed. In this procedure PBMC are depleted of T cells, B cells, NK cells and monocytes, and enriched for HLA-DR cells (Fig. 1A). In contrast to O’Doherty et al., we used a narrow window to select HLA-DRlow cells only (Fig. 1B); this purification resulted in a homogeneous population (Fig. 1B, panel 4 and below). The final purity of the sorted cells ranged from 90 to 98%. These cells are referred to as pre-DC.

Characterization of pre-DC and DC
To reveal the ultrastructure of pre-DC, cells were fixed, and embedded in araldite and epon. Morphological analysis demonstrated that the isolation procedure resulted in a homogeneous population. As can be seen in Fig. 2(A), the pre-DC appear as round cells with only small cytoplasmic processes. The cells contained abundant rough ER, especially apparent at the periphery of the cell.

During 40 h culture of pre-DC in conditioned medium (see Methods), the cells matured into typical DC (Fig. 2B). A representative field of DC after fixation and embedding in araldite and epon is shown in Fig. 2(B); no other cell types could be detected. The cells had enlarged and developed longer cytoplasmic processes. Rough ER was still abundantly present in most of the DC. Furthermore, the number of electronlucent vesicles had increased during culture. Thus, pre-DC freshly isolated from human blood as described above matured into cells that can be morphologically defined as DC.

Mature DC display a high surface expression of MHC class Fig. 2. Morphology of pre-DC and DC. Pre-DC were fixed using 1.5% glutaraldheyde, and embedded in a mixture of araldite and epon (1:1). Ultra-thin sections were stained with uranyl acetate and lead citrate (A). The same procedure was followed after 40 h culture in conditioned medium (B). Magnification: ×11,450. N, nucleus; arrow, rough ER.
II (26). To investigate the expression of MHC class II on pre-DC and DC, both populations were incubated with anti-HLA-DR–PE and analyzed using flow cytometry. The intensity of HLA-DR surface staining increased during culture of pre-DC (Fig. 3A and B, left profiles). Furthermore, to reveal surface and intracellular expression, cells were fixed with 3% paraformaldehyde, treated with 0.5% saponin and stained with anti-HLA-DR–PE. The difference between the left and the right profile gives an indication of the intracellular MHC class II expression. Intracellular MHC class II molecules were present in both pre-DC and DC; the amount of intracellular MHC class II was higher in pre-DC during maturation (Fig. 3).

To visualize the distribution of MHC class II molecules in pre-DC and DC, cells were adhered to poly-L-lysine-coated glass slides, fixed, and permeabilized and stained with polyclonal anti-MHC class II antiserum, followed by a FITC-labeled secondary antibody.

As shown in Fig. 3(C), pre-DC contained low amounts of MHC class II molecules on the cell surface, whereas intracellularly they were concentrated at a perinuclear location. Consistent with the flow cytometry analysis, the expression of MHC class II molecules on the plasma membrane had increased during maturation (Fig. 3D).

Thus, the pre-DC described here are characterized by a low surface MHC class II expression which increased during in vitro maturation.

To relate the differentiation stadia of pre-DC and DC with their T cell stimulatory capacities, both populations were tested for their ability to stimulate allogeneic T cell proliferation in a mixed lymphocyte reaction. Irradiated pre-DC and DC were incubated with T cells at ratios varying from 1:1000 to 1:10. After 6 days, proliferation of T cells was determined by [3H]thymidine incorporation as described in Methods. As is shown in Fig. 4, the stimulatory capacity of pre-DC increased ~150-fold during culture, most probably as a result of higher surface expression of MHC class II, as well as the up-regulation of co-stimulatory molecules (29).

Taken together, the in vitro maturation of pre-DC resulted in morphologically defined DC with a high capacity to stimulate T cell proliferation.

