Mobilization of MHC class I molecules from late endosomes to the cell surface following activation of CD34-derived human Langerhans cells

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Langerhans cells are a subset of dendritic cells (DCs) found in the human epidermis with unique morphological and molecular properties that enable their function as ''sentinels'' of the immune system. DCs are pivotal in the initiation and regulation of primary MHC class I restricted T lymphocyte immune responses and are able to present both endogenous and exogenous antigen onto class I molecules. Here, we study the MHC class I presentation pathway following activation of immature, CD34-derived human Langerhans cells by lipopolysaccharide (LPS). LPS induces an increase in all components of the MHC class I pathway including the transporter for antigen presentation (TAP), tapasin and ERp57, and the immunoproteasome subunits LMP2 and LMP7. Moreover, in CD34-derived Langerhans cells, the rapid increase in expression of MHC class I molecules seen at the cell surface following LPS activation is because of mobilization of MHC class I molecules from HLA-DM positive endosomal compartments, a pathway not seen in monocyte-derived DCs. Mobilization of class I from this compartment is primaquine sensitive and brefeldin A insensitive. These data demonstrate the regulation of the class I pathway in concert with the maturation of the CD34-derived Langerhans cells and suggest potential sites for antigen loading of class I proteins.

Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system and are essential for the initiation and control of an immune response (1, 2). Although resident in peripheral tissues, DCs are particularly well suited for antigen capture. As sentinel cells, they survey the immediate environment for incoming pathogens through macropinocytosis as well as receptor-mediated uptake mechanisms (3). Following contact with inflammatory mediators, DCs undergo a regulated maturation process and switch from an antigen-capturing to antigen-presenting mode. This activation process involves the secretion of chemokines, cytokines, and migration to the local lymphoid organs where DCs recruit circulating T and B cells (4). During this process, DCs become specialized in antigen presentation for T cell activation and up-regulate MHC class I and II, adhesion, and costimulatory molecules. The cellular consequences of DC activation have been particularly well studied for the MHC class II pathway (5, 6). Class II molecules accumulate in late endosomal and lysosomal compartments (collectively termed MIICs) of immature DC and are expressed at the cell surface following maturation. This is a regulated process controlled by the cleavage of the invariant chain and, in mature DCs, results in efficient delivery of class II to the cell surface (7). In contrast, there is less information on how DCs modulate their class I antigen presentation pathway upon maturation. Bacterial products up-regulate synthesis and stabilize class I molecules in human and murine DC (8, 9), and the kinetics are reported to be slower than for class II molecules. It is well recognized that in their role in priming CD8 T cell responses, DCs have the unusual capability of presenting not only endogenous but also exogenous antigen onto their class I molecules (10). This unique aspect of DCs and some macrophages is not seen in other nucleated cells and, both TAP-dependent and independent routes of class I antigen presentation have been identified. The powerful ability of DCs to initiate primary immune responses has generated great interest in their therapeutic potential, in particular the capacity of DCs to generate T cell responses to tumor antigens. The realization of this therapeutic potential requires a thorough understanding of the different DC types as well as their antigen presentation pathways. DCs represent a heterogeneous population with distinct lineages and functions (11). In the majority of studies on human DCs, peripheral blood $CD14^+$ monocytes are cultured in the presence of granulocyte macrophage–colony-stimulating factor and IL-4 to promote differentiation into monocyte-derived DCs (12). More recently, DCs have been grown from $CD34^+$ stem cells, and of particular interest is the use of $CD34^+$ cells as a source of DCs that closely resembles epidermal Langerhans cells (LCs) (13, 14). In this study, we compared the class I antigen presentation pathway from two distinct DC populations, monocyte-derived DCs from $CD14^+$ monocytes in peripheral blood, and CD34-derived LCs from $CD34^+$ stem cells obtained at leukapheresis. Activation of immature CD34-derived LCs by inflammatory mediators affected MHC class I, TAP, tapasin, Erp57, LMP2, and LMP7, all INF-γ inducible components of the class I pathway. We find that immature CD34-derived LCs, in contrast to monocyte-derived DCs, accumulate class I molecules in endosomal compartments. Following activation of CD34-derived LCs with LPS, there is a rapid and sustained increase in MHC class I cell-surface expression. This increase is associated with an induction of both class I and its regulatory components. However, the immediate rapid increase in cell-surface class I expression is a consequence of mobilization of class I molecules from intracellular vesicles to the cell surface.

