Human Dendritic Cell Expression of HLA-DO Is Subset Specific and Regulated by Maturation[1]

Tara M. C. Hornell,* Timo Burster,* Frode L. Jahnsen,† Achal Pashine,[2]* Maria T. Ochoa,‡ James J. Harding,* Claudia Macaubas,* Andrew W. Lee,* Robert L. Modlin,‡ and Elizabeth D. Mellins[3]*

Expression of HLA-DO (DO) in cells that express HLA-DM (DM) results in an altered repertoire of MHC class II/peptide complexes, indicating that DO modulates DM function. Human and murine B cells and thymic epithelial cells express DO, while monocytes/macrophages do not. Monocyte-derived dendritic cells (DC) also have been found to be DO-negative, leading to the assumption that DC do not express DO. In this study, we report that, in fact, certain types of human primary DC express DO. These include Langerhans cells (LC) and some subtypes of circulating blood DC. Specifically, the majority of BDCA-3$^+$ DC, a small subset of uncertain function, are DO$^+$, while smaller proportions of CD11c$^+$, BDCA-1$^+$ (myeloid) DC, at most a minority of CD123$^+$/BDCA-2$^+$ (plasmacytoid) DC, and no detectable CD16$^+$ (myeloid) DC, express DO. Immunohistochemistry of human tonsil sections demonstrates that tonsillar interdigitating DC are also DO$^+$. In a subset of immature LC with higher DO expression, an increased fraction of surface DR molecules carry CLIP peptides, indicating that DO functions as a DM inhibitor in these cells. LC expression of DO is down-regulated by maturation stimuli. DM levels also decrease under these conditions, but the DM:DO ratio generally increases. In the myeloid cell types tested, DO expression correlates with levels of DOβ, but not DOα, implying that modulation of DOβ regulates DO dimer abundance in these cells. The range of APC types shown to express DO suggests a broader role for DO in immune function than previously appreciated. *The Journal of Immunology,* **2006, 176: 3536–3547.**

The recognition of antigenic peptides bound to class II MHC molecules by TCRs expressed on CD4$^+$ T cells is critical for immune responses to pathogens and tumors. Several molecules regulate the formation of stable class II MHC/peptide complexes and their expression on the cell surface (reviewed in Ref. 1). Invariant chain (Ii)[4] directs class II molecules from the endoplasmic reticulum (ER) to late endocytic peptide-loading compartments (here referred to as MIIC) and occupies the peptide-binding groove of the class II molecule, generally preventing peptide loading before MIIC localization. In MIIC, proteases successively degrade Ii until a series of nested peptides termed CLIP remains. The nonclassical class II MHC molecule DM acts on CLIP/class II MHC complexes to catalyze CLIP release and stabilize empty class II molecules. DM also serves as a peptide editor, likely by releasing peptides from class II MHC until a stable peptide/MHC complex is formed. In the absence of DM, most class II alleles accumulate at the cell surface with CLIP still bound.

HLA-DO, another nonclassical class II MHC molecule, associates tightly with DM in the ER and localizes to MIIC in a DM-dependent manner (2). Investigations of overexpression of DO in transfected cells and transgenic mice argue that DO inhibits the function of DM (3, 4); evidence from human B cells supports this conclusion (5, 6). In vitro studies with purified molecules indicate that the inhibition is pH-dependent and is reduced at pH $<$6.0 (7–9). DO expression alters the repertoire of peptides bound to class II MHC molecules, presumably through modulating DM function (10, 11).

Class II MHC, DM, and Ii are constitutively expressed in all professional APC, but expression of DO dimer has been reported only in B cells and thymic epithelial cells (12–14). Naive and memory B cells express more DO and a lower DM:DO ratio than germinal center B cells (5, 6, 15). High DO expression correlates with increased CLIP surface expression in B cells (5, 6). Down-regulation of DO in germinal center B cells has been proposed to contribute to their enhanced ability to present Ag to T cells (5, 6, 15).

Examination of DO expression in human dendritic cells (DC) has been limited, with the exception of monocyte-derived DC, which have been found to lack DO expression (4, 5, 16). In the course of studying the class II MHC pathway in Langerhans cells (LC) derived from CD34$^+$ hematomopoietic stem cells, we discovered that these cells express DO dimers. This finding led us to examine DO expression in several different types of human primary APC. We find that certain types of primary human DC express DO, that DO functions to inhibit DM in these cells, and that DO expression is regulated by differentiation and activation.

*Department of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305; †Laboratory for Immunochemistry and Immunopathology, Rikshospitalet University Hospital, Oslo, Norway; and ‡Division of Dermatology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA 90095

Received for publication September 2, 2005. Accepted for publication December 28, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the National Institutes of Health (PO1-AI48212 to E.D.M.), an Immunology Training Grant (T32AI07290-16 to T.M.C.H.), a Stanford University Dean's fellowship (to T.M.C.H.), the Cancer Research Institute (to T.M.C.H.), the Deutsche Forschungsgemeinschaft (BU 1822/1-1 to T.B.), and the Howard Hughes Medical Institute (to J.J.H.).

[2] Current address: Roche Palo Alto LLC, 3431 Hillview Avenue, Palo Alto, CA 94304.

[3] Address correspondence and reprint requests to Dr. Elizabeth D. Mellins, Department of Pediatrics, Stanford University School of Medicine, 269 Campus Drive, CCSR Building 2115c, Stanford, CA 94305. E-mail address: mellins@stanford.edu

[4] Abbreviations used in this paper: Ii, invariant chain; ER, endoplasmic reticulum; LC, Langerhans cell; mLC, mature LC; iLC, immature LC; B-LCL, B lymphoblastoid cell line; rt, room temperature; DC, dendritic cell; PAS, protein A-Sepharose; MFI, mean fluorescence intensity.

0022-1767/06/$02.00

Materials and Methods

Cells

Primary human monocytes were isolated from PBMC as described previously (17). For some experiments, purified monocytes were stimulated with 800 U/ml GM-CSF (R&D Systems) or 100 U/ml IFN-γ (Roche) for 24 h for analysis of RNA and 48 h for protein analysis, or for 20 h with 400 U/ml IL-10 (R&D Systems) for protein analysis.

Immature monocyte-derived DC were derived from purified monocytes by culture with GM-CSF and IL-4 as described elsewhere (17), and matured on day 7 by addition of 800 U/ml GM-CSF, 500 U/ml IL-4 (R&D Systems), and 10 ng/ml TNF-α (R&D Systems). Monocyte-derived macrophages were derived by incubation of purified monocytes with 800 U/ml GM-CSF (R&D Systems) for 7 days and the adherent cells were harvested (18). Monocyte-derived LC were derived by incubation of purified monocytes with 800 U/ml GM-CSF, 1000 U/ml IL-4, and 10 ng/ml TGF-β for 5 days (19).

G-CSF-mobilized peripheral blood $CD34^+$ progenitors were purchased from BioWhittaker or obtained through the National Heart, Lung, and Blood Institute, Programs of Excellence in Gene Therapy Hematopoietic Cell Processing Core (PEGT-HCPC), courtesy of Dr. S. Heimfeld (Fred Hutchinson Cancer Research Center, Seattle, WA). LC were derived as described (20, 21). To generate mature LC (mLC), purified immature LC (iLC) clusters were disaggregated by pipetting and seeded at 1.6×10^5 cells/ml in X-VIVO 15 containing 10% FBS, 200 ng/ml trimeric CD40L (CD40LT; Amgen), and 1500 IU of GM-CSF/ml for 2 days. In some experiments, 1 μg/ml LPS (Sigma-Aldrich) or 10 ng/ml TNF-α (PeproTech or R&D Systems) was added instead of CD40L.

For some experiments, iLC and mLC were enriched by positive selection of cells labeled with anti-CD1a-PE or anti-CD83-PE, respectively, followed by anti-PE microbeads (Miltenyi Biotec), per manufacturer's directions. Purity was assessed by flow cytometry (anti-CD1a or -CD83) and was >70%.

Tonsillar B cells were purified from tonsils, obtained with Stanford IRB approval from patients undergoing tonsillectomies. B cells were purified with RosetteSep B cell enrichment reagent (StemCell Technologies) per manufacturer's instructions. Cells were >90% pure by flow cytometry (anti-CD19).

The B lymphoblastoid cell lines (B-LCLs) Raji, 8.1.6, 6.1.6, and 5.2.4 were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FBS (HyClone Laboratories) and 2 mM L-glutamine (Invitrogen Life Technologies). 8.1.6 carries a hemizygous deletion in the MHC II region between the *DMB* and *DRA* genes (22). 6.1.6 has lost expression of all coregulated class II genes, due to mutation of a class II transcription factor (23). 5.2.4 harbors a homozygous deletion in the MHC, affecting *DOB* genes (24).

Antibodies

The following Abs were used for flow cytometry. For some experiments, anti-DR (L243) and anti-CLIP-loaded class II molecules (CerCLIP) followed by FITC-labeled goat $F(ab')_2$ anti-mouse IgG (Caltag Laboratories) were used. Anti-DR-FITC, anti-CD1a-FITC and -PE, and anti-CD83-allophycocyanin and the appropriate isotype controls were purchased from Caltag Laboratories. Purified IgG2b was purchased from Caltag Laboratories, and purified anti-DO (DOB.L1) was purchased from BD Pharmingen. The following directly conjugated Abs and the appropriate isotype controls were purchased from BD Pharmingen: anti-DO-FITC and -PE (clone DOB.L1), anti-DR-PerCP, anti-CD83-FITC, -PE, and -allophycocyanin, anti-CD11c-allophycocyanin, anti-CLIP-FITC (clone CerCLIP), and anti-DM-PE. The lin 1 cocktail (CD3, CD14, CD16, CD19, CD20, CD56)-FITC and the custom lin 2 cocktail (CD3, CD14, CD19, CD20, CD56)-FITC were obtained from BD Biosciences. Anti-BDCA-1-allophycocyanin, anti-BDCA-2-FITC and -allophycocyanin, anti-BDCA-3-FITC and -allophycocyanin, and anti-CD123-allophycocyanin were purchased from Miltenyi Biotec. The dimer-specific anti-DO mAb, Mags.DO5-FITC, was a gift from L. Denzin (Cornell University, New York, NY), and IgG1-FITC purchased from Caltag Laboratories was used as an isotype control.

The following DM- and DO-specific Abs were gifts: 5C1 (DMα; J. Trowsdale, University of Cambridge, Cambridge, U.K.), 47GS4 (DMβ; S. Pierce, Northwestern University, Chicago, IL), K571 (DOβ; L. Karlsson, R. W. Johnson Pharmaceutical Research Institute, San Diego, CA), Mags.DO2 (DO dimer; L. Denzin), and an antiserum specific for DOα (J. Thibodeau, University of Montreal, Montreal, Canada). DOB.L1 (DOβ) was purchased from BD Pharmingen and we have previously described the antisera SU36 (DM; Ref. 25), SU46 (DOβ; Ref. 26), and SU66 (DOα; Ref. 17). The anti-calnexin Ab, AF8 (27), was a gift from M. Brenner (Harvard University, Cambridge, MA).

