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Expression Patterns of H2-O in Mouse B Cells and Dendritic Cells Correlate with Cell Function

Jennifer L. Fallas,* Woelsung Yi,§ Nicole A. Draghi,† Helen M. O’Rourke,§ and Lisa K. Denzin2‡§

In the endosomes of APCs, the MHC class II-like molecule H2-M catalyzes the exchange of class II-associated invariant chain peptides (CLIP) for antigenic peptides. H2-O is another class II-like molecule that modulates the peptide exchange activity of H2-M. Although the expression pattern of H2-O in mice has not been fully evaluated, H2-O is expressed by thymic epithelial cells, B cells, and dendritic cells (DCs). In this study, we investigated H2-O, H2-M, and I-Aβ-CLIP expression patterns in B cell subsets during B cell development and activation. H2-O was first detected in the transitional 1 B cell subset and high levels were maintained in marginal zone and follicular B cells. H2-O levels were down-regulated specifically in germinal center B cells. Unexpectedly, we found that mouse B cells may have a pool of H2-O that is not associated with H2-M. Additionally, we further evaluate H2-O and H2-M interactions in mouse DCs, as well as H2-O expression in bone marrow-derived DCs. We also evaluated H2-O, H2-M, I-Aβ, and I-Aβ-CLIP expression in splenic DC subsets, in which H2-O expression levels varied among the splenic DC subsets. Although it has previously been shown that H2-O modifies the peptide repertoire, H2-O expression did not alter DC presentation of a number of endogenous and exogenous Ags. Our further characterization of H2-O expression in DCs, as well as the identification of a potential free pool of H2-O in mouse splenic B cells, suggest that H2-O may have a yet to be elucidated role in immune responses. The Journal of Immunology, 2007, 178: 1488–1497.

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Laboratory. 139. H2-Oa further characterization of H2-O expression patterns in DCs suggest endogenous Ags (24). Thus, our studies revealed that there are similarities and fluid phase endocytosed Ags. Our results support a recent study presentation of a limited number of previously unexamined endogenous DCs. Finally, we found that H2-O expression did not alter DC pre-lymphoid DCs. We also report in this study the expression of H2-O in cells. We observed H2-O expression in mouse lymphoid and myeloid splenic DC (sDC) populations, as recently reported (24), with high expression levels of H2-O and a low relative H2-M:H2-O ratio in lymphoid DCs. We also report in this study the expression of H2-O in splenic plasmacytoid DCs and in bone marrow-derived DCs (bmd-DCs). Finally, we found that H2-O expression did not alter DC presentation of a limited number of previously unexamined endogenous and fluid phase endocytosed Ags. Our results support a recent study examining the effect of H2-O on DC presentation of two other exogenous Ags (24). Thus, our studies revealed that there are similarities in H2-O and DO expression levels in B cell subsets during steady-state development and during an immune response. However, our finding of a pool of H2-O not associated with H2-M in B cells and further characterization of H2-O expression patterns in DCs suggest that H2-O may have additional functions that remain to be elucidated.

Materials and Methods

Mice and immunizations

C57BL/6 (B6) and H2-Ma−/− mice were purchased from The Jackson Laboratory. 129.H2-Oa−/− mice were provided by L. Karlsson (Johnson and Johnson Pharmaceutical Research and Development, San Diego, CA) and backcrossed 10 generations to the B6 background to generate the B6.H2-Oa−/− (H2-Oa−/−) mice used for these studies. All mice were bred and maintained under specific pathogen-free conditions in the Memorial Sloan-Kettering Cancer Center’s (MSKCC) animal facility. Use of animals was in accordance with MSKCC’s Institutional Animal Care and Use Committee guidelines. Age- and sex-matched 6- to 9-wk-old B6 mice were immunized i.p. with 50 μg of (4-hydroxy-3-nitrophenyl)acetyl (NP) conjugated to chicken γ globulin (CGG) (Biosearch Technologies) precipitated in alum (Pierce) and 14 days later mice were analyzed by FACS or immunohistochemistry.

Generation of bmdDCs

DCs were derived in vitro from B6 and H2-Oa−/− mice by culturing bone marrow cells for 7 days with RPMI 1640 supplemented with 25 ng/ml GM-CSF as described previously (36, 37). Where indicated, bmd-DCs were harvested on day 4 (immature bmdDCs) or on day 6 and treated with 5 μg/ml LPS (Escherichia coli type 0111.B4; Sigma-Aldrich) overnight to generate mature bmdDCs.