Materials and Methods

Purification of $CD34^+$ Hemopoietic Stem Cell Leukapheresis Products. Patients undergoing granulocyte colony-stimulating factor treatment for stem cell mobilization with >1% $CD34^+$ cells as a constituent of their peripheral blood mononuclear cells were asked to donate part of a standard (60 ml) leukapheresis. Informed consent was obtained from all patients, and the protocol was approved by the Cambridge Local Research Ethics

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Abbreviations: DC, dendritic cells; LPS, lipopolysaccharide; LC, Langerhans cells; HSP, heat shock protein.

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Committee. $CD34^+$ stem cells were purified form the leukapheresis material by using a midi-MACS positive selection system (CD34 progenitor isolation kit). The protocol used was as specified by the manufacturer (Miltenyi Biotec, Auburn, CA). The purity of $CD34^+$ cell postselection varied from 80% to 95%. The yield of $CD34^+$ cell was ≈50% expected. Purified $CD34^+$ cells were frozen at concentrations of 5×10^6/aliquot.

Generation of Immature Langerhans Cells from $CD34^+$ Precursors. The purified $CD34^+$ cells were cultured at 3×10^4/ml in 24-well plates (Nunc) at 37°C/5% CO_2 for 7 days. The growth media used was serum free X-VIVO 15 (BioWhittaker). The cytokine milieu used to differentiate the $CD34^+$ cells was as defined (13, 15) and included Flt 3 ligand (FLt3L) (100 ng/ml), granulocyte macrophage–colony-stimulating factor (100 ng/ml), TGF-β1 (0.5 ng/ml), TNF-α (50 units/ml) (all from Peprotech), and stem cell factor (20 ng/ml) (a kind gift from A. Green, Cambridge Univ., Cambridge, U.K.). At day 0, $CD34^+$ cells were in single cell suspension with clustering evident by day 3–4 and loosely adherent cells by day 7. Total cell numbers increased 50–100-fold during this culture period, with 50–65% of the cells staining for CD1a/HLA-DR and CLA 1, markers associated with human LCs. As observed by Gatti and colleagues (13), the immature CD34-derived LCs were concentrated in large proliferative clusters. These were purified by using $1 \times g$ sedimentation on 7.5% BSA columns and contained cells that were 85–90% $CD1a^+/CLA^+$, 99% $HLA\text{-}DR^+$, with 85–90% of cells staining positively with the unique LC marker Langerin by immunofluorescence. The purified CD34-derived LCs could be cultured at 5×10^5/ml in 24-well plates for a further 2 days. Activation/maturation of the immature CD34-derived LCs was achieved by addition of LPS (0.5 μg/ml). Brefeldin A (Sigma) was used at a concentration of 10 μg/ml for the initial hour and at 1 μg/ml thereafter. Primaquine (Sigma)-treated cells were pretreated with 100 μm for the initial hour and at 10 μm for up to 24 h.

Generation of Monocyte-Derived DC. Monocyte-derived DCs were generated from $CD14^+$ peripheral blood monocytes following culture in 100 ng/ml human granulocyte macrophage–colony-stimulating factor (Schering-Plough) and 1,000 units/ml IL-4 (Peprotech) (12). After 6 days in culture, 95–99% of cells expressed markers associated with monocyte-derived DC, HLA-DR, CD83, and CD86.

Antibodies. The following antibodies were purchased: anti-human CD40, CD80, CD86, and isotype controls (Serotec–Coulter); LMP2, LMP7, and MCP20 (Affiniti, Exeter, U.K.); anti-LAMP1 (PharMingen); anti-CD63 (BioDesign, Kennebunk, ME); and all secondary mAbs (The Jackson Laboratory). The following antibodies were kind gifts: HB15a anti human CD83 (T. Tedder, Harvard Medical School, Boston), 10D12 anti human CD1a (D. Olive, Institut Paoli Calmettes, Marseilles, France, ref. 16), Mem18 anti-human CD14 (M. Labeta, University of Wales College of Medicine, Cardiff), polyclonal anti-MPR (S. Pfeffer, Stanford University, Stanford, CA), anti-human LAG (M. Velleca, Yale University, New Haven, CT), anti-human ERP57 (J. Lindquist, Cambridge University), HC10 (H. Ploegh, Harvard Medical School), rabbit anti-class I heavy chain antisera (J. Neefjes, The Netherlands Cancer Institute, Amsterdam) (17), HLA-DR (R.DRAB), HLA-DM (R.DMB-C) (18), and R.RING4C and R.gp48N (ref. 19; P. Cresswell, Yale University). The β_2M-dependent mAbs w632 and 4E were used for the analysis of pan-class I and HLA-B, respectively.