Flow cytometric staining of human blood DC

Peripheral blood samples collected from healthy donors in Vacutainer tubes containing sodium heparin were obtained from the Stanford Blood Center. A total of 250 μl of whole blood was stained as described (28) with some modifications. Briefly, blood was incubated with mAb specific for lin 1 cocktail (CD3, CD14, CD16, CD19, CD20, CD56)-FITC or, for identification of $CD16^+$ DC, the custom lin 2 cocktail (CD3, CD14, CD19, CD20, CD56)-FITC and/or HLA-DR, and an Ab specific for either BDCA-1, BDCA-2, BDCA-3, CD11c, CD16, or CD123 for 30 min at room temperature (rt). A total of 2 ml of FACS Lysing Solution (BD Biosciences) was added to lyse erythrocytes and fix leukocytes. The samples were stored at -80°C until further processing.

Before intracellular staining, the samples were thawed and pelleted. The pellet was washed once with wash buffer (PBS + 0.5% BSA (Sigma-Aldrich) + 0.1% sodium azide (Sigma-Aldrich)), and incubated with 500 μl of FACS Permeabilizing Solution 2 (BD Biosciences) for 10 min at rt. Cell pellets were then incubated with 1 μg of either purified DOB.L1 or purified IgG2b for 30 min at rt. After washing, samples were incubated with DOB.L1-PE for 30 min at rt, washed, and resuspended in 200–300 μl of 1% paraformaldehyde. The samples preincubated with DOB.L1 followed by staining with DOB.L1-PE served as a negative control, since staining with the isotype control (IgG2b-PE) was higher than staining with DOB.L1-PE even on B cell controls (data not shown). Samples (500,000 events each) were analyzed with a FACSCalibur flow cytometer (BD Biosciences). Compensation was performed with CaliBRITE beads and the FACScomp program (BD Biosciences). DC were defined as cells within an expanded lymphocyte gate defined by forward and side scatter that were lin-negative, DR^+ (see below and Ref. 28). Data analyses were performed using CellQuest or CellQuest Pro Software (BD Biosciences).

Flow cytometric staining of LC

Before surface staining, harvested LC were incubated with blocking buffer (PBS + 5% human AB serum (Gemini Bio-Products) + 5% goat serum (Caltag Laboratories)) for 15 min. on ice. The cells were then incubated for 30 min on ice with Ab to surface markers (CD1a, CD83, DR, CLIP/class II) diluted in blocking buffer, washed with PBS + 0.2% BSA (Sigma-Aldrich), and, where appropriate, incubated with secondary Ab and washed again. DO and DM staining was performed after permeabilizing cells that had been surface stained. Cells were incubated for 20 min with BD Biosciences Fix/Perm and washed twice with BD Biosciences Perm solution. Nonspecific binding was blocked by incubating the cells for 15 min with 5% human AB serum + 5% goat serum diluted in BD Biosciences Perm solution. Cells were then stained with anti-DM or anti-DO, or the appropriate isotype controls for 1 h, washed twice with BD Perm solution, and analyzed using a FACScan or a FACSCalibur flow cytometer (BD Biosciences). In some cases, instead of an isotype control, cells were preincubated with purified DOB.L1 before staining with anti-DO-FITC or -PE. Staining for DO was performed both with the β-chain-specific mAb DOB.L1 and the heterodimer-specific mAb Mags.DO5, with equivalent results, showing that staining with DOB.L1 in these cells indicates the amount of DO dimer present. LC were gated by forward and side scatter, using a gate that overlapped with all but a minor population of $CD1a^+$ cells that are not differentiated LC (21). Data analyses were performed using CellQuest or CellQuest Pro Software (BD Biosciences).

Immunohistochemistry

Normal human skin was obtained from the Human Tissue Research Center (HTRC) at the University of California Los Angeles (UCLA) School of Medicine with Institutional Review Board approval. Biopsy specimens were embedded in OCT medium (Ames) and snap-frozen in liquid nitrogen. Sections (3- to 5-mm thick) were acetone-fixed and kept frozen until use. Double immunofluorescence was performed by serially incubating cryostat tissue sections with mouse anti-human mAbs of different isotypes (DCGM4 (anti-Langerin, IgG1; Immunotech/Beckman Coulter) or NA1/34 (anti-CD1a, IgG2a; DakoCytomation) and DOB.L1 (anti-HLA-DO, IgG2b; BD Biosciences)), followed by incubation with isotype-specific, fluorochrome Alexa 488-, or Alexa 568-labeled goat anti-mouse Ab (Molecular Probes/Invitrogen Life Technologies). No staining was detected after incubation with isotype-matched irrelevant Ab. Sections were examined using a Leica-TCS-SP inverted confocal laser-scanning microscope outfitted with krypton and argon lasers at the Carol Moss Spivak Cell Imaging Facility in the UCLA Brain Research Institute. Sections were illuminated with 488 and 568 nm of light after filtering through an acoustic optical device. Images decorated with Alexa 488 or Alexa 568 (Molecular

Probes) were recorded simultaneously through separate optical detectors with a 530-nm band-pass filter and a 590-nm long-pass filter, respectively. Pairs of images were superimposed for colocalization analysis.

We obtained samples of uninflamed palatine tonsils from patients undergoing tonsillectomy with approval obtained from the review board of the Faculty Division, Rikshospitalet, University Hospital (Oslo, Norway). Samples were embedded in OCT (Tissue-Tek; Miles Laboratories) and snap-frozen in liquid nitrogen. Cryosections were cut at 4 μm, dried at rt, and acetone-fixed for 10 min. Multicolor immunofluorescence stainings were performed as described (29). Briefly, anti-DO (DOB.L1, IgG2b, 10 μg/ml) was mixed with either anti-CD11c (KB90, IgG1, 1/10; gift from K. Pulford, John Radcliffe Hospital, Oxford, U.K.), anti-CD19 (HD37, IgG1, 1/10), or anti-CD68 (EBM11, IgG1, 1/400) (both from DakoCytomation) and incubated on sections for 1 h, followed by biotinylated goat anti-mouse IgG2b (10 μg/ml; Southern Biotechnology Associates) for 1.5 h, and finally Cy3-labeled goat anti-mouse IgG1 (2 μg/ml; Southern Biotechnology Associates) mixed with Cy2-streptavidin (1 μg/ml; Amersham Biosciences) for 30 min. In all staining experiments, irrelevant isotype- and concentration-matched primary mAbs served as negative controls.

Real-time RT-PCR

RNA was isolated, reverse-transcribed, and real-time PCR was performed, as described (17). To determine relative quantity, control cDNA made from Raji cell total RNA was used to generate a standard curve. Relative quantities of the gene of interest were determined for unknown samples by comparison with this standard curve, and normalized to GAPDH quantities. Fold changes in expression after LC maturation were determined by dividing the normalized quantity of the gene of interest from mLC by the normalized quantity of the gene of interest from iLC. All real-time RT-PCR experiments were performed three or more times.

Immunoblotting, metabolic labeling, and immunoprecipitation

Cells were harvested and lysed in 50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM $MnCl_2$, 1 mM $CaCl_2$, or 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, plus protease inhibitors. Unextracted material was pelleted, and the amount of protein in the supernatant was quantitated by Bradford assay and normalized between samples. For Western blots, samples were boiled in 2× Laemmli SDS-PAGE sample buffer with 2-ME, run on 12% SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). Membranes were blocked overnight at 4°C in 100 mM Tris-HCl (pH 7.7), 200 mM NaCl, 1% casein (Hammerstein grade; ICN Pharmaceuticals), 0.05% Tween 20, and 0.05% NaN_3, followed by incubation with the appropriate Ab diluted in blocking buffer. After washing in TBS + 0.05% Tween 20, HRP-conjugated secondary Ab (donkey anti-rabbit Ig, Amersham Biosciences; or goat anti-mouse Ig; Caltag Laboratories) were added in TBS-Tween containing 5% nonfat dry milk. Following additional washes, ECL substrate was added (Renaissance; PerkinElmer), and the blots were exposed to film (Hyperfilm ECL; Amersham Biosciences).

For metabolic labeling and immunoprecipitation, 1×10^6 LC were washed twice in methionine-cysteine free RPMI 1640 (Cellgro; Mediatech) containing 10% dialyzed FBS and 2 mM glutamine (starvation medium) to remove unlabeled methionine and cysteine. Cells were then starved for 2.5 h at 37°C in 10 ml of starvation medium, and labeled in 2 ml of starvation medium containing 300 μCi [^{35}S]methionine and cysteine-labeling mix (PerkinElmer) for 2.5 h at 37°C. Cells were then washed twice in complete RPMI medium (RPMI 1640 with 10% BCS + 2 mM glutamine) to remove excess free radiolabel. Labeled pellets were either frozen at −80°C until use or lysed in lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, containing freshly added protease inhibitors (COMPLETE protease inhibitor; Roche). After rotation for 1 h at 4°C, lysates were spun at 13,000 rpm for 15 min at 4°C to remove nuclear and cellular debris. Spun lysates were subjected to at least three rounds of preclearing by incubation with normal mouse serum and heat-killed Strep A cells (Pansorbin; Calbiochem). Cell lysates were then normalized for radioactivity incorporation. Mock immunoprecipitations were done with 40 μl of uncoated protein A-Sepharose (PAS) beads. To immunoprecipitate DO, 40 μl of anti-DOβ mAb DOB.L1- (BD Pharmingen) coated PAS beads were used. To immunoprecipitate DM, 40 μl anti-DMβ mAb 47G.S4-coated PAS beads were used. Immunoprecipitated material was dissociated from the beads by boiling for 10 min in denaturation buffer (25 mM Tris (pH 7.5), 150 mM NaCl, and 1%SDS) and reimmunoprecipitated by overnight incubation with DOB.L1- or 47G.S4-coated PAS beads to isolate DOβ or DMβ single chains. DO-cleared normalized lysates were also used to immunoprecipitate calnexin as a control. All the beads were then boiled in Laemmli sample loading buffer and run on 12% reducing SDS-PAGE gels. Gels were dried under vacuum and subjected to phosphor imager analysis.

Quantification of DM and DO by ELISA

mAb 5C1 (for DM) and DOB.L1 (for DO) were immobilized on ELISA plates. The plates were incubated with 2% BSA in PBS for 2h at 37°C to block nonspecific binding of cell lysate proteins, and washed three times with PBS-T (PBS + 0.05% Tween 20). Titrated amounts of cell lysate from B cell lines and LC were prepared in PBS + 0.01% IGEPAL and incubated with the immobilized Abs for 1 h at 37°C. After washing with PBS-T, the plates were incubated with SU36 (1/250) for DM, and Mags.DO2 (1/100) for DO. After washing three times with PBS + T, the plates were incubated with peroxidase-labeled secondary mAb (1/1000) for 1 h at 37°C. After washing, the plates were developed using the colorimetric substrate ABTS and the absorbance at 405 nm was measured with a 96-well plate reader Wallac 1420 workstation (Wallac).

Table I. *Expression of DO in myeloid APC and precursors*

	RNA[b]		Protein[c]		
Cell Type[a]	DOA	DOB	DOα Western	DOβ Western	DO FACS
Monocyte	Low	Low	Pos	Neg	Neg
m + GM-CSF	Pos	Low	Pos	Neg	Nd[d]
m + IFN-γ	Pos	Pos	Pos	Neg	Nd
m + IL-10	Nd	Nd	Low	Neg	Nd
Macrophage	Pos	Low	Pos	Neg	Nd
i mDC	Pos	Low	Pos	Neg	Nd
m mDC	Pos	Low	Pos	Neg	Nd
$CD34^+$ stem cell	Pos	Low	Nd	Nd	Neg
iLC	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**
mLC	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**

[a] Primary human cells used include, in this order: primary human monocytes, monocytes stimulated with GM-CSF (m + GM-CSF), IFN-γ (m + IFN-γ), or IL-10 (m + IL-10), monocyte-derived macrophages, immature monocyte-derived DC, TNF-α-matured monocyte-derived DC, $CD34^+$ stem cells, $CD34^+$ stem cell-derived iLC, and CD40L-matured $CD34^+$ stem cell-derived LC. All results are representative of at least three experiments with cells from different donors.

