Conformational Variation of Surface Class II MHC Proteins during Myeloid Dendritic Cell Differentiation Accompanies Structural Changes in Lysosomal MIIC[1]

Ilaria Potolicchio,*† Sriram Chitta,‡ Xiaonan Xu,* Dora Fonseca,* Giovanna Crisi,§ Vaclav Horejsi,¶ Jack L. Strominger,‖ Lawrence J. Stern,‡ Graca Raposo,† and Laura Santambrogio[2]*

Dendritic cells (DC), uniquely among APC, express an open/empty conformation of MHC class II (MHC-II) proteins (correctly folded molecules lacking bound peptides). Generation and trafficking of empty HLA-DR during DC differentiation are investigated here. HLA-DR did not fold as an empty molecule in the endoplasmic reticulum/*trans*-Golgi network, did not derived from MHC/Ii complexes trafficking to the cell surface, but was generated after invariant chain degradation within lysosomal-like MHC-II rich compartments (MIIC). In pre-DC, generated from monocytes cultured in the presence of GM-CSF, Lamp-1$^+$MHC-II$^+$ compartments are predominantly electron dense and, in these cells, empty MHC-II molecules accounts for as much as 20% of total surface HLA-DR. In immature DC, generated in presence of GM-CSF and IL-4, empty HLA-DR reside in multilamellar MIIC, but are scarcely observed at the cell surface. Thus, the morphology/composition of lysosomal MIIC at different DC maturational stages appear important for surface egression or intracellular retention of empty HLA-DR. Ag loading can be achieved for the fraction of empty HLA-DR present in the "peptide-receptive" form. Finally, in vivo, APC-expressing surface empty HLA-DR were found in T cell areas of secondary lymphoid organs. *The Journal of Immunology,* **2005, 175: 4935–4947.**

Developmental maturation of myeloid dendritic cells (DC)[3] into the most powerful APC is associated with numerous morphological and functional changes (1–4). Among those quantitatively and qualitatively differences in surface expression of MHC class II (MHC-II) proteins. Immature DC exhibit few MHC-II-peptide complexes, increased surface expression of MHC-II complexed with invariant chain (Ii) and abundant empty MHC class II proteins (5–8). Also total MHC-II immunoprecipitation indicate that in immature DC $>$ 50% of MHC II complexes are SDS unstable at room temperature (9). On the contrary DC stimulated with proinflammatory cytokines express high levels of peptide-loaded MHC-II proteins, no empty molecules and by immunoprecipitation the majority of MHC-II complexes are SDS stable (5–9). Several hypothesis have been advanced to explain these differences; developmentally regulated cleavage of Ii (10), differential surface MHC-II recycling rate (11) and regulated cleavage of molecules involved in endosomal remodeling (12, 13) could all account for the low level of MHC-II peptide complexes in immature cells. On the other hand developmentally regulated phagocytosis and acidification of endosomal compartments could explain the inefficiency in Ag processing and loading, as well as presence of empty MHC-II proteins in immature DC (7, 14, 15).

Ultrastructural analysis of MHC II-rich compartments (MIIC) has revealed variability in their number and morphology among the various types of murine and human APC, between cell lines vs primary cells, and for differences in culturing conditions. In human cells MIIC have been grouped in B cells either as multivesicular bodies (MVB), which have from few to several internal vesicles, or as multilamellar bodies (MLB) (16, 17), which are formed by onion-like concentric lamellae (18). In immature murine DC the most prominent MIIC is the multivesicular type (19, 20). These compartments are reduced in number after LPS treatment as a consequence of a reorganization to vacuolar tubular endosomes (9). In immature human DC, grown in GM-CSF and IL-4, both MVB and MLB are present, although a bias to the MLB type has been observed (21). After DC maturation, the majority of MHC II proteins have been exported at the cell surface, and electron dense unilamellar lysosomes mostly void of MHC-II proteins observed are observed (22). Overall, the relative composition of each of these compartments and their specific contribution to Ag processing and MHC-II loading is not known. Another important aspect of the MIIC biogenesis that is not completely understood is how such compartments originate: whether morphological change of one compartment into another occurs during the developmental maturation of precursors into preimmature and immature DC, or whether new compartments are generated during maturation.

To further understand how empty MHC II molecules would relate to myeloid DC maturation we have investigated 1) the presence of empty/open HLA-DR molecules on human myeloid DC

*Department of Pathology Albert Einstein College of Medicine, New York, NY 10461; †Institut Curie Centre National de la Recherche Scientifique, Unité Mixte de Recherche, Paris, France; ‡Department of Pathology, University of Massachusetts Medical School, Worcester, MA 01655; §Department of Pathology, Baystate Medical Center, Springfield, MA 01199; ¶Institute of Molecular Genetics, Academy of Sciences, Prague, Czech Republic; and ‖Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138

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[2] Address correspondence and reprint requests to Dr. Laura Santambrogio, Department of Pathology Albert Einstein College of Medicine, Forchheimer 538, 1300 Morris Park Avenue, Bronx, NY 10461. E-mail address: lsantamb@aecom.yu.edu

[3] Abbreviations used in this paper: MHC-II, MHC class II; Ii, invariant chain; MIIC, MHC II-rich compartment; MVB, multivesicular body; MLB, multilamellar body; EDB, electron-dense body. ER/TGN, endoplasmic reticulum/*trans*-Golgi network.

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and their precursors, 2) how empty MHC II molecules originate, 3) where they reside intracellularly, 4) how ultrastructural changes in MIIC affect their appearance at the cell surface, and finally 5) their surface loading. We find that open/empty conformations of HLA-DR are expressed on the surface of very immature DC or pre-DC populations and intracellularly are localized in lysosomal but not late endosomal MIIC, from where they can be exported at the cell surface. Ultrastructural modification of lysosomal MIIC occurs during DC maturation. The multilamellar morphology appear to prevent exit of empty/open HLA-DR molecules. Approximately 20% of the empty/open surface molecules could be loaded with exogenously added peptide, a fraction that could be increased in the presence of a HLA-DM-like activity. Finally, pre-DC populations with surface expression of open/empty MHC-II molecules were observed in paracortical and interfollicular areas of secondary lymphoid organs suggesting that the changes in MHC conformation and MIIC morphology observed to accompany DC differentiation in vitro could reflect DC development in vivo.

Materials and Methods

PBMC preparation, culture, and staining

Peripheral blood was obtained by the New York Blood Bank. Lymphomononuclear cells were separated over a Ficoll gradient and either directly stained for analysis or the monocyte population was separated using CD14-conjugated MicroBeads (Miltenyi Biotec). Purified $CD14^+$ cells were cultured in GM-CSF (30 ng/ml) or GM-CSF/IL4 (30 ng/ml plus 10 ng/ml) (R&D Systems) for 5–6 days in all reported experiments unless differently specified. HLA-DRB typing was performed by PCR (Biotest ABDR). Primary Abs used in cytometry were as follows: MEM-265, LB3.1 (27) L243 and CD 74 (BD Bioscience). In some experiments fluorescence values were converted to numbers of receptors per cell by using calibrated flow cytometry beads (QIFIKit; DakoCytomation).

Pulse chase and immunoprecipitation

GM-CSF-differentiated cells were cultured in methionine- and cysteine-free medium complete DMEM containing 5% dialyzed serum for 2 h and then labeled with 1 mCi/ml [^{35}S]methionine (NEN Life Science Products) for 30 min (Pulse). Cells were then washed three times and incubated in complete DMEM supplemented with 10× cold methionine for 4 h (Chase). Cells were lysed in 1% NP40, 150 mM NaCl, 50 mM Tris containing a mixture of protease inhibitors (Complete Mini; Roche Diagnostics) for 30 min on ice, spun at 14,000 rpm for 30 min to remove cell nuclei and debris. The amount of incorporated radioactivity in the postnuclear supernatants was determined using 10% TCA. Equivalent amounts of radioactive lysates were precleared with rabbit serum adsorbed to protein A beads followed by Protein A/G beads alone. Immunoprecipitation was performed using mAb LB-3.1 (10 μg) or a mixture of MEM-266 and MEM-267 (10 μg each) bound to protein A/G beads. The beads were washed three times with lysis buffer and eluted with sample buffer. The eluate, divided as boiled and nonboiled conditions, was resolved by SDS-PAGE.

Peptide binding to surface empty MHC-II

GM-CSF differentiated monocytes were prepared from HLA-DRB1*01 or DRB1*15 donors. Cells were incubated in DMEM containing 0.02% NaN_3 in the presence or in the absence of peptides. In most experiments a 4-h incubation with peptide concentration of 10^{-4} M was used. In other experiments peptide concentrations between 10^{-3} and 10^{-7} M and incubations up to 18 h were used. Peptide sequences HA306–318, PKYVKQNTLKLAT; MBP 86–100, NPVVHFFKNIVTPRT; LR, HSL GKLLGRPDKF. In some experiments 2% isopropanol was also added to the incubation mixture (23). Incubation was conducted at 37°C under tissue culture conditions. After peptide incubation the cells were washed in PBS and stained as usual for surface MEM-265 reactivity. The fraction of peptide-receptive molecules was determined as the ratio of the specific MEM-265 binding for a sample that had been incubated in the presence of peptide to that of a replicate sample incubated in the absence of peptide.

Western blot analysis

Cells were lysed (50×10^6/ml) in 1% NP40, 150 mM NaCl, 50 mM Tris-HCl, protease inhibitor mixture (Complete Mini; Roche Diagnostics) for 30 min on ice. Postnuclear supernatants were normalized for protein content. In each experiment between 50 and 80 μg of total cell lysate were run on a 10% SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane, which was subsequently probed with rabbit serum anti HLA-DR $\alpha\beta$(CHAMP), anti μ1-adaptin (rabbit serum RY/1) gift from L. Traub (University of Pittsburgh, Pittsburgh, PA) and anti-β-tubulin (Sigma-Aldrich). Membranes were then probed with HRP-conjugated secondary mAbs and proteins visualized by chemiluminescence (Pierce).

