Regulated Expression of Human Histocompatibility Leukocyte Antigen (HLA)-DO During Antigen–dependent and Antigen–independent Phases of B Cell Development

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Abstract

Human histocompatibility leukocyte antigen (HLA)-DO, a lysosomal resident major histocompatibility complex class II molecule expressed in B cells, has previously been shown to be a negative regulator of HLA-DM peptide loading function. We analyze the expression of DO in human peripheral blood, lymph node, tonsil, and bone marrow to determine if DO expression is modulated in the physiological setting. B cells, but not monocytes or monocyte-derived dendritic cells, are observed to express this protein. Preclearing experiments demonstrate that ∼50% of HLA-DM is bound to DO in peripheral blood B cells. HLA-DM and HLA-DR expression is demonstrated early in B cell development, beginning at the pro–B stage in adult human bone marrow. In contrast, DO expression is initiated only after B cell development is complete. In all situations, there is a striking correlation between intracellular DO expression and cell surface class II–associated invariant chain peptide expression, which suggests that DO substantially inhibits DM function in primary human B cells. We report that the expression of DO is markedly downmodulated in human germinal center B cells. Modulation of DO expression may provide a mechanism to regulate peptide loading activity and antigen presentation by B cells during the development of humoral immune responses.

Key words: antigen presentation • B cell development • germinal center • HLA-DM • HLA-DO

Introduction

MHC class II αβ molecules assemble with the chaperone protein, invariant chain (Ii),[*] in the endoplasmic reticulum (1). An unstructured segment of Ii, encoded by exon 3, occupies the αβ peptide binding site, which helps stabilize the newly assembled complex. After transport to endosomal compartments, most of the Ii protein is released from αβ through a series of proteolytic cleavage events, leaving a fragment of Ii, class II–associated Ii peptide (CLIP), buried in the peptide binding site (2–4). HLA-DM (H2-M in mice) is a key component of the MHC class II antigen–processing pathway (5). Although this lysosomal resident protein is structurally similar to other class II molecules, it

has limited polymorphism and does not bind peptide antigens. Its major function is to catalyze peptide binding and exchange reactions in other class II molecules (6–8). Cell surface class II molecules are predominantly occupied by CLIP in DM-deficient APC (2, 3, 9–11). Thus, a major function of DM is to catalyze the release of CLIP and the binding of peptide antigens present in endosomal compartments. DM can also catalyze further rounds of peptide exchange, markedly influencing the repertoire of peptides displayed at the cell surface and favoring the most stable peptide complexes (12–14).

Like DM, HLA-DO is a lysosomal resident MHC class II protein with limited polymorphism. However, early studies demonstrated that the murine homologue of DO (H2-O) is selectively expressed in B cells and a subset of thymic epithelial cells, but not in other APC (15, 16). Thus DO must have a specialized function, rather than a general role in the class II antigen–processing pathway. In the absence of DM, DO appears to be an intrinsically unstable

molecule, rapidly degraded (17). DO binds stably to DM in the endoplasmic reticulum, and DO–DM complexes are transported to lysosome-related compartments through a targeting signal in the DMβ chain cytoplasmic domain (17, 18). Transfection experiments and studies with purified DO–DM complexes have demonstrated that DO is a negative regulator of DM catalytic activity (19–22), although there is one report that DO can enhance DM function (23). DO does not completely inhibit DM function, but rather limits the pH range in which DM is active (21, 22). By limiting DM function in all but the most acidic endosomal compartments in B cells, DO might serve to promote the presentation of peptides derived from antigens internalized through the B cell antigen receptor (BCR), which is preferentially targeted to lysosomal compartments (21, 22).

It is appealing to consider the idea that DO may also provide a mechanism to rapidly up-regulate DM activity (24). However, no previous evidence has been provided indicating that DO can be released from DM, or that the ratio of free DM to DM–DO complexes can be modulated in B cells, for example, by activation. Indeed, the fraction of DM molecules bound to DO in primary B cells has not been determined and it is not clear whether DO has a major impact on DM activity in normal B cells. In the present study, we describe the expression of DO in human peripheral blood cells, bone marrow, and secondary lymphoid tissue. Our results demonstrate that DO is selectively expressed in B cells, a major fraction of DM molecules is bound to DO in these cells, and DO expression is modulated during both the antigen-dependent and -independent phases of B cell development.

Materials and Methods

Cells.   Fresh or frozen lymph node cells were derived from healthy cadaveric organ donors. Peripheral blood specimens were derived from the same sources or healthy blood donors. Bone marrow cells were derived from bone marrow aspiration of patients whose specimens were received for pathological evaluation to rule out hematological disorders and diagnosed as "no diagnostic pathology or unremarkable." Fresh tonsil cells for flow cytometry analysis were derived from tonsillectomy specimens. The presence of germinal centers (GCs) in the tonsils was confirmed by subsequent histological evaluation. Peripheral blood mononucleated lymphoid cells were isolated by Ficoll–Paque density gradient (Amersham Pharmacia Biotech). Single cell suspensions of lymphoid tissues and peripheral blood mononucleated lymphoid cells were prepared for four-color flow cytometry analysis.

Antibodies and Flow Cytometry.   Chromogen-conjugated mAb to CD19 (HIB19), CD38 (HIT2), IgD (IA6-2), IgM (G20-127), CLIP (CerCLIP), and HLA-DM (MapDM1); and CD34 (581), CD45 (HI30), CD3 (UCHT1), CD5 (L17F12), HLA-DR (L243), CD14 (M5E2), CD10 (HI10a), and isotype-matched negative control mAbs were purchased from BD PharMingen. Biotinylated CerCLIP antibody was produced in the laboratory at the Emory University School of Medicine. mAb to HLA-DOβ cytoplasmic domain, DOB.L1 (23), provided by H. Kropshofer and A. Vogt (Basel Institute for Immunology, Basel, Switzerland), was FITC conjugated in this laboratory. PE-conjugated mAb to DO was purchased from BD PharMingen. Cell surface staining was performed with a combination of four of each FITC, PE, PerCP, or APC-labeled mAbs according to the standard procedures. Intracellular staining was performed using Fix & Perm kits (Caltag Laboratory) according to the manufacturer's instructions. In brief, cells were washed with 1% PBS–BSA after surface staining and fixed with medium A for 15 min at room temperature. Cells were then thoroughly washed with 1% PBS–BSA and incubated in medium B containing DM or DO antibodies for 15 min at room temperature, followed by washing with 1% PBS–BSA. Stained cells were analyzed on a FACS® Calibur (Becton Dickinson). 10,000–20,000 events were collected per dot plot.