Microscopy. Primary cultures of immature or mature DCs were incubated for 30 min at a concentration of 5×10^5/ml in serum-free growth media on 1% Alcian Blue-coated coverslips. Immunofluorescence confocal microscopy was performed as previously described (20) except that cells were fixed for 24 h in methanol:acetone at −2°C. Images were obtained by using a confocal laser scanning microscope TCS 4D (Leica Lasertechnik). Ultrastructural immunocytochemistry on frozen ultrathin sections was performed as described (20) except that cells were fixed in 8% paraformaldehyde.

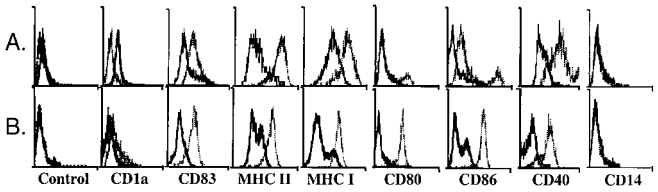

Fig. 1. Surface phenotype of CD34-derived LCs (*A*) compared with CD14 monocyte-derived DCs (*B*). Cells generated under distinct culture conditions were analyzed by flow cytometry for the expression of the cell markers indicated. Thick lines indicate the cells stained in the absence of LPS, whereas the dotted lines show the profiles obtained following activation with LPS (0.5 μg).

Cytofluorographic Analysis. Phenotypic staining of the human DCs was performed by using murine monoclonal antibodies as previously described (21). The labeled cells were analyzed on a fluorescence-activated cell scanner (FACScalibur, Becton Dickinson).

Immunoblotting. Immunoblots were performed as described (19), and reactive bands were detected by chemiluminescence.

Peptide Translocation Assays. Peptide translocation was carried out as previously described on streptolysin O (S. Bhakdi, University of Mainz, Mainz, Germany) permeabilized cells (22).

Results

Generation and Phenotype of CD34-Derived LC and Monocyte-Derived DC. Despite representing an important DC population, the study of human LCs has been hampered by difficulties in isolating cells in sufficient number and purity for detailed analysis. Strobl *et al.* showed that *in vitro* culture of cord blood-derived $CD34^+$ stem cells with Flt3L, stem cell factor, TGF-β, and TNF-α in serum-free conditions allowed differentiation into a DC population that closely resembled LCs (15). They contained abundant Birbeck granules and stained positive for the Birbeck granule-associated C-type lectin, Langerin. Using similar culture conditions, Gatti *et al.* generated a DC population with an LC phenotype from $CD34^+$ cells obtained at leukapheresis (13). Following purification, the cells seemed to represent a homogenous population, remained in an immature state, and could be induced to undergo a controlled and synchronous maturation with inflammatory mediators. Using the same conditions as Gatti *et al.*, we grew immature dendritic cells from $CD34^+$ stem cells and compared their cell-surface phenotype with monocyte-derived DCs grown from peripheral blood. Both cell types were activated with LPS (0.5 μg) for 48 h, and the cell-surface phenotype was determined by cytofluorographic analysis (Fig. 1). Immature CD34-derived LCs expressed Langerin (see Fig. 3*B*), by electron microscopy showed Birbeck granules (not shown), and expressed $CD1a^{dim}$, $CD83^{dim}$, MHC class II^{dim}, $CD80^{dim}$, $CD86^{dim}$, MHC class I, and CD40 and were negative for CD14. Immature monocyte DCs expressed $CD83^{dim}$, MHC class II^{dim}, MHC class I^{dim}, $CD80^{dim}$, $CD86^{dim}$, and $CD40^{dim}$ and were negative for CD1a, CD14, and Langerin. Following activation with LPS for 48 h, both cell types became $CD83^+$, MHC class II^{bright}, and MHC class I^{bright} with the CD34-derived LCs expressing CD40, class I, and class II molecules at extremely high levels.