Real-time PCR for DR1β and CIITA

RNA from monocytes differentiated with GM-CSF and GM-CSF/IL4 was prepared using TRIzol (Invitrogen Life Technologies) and reverse transcribed into cDNA using random primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). Primers and probes for real-time PCR were the following: DR1β forward 5′-GCCAAC CTGGAAATCATGACA-3′, reverse 5′-AGGGCTGTTCGTGAGCACA-3′, and probe 5′-^{FAM6}CAACTATACTCCGATCACCAATGTACCTC^{TAMRA}-3′; GAPDH forward 5′-GAAGGTGAAGGTCGGAGTCA-3′, reverse 5′-GAAGATGGTGATGGGATTTC-3′, and probe 5′-^{FAM6}CAAGCTTCCCG TTCTCAGCC^{TAMRA}-3′; CIITA I forward 5′-CTAGAGAAAGGAGACC TGGATTTG-3′, reverse 5′-TCATAGAAGTGGTAGAGGCACAGG-3′, and probe 5′-^{FAM6} CTGGAGCTTCTTAACAGCGATGCTGACC^{TAMRA}-3′; CIITA III forward 5′-TGGGATTCCTACACAATGCGT-3′, reverse GGGTCA GCATCGCTGTTAAGA, and probe 5′-^{FAM6} CAGAGCCCCAAGGCA GCTCACAGT 3′^{TAMRA}; and CIITA IV forward 5′ CAGCACTCAGAAGC ACGGG-3′, reverse ATCCATGGTGGCACACAGAC, and probe 5′-^{FAM6}TG GTCGCGGCTGTGGCTGTG^{TAMRA}-3′ PCR were performed using the TaqMan PCR core kit.

siRNA for AP-1 μ1 and AP-2 m2 adaptins

A siRNA duplex designed against a conserved sequence of the μ1A protein of the AP-1 complex: AAGGCAUCAAGUAUCGGAAGA and of the μ2 protein of the AP-2 complex: GTGGATGCCTTTCGGGTCA were purchased from Proligo. A sense probe spanning the same sequence was also design for control. Both probes were FITC-labeled at the sense 5′ end. Ten million of GM-CSF-differentiated monocytes were transfected with 120 pmol of the single or duplex probes using Oligofectamine (Invitrogen Life Technologies) as specified by the manufacturer. The transfection mixture was left on the cells for 4 h, after which 1 ml of DMEM/10%FCS was added. After overnight incubation the FITC-high population was sorted, retransfected and incubated for an additional 18 h with 20 ng of recombinant IFN-γ (R&D Systems) or 100 ng of LPS (Sigma-Aldrich). Surface MHC-II and Ii were detected using the anti-MHC-II mAbs MEM-267 and L243 or the anti-Ii mAb to CD74 (BD Biosciences).

Immunogold labeling on ultrathin cryosections

Monocytes grown in presence or absence of GM-CSF or GM-CSF/IL-4 were fixed with a mixture of 2% paraformaldehyde and 4% polyvinylpyrolodone in phosphate buffer 0.2 M (pH 7.4) at 4°C. Fixed cells were processed for ultrathin cryosectioning as described previously (24). Immunogold labeling was performed using primary Abs in combination with protein A coupled either to 10 or 15 nm gold particles. Contrast was obtained with a mixture of 2% methylcellulose (Sigma-Aldrich) and 0.4% uranyl acetate (pH 4) (EMS). Samples were viewed under a CM120 Philips electron microscope (Philips). For the statistical analysis the randomness test was used (25, 26). The following Ab were used: polyclonal anti HLA-DR rabbit sera raised against purified HLA-DR1(42), MEM-265 mouse IgG2b specific for empty/open HLA-DR molecules (27), rabbit serum against the C-terminal of Ii (28), and PIN1 mAb to the N-terminal of Ii.

Immunohistochemistry

Snap-frozen tissue sections, cut at 4 μm, were fixed in form alcohol before immunohistochemical studies. Immunoperoxidase staining was done using the DakoCytomation Envision$^+$ kit (DakoCytomation). Slides were incubated with the primary Abs for 30 min at room temperature, rinsed in 1× Tris-buffered saline, and labeled polymer was applied for 25 min. Ag-Ab reaction was visualized using diaminobenzidine chromogen applied for 7 min, after which sections were counterstained in hematoxylin for 5 min and dehydrated in EtOH and in xylene before manual coverslipping. Abs used were as follows: MEM-265 at 10 μg/ml (mouse IgG2b; Ref. 27), CD11c, a broad spectrum dendritic cell marker at 1/200 (clone KB90, mouse IgG1; DakoCytomation) and control mouse IgG1 or IgG2b isotypes (DakoCytomation). In some experiments the epitope peptides recognized by the MEM Abs were used at 50 μM to block the Ab binding, briefly HLA-DRB1*0101 β 50–69 peptide (VTELGRPDAEYWNSQKDLLE) and MEM-265 were incubated at room temperature for 30 min before applying to the test slides.

Results

Conformational difference in MHC II surface proteins between monocytes cultured in GM-CSF and GM-CSF+IL4

Circulating human monocytes can be induced to differentiate *in vitro* into immature DC. Several culturing methods are used in this regard, and the most widely used for human cells is a combination of GM-CSF and IL-4 (4). For mouse cells, the most common practice is to differentiate cultured bone marrow with GM-CSF (29). Analysis of the surface phenotype of the differentially grown human monocytes indicate that GM-CSF-differentiated cells have a transitional phenotype between monocytes and typical GM-CSF/IL-4-differentiated immature DC (S. Chitta, L. Santambrogio, and L. J. Stern, unpublished observations).

Under GM-CSF culture conditions we previously reported the presence of an empty/open conformation of MHC II molecules at the surface of murine bone marrow DC using an Ab specific for the β1 domain (aa 58–69) of $IA^{s,k}$ (7) (Fig. 1*a*). This result raised the question of whether differences in MHC-II conformation could result from differences in DC culture conditions. Monocytes from