Immunohistochemistry.   Representative formalin-fixed, paraffin-embedded tissue sections of tonsillectomy specimens were dewaxed and subjected to antigen retrieval in citrate buffer, pH 6, using an electric pressure cooker (Biocare Medical) set at 120°C for 5 min (25). Sections were incubated for 5 min in 3% hydrogen peroxide. Immunohistochemistry was performed using primary antibodies directed against DO (DOB.L1), DMβ cytoplasmic domain (clone 4.7GS; provided by S. Pierce, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Rockville, MD), CD10 (56c6; Novocastra), DR (TAL.1B5; Dako), CD20 (Bly1; Dako), CD3 (T3-4B5; Dako), and Mib-1/Ki-67 (Mib-1; Dako). After 25-min incubations with primary antibody, sections were washed and treated with commercial biotinylated secondary anti-immunoglobulin, followed by avidin coupled to biotinylated horseradish peroxidase, according to the manufacturer's instructions (LSAB2 kit for mouse primary antibodies; Dako). The immunohistochemical reactions were visualized using diaminobenzidine as a chromogenic peroxidase substrate. Sections were counterstained with hematoxylin.

Immunoprecipitation and Western Blot Analysis.   CD14$^+$ monocytes and CD19$^+$ B cells were isolated from PBMC using the MACS® separation system (Miltenyi Biotec) according to the manufacturer's guidelines. In brief, blood mononuclear cells fractionated via Ficoll-Paque density gradient were resuspended in MACS® buffer (PBS without Ca^{2+} supplemented with 0.5% BSA and 2 mM EDTA) containing the appropriate amount of anti-CD14 or anti-CD19 antibody-conjugated magnetic microbeads. After 30 min of incubation on ice, CD14$^+$ and CD19$^+$ cells were isolated from the cell suspensions using an LS$^+$ column (Miltenyi Biotec). Greater than 95% of the purity of the cell separations was confirmed by flow cytometry. To generate dendritic cells, CD14$^+$ cells were resuspended in RPMI 1640 (10% FCS, 100 U/ml penicillin, and 100 U/ml streptomycin; Life Technologies) containing 10 ng/ml rhIL-4 and 10 ng/ml rhGM-CSF (Pepro-Tech) and cultured for 6 d. Mature dendritic cells were generated by stimulating the immature dendritic cells with 100 ng/ml LPS for 24 h. For immunoprecipitation and immunodepletion experiments, 5×10^6 cells were lysed in 0.25 ml of lysis buffer (PBS, 1% CHAPS, 1 mM DTT, 20 μM p-APMSF, and 1 mM EDTA) for 30–40 min. After clarification in a microfuge for 10 min at 14,000 rpm, the lysates were incubated for 1.5 h on a rotating platform at 4°C with specific antibody (6 μg per sample), followed by incubation with 60 μl of the protein A–Sepharose (Amersham Pharmacia Biotech) for 1 h. The protein A–Sepharose pellet was washed six times at room temperature with 1 ml of the washing buffer (PBS, 0.5% CHAPS) and resuspended in 30 μl of Laemmli buffer followed by SDS-PAGE. The primary mAbs used in the immunoprecipitation and Western blot experiments were DOB.L1 (23) against HLA-DOβ cytoplasmic domain, 4.G7S against HLA-DMβ cytoplasmic domain, 9E10 against c-MYC epitope (American Type Culture Collection), and polyclonal rabbit antisera to HLA-DRαβ.

GC and non-GC B cells were isolated from single cell suspensions of human tonsils. Cells were first stained with 10 μg/ml of mouse anti–human CD10 mAb (HI10a/IgG1-κ; BD PharMingen) followed by MACS® rat anti–mouse IgG1 MicroBeads (Miltenyi Biotec). CD10$^+$ cells were selected by passing cell suspension through a magnetic separator (Miltenyi Biotec) according to the manufacturer's instructions. The CD10-depleted tonsil cells were then stained with MACS® anti-CD19 MicroBeads, and CD19$^+$ cells were selected using the magnetic separator. The purity of each cell preparation was evaluated by flow cytometry.

Results

Expression of HLA-DO in Peripheral Blood B Cells but Not Monocytes or Dendritic Cells. Cell surface CLIP expression serves as a surrogate marker of HLA-DM function in APC. DM is required for the efficient release of CLIP from MHC class II molecules and high concentrations of CLIP–class II complexes are present on the surface of DM-deficient APC (2, 3, 9–11). We observed that CLIP is readily detected on peripheral blood B cells by flow cytometry, whereas relatively little CLIP is detected on monocytes (Fig. 1 a). There is considerable variation in the levels of CLIP expressed on B cells from different donors, probably due to differences in the stability of the binding of CLIP to different DR allotypes. However, CLIP expression is always much lower on monocytes. Surface HLA-DR ex-

pression is also lower on monocytes compared to B cells, but the reduction in DR is insufficient to account for the difference in CLIP expression. Based on the analysis of 15 independent samples, the mean fluorescence intensity of DR staining was 824 ± 281 (B cells) and 489 ± 251 (monocytes). By contrast, the mean fluorescence intensity of CLIP staining was 331 ± 98 (B cells) and 16 ± 9 (monocytes). The possibility that reduced HLA-DM expression could account for higher CLIP levels in B cells was addressed by intracellular staining. DM expression was observed to be one- to threefold higher in B cells compared to monocytes, depending on the sample (Fig. 1 a). By contrast, HLA-DO expression was only observed in B cells and not in monocytes. The latter result was confirmed by Western blot analysis of lysates of purified B cells and monocytes (Fig. 1 b). Notably, DO expression was undetectable in immature and LPS-activated monocyte-derived dendritic cells by Western blot analysis (Fig. 1 b) and flow cytometry (unpublished data), despite very high expression levels of DR (unpublished data) and DM (Fig. 1 b). These results demonstrated a correlation between CLIP expression and DO expression in DR$^+$ peripheral blood mononuclear cells, which suggests that DO functions specifically in B cells to inhibit DM activity.