**Fluid-phase endocytosis in pre-DC and DC**

Peptides presented by MHC class II molecules are usually derived from protein antigens that enter the cell through endocytosis. DC were recently shown to be highly active in endocytosis (34). The endocytic capacity of the pre-DC and DC described here was analyzed by quantitative immunocytochemistry. Cells were incubated with 0.1 mg/ml DNP-albumin for 2 h at 37°C. Subsequently, cells were fixed and embedded in Lowicryl-HM20. Ultra-thin sections were labeled with anti-DNP antibodies followed by Protein A gold. The mean number of gold particles in 20 cell profiles relative to background labeling on the nucleus was quantified. In both pre-DC as well as in DC, DNP-albumin uptake could be detected. As is shown in Fig. 5, the uptake of DNP-albumin in DC was up to 3 times higher than in pre-DC. These data do not distinguish between the various routes of internalization; they do, however, indicate an overall increase of endocytic activity upon maturation of pre-DC.

![Fig. 3. Surface and intracellular expression of MHC class II. For surface staining, 10⁴ pre-DC (A) and DC (B) were incubated with HLA-DR–PE and washed (left profile). For intracellular and surface staining, 10⁴ cells were fixed in 3% paraformaldehyde for 20 min at 4°C, treated with 0.5% saponin and incubated with HLA-DR–PE (right profile). Samples were analyzed using a FACScan flow cytometer. For confocal analysis, pre-DC (C) and DC (D) were adhered to glass slides coated with poly-L-lysine, fixed and permeabilized. After quenching, the cells were incubated with polyclonal anti-MHC class II antiserum, followed by FITC-labeled secondary antibodies. Slides were analyzed using a confocal laser scanning microscope system. Magnification: ×4500.](image-url)
MHC II–Ii biosynthesis and transport during DC maturation

Fig. 4. Stimulation of allogeneic T cells. Pre-DC and DC were co-cultured with $2 \times 10^5$ allogeneic T cells at the indicated ratios for 6 days. $[^{3}H]$Thymidine incorporation was determined as described in Methods and is depicted as average c.p.m. of triplicate cultures $\pm$ SEM: Pre-DC alone incorporated, 113 $\pm$ 99 c.p.m.; DC alone, 254 $\pm$ 135 c.p.m.; and T cells alone, 569 $\pm$ 78 c.p.m.

Fig. 5. The endocytic capacity of pre-DC and DC. Pre-DC and DC were incubated with 0.1 mg/ml DNP-albumin for 2 h at 37°C and 5% CO$_2$. Subsequently, cells were fixed and embedded in Lowicryl-HM20. Ultra-thin sections were labeled with anti-DNP antibodies followed by Protein A gold. The number of gold particles in 20 cell profiles was determined relative to background labeling on the nucleus and expressed as mean $\pm$ SD: Pre-DC, 49 $\pm$ 31.4; DC, 134 $\pm$ 64.9; $P < 0.001$.

Fig. 6. Biosynthesis of MHC class II and Ii. Pre-DC and DC were metabolically labeled for 20 min using $[^{35}S]$methionine/cysteine, and lysed as described in Methods. Proteins were immuno-precipitated with anti-class II antibodies (cl II) or with anti-invariant chain antibodies (II). Shown are autoradiographs after SDS–PAGE and fluorography; each lane corresponds to 5000 cells (A). Quantification of the amount of radioactivity was performed using a PhosphoImager (B). Shown are the counts of the total radioactivity after immunoprecipitation with anti-class II antibodies (cl II), and the counts of II$\beta$3 and II$\beta$4 after immunoprecipitation with anti-II antibodies.

Biosynthesis of MHC class II and Ii

In addition to up-regulation of endocytic capacity, antigen presentation by DC might also be enhanced by induction of biosynthesis of MHC class II molecules and Ii. To test this, the synthesis of these glycoproteins was studied using metabolic labeling. Pre-DC and DC were metabolically labeled with $[^{35}S]$methionine/cysteine for 20 min, lysed and proteins were immunoprecipitated with antibodies to respectively MHC class II and Ii molecules. The results after SDS–PAGE and autoradiography are shown in Fig. 6(A).

Newly synthesized MHC class II molecules could not be detected in pre-DC, although these cells did synthesize low amounts of the P33 isoform of Ii (Fig. 6A). In contrast, after maturation, DC synthesized MHC class II molecules that were associated with the two isoforms of Ii, II$\beta$3 and II$\beta$4 (Fig. 6A).