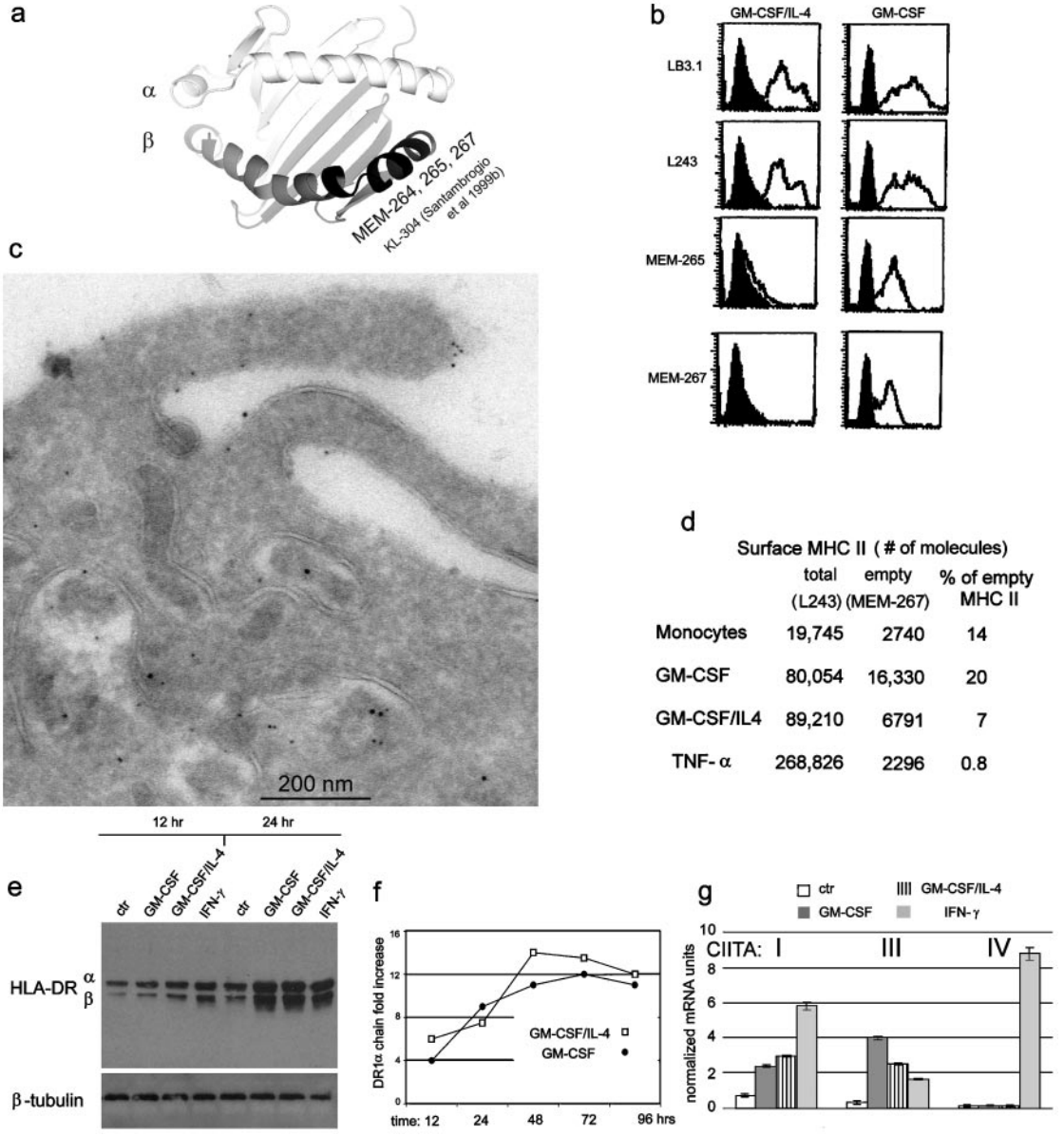

FIGURE 1. GM-CSF but not GM-CSF/IL4 differentiated monocytes express an open/empty conformation of MHC II molecules. *a*, Ribbon diagram of HLA-DR peptide binding site, with α-chain shaded light, β-chain shaded dark, and epitope for Abs that specifically recognize the empty/open conformation MEM-264, -265, and -267 shown in black (27). *b*, Circulating monocytes purified using CD14-coated magnetic beads were cultured for 6 days in GM-CSF (10 ng/ml) or GM-CSF/IL-4 (10 and 5 ng/ml), and differentiated cells were stained with different anti-MHC-II mAb and analyzed by flow cytometry. One of over 12 experiments is shown. *c*, Ultrathin cryosection of monocytes treated with GM-CSF for 6 days. Surface empty/open MHC-II molecule (MEM-265 10 nm of gold) and HLA-DR (5 nm of gold) are indicated. d) Quantification of total (LB3.1) and empty/open (MEM-265) HLA-DRB1*0101 molecules at the surface of monocytes, GM-CSF, or GM-CSF/IL-4-differentiated monocytes or immature DC further differentiated with TNF-α. Data are reported as the actual number of total (LB3.1) or empty (MEM-265) HLA-DR molecules per cell (*Materials and Methods*). *e*, Total cell lysates from monocytes cultured for 12 or 24 h with GM-CSF, GM-CSF/IL-4, or IFN-γ were resolved over SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was probed using a rabbit serum recognizing both α and β subunits of HLA-DR1, and also for β-tubulin as an internal loading control. One of three experiments is shown. *f*, Time course of DRα up-regulation, determined by Western blotting and densitometric analysis. *g*, Real-time PCR of monocytes treated as in *e* for mRNA encoding for the transcription factor CIITA type I III and IV indicates similar level of mRNA between GM-CSF and GM-CSF/IL-4. One of three experiments is shown.

an HLA-DRB1*01 donor were cultured with either GM-CSF or with a combination of GM-CSF and IL-4. After 6–8 days cells were stained with mAbs specific for the β-subunit of human HLA-DR molecules that recognize only the empty or open conformation but not the peptide-loaded form (27). Both GM-CSF and GM-CSF/IL-4-treated cells stained positively with LB-3.1 and L243 Abs, which bind both empty and peptide-loaded HLA-DR1 (30) (Fig. 1*b*), but only GM-CSF-treated cells bound MEM-265 and 267, which recognize the open/empty form of HLA-DR1 (Fig. 1*b*) (27).

The amount of cell surface expression of open/empty MHC-II molecules (MEM-265) and total HLA-DR (LB3.1) was quantified for monocytes, GM-CSF-differentiated or GM-CSF/IL-4-differentiated monocytes, and immature monocyte-derived DC further activated with TNF-α by performing surface staining with saturating amounts of mAbs conjugated with a known number of FITC molecules (Fig. 1*d*). Fluorescence values from each mAb were converted to numbers of receptors per cells using calibrated flow cytometry beads (QIFIKit; DakoCytomation) (Fig. 1*d*).

Only monocytes and monocytes differentiated with GM-CSF expressed a substantial fraction of their cell surface MHC II in the empty/open form (20% of total surface MHC-II for GM-CSF-treated cells) (Fig. 1*d*).

Immuno electron microscopy using gold-labeled anti-DR1 and MEM-265 Abs, verified the presence of HLA-DR complex and empty/open MHC-II molecules at the surface of GM-CSF-treated monocytes (Fig. 1*c*).

Differences between peptide occupancy of HLA-DR in GM-CSF- and GM-CSF+IL-4-treated monocytes are not the result of quantitative differences in MHC-II expression

These findings prompted us to develop experiments to further understand the differences in MHC II expression/conformation between monocytes cultured in GM-CSF and those cultured in GM-CSF/IL-4. As a first approach the steady state level of total MHC-II produced under the two differentiating conditions was examined. Circulating monocytes were purified using CD14 coated magnetic beads, and used immediately or after culture with GM-CSF or with GM-CSF/IL-4. A low dosage of IFN-γ (5 ng/ml) was used as positive control for MHC-II induction. Total cell lysates normalized for protein concentration and analyzed by Western blotting indicated an increase of MHC-II protein expression observed as early as after 12 h of GM-CSF or GM-CSF/IL4 treatment (Fig. 1*e*). The maximal MHC II expression was observed after 48 h in both treatments and persisted with no reduction up to the last time point analyzed (96 h) (Fig. 1, *e* and *f*, and data not shown). GM-CSF and GM-CSF/IL-4 treatments induced similar amounts of MHC-II up-regulation.

Next, the question whether the MHC II protein up-regulation was due to an increase in mRNA level was addressed. Real-time RT-PCR analysis was conducted using RNA extracted from monocytes either untreated or treated for different periods of time with GM-CSF, with GM-CSF/IL4, or with a low dosage of IFN-γ, both GM-CSF and GM-CSF/IL-4 similarly increased mRNA encoding for DR1 β-chain, although at a lower level than observed after IFN-γ treatment (data not shown). The transcriptional up-regulation of MHC-II by GM-CSF was, as previously reported, mediated by CIITA I and II induction (31). Again no significant differences were observed between GM-CSF and GM-CSF/IL-4 treatments (Fig. 1*g*). This series of experiments established that while the total amount of MHC II protein increased upon monocyte differentiation, no quantitative differences were observed between cells treated with GM-CSF or GM-CSF/IL-4. However, as noted above, cell surface staining indicated large differences in MHC II conformation between the two DC populations. Thus, inclusion of IL-4 in the culture medium greatly diminished surface open/empty MHC-II molecules without a change in overall protein expression.

The MEM-reactive species corresponded to peptide-free HLA-DR in the open/empty conformation

To address biochemically the peptide-occupancy of MHC class II molecules in the open/empty conformation, pulse/chase experiments were conducted in monocytes from a HLA-DRB1*01 donor after differentiation in GM-CSF for 6 days. Cells were labeled with [^{35}S]methionine/cysteine for 45 min (pulse) followed by a chase in 10$\times$ cold medium for 4 h. Immunoprecipitation with LB-3.1 after the 45 min pulse pulled down $\alpha\beta$Ii heterotrimers, which as expected dissociate in SDS to the individual α, β, and Ii subunits (Fig. 2*a*). Immunoprecipitation with LB3.1 after 4 h of chase revealed mostly SDS-stable $\alpha\beta$/peptide complexes, which are resistant to SDS-induced subunit dissociation at room temperature (Fig. 2*a*, nonboiled (nb)) but which dissociate into α and β-chains after boiling in SDS (Fig. 2*a*, boiled (b)). By contrast, immunoprecipitation with a mixture of MEM-266 and MEM-267 failed to pull down any complex after the 45-min pulse (only small amounts of free β-chain was visible), indicating that these Abs do not recognize the $\alpha\beta$Ii heterotrimers present in this sample as shown by immunoprecipitation with LB3.1. After 4 h of chase, SDS unstable α-β heterodimers were precipitated by MEM-266/267 (Fig. 2*a*).

It is generally accepted that SDS-stability assays can provide information about the occupancy of the class II MHC peptide binding groove. For HLA-DR, SDS-stable complexes generally contain tightly bound peptides, while SDS-sensitive complexes are either empty, occupied with low-affinity or poorly binding peptides or else are bound to the chaperone Ii or its fragments (32, 33). Thus, from the pulse-chase experiments we concluded that the MEM Abs did not interact with MHC-Ii or MHC-peptide complexes, consistent with previous experiments using purified soluble MHC proteins (27), and that the MEM-reactive open/empty species found in GM-CSF-treated monocytes corresponded to SDS-sensitive HLA-DR free of tightly bound peptides.

Surface loading of open/empty MHC-II molecules

Empty MHC-II populations have been previously characterized in vitro as a mixture of peptide-receptive and peptide-averse molecules. In vitro, peptide-receptive molecules can be generated by release of low-affinity peptides (34, 35). The receptive form rapidly equilibrates into a mixture of species containing predominately the less-receptive peptide-averse state, but the receptive form can be rescued by HLA-DM (36, 37) or HLA-DM like activity (38). We evaluated the availability of empty MHC-II molecules on GM-CSF-derived cells for peptide loading. Biotinylated antigenic peptide was observed to bind at the cell surface of GM-CSF-treated monocytes (data not shown). To evaluate whether this activity was due to binding of peptides to cell surface empty/open molecules or to peptide exchange with previously loaded molecules, we stained cells with MEM-265 before and after peptide loading. Cells from HLA-DRB1*15 or HLA-DRB1*01 individuals were incubated with peptides known to bind with high affinity to these allotypes, MBP 86–101 and HA 306–318, respectively, and staining with MEM-264 or MEM-265 was performed to determine the residual amount of open/empty MHC-II surface molecules (Fig. 2, *b–d*). A 20% reduction in MEM-264 surface staining was observed after 4-h incubation of HLA-DRB1*01 GM-CSF-differentiated monocytes with 10 μM HA 306–318 peptide (Fig. 2*b*). No differences were observed under the same experimental conditions in LB3.1 staining (Fig. 2*b*). Four-hour incubation with 10 μM specific MBP or HA peptide but not with nonspecific LR peptide resulted in consistent inhibition of ~20% of