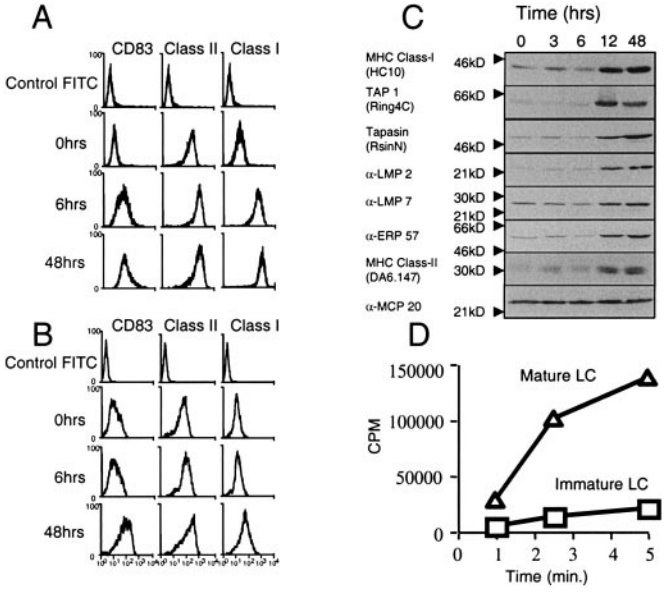

Fig. 2. LPS induced changes in the MHC class I antigen presentation pathway in DCs. Immature CD34-derived LCs (*A*) and monocyte-derived DCs (*B*) were activated by addition of 0.5 μg of LPS. At time 0, 6, and 48 h post-LPS, cells were harvested and fixed, and the expression of DC activation marker CD83, MHC class II, and MHC class I was monitored by flow cytometry. (*C*) LPS mediated a coordinate induction of MHC class I components. CD34-derived LCs were induced with LPS for 0–48 h, after which the cells were extracted in 1% Triton X-100, and membranes from separated lysates were probed with antibodies specific for the indicated proteins. MCP20 is a constitutive, noninducible component of the human proteasome and serves as a loading control for each time point. (*D*) LPS increases TAP-mediated peptide transport in CD34-derived LCs. Streptolysin O-permeabilized immature (□) and LPS-matured (△) CD34-derived LCs were incubated with the iodinated RRYQNSTEL peptide at 37°C for the indicated time periods. Translocation was assessed by binding of the hot, glycosylated reporter peptide to Con A-Sepharose beads.

LPS-Induced Maturation of Immature CD34$^+$ Stem Cell-Derived Langerhans Cells Causes a Rapid Increase in Cell-Surface MHC Class I and Class II Expression. To examine the LPS-induced changes in cell-surface expression of MHC class I, class II, and the DC activation marker CD83, we performed a time course of maturation on immature CD34-derived LCs and monocyte-derived DCs. The cell-surface expression of these molecules was investigated by flow cytometry at 0, 6, 12, 24, and 48 h post-LPS stimulation. We have selected the, 0-, 6-, and 48-h time points to illustrate the observed changes (Fig. 2*A* and *B*). By 48 h, in CD34-derived LCs, expression of CD83 had increased 7–8-fold, MHC class II expression 10-fold, and MHC class I expression 50-fold. Even after 6 h of LPS, a marked increase in not only CD83 and MHC class II but also MHC class I was seen (5-fold, 3-fold, and 8-fold, respectively). These findings were reproducible by using CD34-derived LCs generated from six unrelated donors. The increased cell-surface class I and class II was already evident 3 h after activation and was seen with other inflammatory stimuli, including poly(I·C) (data not shown). In contrast, LPS stimulation of monocyte-derived DCs resulted in a 2-fold increase in class II staining by 6 h, but up-regulation of cell-surface MHC class I was not seen until 12 h of stimulation with LPS and was maximal at 48 h (Fig. 2*B*).

The rapid increase in cell surface class I expression in maturing CD34-derived LCs was surprising, and we wanted to determine how the increased class I expression reflected changes in components of the class I antigen presentation pathway. Immunoblots were performed on detergent cell lysates of LPS-activated immature CD34-derived LCs at 0, 3, 6, 12, and 48 h following stimulation and probed with antibodies to TAP1, tapasin, MHC class I, MHC class II, LMP2, LMP7, ERp57, and MCP20. The noninducible proteasomal subunit MCP20 was used as a loading control. Immature CD34-derived LCs showed low constitutive expression of all class I components. The immunoproteasome components LMP2 and LMP7 were low but clearly expressed. A marked induction of all class I dedicated assembly proteins and MHC class II was seen following activation with LPS (Fig. 2*C*). The increased protein expression was first seen at 12 h post-LPS and was maximal by 48 h. To ascertain that higher TAP levels were associated with increased peptide translocation, we used an iodinated reporter peptide to assess TAP-mediated peptide translocation in immature and LPS-matured CD34-derived LCs (Fig. 2*D*). There was a marked increase in both the absolute level and rate of TAP-mediated peptide translocation, which correlates with the LPS-induced increase in TAP expression.