Fraction of HLA-DM Molecules Bound to HLA-DO in Primary B Cells. Available evidence indicates that HLA-DO molecules are unstable in the absence of DM and that all DO present in post-Golgi compartments is stably bound to DM (17). Kropshofer et al. (23) reported that 60–70% of DM molecules in the WT-100 human B cell line are coprecipitated with mAb to DO. However, the fraction of DM molecules bound to DO in primary B cells has not been reported. This is an important issue when considering the potential biological roles for DO. If only a small fraction of DM molecules are occupied by DO, one must seriously consider the possibility that DO has some unique function other than inhibiting DM activity. We addressed this issue by preclearing detergent lysates of B cells with anti-DO mAb and measuring residual free DM in Western blots. As demonstrated in Fig. 2 a, a substantial fraction of total DM in the Raji B cell line is removed by depletion with antibodies to DO. Titration of lysates precleared with anti-DO versus control antibody demonstrated that ∼50% of DM in Raji cells is stably bound to DO. Similar experiments were performed with purified peripheral blood B cells, demonstrating that ∼50% of DM is stably bound to DO in these cells (Fig. 2 b). We conclude that a substantial fraction of DM is associated with DO in primary human B cells.

HLA-DO Expression Occurs Late During B Cell Development in Adult Human Bone Marrow. It has previously been demonstrated that MHC class II molecules are not expressed on pro-B cells in fetal mice, but rather in adult mice (26, 27). We were interested in determining if components of the class II antigen–processing pathway are expressed in human B cell precursors. Pro-B (CD19$^+$CD10$^+$CD34$^+$) and pre-B (CD19$^+$CD10$^+$CD34$^-$) populations (28) can be distinguished from B cells (CD19$^+$CD10$^-$CD34$^-$) by differential expression of CD45 (Fig. 3,
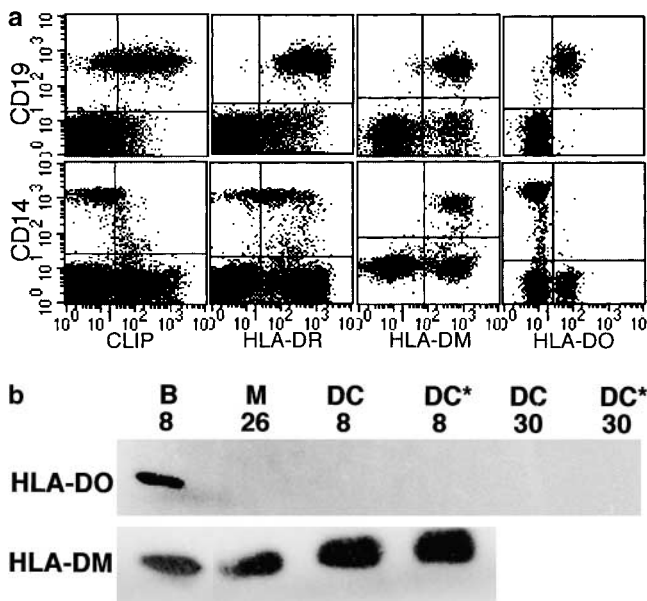

Figure 1. Expression of HLA-DO in peripheral blood B cells but not in monocytes or dendritic cells. (a) Analysis of CLIP, DR, DM, and DO expression in B cells (CD19$^+$) versus monocytes (CD14$^+$) using four-color flow cytometry. DM and DO expression were measured by staining permeabilized cells as previously described in Materials and Methods. (b) DM and DO expression in purified B lymphocytes (B), monocytes (M), immature dendritic cells (DC), and mature dendritic cells (DC*) were evaluated by Western blot analysis as previously described in Materials and Methods. Numbers indicate the quantity (cell equivalents × 10^{-4}) loaded in each lane. Blots were developed with anti–HLA-DM (4.G7S) and anti–HLA-DO (DOB.L1) mAb.
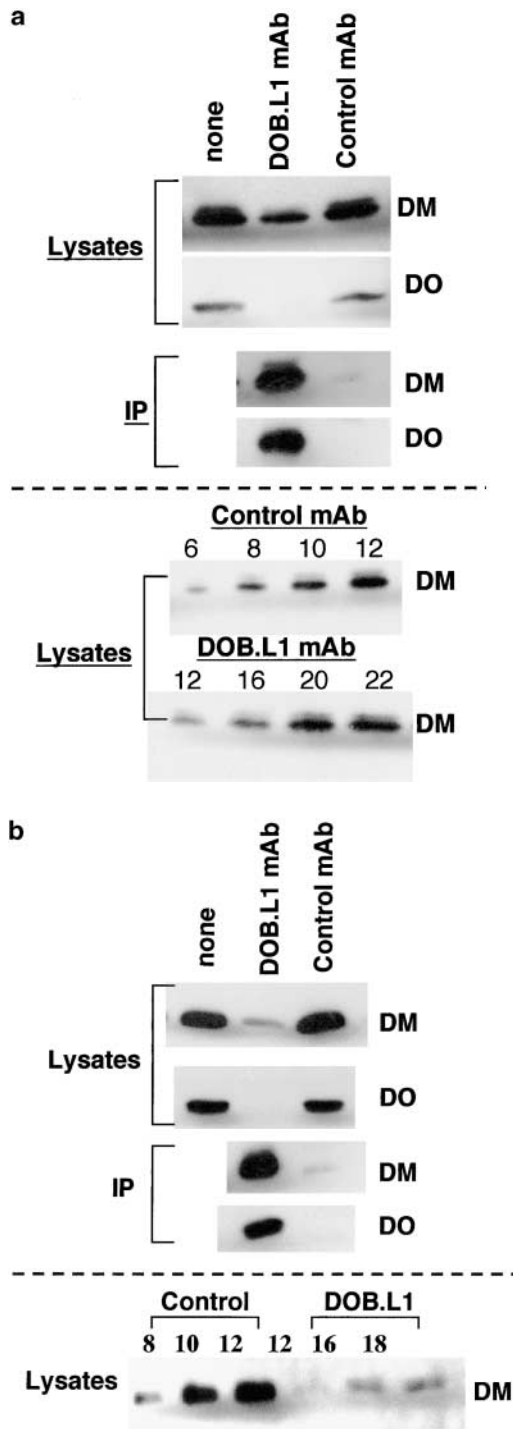
Figure 2. Fraction of HLA-DM molecules bound to HLA-DO in B cells. (a) The top panels (lysates) show Western blot analysis of Raji B cell lysates before or after preclearing with anti-DO or control mAb, as indicated. The middle panels (IP) show Western blot analysis of immunoprecipitates generated with the anti-DO (DOB.L1) or control mAb. Blots were stained with mAb 4.7GS (DM) or DOB.L1 (DO). The bottom panels show DM Western blot analysis of titrations of Raji cell extracts depleted with anti–HLA-DO or with control antibody. Numbers indicate sample quantity (cell equivalents $\times$ 10^{-4}) loaded in each lane. (b) Similar analysis with purified peripheral blood B cells.