[b] Relative expression of DOA and DOB transcripts was determined by real time RT-PCR. Low indicates transcript levels above levels found in the DOα- and DOβ-deficient cell lines 6.1.6 and 5.2.4, respectively, but, on average, <0.1-fold of the levels expressed by the B cell line Raji. Positive indicates transcript levels >0.1-fold of the levels expressed by the B cell line Raji.

[c] Expression of DOα and DOβ protein was determined by immunoblotting. In addition, cellular DO levels were measured by flow cytometry using the DO dimer-specific Ab Mags.DO5 or the DOβ-specific Ab DOB.L1. Results are representative of the majority of samples tested for each cell type.

[d] Nd, Not done.

Statistics

Paired and unpaired *t* tests were performed to determine statistical significance of differences. Statistical analyses were performed using GraphPad Prism (GraphPad Software).

Results

Expression of DO in primary myeloid APC

We detected RNA and protein for DO α- and β-chains in $CD34^+$ stem cell-derived cultures of iLC and mLC (Table I and Fig. 1, *A* and *B*). Intracellular staining with the conformationally sensitive mAb, Mags.DO5, indicated that the monomeric chains form heterodimers (Fig. 1*C*). The level of staining observed is a conservative estimate, because we set the threshold for nonspecific staining of cells as the level of labeled anti-DO Ab bound, after blockade with unlabeled anti-DO Ab. Double staining with the anti-DO mAb and the LC markers CD1a and CD83 for iLC and mLC, respectively, demonstrated that only the LC within the cultures (20–40% of total nonadherent cells) express DO heterodimers (data not shown). $CD34^+$ stem cells themselves express DOA RNA, but only negligible levels of DOB RNA and no detectable DO dimer by flow cytometry (Table I).

To determine whether DO expression varies in a donor-dependent manner, we compared iLC cultures derived from progenitor cells from 10 different donors. By flow cytometry, the mean fluorescence intensity (MFI) of DO staining varied between donors by as much as 10-fold with a median MFI of 5.29, and the percentage of DO^+ iLC varied between donors by as much as 12-fold, with a median of 40% (data not shown). Fig. 1*C* shows two examples with varied DO expression. The variation in the percentage of DO^+ LC from different donors is substantially higher than the variation in the percentage of DO^+ LC (maximum 3.4-fold) in different cultures derived from stem cells from the same donor (data not shown). Thus, iLC cells from all donors examined expressed DO dimers, but there are donor-dependent differences in the level of DO expression.

To further assess expression of DO in primary human APC, we examined a panel of cells, including blood monocytes, monocyte-derived macrophages, monocyte-derived DC, and monocyte-derived LC (Table I). We find DOα RNA and/or protein in all of these cells, but DOβ protein is not detectable by immunoblotting in any of them. We also tested whether monocyte expression of DO could be induced by factors known to affect monocyte activation. GM-CSF treatment, which we have shown increases monocyte expression of class II MHC, DM, and Ii (17), increases expression of DOα RNA and protein levels, but does not affect DOβ expression (Ref. 17 and Table I). IFN-γ treatment of monocytes leads to increased RNA and protein levels of DOα and increased levels of DOβ RNA; however, DOβ protein is not detected by immunoblot (Table I). IL-10 treatment of monocytes does not induce DOβ protein expression (Table I), nor does the combination

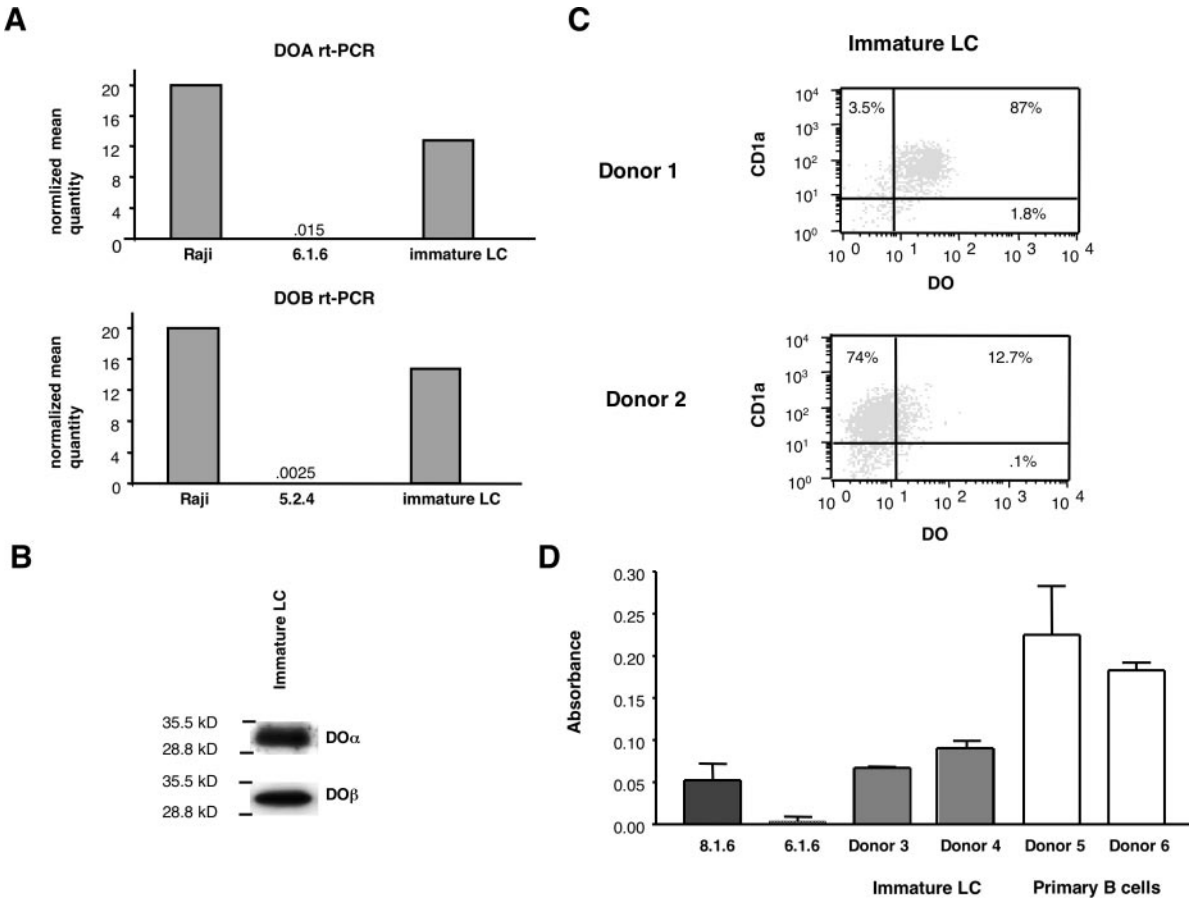

FIGURE 1. In vitro-derived LC express DO. *A*, Real-time RT-PCR of DOA (*top*) and DOB (*bottom*) mRNA expressed by iLC. Relative mean quantities, normalized to GAPDH expression, are shown (see *Materials and Methods*). RNA from B-LCL 5.2.4 and 6.1.6 are negative controls for DOB and DOA, respectively. Results from 1 donor (experiment), representative of 17 experiments (8 donors), are shown. *B*, LC protein expression of DOα and DOβ by immunoblotting with polyclonal antisera specific for DOα and mAb DOB.L1, respectively. Results are representative of 6 donors. *C*, DO expression by iLC, determined by flow cytometry using the anti-DO mAb, MagsDO5, and anti-CD1a. Results are from 2 of 10 donors. *D*, DO dimer expression in cell lysates (40 μg of protein) from enriched iLC, B-LCL 8.1.6 and 6.1.6 (negative control), and primary tonsillar B cells, determined by ELISA.

of GM-CSF, IL-4, and TGFβ, which has been reported to stimulate monocyte differentiation into Langerhans type DC (Ref. 19; data not shown) Taken together, our results argue that DO induction is blocked in monocytes and monocyte derivatives and requires $CD34^+$ stem cells and/or the specific mixture of cytokines that differentiate them toward LC. Expression of DOβ correlates with expression of DO heterodimers, implying that DOB modulation is a mechanism of regulating DO protein levels.

To gauge LC expression of DO relative to other DO-expressing APC, we compared the level of DO expressed by enriched LC (see *Materials and Methods*) to that expressed by B-LCL, a model of activated B cells, and primary tonsillar B cells, which are a mix of resting and activated B cells. We measured DO levels in 40 μg of protein from the different cell types (Fig. 1*D*). Protein from enriched iLC contains similar levels of DO as B-LCL 8.1.6, but significantly lower levels than comparable amounts of protein from primary B cells ($p < 0.02$).

Normal human skin LC express HLA-DO

To determine whether LC found in situ also express DO, we examined normal skin sections for DO^+ LC. Sections were stained with Abs to CD1a or langerin to identify DC ($CD1a^+$) and LC ($CD1a^+$ and $langerin^+$), and DO. As shown in Fig. 2, DO staining colocalizes with CD1a and langerin staining in the skin. Some LC in the epidermis and some $CD1a^+$ DC in the superior dermis express DO. These data corroborate our findings with in vitro-derived LC.

Subset-specific expression of DO in human blood DC

To determine whether other types of primary human DC express DO, we tested freshly isolated circulating DC. To identify these DC, we stained whole blood with a FITC-labeled mixture of mAbs to T cells, monocytes, neutrophils, B cells, and NK cells (lin 1: anti-CD3, -CD14, -CD16, -CD19, -CD20, -CD56) and with an anti-DR mAb conjugated to PerCP. To allow assessment of $CD16^+$ DC (see below), we also used a lineage cocktail (lin2) lacking anti-CD16. We defined DC in a standard manner, as those cells that fall within an expanded lymphocyte gate by forward and side scatter, are lin-negative, and express high surface levels of DR (Fig. 3*A* and Ref. 28). Intracellular staining for DO showed that DO^+ DC are present in 17 of 17 donors examined, but represent a minority of the total ($lin1^-$) DC: 10.6–31.3% of lin 1^-, DR^+ cells are DO^+ with a median of 19.5% (Fig. 3*B*, histogram, and data not shown). In addition, the level of DO expression is quite variable across donors, with a range in MFI of 16.7–74.03 and a median of 36.2. In samples from some donors, we compared the frequency of DO^+ DC and the level of DO expression in DO^+ DC to the frequency of circulating DO^+ B cells and their level of DO expression. The proportion of DO^+ DC varied from 11 to 28% in four donors, while 76–85% of circulating B cells in these donors expressed DO (data not shown). We found that the DO^+ DC express on average 50% of the level of DO per cell (based on MFI) as B cells (not shown). This is consistent with the reduced levels of DO in in vitro-derived LC compared to primary B cells (Fig. 1*D*).