Confocal Microscopy Analysis of Immature and Mature CD34-Derived LCs. The rapid increase in MHC class I cell-surface expression is not matched by a comparable increase in class I dedicated assembly proteins over the 6-h time period. We wondered whether MHC class I molecules in intracellular stores may gain rapid access to the cell surface following activation. We therefore used confocal immunofluorescence microscopy to visualize class I expression. Initial experiments with the class I conformation-specific mAb w6/32 suggested that much of the class I was in intracellular vesicular structures (Fig. 3*A*). Further studies showed that the class I exhibited a high degree of colocalization with both HLA-DR (MHC class II) and HLA-DM molecules (Fig. 3*A*), and these structures most likely represent MIIC. In striking contrast, immature monocyte-derived DCs showed predominantly cell-surface class I staining, and no colocalization with HLA-DR was seen (Fig. 3*A*). LPS activation of CD34-derived LCs induced dramatic morphological changes, with the development of abundant filopodia already evident 6 h post-stimulation (Fig. 3*A*). In addition, following LPS stimulation, activated cells showed a loss of intracellular MHC class I staining, with increased cell-surface class I staining (Fig. 3*A*). To determine whether unfolded and potentially peptide-receptive class I colocalizes in the MHC class I/HLA-DM positive endosomal compartments, we used the mAb HC10, which recognizes free class I heavy chains. HC10 reactive class I heavy chains were also found to colocalize within the HLA-DM positive compartment (Fig. 3*A*). Taken together, these results suggest that during LC activation, MHC class I molecules are mobilized to the cell surface.

To further define the nature of the compartment stained with class I antibodies, we used markers that distinguish early from late endocytic compartments. Immature CD34-derived LCs were costained for class I and CD1a, an immunological marker for early endosomes (23), and LAMP1, a marker for late endosomes/lysosomes. Class I molecules showed considerable overlap staining with LAMP1 (Fig. 3*B*), whereas little colocalization was seen between the CD1a and HLA-DM/class I compartments (Fig. 3*B*). These results provide further evidence that the class I are in a late endosomal compartment, which is distinct from recycling early endosomes, and that MHC class I/HLA-DM compartments are components of the late endocytic pathway. CD34-derived LCs were also analyzed with anti-Lag, an antibody which recognizes Langerin, a protein specific for the Birbeck granules of Langerhans cells (Fig. 3*B*) (24). LAG staining of Birbeck granules was clearly separate from HLA-DM positive compartments, suggesting that neither HLA-DM nor class I is present in Birbeck granules.

Immunoelectron microscopy of double immunolabeled ultrathin cryosections from immature CD34-derived LCs confirmed the colocalization of MHC class I with MHC class II molecules