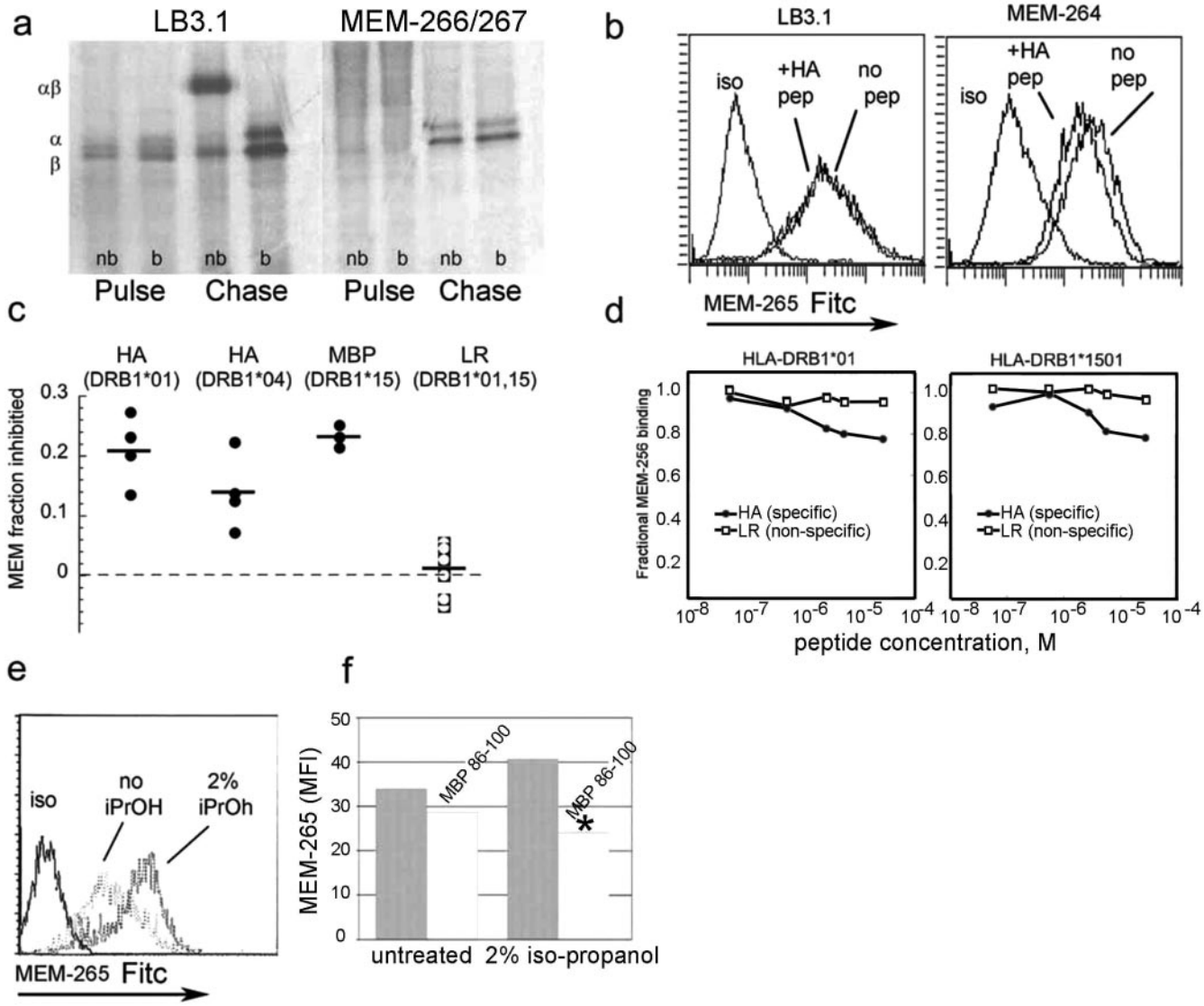

FIGURE 2. Empty/open HLA-DR molecules are mostly present in a peptide averse conformation. *a*, Human DRB1*01 monocytes differentiated for 6 days in GM-CSF were pulsed for 45 min with S35 methionine/cysteine and chased for 4 h. Immunoprecipitation was conducted with LB3.1 (total DR) or a mixture of MEM-266 and MEM-267 (empty conformation only). Samples in alternate lanes were boiled (b) or not (nb) before loading; an SDS-resistant band in the nb lanes indicated MHC-peptide complexes. One of four experiments is shown. *b*, HLA-DRB1*0101 GM-CSF-differentiated monocytes were incubated for 4 h at 37°C in the presence or in the absence of HA 306–318 peptide (10^{-4} M) and then stained with LB3.1 (which recognizes both empty and loaded HLA-DR) or MEM-264 (specific for empty HLA-DR). One of four experiments is shown. *c*, The inhibition of MEM binding induced by peptide was observed for samples from different donor. Cells from HLA-DRB1*01 and DRB1*04 donors were incubated with specific HA or control LR peptide, cells from HLA-DRB1*15 donors were incubated with specific MBP or control LR peptide before staining with MEM Abs. A peptide concentration of 10^{-4} M was used. Data are reported as fraction of MEM staining inhibited by peptide binding calculated as: $1 - (MFI_{MEM,+peptide} - MFI_{iso})/(MFI_{MEM,no\ peptide} - MFI_{iso})$ where $MFI_{MEM,\ no\ peptide}$ is the mean fluorescent intensity (MFI) of MEM staining, $MFI_{MEM,+peptide}$ is the MFI of MEM staining after treatment with peptide, and MFI_{iso} is the MFI of staining observed for an isotype-matched control Ab. *d*, Incubation of HLA-DRB1*01 and DRB1*04 with increasing amounts of HA 306–318 or MBP 86–101, respectively. Fractional MEM binding observed after incubation with different concentrations of peptide was calculated as $(MFI_{MEM,+peptide} - MFI_{iso})/(MFI_{MEM,no\ peptide} - MFI_{iso})$ where $MFI_{MEM,\ no\ peptide}$ is the MFI of MEM staining, $MFI_{MEM,+peptide}$ is the MFI of MEM staining after treatment with peptide, and MFI_{iso} is the MFI of staining observed for an isotype-matched control Ab. *e*, Increase of surface MEM-265 reactivity in GM-CSF-differentiated monocytes after incubation for 2 h with 2% isopropanol. One of four experiments is shown. *f*, Surface Ag loading of MBP 86–100 (10^{-4} M) is promoted by incubation with 2% isopropanol. Peptide binding is detected as a decrease in MEM-265 reactivity. * indicates a statistically significant difference ($p < 0.0001$) in the MEM-265 MFI between isopropanol-treated cells incubated or not with MBP 86–100. One of four experiments is shown.

the MEM binding for cells from different individuals (Fig. 2*c*). No further inhibition was observed even at high peptide concentration (Fig. 2*d*). This nonreceptive fraction was similar to that observed for purified empty human MHC-II in vitro (34, 35, 39) and for exogenously expressed empty murine MHC-II (40). Previous studies have shown that isopropanol and other small molecules can facilitate conformational transition of peptide-averse HLA-DR into a peptide receptive state (23, 41). Incubation of GM-CSF-differentiated monocytes (HLA-DRB1 01–15 or HLA-DRB1 01–01) for 4 h in the presence of 2% isopropanol resulted in an increase in MEM cell surface staining (Fig. 2*e*) indicating formation of surface empty HLA-DR molecules. Under these conditions a statistically significant increase in peptide binding was also observed indicating formation of peptide-receptive molecules in the isopropanol treated samples (Fig. 2*f*). In conclusion, ~20% MEM-reactive open/empty HLA-DR molecules on the surface of GM-CSF-differentiated monocytes were able to readily bind peptide, with the remainder present in the peptide-averse form, treatment with H-donor compound facilitate

conformational transition of peptide-averse HLA-DR into a peptide receptive state, thus ultimately peptide binding.

Empty/open HLA-DR is not found in the endoplasmic reticulum (ER) or trans*-Golgi network (TGN) and does not derive from HLA-DR Ii complexes that travel at the cell surface*

The next series of experiments was designed to investigate the generation/trafficking of empty MHC-II molecules. A series of hypothesis were considered 1) HLA-DR could fold as empty protein in the ER (we considered this possibility very unlikely), 2) empty HLA-DR could derived from HLA-DR/Ii complexes traveling from the TGN to the cell surface before internalization in endosomal compartment, 3) empty HLA-DR could form in endosomal compartment and then exported as empty protein at the cell surface, and 4) empty molecules could derive from surface MHC-II with loosely bound peptides.

To assess whether HLA-DR could fold as empty protein in the ER/TGN an ultrastructural analysis of GM-CSF-differentiated DC was performed using MEM-265 in combination with 10 nm of protein A-gold particles. Controls on empty HLA-DR negative cell lines were performed to ensure that the fixation procedure used for the ultrastructural analysis would not alter HLA-DR binding epitopes (data not shown). Immunogold labeling could not be observed using MEM-265 at either the ER or TGN (Fig. 3, *a* and *b*). On the contrary, a positive labeling using Abs directed against different domains of the MHC-II chaperone Ii was clearly evident in the ER and the Golgi/TGN (Fig. 6c^1). Positive staining was also observed using a rabbit anti HLA-DR serum (data not shown). Several conclusions can be drawn from the lack of MEM-265 staining at the ER/TGN: 1) MEM-265 does not recognize HLA-DR/Ii heterotrimers, as also indicated by pulse chase experiments (Fig. 2*a*), 2) MEM-265 does not bind unfolded or partially denaturated protein that might be present in the ER (MEM-265 will bind unfolded HLA-DR1β, but with ~100-fold lower affinity than for the folded empty protein (27), and finally 3), HLA-DR does not fold as empty molecules in the ER or TGN.

The second hypothesis to be considered was that empty HLA-DR could form from MHC II-Ii complexes traveling to the cell surface once Ii was processed extracellularly because increased flux of MHC-Ii from the TGN to the cell surface instead of to endosomes has been reported for immature DC (8). Access of MHC II-Ii to endosomes is controlled by the adaptor complex AP-2 (42, 43), whereas retention of partially processed Ii during endosomal remodeling in maturing DC is controlled by the adaptor complex AP-1 (13). AP-2 silencing increases surface Ii of several folds in transfected HELA cells (42), whereas silencing of AP-1 increases surface Ii of only around 3-folds in maturing DC (13). Both adaptors are not involved in MHC II-peptide trafficking. Because silencing of both adaptors increase surface expression of MHC II-Ii we used this as a model to dissect whether empty HLA-DR could derived from surface processing of Ii. In this scenario, adventitious cleavage of MHC-Ii at the cell surface could lead to MHC-clip or related species that might easily lose peptide to generate the empty form.