Purification of B cells and sDCs

bmdDCs from day 6 cultures (not treated with LPS) or splenocytes from B6 or H2-Oa−/− mice were stained with PE-anti-CD11c Abs specific for CD11c or CD19 (BD Pharmingen), respectively, and purified by MACS (Miltenyi Biotec) using anti-PE- conjugated microbeads according to the manufacturer’s protocol. sDCs were purified by digestion of spleens in 400 U/ml collagenase D (Roche Applied Science) and 100 μg/ml DNase I (Roche Applied Science) for 30 min at 37°C followed by MACS purification using anti-CD11c-conjugated microbeads (Miltenyi Biotec) according to the manufacturer’s protocol. Purified B cells, bmDCs, and sDCs were >95, >95, and >90% pure, respectively, as confirmed by FACS (data not shown).

Antibodies

Anti-mouse Abs used for FACS analyses and cell sorting and purchased from BD Pharmingen (unless otherwise noted) were as follows: FITC-conjugated anti-Ab-CLIP (AF6-120.1), anti-CD21/CD35 (CR2/CR1; 7G6), anti-β2-microglobulin (GL7; PE-conjugated anti-Ab), anti-H2-M (HL3), anti-CD19 (1D3), anti-CD23 (FczR; B3B4), anti-IgD (11-26; eBioscience), anti-TCRβ (H57-597); CyChrome-conjugated anti-CD8α (53-67), anti-TCRβ (H57-597); PerCP-Cy5.5-conjugated anti-CD19 (1D3); PE-Cy7-conjugated anti-CD8α (53-67), anti-CD45R (B220; RA3-6B2), anti-CD19 (1D3); allopophysycocyanin-conjugated anti-CD93 (Cl1Rq; A44.1; eBioscience), anti-CD11b (M1/70), anti-CD19 (1D3), anti-MHC class II (I-A/II-E; M5/114.15.2; eBioscience); allopophysycocyanin-Cy7-conjugated anti-CD45R (B220; RA3-6B2; eBioscience), anti-CD4 (GK1.5); biotinylated anti-CD8 (B7-2; GL1), anti-CD93 (Cl1Rq; A44.1; eBioscience), anti-CD95 (Fas/APO-1; Jo2), anti-DEC-205 (NLCD-145; MSKCC mAb Core Facility), anti-IgM (goat F(ab’2’); Southern Biotechnology Associates); purified anti-rat IgG (R3-34; isotype control), anti-MHC class II (I-A; 212.A1; MSKCC mAb Core Facility), and anti-H2-M (2C3A) (produced in the laboratory). Biotinylated mAbs were detected with streptavidin-conjugated PerCP (BD Pharmingen) or PE-Cy7 (Caltag Laboratories). The hybridoma cell lines 15G4 (anti-Aβ-CLIP) and 2C3A (anti-H2-M) were provided by A. Rudensky (University of Washington, Seattle, WA) and L. Karlsson (Johnson and Johnson Pharmaceutical Research and Development, respectively). mAbs 15G4, 2C3A, 212.A1, Mags.Ob1, and Mags.Ob3 were purified from bioreactor supernatants using immobilized protein G-Sepharose (Amersham Pharmacia Biotech) or protein A-Sepharose Sigma-Aldrich) affinity chromatography and used as purified mAbs or conjugated with Alexa Fluor 488 (15G4, 2C3A, 212.A1, and Mags.Ob1), Alexa Fluor 633 (Mags.Ob3 and 2C3A), or Alexa Fluor 647 (15G4) (all obtained from Molecular Probes) according to the manufacturer’s protocol.

The mouse mAb YoDMA.1, specific for denatured H2-Mo has been described (20). Hamster mAbs specific for the cytoplasmic tail of H2-O (C-KASVETQPNEASRESLHSQP; Ob/c) were generated by injecting hamsters s.c. with keyhole limpet hemocyanin-Ob/c conjugate emulsified in Titermax and screened for an immune response by ELISA. Splenocytes from an Ob/c-reactive hamster were fused to Ag14 myeloma cells, and culture supernatants from the hybridoma clones were screened by immuno-fluorescence and Western blot using splenocytes from B6 and H2-Oa−/− mice. Two hybridomas secreted mAbs that recognized free H2-O (Mags.Ob1 and Mags.Ob3). Hamster preimmune serum was used as control for B cell co-immunoprecipitations (IP). To produce anti-mouse H2-O polyclonal Abs (R.Ob/c), rabbits were immunized with the keyhole limpet hemocyanin-Ob/c conjugate, and peptide-specific Abs were purified from the sera by affinity chromatography with an Ob/c peptide column.