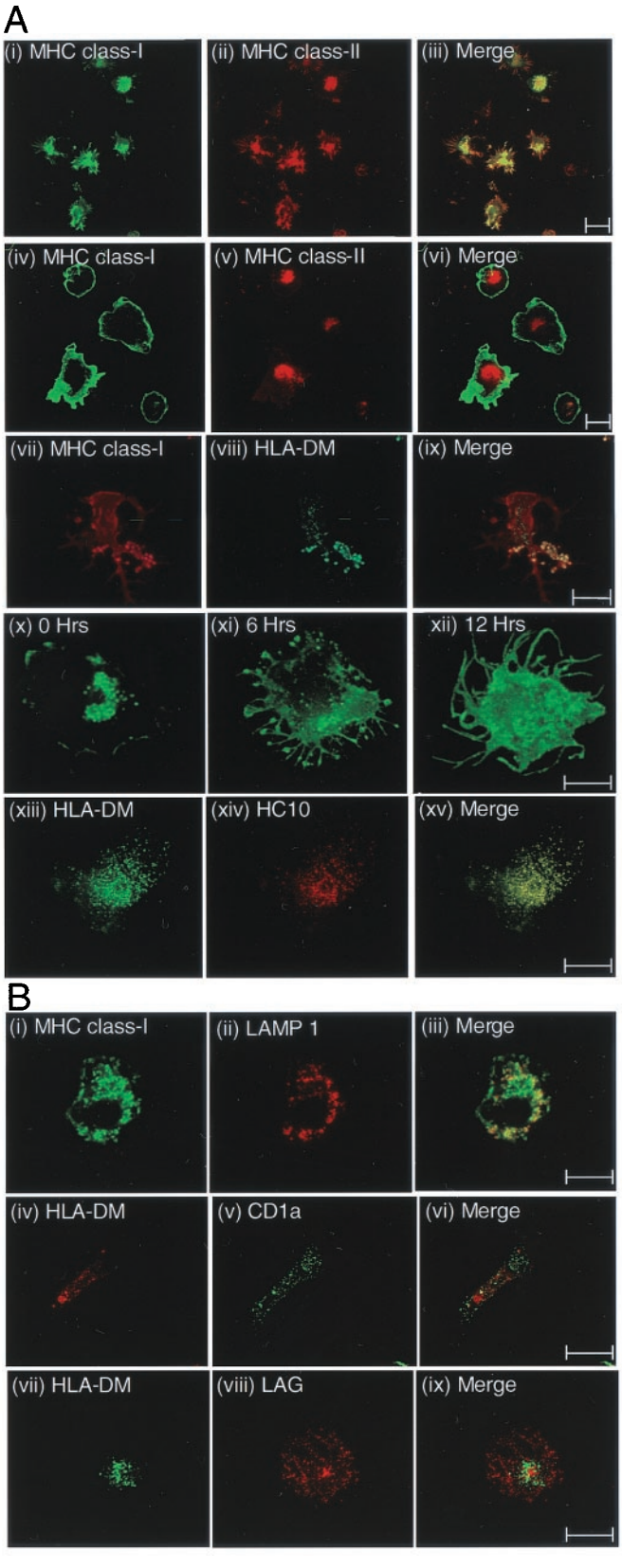

Fig. 3. Cellular localization of MHC class I molecules in DCs. (*A*) MHC class I colocalizes in MIIC. (*i–iii*) Day 7 immature CD34-derived LCs and (*iv–vi*) monocyte-derived DCs were fixed, stained with R.DRAB (anti-class II $\alpha\beta$) and 4E (anti-class I), and examined by confocal immunofluorescence microscopy. The class I and class II molecules show colocalization in CD34-derived LCs (*iii*) but not in the monocyte-derived DCs (*vi*). Day 7 immature CD34-derived LCs (*vii–ix*) were stained with 4E (anti-MHC class I) and R.DMB-C (anti-HLA-DM). Merged images show colocalization of class I with HLA-DM. (*x–xii*) Time course of activation of immature CD34-derived LCs with LPS. 6 h postactivation, intracellular class I staining (rabbit anti-class I heavy chain antisera) is less apparent, and by 12 h class I is seen predominantly at the cell surface. (*xiii–xv*) Immature CD34-derived LCs stained with antibody recognizing free class I heavy chains (HC10) and HLA-DM (R.DMB-C) show colocalization. (*B*, *i–iii*) Immature CD34-derived LCs were stained for immunological markers for late endosomes/lysosomes LAMP1 (H4A3) (*i–iii*), for early endosomes, CD1a (10D12) (*iv–vi*), and for Birbeck organelles LAG (anti-LAG) (*vii–ix*). Merged images show colocalization between MHC class I and LAMP1, but no colocalization between HLA-DM and the early endosome marker, CD1a, or the Birbeck granule marker, Langerin. Size markers represent 10 μm.

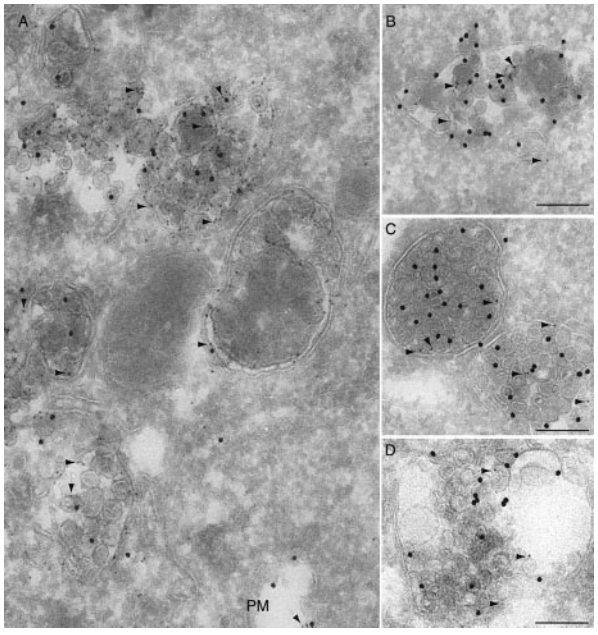

Fig. 4. Class I colocalizes in MIICs. (*A*) Ultrathin cryosections of immature CD34-derived LCs were double immunolabeled for MHC class II (rabbit anti-human HLA-DR, small gold) and MHC class I (rabbit anti-heavy chain serum, large gold). PM, plasma membrane. (*B–D*) Endosomes, double immunolabeled with class I (small gold), together with HLA-DR (large gold) (*B*), class I (large gold) together with CD63 (small gold) (*C*), and mannose-6-phosphate receptor (small gold) (*D*). Large gold particles are 15 nm and small gold particles (arrows) 5 nm; size bars represent 200 nm.

in the MIICs of immature CD34-derived LCs (Fig. 4). Double staining showed class I and class II molecules on the plasma membrane, but the majority of both class I and class II molecules are seen within multivesicular structures, which show staining of class I on both the inner and limiting membranes (Fig. 4 *A* and *B*). These vesicles also contain the late endocytic compartment markers CD63 (Fig. 4*C*) and the mannose-6-phosphate receptor (Fig. 4*D*).