To analyze the total cohort of Ii molecules being synthesized in DC, a mixture of antibodies against the N- and C-terminal domain of Ii was used (see Methods) As shown in Fig 6(A), both the II$\beta$3 and II$\beta$4 isoforms were detected in DC; in addition, a molecule of 66 kDa mol. wt, probably representing the II$\beta$3 dimer, was present in the anti-II immunoprecipitates.

Although in mature DC newly synthesized class II–Ii complexes were readily detected in lysates prepared from as little as 5000 metabolically labeled cells (Fig. 6A, lane 3 and 4), pre-DC did not synthesize any measurable class II molecules (Fig. 6A, lane 1). However, it cannot be excluded that small amounts of newly synthesized molecules escaped detection. To account for the limitations posed by fluorography (optical density is not linear with exposure times $>60$ h), we determined directly the amount of radioactivity associated with the proteins present in the dried gel by exposure to a PhosphoImager screen. This analysis confirmed the absence of MHC class II synthesis in pre-DC (Fig. 6B). During DC maturation MHC class II synthesis is induced, whereas II$\beta$3 (13-fold) and, most notably, II$\beta$4 (30-fold) biosynthesis is dramatically up-regulated.

To control for non-specific induction of MHC class II–Ii biosynthesis, the HLA-DR–PE$^+$ non-DC markers–FITC$^+$ population (containing T cells, B cells and monocytes) was
MHC II–Ii biosynthesis and transport during DC maturation

Fig. 7. Intracellular transport of MHC class II and Ii. Pre-DC (A), DC (B) and B (C) cells were labeled for 20 min with [35S]methionine/cysteine and proteins were immunoprecipitated with anti-MHC class II antibodies (cl II), with anti-Ii antibodies (i) or with anti-TfR antibodies either directly (0 h) or after 4 h of chase (4 h). Half of each sample was incubated for 16 h with 2 mU Endo H at 37°C (+). Shown are autoradiographs after SDS–PAGE and fluorography. Arrow: 30 kDa molecule co-precipitated with Ii. I: P33*: Endo H sensitive form of I; P33. Counts were calculated as a percentage of the total counts in each lane. In D, the amount of radioactivity associated with endo H resistant α-chains of MHC class II in the dried gels is depicted after quantification using a PhosphoImager.

Intracellular transport of MHC class II and Ii

MHC class II–peptide complexes are predominantly formed intracellularly in MHC class II compartments (12–14). MHC class II compartments are found in large amounts in DC (33,34). Newly synthesized MHC class II–Ii complexes are transported from the ER to these compartments where Ii is degraded and peptides are loaded on MHC class II molecules (12–14,41). To establish the fate of the newly synthesized MHC class II and Ii in pre-DC and DC, pulse–chase analysis was performed. Cells were metabolically labeled with [35S]methionine/cysteine for 20 min, washed, and cultured for an additional 4 h prior to lysis and immunoprecipitation using anti-MHC class II and anti-Ii antibodies. Immuno-isolated complexes were treated with Endo H that selectively cleaves high mannose oligosaccharides that are acquired in the ER and are converted to complex oligosaccharides in the Golgi complex. Although not all carbohydrates acquire Endo H resistance upon transit through the Golgi complex, at least in human cells, MHC class II molecules have been shown to become Endo H resistant after export from the ER (11,42).

Results after pulse–chase and Endo H treatment are shown in Fig. 7.

As was shown above, biosynthesis of MHC class II molecules was not detectable in pre-DC (Figs 6 and 7A). In pre-DC, newly synthesized IiP33 remained in the ER during a 4 h chase (Fig. 7A), consistent with the fact that Ii remains largely in the ER in the absence of MHC class II molecules (2).

When transport of newly synthesized MHC class II molecules was analyzed in DC, only 50% of the MHC class II molecules were transported out of the ER during a 4 h period, as analyzed by the acquisition of Endo H-resistant glycans (Fig. 7A and D, left panel). Thus, despite the large excess of newly synthesized Ii over MHC class II molecules in DC, that functions in the export of MHC class II molecules from the ER in other cell types, MHC class II molecules are being retained in the ER. This retention was specific for MHC class II molecules; after a 4 h chase, all TfR molecules synthesized during a 20 min pulse were transported out of the ER as judged by an apparent higher mol. wt (Fig. 7B). Furthermore, all newly synthesized MHC class II molecules in freshly isolated B cells were exported from the ER as judged by their acquisition of Endo H resistance (data not shown), indicating that the retention of MHC class II molecules is specific for DC.