top). As illustrated in Fig. 3, all CD19^{+} B lineage cells in adult human bone marrow express HLA-DR, although it is clearly up-regulated at the pro-B (R2) to pre-B (R3) transition. Pre-B cells express DR at a level similar to B cells (R4). Remarkably, CLIP expression is largely limited to B cells, with little or no expression on B cell precursors. Intracellular staining demonstrates that all CD19^{+} B lineage cells express DM. Two distinct populations are observed, which express low versus high levels of DM. Pro-B and pre-B cells uniformly express low DM, whereas B cells are divided into both groups. A minority of B cells express low DM and the major population expresses high DM. By contrast, DO is only expressed in B cells and is not detected in B cell precursors.

A subpopulation of the B cells is negative for DO, as illustrated in Fig. 3. Notably, all B cells that express high levels of DM also express DO, whereas cells with lower DM expression are negative for DO. Using CD10 expression to distinguish immature (CD10^{+}) and mature (CD10^{−}) B cells, we observed that DO up-regulation occurs at the transition from immature to mature B cells. Both populations express similar levels of surface IgM. CLIP expression is also substantially up-regulated at the transition from immature to mature B cells. Thus we conclude that both DR and DM are expressed beginning at the pro-B stage, whereas DO protein expression is initiated only after B cell maturation is complete. In addition, these results provide another example of a correlation between the expression of DO and CLIP, adding support to the hypothesis that DO has a substantial impact on DM functional activity in primary human B cells.

Expression of HLA-DO Is Down-regulated in GC B Cells. The major role of the antigen-processing and presentation pathway in B cells is to provide a mechanism for cognate T–B cell interactions, which are required to drive T cell–dependent humoral immune responses. These interactions occur in secondary lymphoid tissues and are required for the initiation, and possibly propagation, of GC reactions. We were interested in the possibility that DO expression might be modulated during antigen-driven B cell differentiation. Strikingly, immunohistochemistry experiments with human tonsil demonstrated that DO expression was markedly reduced in GC B cells. In these studies, DO was detected only in a few scattered cells in the GC, whereas mantle zone B cells and B cells in other sites were strongly positive (Fig. 4). Similar results were obtained in a reactive lymph node sample (unpublished data). By immunohistochemistry, human GC B cells, which can be distinguished by CD10 expression, express both DR and DM, although DM expression appears to be lower in GC B cells compared to non-GC B cells. Both highly proliferating Mib-1^{+} centroblasts (29) and Mib-1^{−} centrocytes down-regulate DO.