AP-1 or AP-2 knockdown was performed by silencing the mRNA encoding for the μ1 and μ2 subunits of the adaptor complexes, respectively. Monocytes differentiated with GM-CSF for 6 days (HLA-DRB1*01 donor) were transfected with the duplex sense/antisense RNA (dx) to block μ1 or μ2 translation. The sense probes (s) were used under the same transfection conditions as negative control. Percentage of positively transfected GM-CSF-differentiated monocytes with both sense or duplex sense/antisense probes are reported (Fig. 3, *c* and *d*). Effective inhibition of protein translation was confirmed by Western blot analysis (Fig. 3, c^1 and d^1).

Cell surface staining for MHC II or Ii was performed 24 h after transfection. Blocking translation of either μ1 or μ2 proteins did not significantly change surface MHC II protein expression as assessed by L243 or MEM-267 staining (Fig. 3, *c* and *d*). These mAbs do not bind HLA-DR-Ii (Ref. 44 and this article). On the other hand, as previously reported (13, 42, 43) an increase in surface expression of Ii (CD74) was observed after blocking μ2 and to much lesser extent μ1 translation. Because increasing the traffic of HLA-DR/Ii to the plasma membrane did not result in increased cell surface MEM-267 reactivity, we conclude that the MEM-267-reactive MHC-II is not derived from MHC-Ii complexes trafficking directly to the cell surface.

Empty HLA-DR is formed in endosomal MIIC

Having determined that HLA-DR does not fold as empty in the ER/TGN and does not derive from surface MHC II/Ii complex, the third hypothesis to investigate was whether empty MHC II molecules were generated in endosomal compartment following the conventional pathway of Ii degradation by cathepsins.

An ultrastructural analysis of MHC-II-positive intracellular compartments (MIIC) was performed, using anti-DR1 and anti-Lamp-1 Abs in combination with 10 and 15 nm protein A-gold particles, respectively. In GM-CSF-treated monocytes the majority of Lamp-1-positive compartments were spherical corresponding to electron dense bodies (EDB) with an estimated area 0.015 μm^2/compartment (Fig. 4, *a* and *b*). EDB had a classical lysosomal morphology and structurally resembled closely the peroxidase-positive and -negative granules previously characterized in human monocytes, the latter being secretory lysosomes (45). In addition electron lucent MVB, also MHC-II positive, with a variable amount of internal vesicles or poorly resolved internal structure were visible (19) (Fig. 4*b*). The ratio between electron lucent MVB and EDB was strictly dependent on the differentiation state of the cells. Indeed, in GM-CSF-treated cells that by ultrastructural analysis expressed low-surface MHC-II, ~88% of the MIIC were EDB (an average of 35.6 compartments/cell), with the remainder MVB (5 compartments/cell) (Fig. 4*c*). In GM-CSF-treated cells that expressed higher levels of surface MHC-II molecules, the number of MIIC with electron lucent MVB represented almost the totality of the MIIC (Fig. 4*c*). MLB were not observed in GM-CSF-treated monocytes.

GM-CSF/IL-4-treated monocytes were similarly assayed for the presence of Lamp1/MHCII compartments. In these samples, multilamellar compartments (MLB) were present together with electron lucent bodies, which were MVB-like, having indistinct internal contents or a small number of small vesicles (M. J. Kleijmeer, unpublished observations) (Fig. 4*d*, MLB and MVB labeled).

Next, MHC-II compartments were labeled with a polyclonal anti-HLA-DR rabbit Ab raised against purified HLA-DR1 (46), and with MEM-265 mAb specific for open/empty HLA-DR molecules (27). Among the MHC class II positive compartments we found expression of open/empty MHCII molecules within the EDB identified in GM-CSF-treated monocytes (Fig. 5, *a* and a^1, EDB labeled). Conversely, electron lucent MHC-II compartments of the MVB-like type, which are present in GM-CSF and GM-CSF/IL-4 differentiated monocytes, were mainly negative for MEM-265 labeling (Fig. 5, a^1, *b*, and b^1, MVB labeled) although they did label strongly with the polyclonal anti-HLA-DR serum. In immature GM-CSF/IL-4 grown DC the classical multilamellar MHC-II compartments (MLB) were highly positive for open/empty MHC-II molecules (Fig. 5, b^2 and b^3, MLB labeled). The average number of gold particles counted per compartment is reported in Fig. 5*c*. The preferential distribution of the MEM-265 epitope in MLB was statistically significant ($p < 0.001$) as assessed by the randomness test (25). In conclusion open/empty MHC II protein are observed in MIIC albeit with a differential distribution in different compartments. Almost no empty HLA-DR are present in MVB, whereas several open/empty molecules are present in MLB. From these last series of experiments we concluded that open/empty HLA-DR are

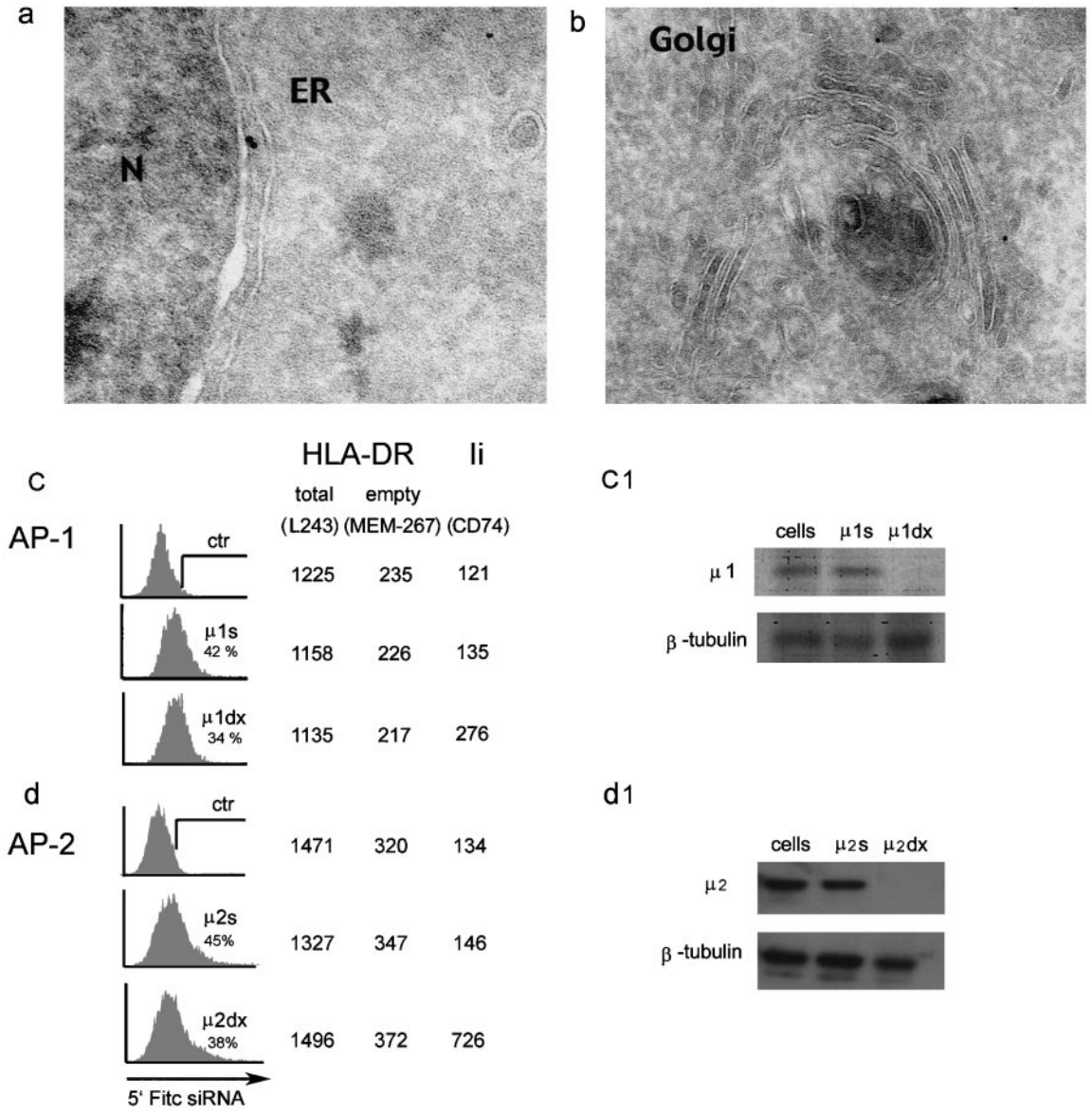

FIGURE 3. The open/empty conformation of HLA-DR is not observed in the ER/TGN and does not derive from $\alpha\beta$/Ii complex trafficking at the cell surface. Ultrathin cryosection of ER (*a*) and TGN (*b*) in monocytes differentiated with GM-CSF (shown) or GM-CSF/IL-4 (not shown). Immunolabeling was performed with MEM-265 (15 nm of gold) specific for open/empty HLA-DR (27). Immunostaining with MEM-265 was not observed in the ER/TGN. *c* and *d*, GM-CSF differentiated monocytes were transfected with FITC-labeled single (s) or duplex (dx) RNA, which targeted the translation of *c*, the AP-1 adaptor complex μ1 protein, or *d*, the AP-2 adaptor complex μ2 protein. Numeric percentage near the FACS histogram refers to number of FITC-siRNA positively transfected cells. The surface amount of empty (MEM-267) or total (L243) HLA-DR protein as well as Ii (CD74) were analyzed by flow cytometry. Data are reported as MFI of the specific Ab minus MFI of the isotype control. c1, d1) Total cell lysates from sorted cells as described in c and d were solved on SDS-PAGE. Membranes were probed with anti μ-1 or μ2 rabbit sera, to determine efficacy of AP-1 silencing, or anti β-tubulin as loading control.

formed inside the Lamp-1^+ lysosomal-like MIIC. At the moment we cannot completely exclude our last hypothesis that empty/open molecules are also formed on the cell surface from HLA-DR associated with loosely/low affinity peptides.