Immunoblot analysis and quantitation

Nonquantitative and fluorescence-based quantitative immunoblot analyses were performed, as described previously (28), with the following Abs: rabbit anti-H2-Oa polyclonal tail (R.Ob/c) and YoDMA.1. For nonquantitative immunoblot analyses, primary Abs were detected with HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG secondary Abs (Jackson ImmunoResearch Laboratories), and blots were developed with SuperSignal West Fico chemiluminescent peroxidase substrate (Pierce Biotechnology). For fluorescence-based quantitative immunoblot analyses, primary Abs were detected with alkaline phosphatase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG secondary Abs (Jackson ImmunoResearch Laboratories) and developed with Vistra ECF substrate (Amersham Pharmacia Biotech). Fluorescence was quantitated with a Molecular Imager FX System (Bio-Rad). Cell numbers used for each blot are indicated in each figure legend.

Endo H digestions

Purified B cells, mature bmdDCs (day 7 + LPS), and sDCs from B6 or H2-Oa−/− mice were extracted in lysis buffer at a pH of 7.0. Following the removal of nuclei and cellular debris by centrifugation, lysates were de-natured and incubated in the presence or absence of 5000 U of Endo H (New England Biolabs) according to the manufacturer’s protocol. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and analyzed by immunoblotting. Cell numbers used for each blot are indicated in each figure legend.

IP and immunodepletion of H2-O and H2-M

Purified B cells, sDCs, and immature and mature bmdDCs were extracted in lysis buffer at a pH of 7.0. Following the removal of nuclei and cellular debris by centrifugation, lysates were incubated with specific Ab (15 μg/
CD11b, H2-O

H2-O was expressed in CD19 analysis of primary spleen cells and confirmed, as expected, that two equal aliquots were separated by SDS-PAGE, transferred to PVDF membranes, and analyzed for H2-M expression in primary cells. Ag presentation assays were performed as described previously (20). Pigeon cytochrome C were purified by negative selection from D10 (43) University, New Haven, CT). Naive T cells specific for conalbumin and DMA.1 and R.Ob/c, respectively. The primary mAbs used for IPs were hambranes, and analyzed for H2-Mα expression in primary cells and transport of H2-M/O complexes requires both H2-O expression we confirmed by Endo H digestion of B cell and sDC lysates. (left) and CD11c+ DCs (right) from B6 (black line) and H2-Oa−/− (gray shaded) mice. The dotted line on each histogram shows staining with a nonspecific control mAb. C, H2-OB detected in H2-Oa−/− B cells and sDCs resides in the ER. Detergent lysates from purified splenic B cells (1 X 10^6 cells/lane) (top panel) and sDCs (bottom panel) (1 X 10^6 cells/ lane) from B6 and H2-Oa−/− mice were denatured and treated (+) or not treated (−) with Endo H, separated by SDS-PAGE, transferred to PVDF membranes, and probed with an Ab to the cytoplasmic tail of H2-OB (R.Ob/c). The data shown are representative of three independent experiments. (H2-OBglycos, Endo H-resistant; H2-OBdeglycos, Endo H-sensitive).

CD11b+ macrophage population was observed due to the autofluorescent nature of macrophages relative to T cells.

To further evaluate the specificity of our anti-H2-OB α mAb, we compared intracellular H2-O expression levels by FACS in splenic B cells and DCs from wild-type (B6) and B6.H2-Oa-deficient mice. Although H2-OB was detected in H2-Oa−/− B cells and DCs, levels were lower than in wild-type cells (Fig. 1B). H2-Oa−/− mice lack only the H2-Obn-chain (18), and our anti-H2-O mAb is specific for H2-OB. The residual H2-OB expression we detected in H2-Oa−/− cells was likely due to ER-resident H2-OB protein that cannot egress from the ER in the absence of H2-0α. We confirmed by Endo H digestion of B cell and sDC lysates followed by Western blot analyses that, as predicted, the majority of H2-OB in wild-type cells was Endo H-resistant (Fig. 1C, top band: H2-OBglycos), whereas H2-OB detected in H2-Oa−/− B cells and sDCs was completely Endo H-sensitive (Fig. 1C, bottom band: H2-OBdeglycos), supporting the ER-localization of H2-OB in H2-Oa−/− B cells and sDCs.