DO expression is markedly reduced but not absent in CD19^{+}CD10^{+} GC B cells, as judged by flow cytometry (Fig. 5, top). A small fraction of GC B cells express DO at levels similar to CD19^{+}CD10^{−} non-GC B cells. These probably correspond to the scattered DO^{+} cells observed in
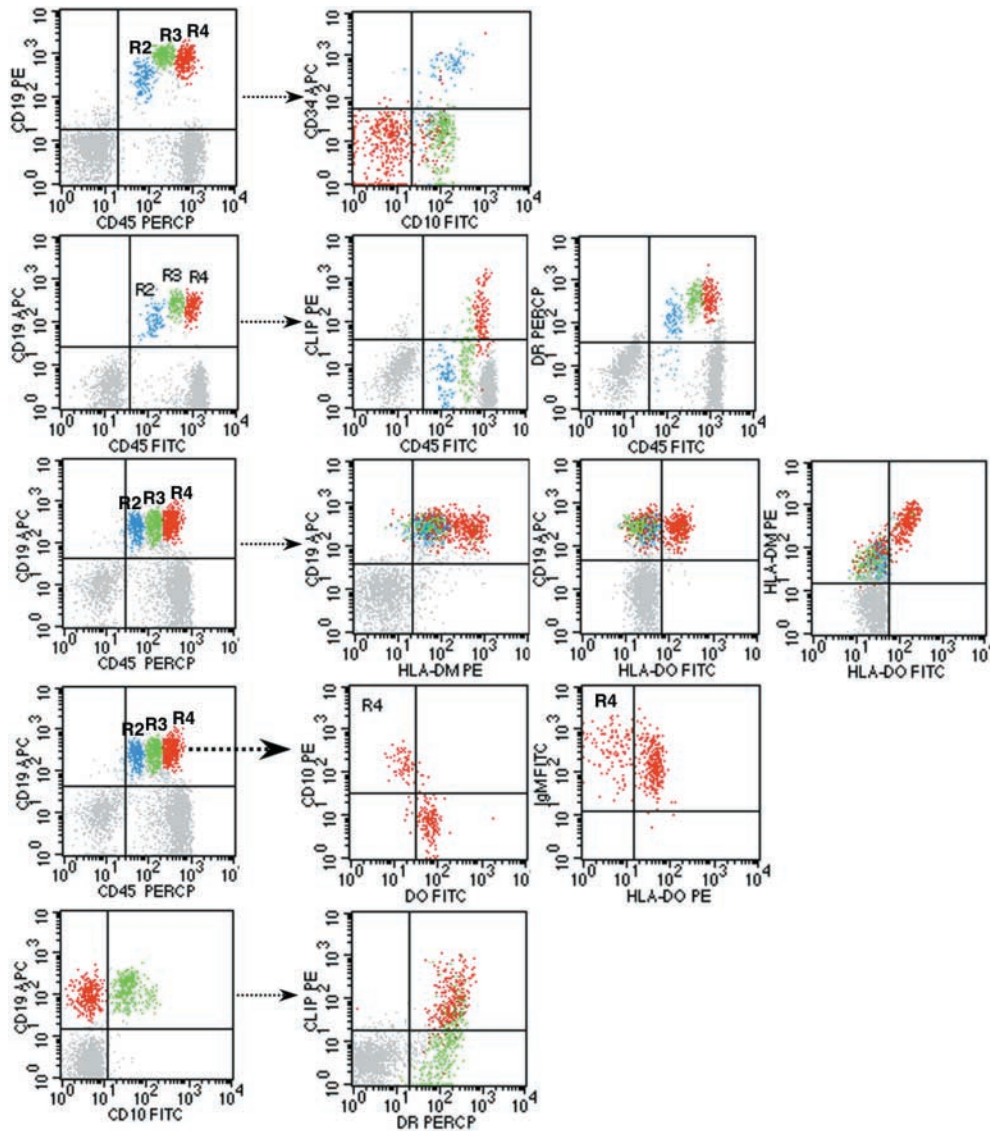
Figure 3. Expression of the components of the class II antigen–processing pathway during B cell development in adult human bone marrow. The top panels show pro-B (R2; CD19$^+$CD45^{low}CD34$^+$CD10$^+$), pre-B (R3; CD45^{intermediate}CD19$^+$CD34$^-$CD10$^+$), and B cell (R4; CD19$^+$CD45^{high}CD34$^-$CD10$^+$ or CD10$^-$) subpopulations as defined by four-color flow cytometry. The other panels show the expression of CLIP, DR, DM, and DO in these three subpopulations (DM and DO were measured by staining permeabilized cells). B cells (R4) are additionally analyzed for DO expression based on the expression of CD10. The bottom panels show CLIP and DR expression in CD10$^+$ (pro-B, pre-B, and immature B cells) and CD10$^-$ (mature B cells).

GCs by immunohistochemistry. DM expression is slightly reduced but there is substantial overlap in the fluorescence intensities of the GC and non-GC B cells. GC and non-GC B cells were purified and analyzed in Western blots to obtain a firmer estimation of the relative extent of down-regulation of DO and DM. DR and DM expression levels were similar in the two populations (Fig. 6). By contrast, DO expression was markedly reduced in GC B cells. DO protein expression is reduced at least threefold based on comparison of titrated quantities of cell lysates.

The fraction of DR molecules bearing CLIP is reduced in GC B cells as determined by flow cytometry. This is best illustrated by comparing the two populations in a plot of DR versus CLIP staining (Fig. 5). There is a wide distribution of DR expression levels in each population, but CLIP expression is always reduced on GC B cells compared to non-GC B cells expressing similar levels of DR. Thus, net DM functional activity appears to be increased

in GC B cells. Notably, GC B cells up-regulate expression of the costimulatory molecules CD80 and CD86, as well as CD40, supporting a possible role for GC B cells as APC (Fig. 5).

The scattered high DO B cells in the GC could represent cells that recently entered the GC and have yet to down-regulate DO, or cells that up-regulate DO expression before exiting the GC. IgD and CD38 can be used as cell surface markers to define discrete subpopulations of human tonsillar B cells (30). As shown in Fig. 7, DM expression is essentially constant in tonsillar B cell subpopulations. IgD$^+$CD38$^-$ naive follicular mantle B cells uniformly express DO at levels similar to peripheral blood B cells. By contrast, DO expression is uniformly reduced in classic IgD$^-$CD38$^+$ GC B cells, which include both centroblasts and centrocytes. The IgD$^+$CD38$^+$ subpopulation has been proposed to include the GC founder cells based on several criteria, including the coexpression of naive B
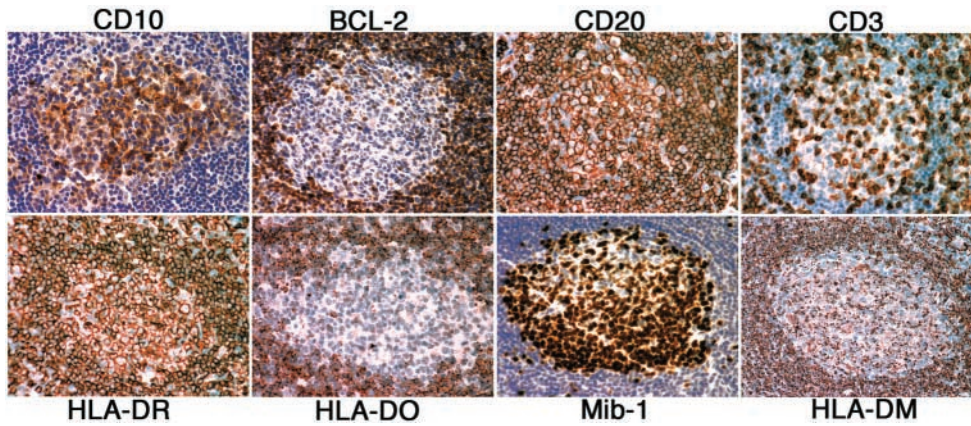
Figure 4. Downmodulation of DO expression in GC B cells. GCs in human tonsil tissue sections were analyzed by immunohistochemistry with mAb specific for the indicated markers. The pattern of staining for each marker is representative of all GCs in the section.