Primaquine and Endosomal Inhibitors Prevent the Rapid Surface Expression of MHC Class I in LPS-Stimulated CD34-Derived LCs. To determine whether the initial increase in cell-surface class I expression results from the mobilization of class I molecules from HLA-DM positive endosomal compartments, we investigated how agents that disrupt the endocytic pathway affected the LPS-induced increase in class I expression. Primaquine, a potent inhibitor of membrane transport from endosomes to the plasma membrane (25), as well as chloroquine, leupeptin, bafilomycin A, and cycloheximide were compared with brefeldin A, which causes disassembly of the Golgi complex and inhibits the secretory pathway (26). Immature CD34-derived LCs were activated

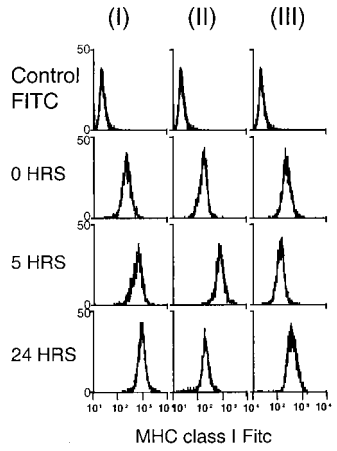

Fig. 5. Primaquine inhibits the rapid LPS-induced increase in cell-surface MHC class I molecules. Day 7 cluster purified immature DC were incubated with LPS alone (I), LPS and brefeldin A (II), and LPS and primaquine (III). Cells were harvested at 0, 5, and 24 h after addition of the LPS and fixed; expression of MHC class I molecules was examined by flow cytometry.

over 24 h with LPS in the presence or absence of inhibitors, and the surface expression of class I was determined by flow cytometry at 5 and 24 h poststimulation. In the absence of inhibitors, a 5-fold increase in surface MHC class I expression was seen 5 h after LPS activation (Fig. 5, lane I). This compared with a 4-fold increase in the cells treated with brefeldin A (Fig. 5, lane II). In contrast, the primaquine-treated cells showed a 3-fold decrease in cell-surface class I expression (Fig. 5, lane III). Hence, the rapid increase in cell-surface class I expression is primaquine sensitive and brefeldin A insensitive. This situation was reversed at 24 h poststimulation. By 24 h, cell-surface class I expression on control cells had continued to rise (lane I). The primaquine-treated cells now showed a 4-fold increase in surface expression when compared with cells at the 5-h time point (lane III). In contrast, after 24 h, the cells treated with brefeldin A showed a 4-fold decrease in level of MHC class I cell-surface expression from the 5-h time point, and no increase from the 0 time point (lane II). Hence, primaquine inhibited the rapid increase in cell-surface expression of MHC class I, but this effect was lost over a longer period of activation. This same effect was also seen with chloroquine, leupeptin, and bafilomycin, although the effect was most marked with primaquine (data not shown). In contrast, brefeldin A and cycloheximide (data not shown) only inhibited the up-regulation of class I seen over the 24-h time period. Taken together, these results suggest that the rapid increase in cell-surface class I comes from a primaquine-sensitive, endosomal class I-containing compartment, whereas the later sustained increase is a consequence of newly synthesized class I molecules that are sensitive to brefeldin A. A pulse–chase analysis confirmed that brefeldin A inhibited maturation of newly synthesized class I molecules through the Golgi in DC (data not shown).

Discussion

In this study, we show that by using similar tissue culture conditions as described by Gatti and colleagues, we can differentiate $CD34^+$ stem cells into a homogenous population of DCs with the phenotype of epidermal LCs (13). The ability to generate a purified LC population with a stable immature phenotype allowed us to examine how inflammatory mediators affected the MHC class I presentation pathway in different DC populations. Treatment of immature CD34-derived LCs with LPS causes a rapid rise in cell-surface class I molecules, already evident 3 h poststimulation. Although the increased cell-surface class I was associated with increased levels of TAP, tapasin, Erp57, and two inducible components of the immunoproteasome, induction of these components was not apparent until 6–12 h post-LPS treatment. Therefore, their induction cannot account for the initial rapid rise in surface class I. Using confocal immunofluorescence and immunoelectron microscopy, class I molecules were seen in intracellular vesicular compartments that colocalized with HLA-DM, HLA-DR, and LAMP1 and most likely represent MIICs (27). Sorting of class I to the cell surface was primaquine sensitive, as primaquine prevented the rapid increase in surface class I, whereas brefeldin A only prevented the later accumulation.