RESULTS

H2-O expression in primary cells

To directly examine H2-O expression, we generated Abs specific for the cytoplasmic tail of H2-OB that can be used for FACS, histology, immunofluorescence, IP, and Western blotting. The formation and transport of H2-M/O complexes requires both H2-0α and H2-OB (16), and thus measurement of H2-OB protein levels should provide an accurate assessment of overall H2-O levels. We confirmed the specificity of the H2-OB Ab by intracellular staining and FACS analysis of primary spleen cells and confirmed, as expected, that H2-O was expressed in CD19+ B cells and CD11c+ sDCs, but not in CD11b+ macrophages or T cells (Fig. 1A). A small population of CD11b+ cells were H2-O+ (Fig. 1A); however, these cells uniformly expressed B220, suggesting that these cells are a subset of B cells (data not shown) (45). A slight shift in the H2-0-negative peak for the

Immunohistology

Spleens from mice immunized 14 days earlier with NP-CGG were embedded in Tissue-Tek OCT compound (VWR Scientific Products), snap-frozen, and stored at −80°C. Frozen embedded spleens were sectioned with a cryostat (6 μm), air-dried for 2 h at room temperature, fixed using cold acetone for 15 min, and stored at −80°C. Acetone-fixed sections were blocked with 3% BSA (Sigma-Aldrich) in PBS for 30 min at room temperature, incubated with the following mAbs: GL7-FITC, IgD-FITC, IgD-BD Pharmingen), and then stained according to the manufacturer’s protocol. FACS analysis of sDCs was performed following digestion of spleens in 400 U/ml collagenase D (Roche Applied Science) and 100 μg/ml DNase I (Roche Applied Science) for 30 min at 37°C.

Ag presentation assays

T hybridoma cell lines specific for I-Aκb complexed with peptides from IgM377–392 (77.1), β2-microglobulin (β2m), and actin 163–177 (15.10) (40, 41) were provided by A. Rudensky (University of Washing-

Flow cytometry

Samples were stained for FACS analyses as previously described (38) and analyzed using an LSR flow cytometer (BD Biosciences) or CyAn flow cytometer (DakoCytomation). Dead cells were excluded from analysis by the addition of the cell vital dye 4′,6-diamidino-2-phenylindole, and cell

FIGURE 1. H2-O is expressed in B cells and splenic CD11c+ DCs, but not in macrophages or T cells. A and B, Spleen cells from B6 and B6.H2-Oa−/− mice were stained with mAbs for the surface expression of CD11c, CD19, H37, and CD11b, permeabilized, stained for intracellular H2-O, and analyzed by four-color flow cytometry. Cells were electronically gated to identify cell populations and H2-O expression was analyzed (A, left). Cells were gated on H2-O−/− cells and staining of CD11c+ DCs relative to CD19+ B cells is shown (A, right). Data are representative of three independent experiments. B, A comparison of H2-O intracellular levels in CD19+ B cells (left) and CD11c+ DCs (right) from B6 (black line) and H2-Oa−/− (gray shaded) mice. The dotted line on each histogram shows staining with a nonspecific control mAb. C, H2-OB detected in H2-Oa−/− B cells and sDCs resides in the ER. Detergent lysates from purified splenic B cells (1 X 10^6 cells/lane) (top panel) and sDCs (bottom panel) (1 X 10^6 cells/ lane) from B6 and H2-Oa−/− mice were denatured and treated (+) or not treated (−) with Endo H, separated by SDS-PAGE, transferred to PVDF membranes, and probed with an Ab to the cytoplasmic tail of H2-OB (R.Ob/c). The data shown are representative of three independent experiments. (H2-OBglycos, Endo H-resistant; H2-OBdeglycos, Endo H-sensitive).
H2-O, H2-M, I-Ab, and I-Ab-CLIP expression in vivo during B cell development

We first characterized the expression levels of H2-O and other class II pathway molecules in primary mouse B cell subsets in the bone marrow and spleen by FACS. Cells were stained with panels of mAbs to well-defined cell surface markers that allowed for the identification of distinct B cell developmental subsets as described previously (46, 47). Cells were also simultaneously stained for surface I-Ab and I-Ab-CLIP or fixed and permeabilized before staining for H2-O and H2-M.