Given the heterogeneity of circulating DC, we were interested in whether DC subtypes differ in DO expression. The two major subsets of blood DC are myeloid DC, which stimulate Th1 responses to bacterial infection and plasmacytoid DC, which produce large amounts of type I IFN in response to viruses (reviewed in Refs. 30 and 31). We used a mAb specific for the integrin CD11c to delineate myeloid ($CD11c^+$) and plasmacytoid ($CD11c^-$) DC. A fraction of both $CD11c^+$ and $CD11c^-$ ($lin1^-$) DC are DO^+ (Fig. 3*B*). However, a greater percentage of $CD11c^+$ DC express DO compared with $CD11c^-$ DC (mean of 31.5% vs 12.1%, $n = 4$, $p = 0.007$). We also identified plasmacytoid DC by their expression of two different cell surface markers, CD123 and BDCA-2. Using these markers (Fig. 3*C*), we find a minor percentage of plasmacytoid ($lin1^-$) DC ($CD123^+$ or $BDCA\text{-}2^+$) express DO: 5.1–17% of the $CD123^+$ DC with a median of 11.6%, $n = 4$; 1.5–19.2% of the $BDCA\text{-}2^+$ DC with a median of 7.6%, $n = 11$. Further, in the small percentage of plasmacytoid DC that express DO, the level of expression of DO per cell is lower than myeloid DC; the median MFI of DO^+ plasmacytoid compared to myeloid DC is 38.2 vs 52.4, $p = 0.009$.

Myeloid ($CD11c^+$) DC can be further differentiated into $CD1c^+$/$BDCA\text{-}1^+$, $BDCA\text{-}3^+$, and $CD16^+$ DC (32). BDCA-1 is expressed on a large population of $CD11c^{bright}$ DC that have the highest allostimulatory capacity of the blood DC subsets (32). $BDCA\text{-}3^+$ DC constitute a small subpopulation that express reduced levels of CD11c, and lack expression of CD2 and several FcRs (33). The subset of DC-expressing CD16 (FcγRIII) (34) expresses low levels of CD14, and may comprise the same population as "$CD16^+$ monocytes" (35, 36). We examined DO expression in each of these subsets (Fig. 3*D*). $CD16^+$ (lin 2^-) DC do not

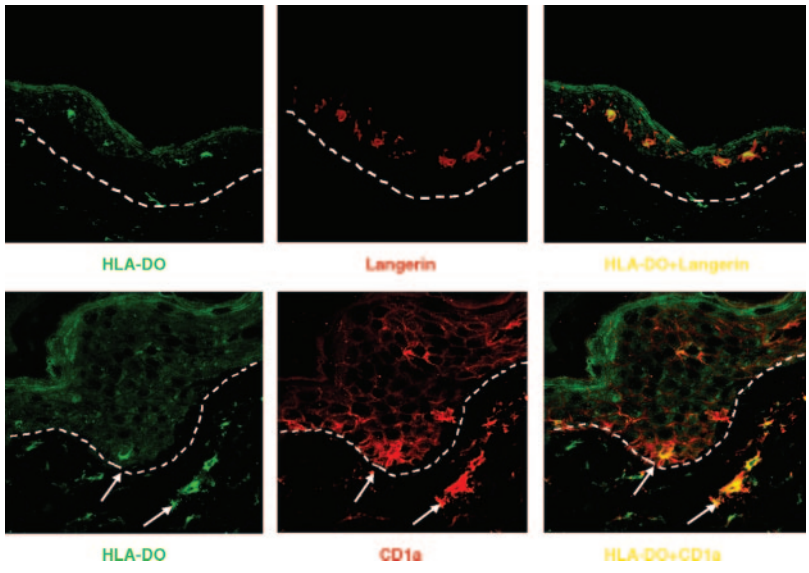

FIGURE 2. Normal skin LC express DO. Immunohistochemistry of normal human skin sections using Abs specific for DO (green, *left*) and either CD1a (*bottom*) or Langerin (*top*) (red, *center*) to detect DC ($CD1a^+$, $Langerin^-$) and LC ($CD1a^+$, $Langerin^+$). Colocalization is indicated by yellow staining (*right*). The white dotted line indicates the margin between the epidermis (*top*) and the dermis (*bottom*). The stratum corneum shows autofluorescence. Original magnification, $\times$400.

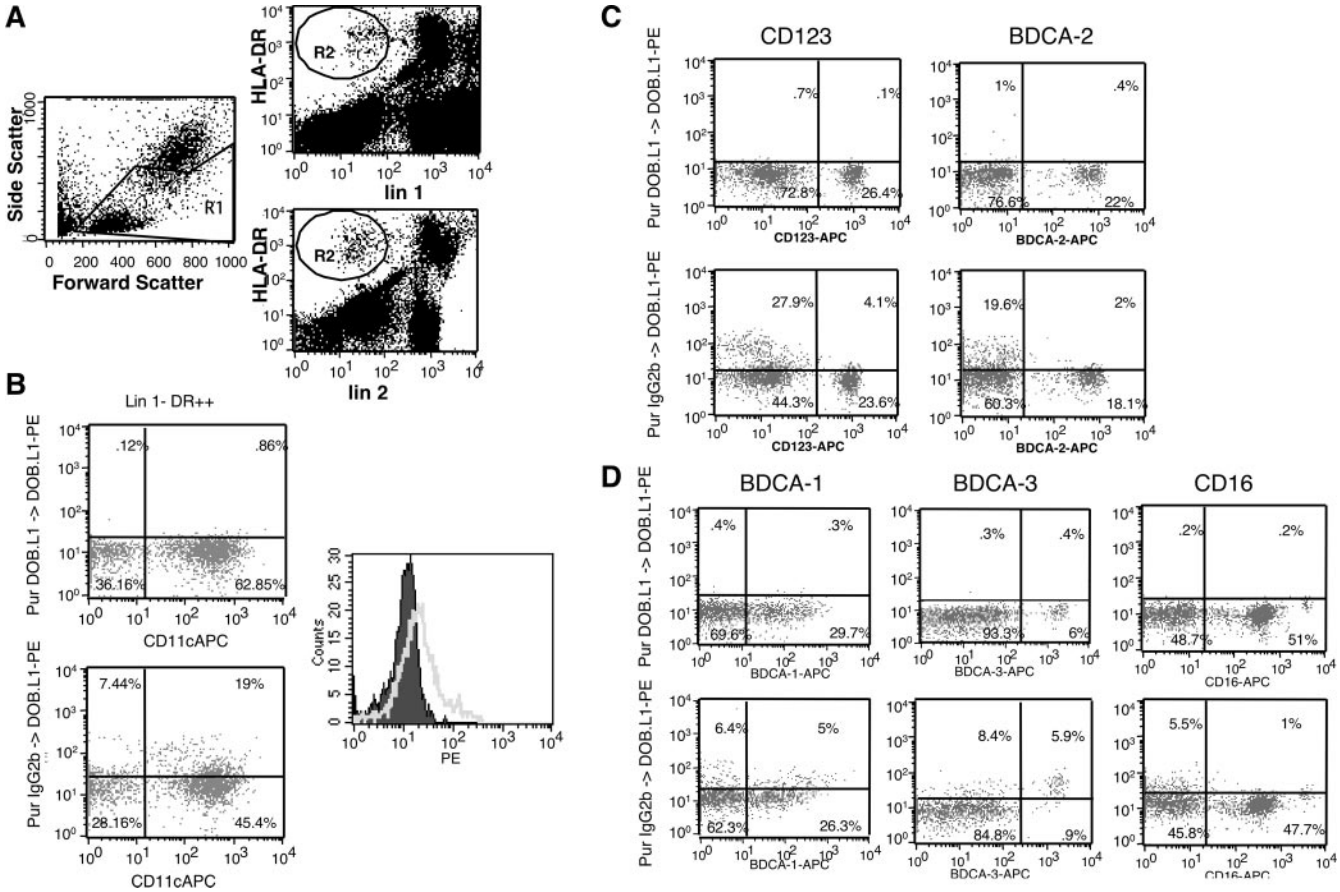

FIGURE 3. Human blood DC express HLA-DO. Flow cytometric analyses of DC subsets in whole blood. *A*, DC were defined as cells within an expanded lymphocyte gate defined by forward and side scatter parameters (R1) and lin$^-$, DR$^+$ (R2). Cells were stained with lin 1 cocktail (mAb to CD3, CD14, CD16, CD19, CD20, CD56) or, for CD16$^+$ DC, lin 2 cocktail (CD3, CD14, CD19, CD20, CD56), DR, DO) and CD11c (*B*), CD123 or BDCA-2 (*C*), or BDCA-1 or BDCA-3 or CD16 (*D*) and analyzed by flow cytometry. The *upper dot plots* in *B–D* show staining with purified anti-DO followed by PE-labeled anti-DO as a negative control; the *lower plots* show cells labeled with purified IgG2b followed by PE-labeled anti-DO. *B*, The dot plots show DO expression by CD11c$^+$ vs CD11c$^-$ DC. The histogram plot shows DO expression on all DC, defined as lin1$^-$, DR$^+$. The shaded histogram is staining with purified anti-DO followed by PE-labeled anti-DO, and the open histogram is staining with purified IgG2b followed by PE-labeled anti-DO. Results from 1 of 4 donors examined are shown. *C* and *D*, Expression of DO by plasmacytoid (*C*) and myeloid (*D*) blood DC subsets. Each plot shows cells from 1 donor; 4 were examined for BDCA-1, CD16, and CD123, 11 for BDCA-2, and 13 for BDCA-3. The plots are from different donors, with the exception of BDCA-1 and CD16.

express DO (the percentage of DO$^+$, CD16$^+$ DC $<2\%$, $n = 4$). A small percentage of the CD1c$^+$/BDCA-1$^+$ (lin1$^-$) DC are DO$^+$ (8.3–21%, median 16%, $n = 4$), whereas the majority of BDCA-3$^+$ (lin1$^-$) DC are DO$^+$ (65–93%, median 84%, $n = 13$). Taken altogether, these results indicate that there is a hierarchy of DO expression in circulating DC subsets with BDCA-3 > BDCA-1 > BDCA-2 > CD16.

Expression of HLA-DO by tonsillar DC

To evaluate whether DC in secondary lymphoid tissue express DO, we examined human tonsillar tissue DC. Tonsillar DC are resident cells that are mostly blood-derived and can be subtyped as interdigitating DC, plasmacytoid DC, and germinal center DC (37). We used mAbs specific for DO and CD11c to stain tonsillar tissue. We chose to focus on the CD11c$^+$ interdigitating DC as they can be identified by their high expression of CD11c and distinct morphology. In addition, they may be related to blood myeloid DC, which express more DO than CD11c$^-$ blood plasmacytoid DC (Fig. 3). As controls, we stained with mAb to CD68, which is largely macrophage-specific in situ (29), and CD19, which is specific for B cells. We found that CD11c and DO staining colocalize (Fig. 4, *upper right*), and the double-labeled cells have morphology consistent with DC. In contrast, we see limited colocalization between CD68 and DO staining, indicating that most tonsillar macrophages, like in vitro monocyte-derived macrophages, do not express DO (Fig. 4, *upper left*). Consistent with published reports (5, 6), germinal center B cells (centrocytes/blasts) express less DO than B cells in the mantle zone (Fig. 4, *lower left*). Thus, DO in tonsils is found in B cells and DC, but not macrophages, similar to results with cell lines and circulating cells.