The low constitutive expression of MHC class I and class I components in immature CD34-derived LCs, together with their induction by LPS, is consistent with functional data and studies on MHC class II showing that immature DCs are proficient in antigen capture and require activation to become efficient antigen presenters (12, 28). The induction of all key components of the class I system suggests that TAP-mediated peptide transport and subsequent class I loading is coordinately regulated to optimize class I assembly and cell-surface expression. These findings are consistent with previous studies on class I induction by IFN-γ in epithelial and primary endothelial cells (22). Induction of lmp2 and lmp7 in CD34-derived LCs was more marked than seen in monocyte-derived human DCs (29) and suggests that despite switching to a presentation mode, the cell must retain the ability to process antigen via the immunoproteasome.

To optimize DCs for therapy requires an evaluation of different DC populations as well as a comparison between the capacity of immature and mature DCs to generate effector $CD8^+$ T cells. Several studies suggest mature monocyte-derived DCs to be superior to immature cells in their capacity to activate $CD8^+$ T cells (30). The few published comparisons between DCs from $CD34^+$ stem cells and monocytes have suggested that CD34-derived DCs show a preferential capacity to activate $CD8^+$ T cells (31), but the mechanisms are unclear. It was therefore of considerable interest to identify an endosomal pool of class I molecules in CD34-derived LCs that was not apparent in monocyte-derived DCs. Endocytosis and recycling of class I molecules has been seen on many cell types, including B lymphoblastoid cells (32), monocytes, and macrophages where class I is internalized via coated pits, and up to 30% of class I may be recycled (33). More recently, a role for phosphorylation as a signal for recycling class I molecules has been suggested (34). However, a significant population of endosomal class I has only previously been seen as a consequence of the activity of the HIV-nef protein, which clears class I from the cell surface to lysosomes for subsequent degradation (35). Therefore, the presence of class I molecules in a class II loading compartment of LC raises many questions. Do all of the class I molecules arise from an endocytosed recycling population, or are newly synthesized class I directed to the MIIC in CD34-derived LCs? Nascent class I molecules would require association with another molecule with endosomal sorting motif for endosomal targeting. Although class I molecules can associate with the invariant chain (36), by using immunoprecipitation we have not seen this association in CD34-derived LCs (data not shown).

The rapid LPS-induced mobilization of class I to the cell surface and the inhibition of this process by primaquine suggests a functional role for endosomal class I, beyond providing a route for delivery to lysosomes and degradation. Why class I should accumulate in a class II loading compartment is unclear, but a role in cross-presentation is suggested. The demarcation between presentation of endogenous antigens on class I and exogenous antigens on class II is not absolute. In particular, in DCs that are required for $CD8^+$ T cell priming, there has been great interest in mechanisms of loading antigen on to class I

molecules in the absence of direct infection. Evidence for more than one pathway exists, and class I presentation of exogenously acquired protein may involve routing of antigen through the classical class I pathway (TAP dependent and BFA sensitive) or post-Golgi loading of class I (TAP independent and BFA resistant) (10). For peptides generated in endosomes to be loaded onto class I molecules requires either regurgitation of peptides to the outside of the cell or capture within endosomes by cycling class I molecules (37). Using a green fluorescent protein-tagged class I molecule, Gromme *et al.* (38) recently identified class I in the MIIC of a melanoma cell line and demonstrated that efficient peptide exchange could occur at the low pH of endosomes (5.0). This mechanism would be particularly attractive for the cross-presentation seen with heat shock proteins (HSPs) in DCs (39–41), in which peptide-loaded HSPs or indeed CTL epitopes from HSP fusion proteins are loaded onto class I molecules. Receptor-mediated uptake of several HSPs, including gp96 and Hsc73, has been observed (42). Where these HSP-delivered peptides intersect with and are loaded onto class I molecules is unclear, but loading onto endosomal class I would be a viable option. DCs are some of the most potent APCs, and several unique features of their class I and class II loading pathway have been described. Accumulation of class I in immature DC endosomes and the coordinated regulation of class I components upon activation may contribute to the efficiency of antigen presentation.

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