When the fate of Ii isoforms was analyzed 4 h after biosynthesis, it appeared that most of the IiP33 remained in the ER in DC (Fig. 7A), as is the case in other APC (42). The IiP41 isoform and the IiP33 dimer could not be detected 4 h after biosynthesis. However, during the 4 h chase, a molecule of 30 kDa became associated with IiP33 in the ER (Fig. 7A, arrow). This 30 kDa molecule is a glycoprotein that is retained in the ER as it remained fully sensitive to Endo H treatment, up to 4 h after biosynthesis. Immunoprecipitation of MHC class II molecules after a 4 h chase revealed that this 30 kDa molecule could also be detected in the anti-MHC class II immunocomplexes (Fig. 7A, left panel). In similar pulse–chase experiments on freshly isolated B cells, this 30 kDa molecule was not observed (Fig. 7C).

To analyze the possibility that the 30 kDa protein represented a degradation product of II, proteins from DC were
subjected to SDS–PAGE followed by transfer to nitrocellulose as described in Methods. To detect Ii-related molecules, a mixture of anti Ii N-terminal and C-terminal antibodies was used. As shown in Fig. 8, IiP33 isoforms could be detected; no bands of lower mol. wt are present even after prolonged exposure. This indicates that the MHC class II/Ii associated 30 kD molecule as detected in metabolically labeled lysates (left panel) or a mixture of domain specific mAbs against Ii (right panel), followed by peroxidase coupled secondary antibodies and visualization by ECL.

Discussion

DC are potent APC of MHC class II-mediated immune responses. They originate from bone marrow and migrate via blood towards various tissues. Tissue DC capture and process antigens; during migration to lymph nodes they mature, resulting in the ability to present peptides derived from these antigens to T cells. Recently, a number of studies have addressed the factors involved in the transition of tissue DC to lymph node DC (26, 34, 43). However, the events leading to the maturation of a tissue DC from precursor cells are poorly understood. As knowledge of these various activation stadia is important for understanding the in vivo mechanism of antigen-presenting function, we have analyzed early events of MHC class II-mediated immune responses in DC isolated from human peripheral blood.

Pre-DC were isolated using a modified procedure originally described by O’Doherty et al. (29). This procedure relies on the depletion of T cells, B cells, NK cells and monocytes, and enrichment of HLA-DRαβ cells. In contrast to O’Doherty et al., we selected HLA-DRlow cells only. This purification resulted in a homogeneous cell population as characterized by electron microscopy. Possibly due to this additional purification step, we could not detect the more mature subset of DC that was characterized in this population by CD11c expression and a higher surface expression of HLA-DR. The pre-DC described here express MHC class II molecules, although they do not synthesize MHC class II molecules (see below). A recent report described the presence of HLA-DR on CD34+ bone marrow progenitors that could give rise to T, B, NK and DC (44). Pre-DC lack the expression of CD34 (data not shown). It is conceivable that the pre-DC described here do evolve from these CD34+ progenitor cells, having lost the CD34 marker but retaining MHC class II expression. This indicates that the pre-DC described here are at a very early stage of the DC lineage (Table 1).

**In vitro maturation of the pre-DC resulted in a cell population with DC morphology based on the development of cytolytic processes, a high capacity to stimulate T cell proliferation and endocytic capacity. These characteristics have also been described for Langerhans cells isolated from the skin (reviewed in 26).** Recently, methods have been described that make use of the cultivation of PBMC in IL-4 and granulocyte macrophage colony stimulating factor that have phenotypic and functional characteristics of tissue DC (43, 45). The matured DC described here relate phenotypically and functionally to these tissue DC (Table 1); furthermore, they did not express CD83 (data not shown), a marker for lymph node DC (46–48). Therefore, the isolated pre-DC are highly immature and can be used to analyze factors involved in the induction of tissue DC.