Functional assessment of DO expression in LC

The only known function of DO is to modulate peptide exchange by DM. Therefore to address the function of DO in LC, it was important to evaluate their DM expression. In Fig. 5*A*, we show that iLC express significantly higher levels of DM than primary tonsillar B cells ($p = 0.001$). Inhibition of DM function by DO can be seen as an increase in cell surface CLIP bound to class II MHC (referred to as CLIP/class II levels) and in the ratio of surface CLIP/class II to total surface DR expression (referred to as CLIP:DR ratio, which normalizes for differences in DR surface expression) (8). Relative to B-LCL, iLC express more DM and comparable DO levels, implying they have a high DM:DO ratio and predicting low expression of surface CLIP/class II and a low

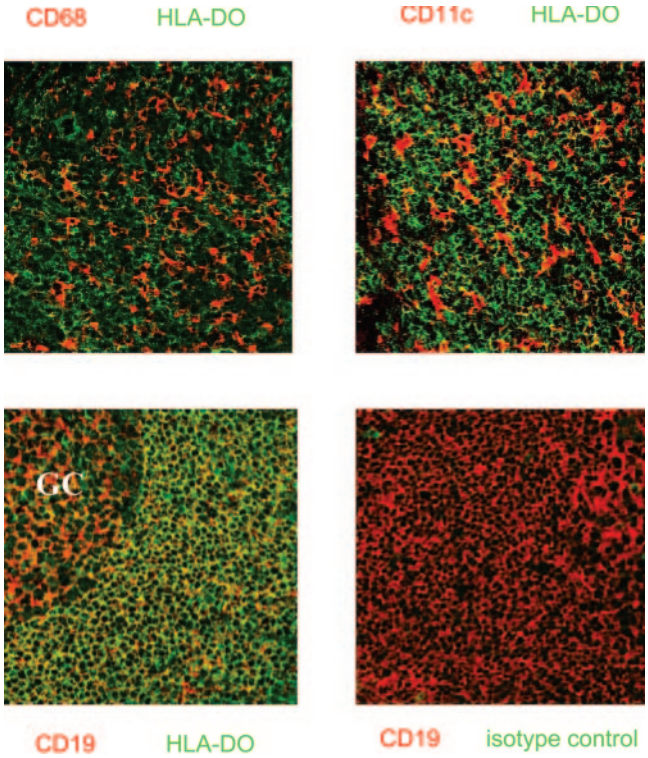

FIGURE 4. Human tonsillar DC, but not macrophages, express DO. Immunohistochemistry of human tonsil sections, using Abs specific for DO detected by Cy2 (green)-labeled secondary reagent or an isotype control, and either CD11c, CD68, or CD19 to detect DC, macrophages, or B cells, respectively, detected by Cy3(red)-labeled secondary Ab. Colocalization is indicated by yellow staining. GC, germinal center.

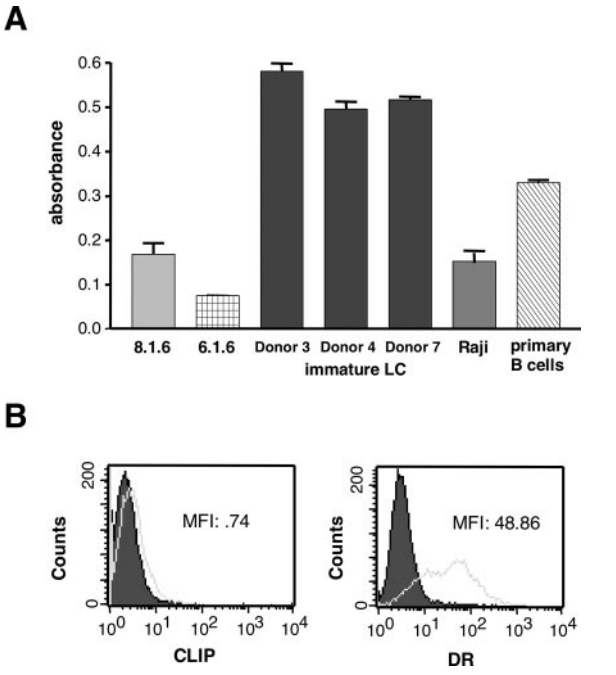

FIGURE 5. iLC express high levels of DM and low levels of surface CLIP/class II. *A*, DM expression in cell lysates (20 μg of protein) from enriched iLC, B-LCL Raji, 8.1.6, and 6.1.6 (negative control), and primary tonsillar B cells, determined by ELISA. *B*, Flow cytometric analyses of iLC, using CerCLIP, mAb to CLIP/class II *(left)*, and L243, mAb to DR dimer (*right*), shown in open histograms. The shaded histograms are staining with isotype controls. LC were gated by size and CD1a expression. One example, representative of 12 analyses (seven donors), is shown.

CLIP:DR ratio. Staining with an anti-CLIP Ab is only slightly higher than isotype control on iLC (MFI of 0.65–3.85, mean of 1.65, $n = 12$, one example is shown in Fig. 5*B*). CLIP:DR ratios vary from 0.6 to 7.2% with a median of 1.9%, which is comparable to B-LCL 8.1.6 supertransfected with HLA-DM (26).

Despite the overall low surface CLIP/class II expression levels on these in vitro-derived LC, we were interested in whether higher DO was associated with higher CLIP:DR ratios, consistent with an inhibitory effect of DO on DM. Therefore, we compared the LC expressing the highest DO levels with the LC expressing the lowest DO levels from the same donor. Gating on $CD1a^{high}DO^{high}$ vs $CD1a^{high}DO^{low}$ iLC (Fig. 6*A*) and then assessing surface CLIP/class II levels, we found that the DO^{high} LC express higher levels of surface CLIP/class II compared with DO^{low} LC. The DO^{high} cells also express higher levels of surface DR; yet despite this, the surface CLIP:DR ratio is higher in the DO^{high} LC compared with the DO^{low} LC in four donors ($p = 0.022$, Fig. 6*C*). These results imply that DO inhibits DM function in iLC. Notably, the DO^{high} LC also express higher levels of DM compared with DO^{low} LC. However the DM:DO ratio is higher in DO^{low} expressing cells than in DO^{high} expressing cells ($p = 0.013$, Fig. 6*D*). Thus, the DM:DO ratio correlates with the CLIP:DR ratio in LC.

A similar analysis of DM^{high} vs DM^{low} expressing LC (Fig. 6*B*) shows that DM^{high} LC express higher levels of surface DR, comparable levels of surface CLIP/class II, and thus lower CLIP:DR ratios than DM^{low} LC ($p = 0.036$, Fig. 6*C*). This is despite slightly higher DO expression by the DM^{high} LC. These results are consistent with the known ability of increased DM to decrease the surface CLIP:DR ratio.

To corroborate these findings, we examined LC that express HLA-DR3 or -DR4 with a mAb that specifically recognizes DR3/CLIP and DR4/CLIP complexes (38) and mAbs that specifically recognize DR3/non-CLIP endogenous peptides or DR4/non-CLIP endogenous peptides (39, 40). The staining with these Ab on iLC from four independent donors (data not shown) showed a consistent and statistically significant increase in the ratio (by MFI) of DR/CLIP to DR/non-CLIP complexes at the surface of DO^{high} compared with DO^{low} cells ($p < 0.006$). Taken together, our data argue that DO inhibits DM function in in vitro-derived iLC and appears to modulate the expression of other endogenous peptides as well.

Maturation leads to decreased expression of DO and DM in LC, but increased DM:DO ratio

Activation of DO-expressing naive B cells and their differentiation into germinal center B cells results in down-modulation of DM and DO expression, but with a greater effect on DO such that the DM:DO ratio increases (5, 6). To determine whether similar changes are associated with maturation of LC, we stimulated iLC with trimers of CD40L and measured the effects on levels of DO and DM protein by intracellular flow cytometric analysis, ELISA, and semiquantitative Western blot. In 16 of 17 experiments (nine donors), the MFI of DO staining either decreases following maturation with CD40L or is unchanged (Fig. 7*A*). On average, there is a significant decrease in DO expression in mLC compared with iLC ($p = 0.0019$). In addition, the percentage of DO^+ cells significantly decreases in mLC relative to iLC (mean 33% $CD1a^+DO^+$ iLC vs 19% $CD83^+DO^+$ mLC, $p = 0.004$; data not shown). DO ELISA and Western blot analyses using titrated amounts of whole cell lysate from enriched iLC and mLC show a similar trend of decreased levels of DO in mLC compared to iLC (Fig. 7*A*). Flow cytometric analyses demonstrate that DM protein

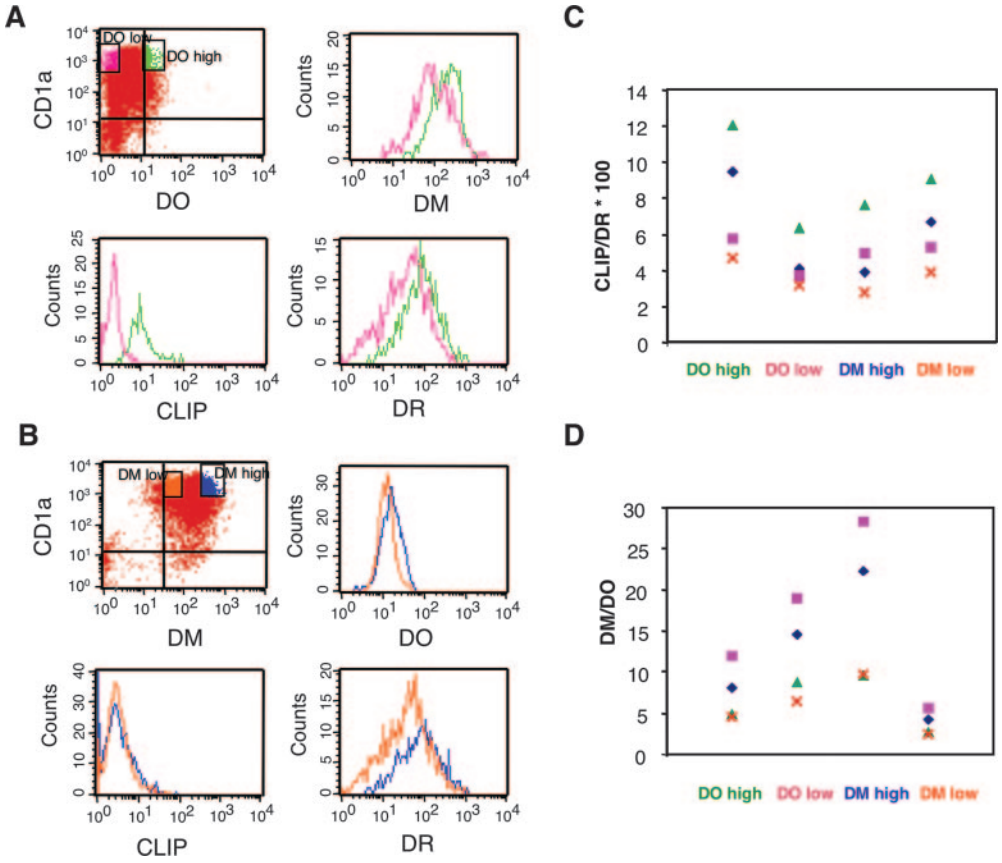

FIGURE 6. DO^{high} LC express a higher ratio of surface CLIP/class II to DR than DO^{low} LC. Flow cytometric analyses of iLC with mAb to 1) CD1a, DO, and CLIP or 2) CD1a, DO, DM, and DR. The highest and lowest DO (*A*) or DM (*B*) expressing iLC were gated as shown and CLIP, DR, and DM (*A*) or DO (*B*) expression in these populations was analyzed. Results from one of four donors with similar results are shown. *C*, The ratio of cell surface CLIP/class II to DR expressed by iLC, based on the MFI for each staining for each population in *A* and *B*. *D*, The ratio of DM:DO expression by iLC, based on the MFI for each staining for each population in *A* and *B*.

levels also decrease in the majority of experiments (7 of 10 from five donors), while in the remainder, DM levels increase slightly following maturation (Fig. 7*B*). On average, there is a significant decrease in DM expression in mLC compared with iLC ($p = 0.03$). DM ELISA and immunoblot analyses of titrated amounts of whole cell lysates also show decreased DM levels in mLC vs iLC (Fig. 7*B*). Although both DM and DO expression decrease following exposure of LC to maturation stimuli, the ratio of DM:DO expression modestly increases in 7 of 10 experiments (five donors), with a decrease in two cultures from the same donor and no change in one (Fig. 7*C*). Overall, there is an increase in the DM:DO ratio upon maturation of iLC ($p = 0.03$).