In fully mature DC, after 24 h of treatment with TNF-α, the amount of open/empty MHC-II molecules within the MLB was strongly reduced. MHC-II-loaded molecules were still present in these compartments even though the majority was now expressed at the cell surface (Fig. 5*d*).

EDB containing empty/open HLA-DR also contain HLA-DM and processed Ii fragments

The spatial relationship between HLA-DM and HLA-DR is important for efficient MHC-II loading (47). To characterize the localization of HLA-DM in the MIIC where empty HLA-DR was found, we performed immunostaining using a polyclonal Ab directed against the transmembrane region of HLA-DM β-chain. (Fig. 6, *a*, a^1, a^2, and *b*). In GM-CSF-treated monocytes the EDB contained HLA-DM, with labeling present on the limiting membrane of the compartments (Fig. 6*a*, EDB labeled). The average number of gold particles in the EDB and MVBs was similar, 1.7–1.9/compartment (Fig. 6*b*) and also equally distributed. In GM-CSF/IL-4 differentiated monocytes there was an increased in the MLB of HLA-DM protein expression, (Fig. 6a^2, MLB labeled), which was redistributed not only on the limiting membrane but all over the inner sheets of the compartments (Fig. 6a^2, MLB labeled). MVB were either negative for the HLA-DM or positive with an average number of gold particles similar to the number observed in the EDB (Fig. 6, a^1 and *b*). This is consistent with the previous observation that HLA-DM is enriched in highly dense lysosomes (48).

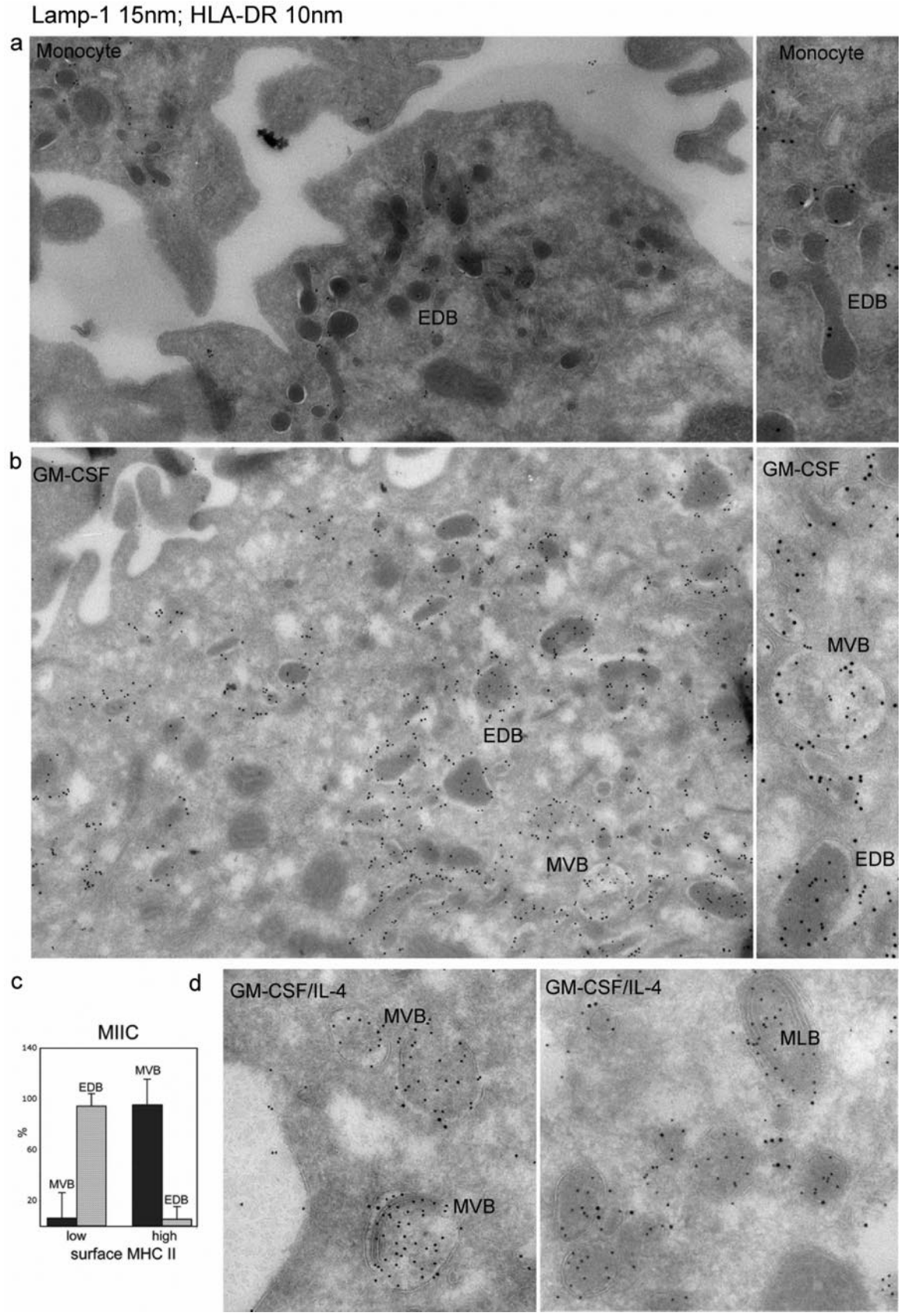

FIGURE 4. Ultrastructural analysis of MIIC in GM-CSF and GM-CSF/IL4 differentiated monocytes. Ultrathin cryosection of monocytes treated or untreated with GM-CSF and GM-CSF/IL-4. Lamp-1 (15 nm of gold) and HLA-DR (10 nm of gold) positive compartments are indicated. *a* and *b*, Lamp-1$^+$ EDB and MVB are observed in monocytes and GM-CSF-differentiated monocytes. *c*, Analysis of EDB and MVB in GM-CSF-differentiated monocytes. In cells that by EM, express low levels of surface HLA-DR the majority of Lamp-1 and HLA-DR-positive compartments are still electron dense (EDB), whereas when cells express high-surface HLA-DR many MVB (Lamp-1 and HLA-DR positive) are observed (24, 25). *d*, Cells grown in GM-CSF/IL-4 mostly have multilamellar MIIC (MLB) and some of the multivesicular type (MVB) (26).

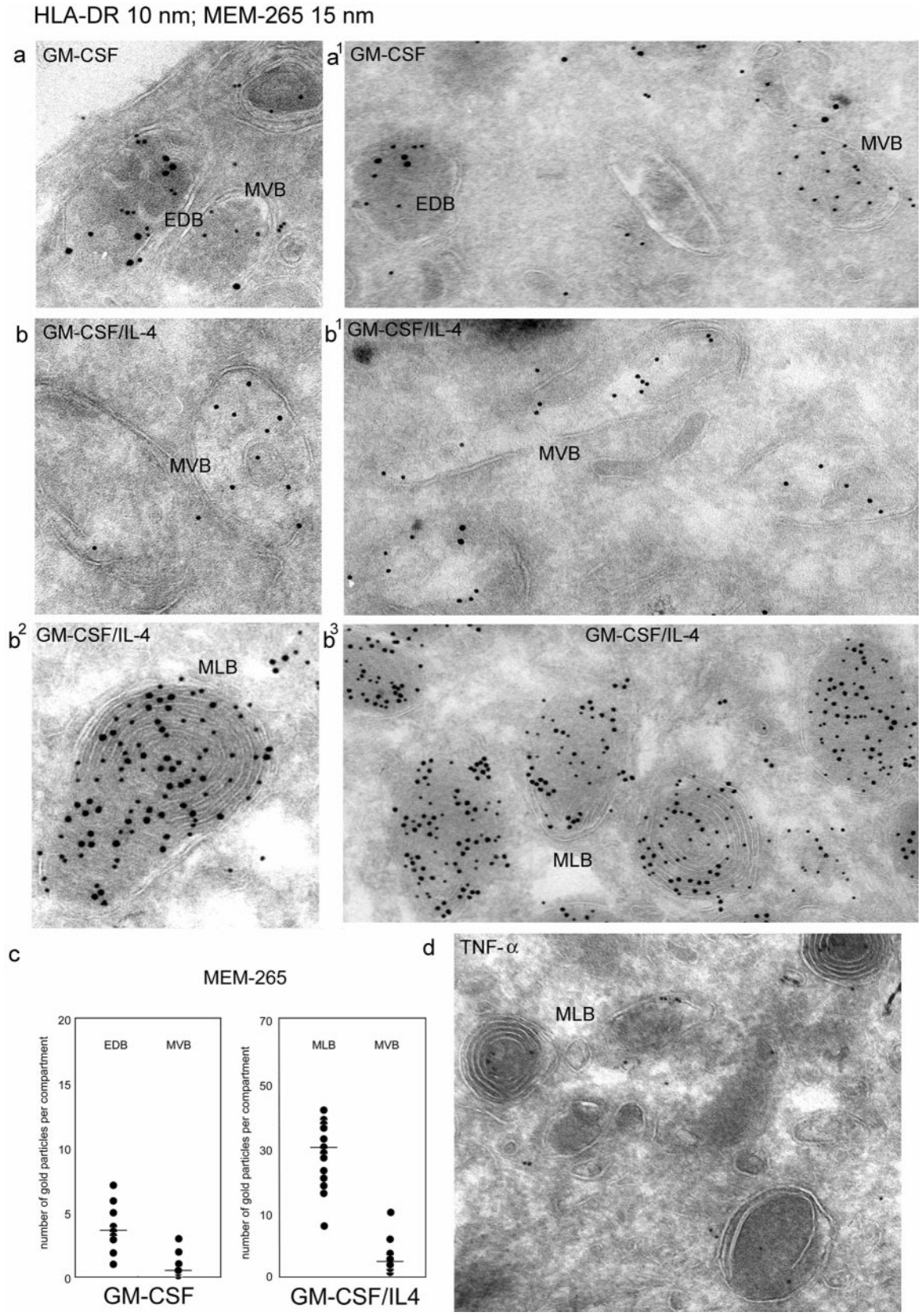

FIGURE 5. The open/empty conformation of HLA-DR is mostly observed in lysosomal electron dense and multilamellar MIIC, but not in late endosomal multivesicular MIIC. Ultrathin cryosection of MIIC in monocytes differentiated with GM-CSF (*a* and a^1) GM-CSF/IL4 (*b*, b^1, b^2, b^3) or further matured with TNF-α (*d*). Immunolabeling was performed with MEM-265 (15 nm of gold) specific for open/empty HLA-DR and with rabbit sera to HLA-DR (10 nm of gold). EDB, MVB, and MLB are labeled. *c*, Quantification of MEM-265 immunostaining in EDB, MVB, and MLB from GM-CSF or GM-CSF/IL-4-differentiated monocytes (25, 26).