cell markers and GC markers, and low levels of IgV gene mutations (30). It is interesting that there appears to be an intermediate level of expression of DO with a possible bimodal distribution in the IgD$^+$CD38$^+$ population (Fig. 7). This needs further investigation, but it raises the possibility that DO expression is down-regulated during the founder cell stage after entry into GC and before the loss of surface IgD expression. High DO expression is observed in the IgD$^-$CD38$^-$ subpopulation and believed to represent memory cells. Thus, DO expression is up-regulated at the transition from GC to memory B cells.

Discussion

In this study, we demonstrate that HLA-DO is not a static component of the B cell antigen-processing pathway, but instead show that its expression is regulated in both the antigen–dependent and antigen–independent phases of B cell development. Intracellular flow cytometry and Western blot analysis of human peripheral blood cells were used to demonstrate that DO is selectively expressed in B cells and not in monocytes. In addition, CD11c$^+$ dendritic cells derived by culturing peripheral blood monocytes with GM-CSF and IL-4, are negative for DO before and after activation with LPS. This result contrasts with a previous report showing DO expression in GM-CSF–induced human dendritic cells (31). Our results support the conclusion that DO is selectively expressed in B cells and not in other peripheral APC in humans.

The fraction of DM molecules stably associated with DO in B cells is a key issue in considering the potential functions of DO. Our results suggest that ~50% of DM molecules are bound to DO in the Raji cell line and in peripheral blood B cells. This could be an underestimate
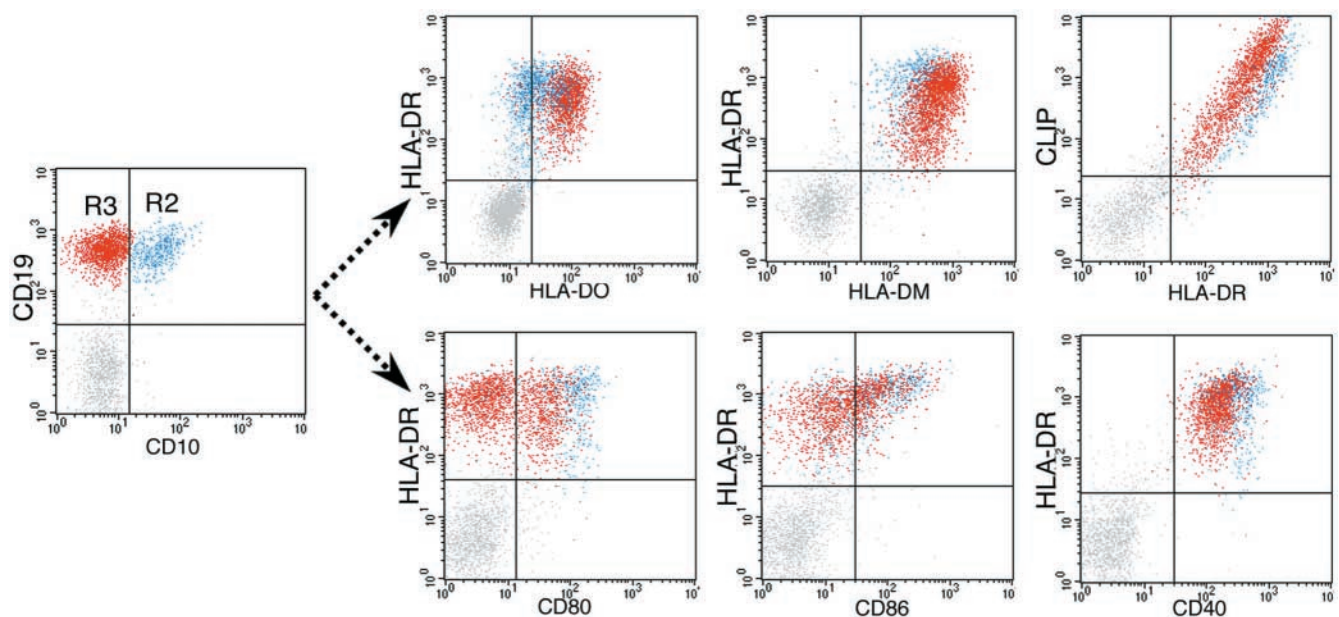

Figure 5. Expression of HLA-DO in cells from human tonsil. GC B cells (R2; CD19$^+$CD10$^+$) express substantially lower levels of DO compared to non-GC B cells (R3; CD19$^+$CD10$^-$) as determined by intracellular staining and four-color flow cytometry. Results are representative of eight tonsillectomy specimens.
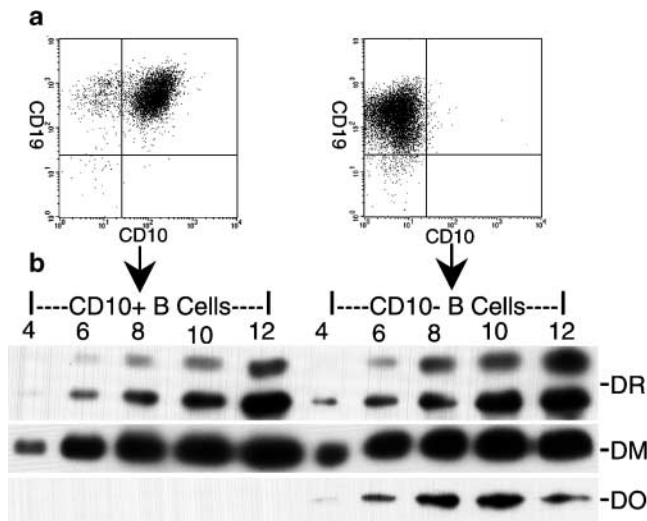
Figure 6. Western blot analysis of GC B cells. GC (CD10$^+$CD19$^+$) and non-GC (CD10$^-$CD19$^+$) B cells were purified from human tonsil as previously described in Materials and Methods. The top panels show flow cytometric analysis of the purified populations. The lower panels show Western blot analysis of DR, DM, and DO expression in CD10$^+$ and CD10$^-$ B cells. Blots were stained with polyclonal rabbit antiserum (DR), mAb 4.7GS (DM), or DOB.L1 (DO). Numbers indicate sample quantity (cell equivalent $\times 10^{-4}$) loaded in each lane.