H2-O expression was not detected in developing pro-B, pre-B, or immature B cells in the bone marrow (Fig. 2 and data not shown). B cell-specific H2-O expression in the bone marrow was detected, however, in mature B cells that had recirculated from the spleen (Fig. 2A). Bone marrow B cells were stained intracellularly for H2-O (Mags.Ob1 (bone marrow and spleen transitional subsets) or Mags.Ob3 (spleen mature subsets)) or H2-M (2C3A) or surface stained for I-Ab (212.A1 (bone marrow and spleen transitional subsets) or M5/114.15.2 (spleen mature subsets)) or I-Ab-CLIP (15G4). Cells were also surface stained with a panel of mAbs to identify distinct B cell subsets, gated (A and B, right) as indicated and analyzed to compare intracellular levels of H2-O and H2-M or levels of surface I-Ab and I-Ab-CLIP staining. Representative staining from one mouse is shown. Data are representative of three independent experiments of three mice each (n = 9). Markers analyzed are indicated in the top right of each histogram. Histograms are coded as indicated on the right.

H2-O is down-regulated in mouse GC B cells. B6 mice were immunized with NP-CGG in alum and 14 days later analyzed by histology (A) or FACS (B). A. Two-color immunohistological analysis of H2-O expression in GC B cells. Abs used in each panel (top left) are indicated. IgD and GL7 were used as markers for FO and GC B cells, respectively. Data are representative of three independent experiments. B. Spleen cells from immunized mice were stained intracellularly for H2-O (Mags.Ob3) or H2-M (2C3A) or surface stained for I-Ab (M5/114.15.2) or I-Ab-CLIP (15G4). Cells were also surface stained with a panel of mAbs to identify distinct B cell subsets, gated (top) as indicated, and analyzed to compare intracellular levels of H2-O and H2-M or levels of surface I-Ab and I-Ab-CLIP staining. Markers analyzed in each histogram (top right) are indicated. Histograms are color coded as indicated in pseudocolor plots (top). Representative staining from one mouse is shown. Bar graphs (right) show plots of the average ratio of the mean fluorescence intensity (MFI) for H2-M staining relative to the MFI for H2-O staining (Ratio: M:O) and the average ratio of the MFI for I-Ab staining relative to the MFI for I-Ab-CLIP staining (Ratio: Ab:Ab-CLIP) for GC and FO B cell populations in three mice from one experiment. Data are representative of two independent experiments.
periphery (Fig. 2A). In the periphery, H2-O expression was detected in all transitional B cell subsets (T1–T3) and expression was maintained in mature B cell populations (Fig. 2B). Thus, H2-O expression is turned on during B cell development as immature transitional B cells traffic from the bone marrow to the spleen.

In the bone marrow, H2-M was expressed at low levels in immature B cells (Fig. 2A) and was up-regulated as cells matured in the periphery from transitional to mature FO and MZ B cells (Fig. 2B). I-Ab expression was first detected in pre-B cells and the cell surface density increased as cells matured, as reported previously (Fig. 2; Ref. 48). I-Aβ-CLIP expression was first observed in pre-B cells and was up-regulated as B cells matured from immature to mature B cells, in parallel with increasing class II levels. I-Aβ-CLIP levels did not increase in H2-O-expressing T1 B cells relative to H2-O-negative immature bone marrow B cells, which supports the fact that surface I-Aβ-CLIP levels are not a functional readout for H2-O expression levels in mouse immune cells (18, 26). Overall, these studies indicate that immature bone marrow B cells may have the ability to process and present Ag to CD4 T cells because they express H2-M and class II. This process may be modulated by the initiation of H2-O expression in immature B cells as they migrate from the bone marrow to the periphery.

In mature spleen cell populations, both H2-O and H2-M expression levels were consistently slightly higher in MZ compared with FO B cells by FACS analysis (Fig. 2B, bottom row). However, quantitative Western blotting of lysates from sorted MZ and FO B cells showed that the average expression levels of H2-O and H2-M in FO and MZ B cells was not substantially different (data not shown). Although H2-M and H2-O levels might be slightly higher in MZ B cells relative to FO B cells as shown by FACS, it is unlikely that this difference is >2-fold, which is likely to be the limit of detection for our quantitative Western blotting assay.