The observed changes in the DM:DO ratio following LC maturation suggest that the CLIP:DR ratio will also be affected as we have shown that DO inhibits DM function in LC. We examined the relationship of these two parameters in six cultures (four donors) (Fig. 7*D*). Upon maturation, surface DR substantially increased in all experiments, regardless of donor, as expected (data not shown). The fold change in the DM:DO ratio predicted the change in the CLIP:DR ratio in all but one case (square symbol). This outlier may express a class II allele with low affinity for CLIP.

The mechanism of maturation induced down-regulation of DM and DO expression in LC

To explore the mechanism of DO and DM down-regulation following LC maturation, we asked whether new synthesis of DO and DM is altered by maturation. We conducted biosynthetic labeling and immunoprecipitation analyses and found a decrease in the amount of nascent DMβ and DOβ in mLC compared to iLC (Fig. 8*A*). This finding suggested that one mechanism for maturation-induced decreases might be decreased levels of DO and DM RNA. Therefore, we measured these levels in iLC and mLC by real-time RT-PCR. Maturation with CD40L results in consistent down-regulation of DOβ mRNA, but not of DOα mRNA levels in LC (Fig. 8*B*; $p < 7 \times 10^{-13}$ and $p = 0.182$, respectively). In fact, diverse maturation stimuli, including LPS, TNF-α, and manipulation alone (mock), result in significantly decreased levels of DOβ mRNA (Fig. 8*C*; $p = 0.04$ (mock), $p = 0.012$ (LPS), $p = 0.008$ (TNF-α)). Maturation with CD40L also significantly decreases DMα and DMβ mRNA levels (Fig. 8*B*; $p < 0.0001$ and $p = 0.0001$, respectively). Taken together with our data on expression of DO in various myeloid APC and their precursors (Table I), these results implicate modulation of DOB transcript levels as one mechanism for the regulation of DO protein expression in APC and precursors.

Discussion

In this study, we demonstrate that primary human DC express DO. This result is in contrast to the current paradigm that DO expression is limited to B cells and thymic epithelial cells. The conclusion that DC do not express DO was based on findings from monocyte-derived DC that express DOα, but DOβ, and thus no DO dimer (Refs. 5 and 14; Table I). We have measured DO expression by multiparameter flow cytometry, real-time RT-PCR, immunoblotting, and immunohistochemistry. We find DO expression in LC, tonsillar interdigitating DC, and subsets of circulating DC.

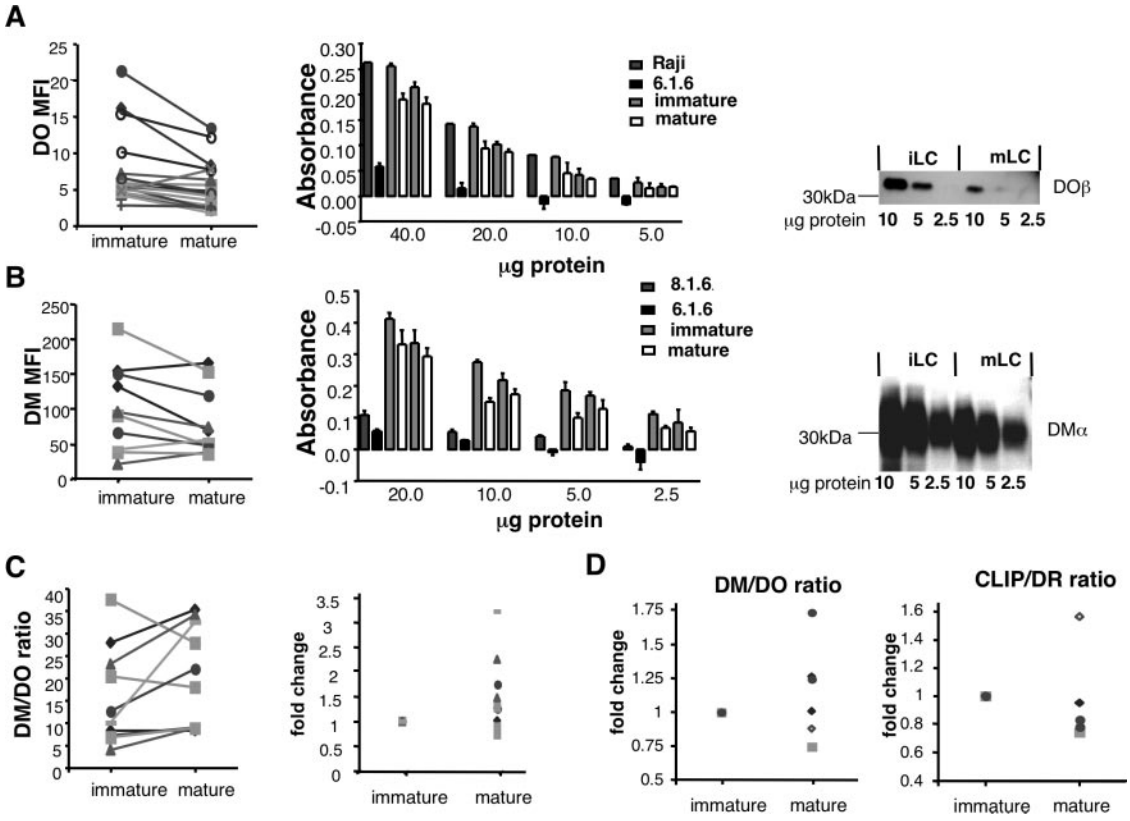

FIGURE 7. Maturation of LC decreases steady state levels of DO and DM leading to an increase in the DM to DO ratio. *A* and *B*, *left panels*, DO levels (*A*) or DM levels (*B*) in iLC and mLC, determined by flow cytometric analysis of cells stained with Abs specific for CD1a (iLC) or CD83 (mLC) and DO or DM. The change in MFI of staining is shown; each stem cell donor is represented by a unique symbol type. *Middle panel*, DO (*A*) or DM (*B*) levels in CD1a or CD83 enriched LC, determined by ELISA from the indicated amount of protein from cell lysates. Results are from two donors of three tested with similar results. *Right panel*, DO (*A*) or DM (*B*) levels in the indicated amount of protein from lysates of CD1a or CD83 enriched LC, as determined by immunoblot using mAb DOB.L1 (*A*) or 5C1 (*B*). Results are from one donor of two tested with similar results. *C*, The ratio of DM to DO expression in iLC vs mLC as determined by flow cytometric analysis in *A* and *B* is shown. The DM:DO ratios (*left*) and the fold change in DM:DO ratio between iLC and mLC (*right*) are shown. *D*, iLC and mLC were stained with Abs to CD1a or CD83, DO, DM, CLIP, and DR. The fold changes in the ratio of DM:DO (*left*) and cell surface CLIP/class II to DR (*right*) staining between iLC and mLC is shown based on the MFI of each staining. There is a significant decrease in the CLIP:DR ratio in mLC compared with iLC only if we remove the one case in which the CLIP:DR ratio increased following maturation ($p = 0.0039$).

Together, these results provide strong evidence that the tissue distribution of DO is broader than was previously appreciated and extends to certain subsets of primary human DC.

Human myeloid DC that express DO include in vitro-derived LC, LC in normal skin, circulating BDCA-3$^+$ DC, a subset of BDCA-1$^+$ DC, and tonsillar interdigitating DC. However, not all human myeloid lineage DC are DO$^+$. Monocyte-derived DC, both interstitial-like (generated with GM-CSF and IL-4) and LC-like (generated with GM-CSF, IL-4, and TGFβ), lack DO expression. In addition, CD16$^+$ DC, which may be equivalent to CD16$^+$ monocytes (35, 36), are DO-negative. Thus, monocyte-derivatives do not express DO even after in vitro differentiation into DC. Monocytes have been shown in mice to give rise to DC (41); whether these in vivo-derived DC also lack DO expression remains to be tested.

Notably, and in contrast to other circulating DC subtypes, the BDCA-3$^+$ human blood DC subset is more uniformly DO$^+$ in all donors examined. BDCA-3$^+$ DC are a small fraction of total blood DC (2.7 $\pm$ 1.4%, Ref. 32). Because of their low frequency, they have not been extensively studied, and their function is unknown. BDCA-3$^+$ DC express higher levels of CD40 and DEC-205 than other circulating DC subsets, lack expression of CD2, FcRs, and CD80, and express low levels of CD86 and Ig-like transcripts (ILT) (32). They also express high levels of CD62L, suggesting that they migrate from the blood into the lymph node (32). BDCA-3 expression can be induced on other subsets of blood DC (BDCA-1$^+$/CD1c$^+$ and CD123$^+$) after culture in vitro, raising the possibility that BDCA-3$^+$ cells are related to these other subsets (33). In addition, monocyte-derived DC matured in the presence of IL-10 up-regulate BDCA-3 expression, but differ from circulating BDCA-3 cells by being ILT^{high} (42). Recently, nectin-like protein 2 (Necl2), a molecule expressed uniquely on human BDCA-3$^+$ blood DC, was found on a rare subset of mouse CD8α^+ DC suggesting that this subset may be the murine equivalent of human BDCA-3$^+$ DC (43). Interestingly, CD8α^+ murine splenic DC express more H2-O than CD8α^- DC (50).

Our finding that blood myeloid DC express more DO than blood plasmacytoid DC argues for DC lineage-specific differences in DO expression, as do the similar differences observed in DO expression in these DC subtypes in the mouse (50). Alternatively, the lack of DO expression by blood plasmacytoid DC could reflect the differentiation state of these cells. Blood plasmacytoid DC are thought to be DC precursors, which may contribute to their low DO expression, as we find that other DC precursors (CD34$^+$ stem cells) also do not express DO.