Figure 7. Analysis of DO expression in tonsillar B cell subpopulations. Samples gated on CD19$^+$ lymphocytes were subdivided based on the expression of IgD and CD38. Mature naive B (IgD$^+$CD38$^-$) and memory B cells (IgD$^-$CD38$^-$) express high levels of DO by intracellular flow cytometry. DO expression is substantially reduced in classical GC B cells (IgD$^-$CD38$^+$). DO expression is intermediate or heterogeneous in IgD$^+$CD38$^+$ cells, which include GC founder cells. DM expression is similar in all subpopulations.

depending on the extent that DM–DO complexes dissociate in cell lysates during detergent solubilization and immunoprecipitation. This estimate represents the total cellular pool. It is possible that the proportion of free DM may vary in different subcellular compartments (23, 32), or between limiting and internal membrane structures of lysosome-related peptide loading compartments (33). Nevertheless, this is an important result. If only a small fraction of DM molecules were bound to DO, one would conclude that DO or DO–DM complexes might have some novel function other than inhibiting DM activity. It appears, however, that a substantial fraction of DM molecules in B cells are not bound to DO. It is not known whether there is an excess of DM in B cells. Therefore, removing half of the DM activity would have little impact on peptide loading activity. Based on previous work, we would predict that a 50% reduction in the concentration of functional DM would be associated with a proportional decrease in DM-catalyzed peptide exchange activity (12). B cells from heterozygous H2-M$^{+/-}$ mice express elevated cell surface class II–CLIP complexes (10, 11), suggesting that a twofold reduction in H2-M has a substantial impact on the catalytic function in B cells from this species. The results presented here provide correlative evidence that physiological levels of DO expression inhibit net DM functional activity in primary human B cells, extending previous studies with transfected cell lines (19, 20, 22). In any given individual, CLIP expression is substantially higher on B cells than monocytes, despite expression of the same array of MHC class II molecules and similar levels of DM. There is a striking relationship between DO expression and CLIP expression during B cell development in adult human bone marrow. Pre-B cells ex-
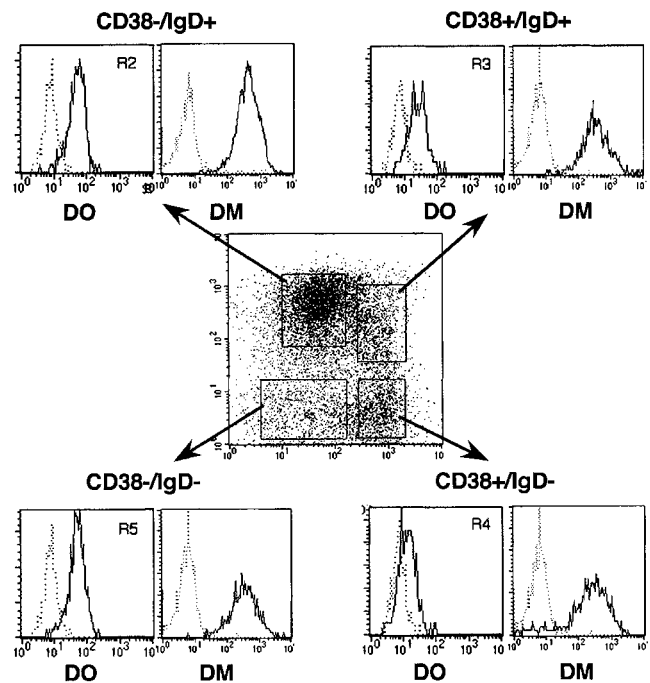
press similar levels of DR and lower levels of DM than B cells, yet CLIP expression is markedly higher in B cells, which correlates with the initiation of DO expression. This relationship is also observed with GC B cells, in which downmodulation of DO is associated with a reduction in the fraction of DR molecules bearing CLIP. In addition, occult follicular lymphoma cells in a lymph node sample were observed to express similar levels of DM and DR, but elevated levels of DO and CLIP compared to normal B cells in the same sample (unpublished data). These results support the conclusion that DO expression has a major impact on net DM activity in primary human B cells. Thus, a substantial fraction of DR molecules do not undergo the first round of DM-catalyzed peptide exchange required to release CLIP. The impact of H2-O may not be as great in mouse B cells, because CLIP expression is low, even on B cells from strains expressing class II molecules with high affinity for CLIP (21). However, as noted, CLIP expression is prominent in B cells from H2-M$^{+/-}$ heterozygotes. Thus, net H2-M activity cannot be far in excess of that required to efficiently mediate CLIP release, and it is likely that subsequent rounds of H2-M–catalyzed peptide exchange are attenuated in mouse B cells.