H2-O expression is down-regulated in GC B cells

We and others have shown that relative to naive and memory B cells, DO expression is dramatically reduced in human GC B cells, and the reduction of DO correlates with low surface levels of class II CLIP and high surface levels of class II peptide in GC B cells (19, 28, 49). To evaluate whether H2-O levels are similarly modulated in B cell populations during T cell-dependent immune responses, wild-type mice were immunized with the model Ag NP-CGG (50). Fourteen days postimmunization, the relative expression levels of H2-O and other class II pathway molecules in splenic GC B cells and other B cell subsets were analyzed by histology and FACS (Fig. 3).

Our results showed that H2-O levels were down-modulated in GC B cells, but not in other B cell subsets during an immune response (Fig. 3), similar to what was previously observed for human GC B cells (19, 28, 49). Immunohistological analysis of spleen sections from NP-CGG-immunized mice showed high levels of H2-O in IgD+ FO B cells (Fig. 3A). H2-O protein expression was not detected in GL7+ GC B cells. Although not apparent from immunohistology, H2-O levels in GC B cells remained detectable above background by FACS analysis (Fig. 3B). H2-O levels were unchanged in FO and non-GC B cells. Importantly, because H2-M levels were not reduced in GC B cells, the relative ratio of H2-M: H2-O was increased in GC B cells compared with FO B cells (Fig. 3B). This mirrored the substantial increase in the relative DM:DO ratio observed for human GC B cells compared with naive and memory B cells (19, 28).

Although we observed a reduction in H2-O levels accompanied by an increase in the relative ratio of H2-M:H2-O in GC B cells, this did not result in an increase in the relative ratio of I-Aβ-I-Aβ-CLIP in GC B cells compared with FO B cells (Fig. 3B). GC B cells exhibited increased surface levels of I-Aβ relative to FO B cells, as expected (28, 49), and I-Aβ-CLIP levels were similarly up-regulated (Fig. 3B). Overall, we conclude that during an
immune response, H2-O levels are down-modulated in GC B cells but not other B cell populations, similar to what has been observed for human B cells (19, 28).

Mouse primary B cells have a pool of H2-O that is not associated with H2-M

We next evaluated H2-M and H2-O interactions in primary mouse B cells. Detergent lysates from CD19<sup>+</sup> splenic B cells were immunoprecipitated with Abs specific for H2-O or H2-M and then analyzed for the presence of H2-M/O complexes by Western blotting. Results showed that H2-M and H2-O are complexed in primary mouse B cells (Fig. 4A), as previously observed in splenocytes by metabolic labeling and two-dimensional gel electrophoresis (16). To further evaluate H2-M/O interactions, we next determined whether all of the H2-M was complexed with H2-O in mouse primary B cells. H2-M/O complexes were either depleted or mock-depleted from primary B cell lysates by IP with an H2-O-specific or control mAb, respectively. H2-O-depletion and the amount of H2-M<sub>o</sub> remaining after H2-O-depletion relative to mock depletion were determined by quantitative Western blotting (Fig. 4B). Results showed that, on average, 33% of cellular H2-M remained in cell lysates postdepletion of H2-M/O complexes (Fig. 4C). Therefore, ~67% of cellular H2-M was associated with H2-O in mouse B cells.

We also depleted H2-M and H2-M/O complexes from B cell lysates to determine whether all H2-O was associated with H2-M (Fig. 4B). Unexpectedly, we found that, on average, 41% of cellular H2-O remained in cell lysates postdepletion of H2-M/O complexes (Fig. 4C). Furthermore, the majority of remaining H2-ββ in H2-M-depleted lysates was Endo H resistant (data not shown). This result indicates that the free H2-O was derived from a post-Golgi compartment and was not simply ER resident H2-ββ, which was in the process of being assembled with H2-M.