In addition to subset-specific differences, we see variability in the proportion of DO$^+$ cells within a given DC subset. DO expression is dependent upon DM expression; in the absence of DM,

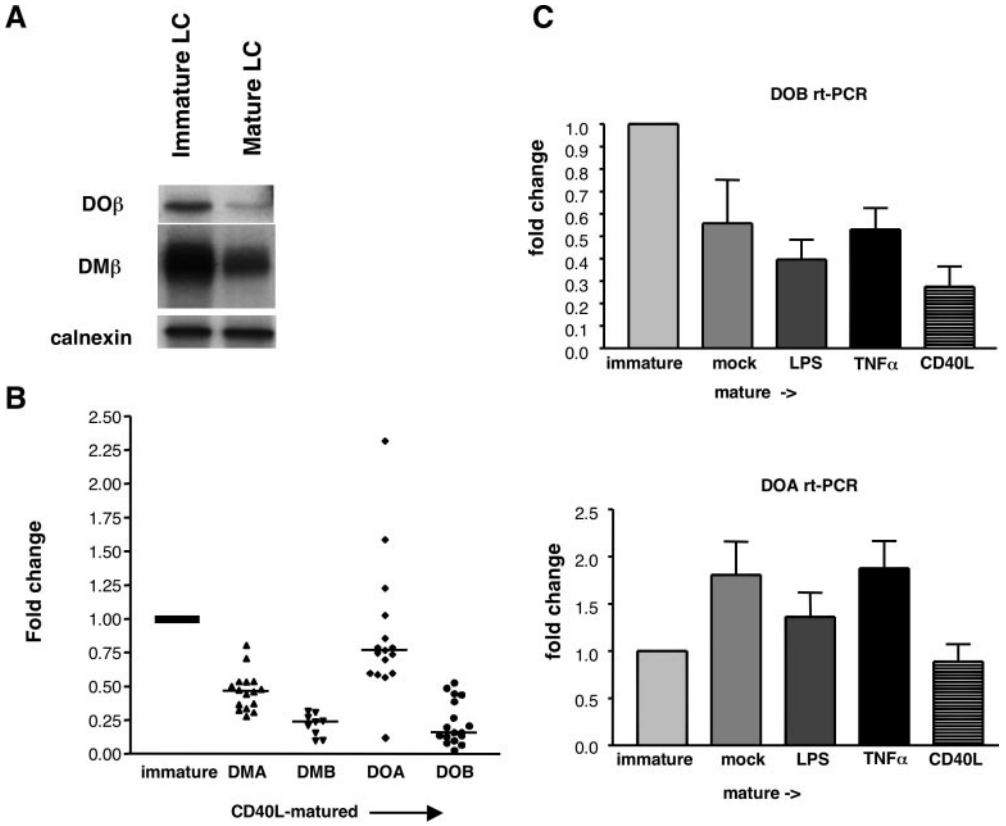

FIGURE 8. Maturation decreases DMα, DMβ, and DOβ, but not DOα, transcript levels in LC and decreases synthesis of DOβ and DMβ. *A*, SDS-PAGE analyses of immunoprecipitated DOβ (using mAb DOB.L1) and DMβ (mAb 47G.S4) from [^{35}S]methionine pulse-labeled iLC and mLC. Calnexin was immunoprecipitated as a control. Data are representative of five experiments testing iLC and mLC from three donors. *B*, Fold change in expression, in CD40L-matured LC compared with iLC, of DMA, DMB, DOA, and DOB mRNA, determined by real-time RT-PCR. The relative quantity of each transcript was determined after normalization to the quantity of GAPDH RNA. The median-fold change is indicated by a bar. *C*, The mean fold change ± SEM in expression of DOA and DOB mRNA in mLC compared with iLC, determined by real-time RT-PCR. RNA was harvested from iLC and mLC matured for 2 days with manipulation alone (mock), LPS, TNF-α, or CD40L. The relative quantity of each transcript was determined after normalization to the quantity of GAPDH RNA.

DO fails to exit the ER (2). Thus, high DO expression may be linked to high DM expression. For example, in three of three donors tested, the BDCA3$^+$ subset has higher DM expression compared with BDCA-2$^+$ DC (data not shown). In CD34$^+$ stem cell-derived LC, we also observe that high DO expression is correlated with high DM and DR expression. Characterization of the differences between the DO$^+$ and DO$^-$ DC within each DC subset may reveal additional heterogeneity within these populations.

We also find evidence that DC expression of DO varies in a donor-dependent fashion in LC cultures. However, here the variability does not appear to correlate with DM protein expression or DO RNA level (data not shown), suggesting that differences in posttranslational regulation of DO may play a role. For blood DC, there is also variability in the percentage of DO$^+$ DC between different donors, perhaps reflecting differences in the relative representation of different DC subsets or polymorphisms affecting DO levels directly. Interestingly, donor-to-donor variability in DO expression has been observed in acute myeloid leukemia blasts; DO:DM ratio correlated with CLIP:DR ratio in these cells, and patients with more DR$^+$/CLIP$^+$ blasts had significantly shorter disease-free survival (44). Whether DO expression in the blasts reflected variations in DO expression in all DO$^+$ cells in the patients or reflected alterations unique to the acute myeloid leukemia blasts was not reported.

DO expression is also regulated by exposure to maturation stimuli in the LC culture system, where CD40L, TNF-α, and LPS induce a dramatic decrease in DOB RNA and nascent DOβ protein levels. In addition, incubation of iLC with PHA-stimulated allogeneic PBMC leads to reduced DO levels in six of six cases; in five of six cases DM:DO ratios increase (data not shown). These results suggest that maturation of iLC most often leads to decreased expression of DM and DO, but modest elevation of the DM:DO ratio. This finding is similar to what has been found for primary human B cells, where treatment with anti-IgM or PMA decreases DO and DM expression (45). In vivo, naive B cells express more DO and DM than germinal center B cells, but germinal center B cells have a higher DM:DO ratio compared with naive B cells (5, 6). However, in contrast to what we find in LC, the decrease in DM and DO expression in germinal center B cells relative to naive B cells does not correspond with a decrease in mRNA levels (14). Thus, it appears that distinct mechanisms are responsible for regulation of DO expression in DC compared to B cells.

Our finding that IFN-γ-treated primary human monocytes upregulate expression of DOA and DOB RNA without evidence of appreciable DO protein provides further support for both transcriptional and posttranscriptional regulatory mechanisms for DO expression. In LC, down-regulation of DO expression following maturation appears to be due to dramatic reduction in DOB message levels, without a change in DOA mRNA levels. This discoordinate regulation of DOα and DOβ expression has been seen in a variety of settings (46, 47). The function of differential regulation of DOα

and DOβ is not clear, but evokes the possibility that the α-chain may have a distinct role and partner, independent of DOβ.

The ratios of surface CLIP:DR are lower on GC B cells than on naive B cells consistent with the increase in DM:DO ratio in GC B cells compared with naive B cells (5, 6). Our data reveal a tendency for mLC to express a lower CLIP:DR ratio compared with iLC. iLC, generated in vitro, express very low ratios of surface CLIP:DR, most likely because of their high levels of DM relative to DO. This provides less of a window in which to observe differences in the CLIP:DR ratio following maturation. Assays to delineate the peptide repertoire may be more sensitive to activation-induced changes in these cells. It also will be interesting to determine whether there are kinetic effects on DM and DO expression following LC maturation.

We find that DO inhibits DM function in LC; DO^{high} cells express a higher ratio of surface CLIP:DR than DO^{low} LC. DM localization, expression level, and activity, as well as the pH of the peptide-loading compartments, regulate the function of DM in DC. Both DM expression and specific activity were reported to be decreased following LPS-induced maturation of immature monocyte-derived DC (16). In LC, we find that DM levels decrease in most cases following maturation, but we find no evidence that similar amounts of DM isolated from iLC and mLC exhibit different activity (T. Burster, data not shown).

How might expression of DO influence Ag presentation function in DC? By inhibiting DM, DO may function to broaden the repertoire of peptides presented by DC (10, 11). This possibility can be tested by comparing the peptides bound to class II MHC molecules isolated from DO^{high} vs DO^{low} expressing LC. DO also may constrain Ag presentation by DC until they are mature/activated. Our finding that DO levels are decreased following maturation indicates that DO expression is highest in immature DC, which are poor APC, but the specific contribution of DO to this is unknown. Studies of ectopic DO expression in mouse and human DC found increased surface CLIP/DR levels and modulation of Ag presentation consistent with DM inhibition (3, 4). However, these studies do not elucidate the physiologic contribution of DO in DC, where DO is regulated by endogenous signals.

The finding that DO is expressed by iLC helps focus models on functions common to iLC, naive and memory B cells, and thymic epithelia. One attractive possibility is a role in the maintenance of self tolerance, as previously proposed (48). In this model, the ability of DM to narrow the surface MHC/peptide repertoire is inhibited by DO in cells that present self-peptides. The MHC/peptide repertoire used to initiate or maintain tolerance is expanded and crypticity is reduced. Interestingly, a recent analysis of PBMC from patients with systemic lupus erythematosus, insulin-dependent diabetes mellitus, rheumatoid arthritis, or multiple sclerosis and their first-degree relatives revealed increased expression of *DMB* compared with healthy controls (49). One can speculate that this may readjust the DM:DO balance in DO-expressing APC in favor of presentation of immunodominant self-peptides, thereby increasing susceptibility to a break in self tolerance.

Acknowledgments

We thank Laura Drohan and Holden Maecker for helpful suggestions with blood DC experiments, Tyson Holmes for statistical advice, and Sujin Roh for discussions. We also acknowledge initial experiments by Vashti Lacaille suggesting that LC express DO. Recombinant human CD40LT was provided by Amgen.

Disclosures

The authors have no financial conflict of interest.