We investigated the intracellular distribution of the MHC class II chaperone Ii, by immunogold staining using Abs directed against different domains (Fig. 6, Ii C-terminal and N-terminal labeled). Staining with a rabbit serum raised against the C-terminal of Ii (28) was clearly evident in the ER in the Golgi/TGN (Fig. 6*c*, Golgi/TGN labeled) and in multivesicular endosomes ((Fig. 6c^1 MVB labeled), as already published (17) but mostly absent in EDB and MLB, (Fig. 6c^1) EDB labeled), indicating that in these compartment the processing of the luminal portion of Ii has occurred. The staining for Ii was then performed using PIN1, an Ab against the N-terminal domain. Again, multivesicular compartments stained positively with PIN1 Ab (Fig. 6d^1) MVB labeled), whereas <1% of EDBs were positive (Fig. 6*d*, EDB labeled) as also observed in the MLB of the GM-CSF/IL-4 differentiated cells. We conclude that empty HLA-DR is present in compartments (MLB and EDB) where Ii is completely processed. Interestingly in the MLB the amount of HLA-DM is five times higher then in the MVB possibly to stabilize empty HLA-DR or to release low-affinity peptides.

Open/empty MHC-II molecules are expressed in secondary lymphoid organs

Finally, MEM-265 was used to probe secondary lymphoid organs to detect whether there was a cellular counterpart expressing empty/open MHC-II molecules similarly to the monocytes differentiated in vitro with GM-CSF. Immunohistochemistry performed on fresh frozen sections of lymph node tissue and tonsil showed MEM-265 immuno-reactive cells with a membranous staining pattern. Morphologically these cells had large nuclei and multiple cytoplasmic processes typical of a DC-like population. MEM-265-reactive cells were predominantly located in paracortical and interfollicular areas (Fig. 7 and data not shown). Some MEM-265-positive cells were also observed in the germinal center with a similar staining pattern. Incubation with the peptide corresponding to the HLA-DR epitope (β1 58–69) recognized by MEM-265 abrogated the immunoreactivity in all tissues tested (Fig. 7 and data not shown). CD11c staining, used to evaluate the total population of myeloid dendritic cells, showed immunoreactive cells present in germinal centers, paracortical areas, and interfollicular areas (Fig. 7). Morphologically these cells had large nuclei and several cytoplasmic processes typical of a dendritic cell population, and very similar or identical to the population stained by MEM-265.(Fig. 7). In addition, a small subset of cells with small nuclei and scant cytoplasm weakly stained for CD11c and MEM-265 in the paracortical area, possibly representing infiltrating monocytes (data not shown). Altogether these data indicate that empty/open MHC-II molecules were expressed at the cell surface of DC-like cells in secondary lymphatic organs

Discussion

Late endosomes and lysosomal organelles are subcellular compartments, which in all cell types are the site of degradation of both

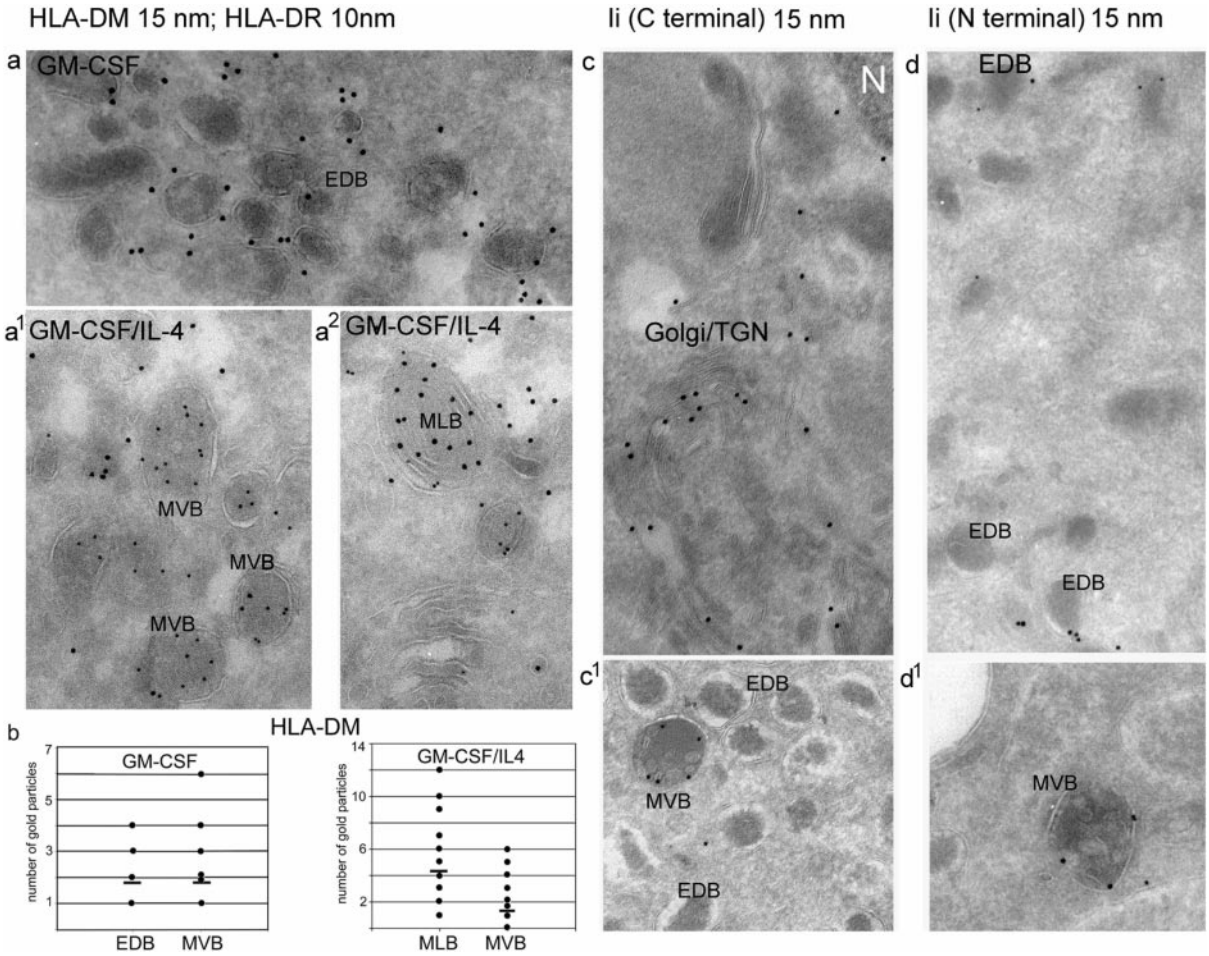

FIGURE 6. HLA-DM and processed Ii are observed in lysosomal EDB. Ultrathin cryosection of MIIC in monocytes, cultured with GM-CSF or GM-CSF/IL-4. *a*, *a*^{1}, and *a*^{2}, Immunolabeling with anti HLA-DM (15 nm of gold) and anti HLA-DR (10 nm of gold) in different MIIC, EDB, and MVB in GM-CSF-differentiated cells and MLB in GM-CSF/IL-4 cultured cells. *b*, Quantification of immunogold staining for HLA-DM in different MIIC in GM-CSF and GM-CSF/IL4 treated cells indicates enrichment of HLA-DM in lysosomal MIIC. *c* and *c*^{1}, Immunolabeling with a rabbit serum anti-Ii (C-terminal), ER/TGN, EDB, and MVB are shown. *d* and *d*^{1}, Immunolabeling with a mAb anti-Ii (N-terminal), EDB, and MVB are shown.

endogenous and exogenous materials. These organelles are characterized by acidic pH, presence of proteases, and expression of Lamp protein family members. Lysosomal compartments are also used in several immune and nonimmune cell-types to perform specific functions that are distinct from protein degradation (49). Indeed, in professional APC late endosomes/lysosomes, enriched in MHC-II proteins, peptide-loading editing molecules (HLA-DM, HLA-DO) and cathepsins are specialized organelles, termed MIIC, where most of Ag processing and MHC-II loading occurs (46, 50). The observation that MIIC appear with different morphologies has long been appreciated even though the biological significance associated with the different morphologies and the exact contribution of each compartment to Ag processing and MHC II loading are still unclear. The multivesicular compartments (MVB) described in both murine and human B cells and DC are conventional late endosomes (17, 51) but the multilamellar compartments (MLB) also present in these cells are lysosomal-like organelles, and whether the presence of MHC-II molecules is connected to their peptide loading or their degradation has been long debated. Notably, in human GM-CSF/IL-4 monocyte derived DC the MLB represent the majority of the MIIC (21). Thus, it seems likely that at least a fraction of MHC-II proteins would be spared from degradation and transported at the cell surface. Also, in patients suffering from Chediak-Higashi Syndrome the absence of MHC-II molecules in B cell multivesicular endosomes and its presence in the multilamellar type have been described. In these subjects there is a delayed MHC-II loading and transport to the cell surface indicating that, under these circumstances, peptide loading and MHC-II trafficking to the cell surface can also occur from the MLB (52).