H2-O expression and interaction with H2-M in DCs

Recent studies have revealed that DCs express H2-O (23, 24) (Fig. 1). To further characterize H2-O expression in DCs, we compared H2-O and H2-M levels in sDCs and B cells by FACs. Our results showed that subsets of sDCs expressed slightly higher and lower levels of H2-O than B cells (Fig. 5A). To determine whether H2-O and H2-M levels are modulated during DC maturation, immature

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(day 4) and mature (day 7 + LPS) bmDCs were analyzed by FACS for intracellular H2-O and H2-M. Both H2-O and H2-M levels were unchanged during bmDC maturation (Fig. 5B). H2-O, the majority of which was Endo H resistant, was also detected in sDCs and bmDCs by Western blotting and immunofluorescence (Fig. 1C and data not shown).

In the absence of H2-M, H2-O does not transport out of the ER and is rapidly degraded (16). Because most of the H2-O in sDCs was Endo H-resistant (Fig. 1C) and immunofluorescence analyses showed that H2-O and H2-M colocalized in DCs (23, 24) and bmDCs (data not shown), we next directly examined H2-O and H2-M interactions in sDCs and bmDCs. IP of H2-O and H2-M from sDC and bmDC lysates with H2-O or H2-M-specific mAbs, followed by Western blotting showed that H2-O and H2-M are associated in DCs (Fig. 5C and data not shown). Our precipitations were performed in Tx-100, a detergent known to disrupt H2-M/O interactions, supporting the idea that H2-M and H2-O interact directly in DCs as is believed to occur in B cells (51).

**H2-O is expressed in all CD11c+ sDC populations**

Because CD11c+ sDCs are a heterogeneous population, we next performed six-color FACS analysis to identify and compare the expression levels of H2-O and associated class II pathway proteins among sDC populations. Spleen cells were stained with a panel of mAbs that specifically recognize surface markers that define distinct CD11c+ mouse sDC populations: lymphoid DCs (CD11c+ CD11b- B220- DEC205+ CD8α-), myeloid DCs (CD11c+ CD11b+ B220- DE205+ CD8α-), and plasmacytoid DCs (CD11c+ CD11b+ B220- DEC205- CD8α+) (52, 53). Staining also included mAbs specific for H2-O, H2-M, class II (I-Aα), or class II (I-Aα)-CLIP. Cells were analyzed by FACS. Our results showed that H2-O is expressed in lymphoid, myeloid, and plasmacytoid DCs (Fig. 5D). Lymphoid DCs expressed ~2-fold more H2-O than myeloid and plasmacytoid DCs. In contrast, H2-M levels were highest in myeloid DCs, whereas plasmacytoid DCs expressed intermediate levels and lymphoid DCs expressed the lowest levels (Fig. 5D). Thus, myeloid DCs possess a high relative ratio of H2-M:H2-O (Fig. 5E) (24), suggesting that myeloid DCs have an active class II Ag processing pathway. This idea is supported by data proposing that these DCs are essential APCs that initiate immune responses (54). The high expression level of H2-O and low relative H2-M:H2-O ratio in lymphoid DCs, also observed by Jensen and colleagues (24), supports the fact that these cells may play a role in tolerance induction in which H2-O actively modulates H2-M-mediated peptide loading in vivo (54). Overall, class II-CLIP levels did not differ between the DC populations and did not correlate with H2-O and H2-M expression levels (Figs. 5, D and E), as expected. A similar analysis of sDC populations performed using a panel of mAbs that recognized an alternative combination of DC surface markers (CD11c, CD11b, CD4, DEC205, and CD8) (53) yielded similar results (data not shown).

**Ag presentation by H2-O+/+ and H2-O−/− bmDCs**

To determine whether H2-O expression affected the presentation of exogenous Ags, we incubated purified bmDCs from B6 and H2-Oa−/− mice with Ags (IgM, OVA, or HEL) and measured presentation by the addition of T hybridoma cells specific for peptides derived from these proteins. We found that H2-O did not alter bmDC presentation of fluid phase endocytosed Ags (data not shown). We also examined the influence of H2-O expression on bmDC presentation of two endogenously expressed Ags by adding T hybridoma cells specific for peptides derived from actin and β2m to titrated numbers of purified B6 and H2-Oa−/− bmDCs. Presentation of epitopes from these endogenous Ags also was not significantly altered by H2-O expression (data not shown). Thus, for the limited numbers of exogenous and endogenous Ags examined in this study, the presence of H2-O had no effect on class II presentation by DCs, supporting recent studies by Chen et al. (24).