The observation that adult bone marrow pro-B and pre-B cells express DR and DM suggests that these cells may have a fully functional MHC class II antigen–processing

pathway. There is no evidence that T cells play a role in the antigen-independent phases of B cell development. It is possible that the antigen presentation machinery in these cells is superfluous, a byproduct of the transcriptional program during B cell development with no functional consequences. Alternatively, antigen presentation by B cell precursors could have some undiscovered role, for example, inducing T cell tolerance to B cell antigens. T cells are present in bone marrow, but their origin and function are poorly understood. B cell precursors do not express costimulatory molecules and CD40 is not expressed until the mature B cell stage (unpublished data). Therefore, interaction with these cells is likely to result in T cell anergy rather than activation. It is particularly interesting to consider the phenotype of immature B cells, which express high levels of DM and DR, but no DO. At this stage, B lineage cells first express BCR (mIgM), providing the potential for BCR-mediated internalization of specific antigen and efficient presentation to cognate T cells. B cells undergo negative and positive selection at this stage (34, 35). A subpopulation of immature cells leaves the bone marrow the same way as transitional B cells that have the potential for interactions with T cells in the spleen (36–39). It is possible that in the presence of antigen, interactions with activated cognate CD4$^+$ T cells might rescue transitional B cells from deletion and promote their recruitment into follicles or GC. In the absence of DO, DM-mediated peptide loading activity might be elevated in immature B cells, increasing the efficiency of antigen processing and compensating for a reduced capacity for BCR-mediated endocytosis resulting from the relatively low expression of total mIg on these IgD$^-$ cells. These ideas will require further investigation.

It is striking that the only physiological situation identified so far in which DO expression is down-regulated in mature B cells is in the context of the GC reaction. DO expression is substantially reduced in GC B cells as determined by immunohistochemistry, flow cytometry, and Western blot analysis. This is true for both centroblasts and centrocytes. By contrast, DM and DR expression levels are very similar in GC and non-GC B cells. There appears to be a marginal reduction in the average DM expression and a slight elevation in the average DR expression. The marked reduction in DO expression appears to be associated with an increase in DM functional activity, as judged by a reduction in CLIP–DR complexes on the surface of GC B cells. We cannot rule out the possibility that components of the antigen-processing pathway, other than DO, are significantly modulated in GC B cells and that this could potentially affect CLIP expression.

The primary role of antigen presentation by B cells is to provide a mechanism to orchestrate selective interactions between B cells and T cells sharing antigen specificity. B cells do not appear to play an important role in activating naive T cells or amplifying CD4$^+$ T cell responses. Cognate T–B cell interactions, dependent upon antigen processing and presentation by B cells, are required to generate GC and drive Ig class switching, somatic hypermutation, and the generation of memory B cells and long-lived plasma cells (40). It is believed that the initial encounter with antigen promotes the migration of B cells from the center of B cell follicles in secondary lymphoid organs to the boundary between the B and T cell zones (41–44). After receiving signals through cognate interaction with previously activated CD4$^+$ T cells, some B cells move back into the center of the B cell follicle and begin a process of rapid proliferation that, over the course of 4–6 d, results in the formation of histologically distinct GC. At what point in this sequence of events is DO expression downmodulated? Our results demonstrate that DO expression is uniformly low in classical IgD$^-$CD38$^+$ GC cells. However, the IgD$^+$CD38$^+$ population in tonsils, believed to include GC founder cells (30), appears to have an intermediate level of expression of DO with a possible bimodal distribution. Thus, it is possible that DO down-regulation occurs after the initial entry of founder cells into the GC, but before the loss of surface IgD. This would imply that the initial T–B cell interaction, leading to the commitment of the B cell to the GC reaction, occurs before the reduction of DO expression. Alternatively, BCR ligation with antigen in the context of the follicle microenvironment might induce DO down-regulation before the initial T cell interaction. The IgD$^+$CD38$^+$ population in tonsil includes an unusual subset of IgM$^-$ cells with a high frequency of Ig mutations (30). The possibility that these cells account for the high DO subpopulation of IgD$^+$CD38$^+$ cells, and that founder cells have uniformly low DO expression, is under investigation. If the latter scenario is correct, DO may be downmodulated before the initial encounter with T cells, providing a means of up-regulating peptide loading and antigen presentation by B cells as a consequence of antigen encounter in the follicle microenvironment.

If DO expression is reduced only after the critical antigen presentation event that results in commitment to the GC reaction, what role might the consequent modulation of the antigen-processing function have in B cell immune responses? Antigen presentation by GC B cells and cognate interactions with T cells could play an important role in driving the continued proliferation and somatic mutation in centroblasts. Alternatively, competition between centrocytes for successful interactions with a limited number of T cells in the GC might play a role in promoting survival and antigen-driven selection in GC. Centrocytes expressing higher affinity BCR would have a competitive advantage internalizing and processing limited quantities of antigen. Total surface BCR expression is markedly reduced in GC B cells because of the loss of expression of IgD, reducing the potential efficiency of BCR-mediated endocytosis. The reduction in DO expression and consequent up-regulation of DM function might compensate to increase the efficiency of antigen processing in GC B cells. In favor of a role for antigen presentation by GC B cells is the observation that the blockade of CD40–CD40L interactions not only inhibits formation of GC, but also results in the dissolution of GC after they are established (45, 46). In addition, the blockade of CD86 was reported to inhibit Ig hypermutation and memory development in established GC (46). As

shown in Fig. 5, GC B cells express increased levels of DR, CD80, and CD86, additionally supporting a role for GC B cells in antigen presentation.

An appealing hypothesis is that the function of DO is to attenuate or limit the antigen-processing function of B cells in all situations outside of the protected environment of the GC. IgD⁻CD38⁻ cells in tonsil uniformly express a high level of DO, similar to naive B cells. This indicates that DO expression is up-regulated in memory B cells before or immediately after exiting GC. DO might simply attenuate the antigen-processing function in non-GC B cells, or it may serve to selectively limit the presentation of peptides derived from antigens internalized through non-BCR–mediated mechanisms (21). Through either mechanism, it may help to prevent aberrant or bystander T–B cell interactions. This protective mechanism may no longer be necessary or desirable in the context of the GC reaction, where contact with T cells is anatomically segregated and limited to a small population of GC T cells that share specificity for common antigens.

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