References

1. Busch, R., C. H. Rinderknecht, S. Roh, A. Lee, J. J. Harding, T. Burster, T. M. C. Hornell, and E. D. Mellins. 2005. Achieving stability through editing and chaperoning: regulation of MHC class II peptide binding and expression. *Immunol. Rev.* 207: 242–60.
2. Liljedahl, M., T. Kuwana, W. P. Fung-Leung, M. R. Jackson, P. A. Peterson, and L. Karlsson. 1996. HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. *EMBO J.* 15: 4817–4824.
3. Fallas, J. L., H. M. Tobin, O. Lou, D. Guo, D. B. Sant'Angelo, and L. K. Denzin. 2004. Ectopic expression of HLA-DO in mouse dendritic cells diminishes MHC class II antigen presentation. *J. Immunol.* 173: 1549–1560.
4. Bellemare-Pelletier, A., J. Tremblay, S. Beaulieu, M. R. Boulassel, J. P. Routy, B. Massie, R. Lapointe, and J. Thibodeau. 2005. HLA-DO transduced in human monocyte-derived dendritic cells modulates MHC class II antigen processing. *J. Leukocyte Biol.* 78: 95–105.
5. Chen, X., O. Laur, T. Kambayashi, S. Li, R. A. Bray, D. A. Weber, L. Karlsson, and P. E. Jensen. 2002. Regulated expression of human histocompatibility leukocyte antigen (HLA)-DO during antigen-dependent and antigen-independent phases of B cell development. *J. Exp. Med.* 195: 1053–1062.
6. Glazier, K. S., S. B. Hake, H. M. Tobin, A. Chadburn, E. J. Schattner, and L. K. Denzin. 2002. Germinal center B cells regulate their capability to present antigen by modulation of HLA-DO. *J. Exp. Med.* 195: 1063–1069.
7. Liljedahl, M., O. Winqvist, C. D. Surh, P. Wong, K. Ngo, L. Teyton, P. A. Peterson, A. Brunmark, A. Y. Rudensky, W. P. Fung-Leung, and L. Karlsson. 1998. Altered antigen presentation in mice lacking H2-O. *Immunity* 8: 233–243.
8. Denzin, L. K., D. B. Sant'Angelo, C. Hammond, M. J. Surman, and P. Cresswell. 1997. Negative regulation by HLA-DO of MHC class II-restricted antigen processing. *Science* 278: 106–109.
9. van Ham, S. M., E. P. Tjin, B. F. Lillemeier, U. Gruneberg, K. E. van Meijgaarden, L. Pastoors, D. Verwoerd, A. Tulp, B. Canas, D. Rahman, et al. 1997. HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr. Biol.* 7: 950–957.
10. van Ham, M., M. van Lith, B. Lillemeier, E. Tjin, U. Gruneberg, D. Rahman, L. Pastoors, K. van Meijgaarden, C. Roucard, J. Trowsdale, et al. 2000. Modulation of the major histocompatibility complex class II-associated peptide repertoire by human histocompatibility leukocyte antigen (HLA)-DO. *J. Exp. Med.* 191: 1127–1136.
11. Perraudeau, M., P. R. Taylor, H. J. Stauss, R. Lindstedt, A. E. Bygrave, D. J. Pappin, S. Ellmerich, A. Whitten, D. Rahman, B. Canas, et al. 2000. Altered major histocompatibility complex class II peptide loading in H2-O-deficient mice. *Eur. J. Immunol.* 30: 2871–2880.
12. Karlsson, L., C. D. Surh, J. Sprent, and P. A. Peterson. 1991. A novel class II MHC molecule with unusual tissue distribution. *Nature* 351: 485–488.
13. Surh, C. D., E. K. Gao, H. Kosaka, D. Lo, C. Ahn, D. B. Murphy, L. Karlsson, P. Peterson, and J. Sprent. 1992. Two subsets of epithelial cells in the thymic medulla. *J. Exp. Med.* 176: 495–505.
14. Douek, D. C., and D. M. Altmann. 1997. HLA-DO is an intracellular class II molecule with distinctive thymic expression. *Int. Immunol.* 9: 355–364.
15. Chalouni, C., J. Banchereau, A. B. Vogt, V. Pascual, and J. Davoust. 2003. Human germinal center B cells differ from naive and memory B cells by their aggregated MHC class II-rich compartments lacking HLA-DO. *Int. Immunol.* 15: 457–466.
16. Rohn, T. A., M. Boes, D. Wolters, S. Spindeldreher, B. Muller, H. Langen, H. Ploegh, A. B. Vogt, and H. Kropshofer. 2004. Upregulation of the CLIP self peptide on mature dendritic cells antagonizes T helper type 1 polarization. *Nat. Immunol.* 5: 909–918.
17. Hornell, T. M., C. G. W. Beresford, A. Bushey, J. M. Boss, and E. D. Mellins. 2003. Regulation of the class II MHC pathway in primary human monocytes by granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 171: 2374–2383.
18. Bertho, N., B. Drenou, B. Laupeze, C. L. Berre, L. Amiot, J. M. Grosset, O. Fardel, D. Charron, N. Mooney, and R. Fauchet. 2000. HLA-DR-mediated apoptosis susceptibility discriminates differentiation stages of dendritic/monocytic APC. *J. Immunol.* 164: 2379–2385.
19. Geissmann, F., C. Prost, J. P. Monnet, M. Dy, N. Brousse, and O. Hermine. 1998. Transforming growth factor β1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J. Exp. Med.* 187: 961–966.
20. Strobl, H., C. Bello-Fernandez, E. Riedl, W. F. Pickl, O. Majdic, S. D. Lyman, and W. Knapp. 1997. flt3 ligand in cooperation with transforming growth factor-β1 potentiates in vitro development of Langerhans-type dendritic cells and allows single-cell dendritic cell cluster formation under serum-free conditions. *Blood* 90: 1425–1434.
21. Hertel, L., V. G. Lacaille, H. Strobl, E. D. Mellins, and E. S. Mocarski. 2003. Susceptibility of immature and mature Langerhans cell-type dendritic cells to infection and immunomodulation by human cytomegalovirus. *J. Virol.* 77: 7563–7574.
22. Morris, P., J. Shaman, M. Attaya, M. Amaya, S. Goodman, C. Bergman, J. J. Monaco, and E. Mellins. 1994. An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. *Nature* 368: 551–554.
23. Gladstone, P., and D. Pious. 1980. Identification of a trans-acting function regulation HLA-DR expression in a DR-negative B cell variant. *Somatic Cell Genet.* 6: 285–298.

24. Mellins, E., S. Kempin, L. Smith, T. Monji, and D. Pious. 1991. A gene required for class II-restricted antigen presentation maps to the major histocompatibility complex. *J. Exp. Med.* 174: 1607–1615.
25. Busch, R., A. Pashine, K. C. Garcia, and E. D. Mellins. 2002. Stabilization of soluble, low-affinity HLA-DM/HLA-DR1 complexes by leucine zippers. *J. Immunol. Methods* 263: 111–121.
26. Pashine, A., R. Busch, M. P. Belmares, J. N. Munning, R. C. Doebele, M. Buckingham, G. P. Nolan, and E. D. Mellins. 2003. Interaction of HLA-DR with an acidic face of HLA-DM disrupts sequence-dependent interactions with peptides. *Immunity* 19: 183–192.
27. Hochstenbach, F., V. David, S. Watkins, and M. B. Brenner. 1992. Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. *Proc. Natl. Acad. Sci. USA* 89: 4734–4738.
28. Drohan, L., J. J. Harding, B. Holm, E. Cordoba-Tongson, C. L. Dekker, T. Holmes, H. Maecker, and E. D. Mellins. 2004. Selective developmental defects of cord blood antigen-presenting cell subsets. *Hum. Immunol.* 65: 1356–1369.
29. Jahnsen, F. L., I. N. Farstad, J. P. Aanesen, and P. Brandtzaeg. 1998. Phenotypic distribution of T cells in human nasal mucosa differs from that in the gut. *Am. J. Respir. Cell Mol. Biol.* 18: 392–401.
30. Colonna, M., G. Trinchieri, and Y. J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat. Immunol.* 5: 1219–1226.
31. Liu, Y. J. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu. Rev. Immunol.* 23: 275–306.
32. MacDonald, K. P., D. J. Munster, G. J. Clark, A. Dzionek, J. Schmitz, and D. N. Hart. 2002. Characterization of human blood dendritic cell subsets. *Blood* 100: 4512–4520.
33. Dzionek, A., A. Fuchs, P. Schmidt, S. Cremer, M. Zysk, S. Miltenyi, D. W. Buck, and J. Schmitz. 2000. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J. Immunol.* 165: 6037–6046.
34. Schakel, K., E. Mayer, C. Federle, M. Schmitz, G. Riethmuller, and E. P. Rieber. 1998. A novel dendritic cell population in human blood: one-step immunomagnetic isolation by a specific mAb (M-DC8) and in vitro priming of cytotoxic T lymphocytes. *Eur. J. Immunol.* 28: 4084–4093.
35. Almeida, J., C. Bueno, M. C. Alguero, M. L. Sanchez, M. de Santiago, L. Escribano, B. Diaz-Agustin, J. M. Vaquero, F. J. Laso, J. F. San Miguel, and A. Orfao. 2001. Comparative analysis of the morphological, cytochemical, immunophenotypical, and functional characteristics of normal human peripheral blood lineage$^-$/CD16$^+$/HLA-DR$^+$/CD14$^{-/lo}$ cells, CD14$^+$ monocytes, and CD16$^-$ dendritic cells. *Clin. Immunol.* 100: 325–338.
36. Passlick, B., D. Flieger, and H. W. Ziegler-Heitbrock. 1989. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood* 74: 2527–2534.
37. Summers, K. L., B. D. Hock, J. L. McKenzie, and D. N. Hart. 2001. Phenotypic characterization of five dendritic cell subsets in human tonsils. *Am. J. Pathol.* 159: 285–295.
38. Patil, N. S., A. Pashine, M. P. Belmares, W. Liu, B. Kaneshiro, J. Rabinowitz, H. McConnell, and E. D. Mellins. 2001. Rheumatoid arthritis (RA)-associated HLA-DR alleles form less stable complexes with class II-associated invariant chain peptide than non-RA-associated HLA-DR alleles. *J. Immunol.* 167: 7157–7168.
39. Johnson, J. P., T. Meo, G. Riethmuller, D. J. Schendel, and R. Wank. 1982. Direct demonstration of an HLA-DR allotypic determinant on the low molecular weight (β) subunit using a mouse monoclonal antibody specific for DR3. *J. Exp. Med.* 156: 104–111.
40. Drover, S., R. W. Karr, X. T. Fu, and W. H. Marshall. 1994. Analysis of monoclonal antibodies specific for unique and shared determinants on HLA-DR4 molecules. *Hum. Immunol.* 40: 51–60.
41. Geissmann, F., S. Jung, and D. R. Littman. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19: 71–82.
42. Velten, F. W., K. Duperrier, J. Bohlender, P. Metharom, and S. Goerdt. 2004. A gene signature of inhibitory MHC receptors identifies a BDCA3$^+$ subset of IL-10-induced dendritic cells with reduced allostimulatory capacity in vitro. *Eur. J. Immunol.* 34: 2800–2811.
43. Galibert, L., G. S. Diemer, Z. Liu, R. S. Johnson, J. L. Smith, T. Walzer, M. R. Comeau, C. T. Rauch, M. F. Wolfson, R. A. Sorensen, et al. 2005. Nectin-like protein 2 defines a subset of T-cell zone dendritic cells and is a ligand for class-I restricted T-cell associated molecule. *J. Biol. Chem.* 280: 21955–21964.
44. Chamuleau, M. E., Y. Souwer, S. M. Van Ham, A. Zevenbergen, T. M. Westers, J. Berkhof, C. J. Meijer, A. A. van de Loosdrecht, and G. J. Ossenkoppele. 2004. Class II-associated invariant chain peptide expression on myeloid leukemic blasts predicts poor clinical outcome. *Cancer Res.* 64: 5546–5550.
45. Roucard, C., C. Thomas, M. A. Pasquier, J. Trowsdale, J. J. Sotto, J. Neefjes, and M. van Ham. 2001. In vivo and in vitro modulation of HLA-DM and HLA-DO is induced by B lymphocyte activation. *J. Immunol.* 167: 6849–6858.
46. Tonnelle, C., R. DeMars, and E. O. Long. 1985. DO β: a new β chain gene in HLA-D with a distinct regulation of expression. *EMBO J.* 4: 2839–2847.
47. Taxman, D. J., D. E. Cressman, and J. P. Ting. 2000. Identification of class II transcriptional activator-induced genes by representational difference analysis: discoordinate regulation of the DN α/DO β heterodimer. *J. Immunol.* 165: 1410–1416.
48. Jensen, P. E. 1998. Antigen processing: HLA-DO–a hitchhiking inhibitor of HLA-DM. *Curr. Biol.* 8: R128–R131.
49. Olsen, N. J., J. H. Moore, and T. M. Aune. 2004. Gene expression signatures for autoimmune disease in peripheral blood mononuclear cells. *Arthritis Res. Ther.* 6: 120–128.
50. Chen, X., L. M. Reed-Loisel, L. Karlsson, and P. E. Jensen. 2006. H2-O expression in primary dendritic cells. *J. Immunol.* In press.