In this report, we have observed EDB as a lysosome-like MIIC present in a pre-DC population together with multivesicular late endosomes. EDB were positive for Lamp-1, MHC-II proteins, HLA-DM and, similarly to what was previously observed in multilamellar lysosomes, the Ii was completely processed such that in $<1\%$ of the EDB the N-terminal domain of Ii could be detected. EDB increased their MHC-II contents once monocytes were differentiated with GM-CSF without undergoing apparent structural modification. On the other hand morphological changes were observed after differentiation with IL-4 resulting in the formation of lysosomal MIIC with a multilamellar organization characteristic of "professional lysosomes." Therefore, a differential lysosomal morphology was associated with different stages of myeloid DC maturation (Fig. 8).

A second observation in this report is the presence of empty HLA-DR in lysosomal MIIC but not in late endosomal multivesicular MIIC. Thus it appear empty HLA-DR is a molecular signature of EDB and MLB but is not observed in MVB. Differences

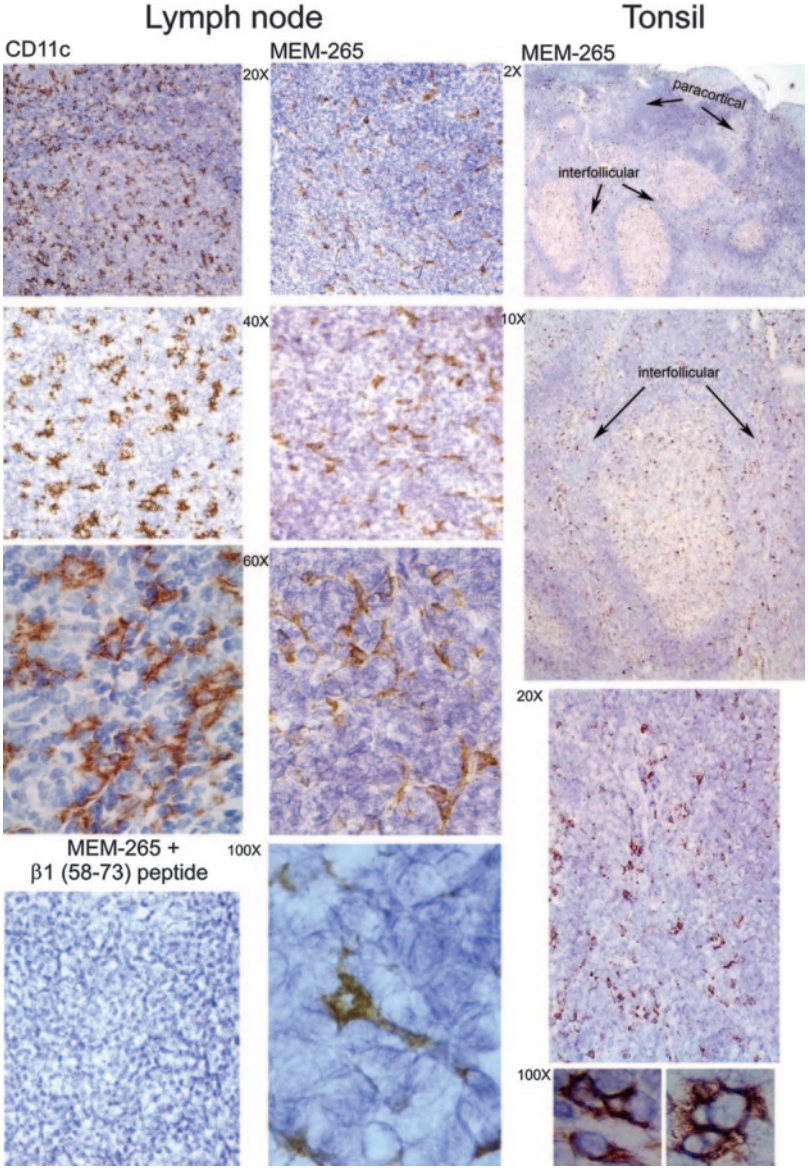

FIGURE 7. Cells expressing empty/open MHC-II molecules are present in secondary lymphatic tissue. Immunohistochemistry of frozen sections from lymph nodes and tonsils using CD11c, a general marker for DC and MEM-265, specific for empty/open MHC-II molecules. Staining indicates the presence of MEM-265-reactive cells in paracortical and interfollicular areas of secondary lymphatic organs.

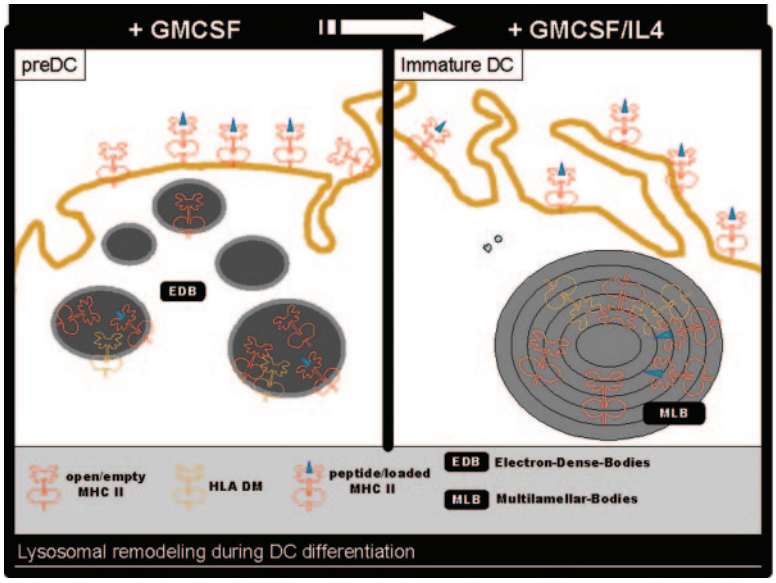

FIGURE 8. Endosomal remodeling during monocyte-derived DC differentiation. EDB are lysosome-like MIIC observed in monocytes and GM-CSF-differentiated cells. In GM-CSF-IL-4-differentiated cells lysosomal MIIC present a multilamellar organization characteristic of "professional MIIC." Both compartments are positive for empty HLA-DR molecules, even though empty HLA-DR molecules are only observed at the cell surface of GM-CSF-differentiated cells.

in Ii processing were previously observed between MVB and MLB (Ref. 17 and this article), the additional result of sequestration of empty HLA-DR uniquely in lysosomal MIIC, together with an increased amount of HLA-DM open interesting questions on differences in Ag processing and HLA-DR loading between the different compartments. Additionally, open/empty HLA-DR comprised as much as 20% of total MHC-II at the surface of pre-DC populations but is almost absent from the surface of GM-CSF/IL-4 differentiated DC, even though abundantly present intracellularly. A possible explanation is that in nonprofessional APC, such as monocytes or early DC precursors, empty HLA-DR reflects an intrinsic inefficiency in Ag processing and MHC-II loading, which will be fully developed when cells mature into "professional APC." In immature cells empty HLA-DR could egress at the cells surface from the EDB, which because they are closely resembling of secretory lysosomes could fuse their limiting membrane with the plasma membrane (45). On the other hand in GM-CSF/IL-4-differentiated cells empty HLA-DR is abundantly expressed intracellularly in MLB. It has been recently reported that in the multilamellar MIIC efficient Ag loading is achieved in the inner lamellae where HLA-DM pairs HLA-DR for peptide loading, probably facilitated by tetraspans CD63 and CD82 (47). Thus, in these professional compartments empty HLA-DR could be effectively loaded before egressing on the cell surface.

The finding that open/empty MHC II molecules are present on APC in secondary lymphatic organs raises the question of their functionality in Ag loading and more generally during an immune response. Peptides of different length generated extracellulary by several secreted protease (53) or unfolded proteins could bind to open/empty MHC-II in a model of "determinant capture" previously suggested by Sercarz and colleagues (54, 55). Indeed, in some cases intact proteins have been shown to bind MHC-II molecules with high affinity (RNase and MBP to HLA-DR1 and myoglobin to I-E) (56). In the case of full-length purified MBP Ag loading for T cell presentation was shown to be extracellular with no requirement for of internalization or endosomal processing (57).

HLA-DR conformational differences are important because they can affect T cell reactivity. It has long been appreciated that MHC II Ag loading can occur at the cell surface, in early endosomes and in late endosomes (58, 59). However, only recently it has been proven that different Ag-processing and -loading pathways produce distinct MHC-II/peptide conformers which differentially prime T cells (60, 61). MHC-II/peptide complex formed in late endosomal/lysosomal compartments at low pH through the action of DM are conformationally different from MHC-II complexed with the same peptide generated at a more neutral pH (cell surface or early endosomes) (60). Surface open/empty MHC-II could function in the latter pathway.

In conclusion we show here that conformational variants of MHC-II molecules are observed in different MIIC and that maturation from a non professional to a professional MIIC correlates with retention and endogenous loading of empty MHC-II molecules. Surface empty MHC-II molecules found on pre-DC could function as an Ag receptor for T cell presentation of peptides/proteins loaded extracellularly or serve as a mechanism to capture external Ag for delivery to endosomal compartments that are on their way to becoming professional MIIC. Either way, open/empty MHC-II proteins at the cell surface add an additional variable to the exogenous pathway for Ag processing and presentation (59).

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Disclosures

The authors have no financial conflict of interest.

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