Recent studies have elucidated some of the mechanisms that contribute to the very potent immunostimulatory capacity of DC. These include uptake of antigens via mannose receptor-mediated endocytosis and high levels of macrophocytosis (34; 36). In accordance with this, we show an up-regulation of endocytic capacity during maturation of pre-DC. Large numbers of MHC class II compartments have also been described in DC (31, 34). An important factor that might regulate efficient antigen presentation is induction of biosynthesis of MHC class II and Ii. We have investigated MHC class II and Ii biosynthesis during maturation of pre-DC into mature DC. As a result of the low frequency of DC in blood and the high degree of purity following the isolation procedure used, very low cell numbers were obtained. Despite these low cell numbers, however, MHC class II and Ii expression could be readily detected in mature DC. Based on quantitation by Phospholmager analysis, our results show an induction of synthesis of MHC class II molecules and a 13-fold increase in Ii synthesis when pre-DC mature into DC. This suggests that antigen processing and presentation by DC requires newly synthesized MHC class II molecules, and is not merely reflected by redistribution (33) or recycling (49, 50) of pre-existing MHC class II molecules.

After synthesis of MHC class II molecules, the αβ complexes associate with Ii (2). Ii assists in transport of the class II complexes from the ER to MHC class II compartments in which Ii is degraded and peptides are loaded onto MHC class II molecules (6–15, 51). In various cell lines, export of MHC class II–Ii complexes out of the ER and degradation of associated Ii occurs within 4 h (10, 52). In DC, however, despite a large excess of newly synthesized Ii, only 50% of the MHC class II molecules are transported out of the ER within 4 h. During this time MHC class II and Ii molecules in the ER become associated with a 30 kD molecule. This 30 kDa molecule was not immunologically related to either MHC class II or Ii molecules, as analysed using a panel of mono- and polyclonal antibodies. At present we cannot exclude the possibility that P30 represents an Ii degradation product.
product. However, the selective association of this molecule with MHC class II and Ii molecules in the ER of DC and not in other APC suggests a function of P30 in the transport of MHC class II molecules in DC. This 30 kDa molecule could function in the observed retention of the MHC class II–Ii complexes in the ER and possibly in preventing degradation of MHC class II–Ii complexes. The retarded transport of MHC class II molecules out of the ER would result in a slow release of MHC class II molecules to intracellular loading compartments, enabling DC to load peptides on MHC class II molecules during a longer period of time.

DC synthesize IiP41 isoforms parallel to large amounts of IiP33, as has also been shown for Langerhans cells (53–55). In addition to the functions performed by IiP33 (20–22), IiP41 has been implicated in altering the transport characteristics of both IiP33 and MHC class II complexes (17,18,56). Furthermore, recent studies suggest a regulatory function of IiP41 in antigen processing; The alternatively spliced exon (6B) present in IiP41 associates with the endosomal protease cathepsin L and inhibits its protease activity (23,24). Such inhibition of endosomal/lysosomal proteolysis might alter class II–Ii trafficking pathways, e.g. retain class II–Ii complexes for prolonged times within intracellular sites (23) as well as generation of epitopes to be loaded on class II molecules. The fact that in DC IiP41 is 30-fold up-regulated suggests that this property of IiP41 plays an important role in the generation of class II–peptide complexes in DC.

Taken together, the results presented here and the recent demonstration of the high levels of endocytosis of DC and the existence of MHC class II compartments (33,34) lead us to propose the following model (Table 1). Precursor DC migrate in a HLA-DRlow, non-functional state via the blood to various tissues. There, biosynthesis of MHC class II molecules is induced, as is the synthesis of Ii P33 and, most notably, IiP41. Simultaneously, DC capture antigens via several highly active endocytic mechanisms (34). Due to the slow transport of MHC class II molecules from the ER and the synthesis of large amounts of IiP41 that may result in the retention of class II complexes intracellularly, DC are able to load peptides on MHC class II molecules during a longer time course. Together with an up-regulation of co-stimulatory molecules (28,29) and an increase in cytoplasmic processes, this enables DC to exhibit their potent capacity to activate T cells after migration to lymph nodes.

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