**Discussion**

Studies to date have yielded inconclusive results concerning the function of DO/H2-O in the class II Ag processing pathway (34). In vitro biochemical assays have mostly shown that the DM/DO complex is inactive in terms of its ability to catalyze peptide loading of class II molecules (18, 25, 29). In contrast, in vitro Ag presentation assays have shown that DO/H2-O expression can promote, inhibit, or have no effect on class II Ag presentation, perhaps due to different experimental systems and/or the Ags examined (18, 24, 26, 27, 30–32). In vivo studies to date, comparing the immune response in H2-O-sufficient and -deficient mice (18, 35) have also not defined a specific role for DO/H2-O. Thus, the function of H2-O remains elusive. In this study, we present an extensive analysis of H2-O expression in vivo and also examine H2-M/O interactions. We show, for the first time, the modulation of H2-O expression in mouse B cells during development and activation. Additionally, our studies suggest that splenic B cells possess a pool of H2-M-free H2-O. Additionally, our data corroborate and expand upon the recently reported observations of DO/H2-O expression in mouse and human DCs (23, 24). H2-O expression in DCs suggests that this molecule may have an important, but yet not defined role in immune responses given the importance of DCs in initiating and controlling immune responses.

Our studies showed that during B cell development, MHC class II and H2-M, but not H2-O, are expressed in immature bone marrow B cells, as previously observed for human bone marrow B cells (Fig. 2A and Ref. 19). Upon migration from the bone marrow to the spleen, detectable levels of H2-O protein are first observed in developing transitional B cells (Fig. 2B), and expression is maintained along with H2-M and class II expression in all three splenic transitional B cell subsets, as well as in mature B cells (Fig. 2B). Because immature bone marrow and splenic B cells also express surface IgM, H2-M, and class II, they are most likely capable of efficient processing and presentation of BCR-internalized Ags via class II, an idea that is supported by in vitro Ag presentation assays (55). Thus, it is tempting to speculate that in immature splenic B cells, H2-O may play a role in the specificity-based positive selection of immature splenic B cells, perhaps by modulating the class II-peptide repertoire derived from BCR-internalized Ags and presented by these cells.

MZ and FO B cells express similar levels of H2-O and H2-M (Fig. 2B), suggesting that these molecules may play a similar role in these mature B cell populations. Thus, we were surprised that in vivo competition assays between H2-O+/+ and H2-Oa−/− B cells revealed that H2-O+/+ B cells repopulated the MZ compartment of the spleen 2- to 3-fold over H2-Oa−/− B cells (J. L. Fallas, unpublished data). This suggests that H2-O expression is somehow advantageous to this B cell population. However, whether H2-O confers this advantage through modulation of the conventional MHC class II Ag processing pathway or via some other pathway remains to be determined.

Our studies show that whereas H2-O is expressed at similarly high levels in FO and MZ B cells, H2-O expression is significantly down-regulated in mouse GC B cells (Fig. 3), analogous to the down-modulation of DO in human GC B cells (19, 28, 49). However, the physiological significance of H2-O/DO down-regulation in GC B cells remains unknown. GC B cells do not survive positive selection without Ag-specific T cell stimulation (56). It is
presented peptides derived from IgM, OVA, HEL, actin, and other Ags and epitopes (24). Importantly, another recent study showed that sDCs from H2-O-/- mice produced a more robust allogenic CD4 T cell response in MLRs than wild-type sDCs, showing that H2-O expression in sDCs alters the class II peptide repertoire (24). Future studies are needed to better characterize the nature of the H2-M/O complex as well as to examine H2-O function in mouse DCs.

Because splenic CD11c+ DCs are a heterogeneous population (53, 60), we characterized the levels of H2-O and other class II pathway molecules in different DC subsets (Fig. 5, D and E). Although the comparison of H2-O and H2-M expression levels in different sDC populations by three-color FACS analysis was recently reported (24), we approached this question by performing six-color FACS analysis, using the expression of five well-defined markers (CD11c, CD11b, B220, DEC205, and CD8α or CD11c, CD11b, CD4, DEC205, and CD8α) (52, 53) to gate distinct sDC populations to compare H2-O, H2-M, class II, and class II-CLIP expression levels among all sDC populations in the same sample (Fig. 5D). Overall, our studies agreed well with the data reported by Chen et al. (24), except that we also detected H2-O expression in plasmacytoid DCs (Fig. 5D). This difference is most likely due to our increased resolution of sDC populations but may also be due to the use of different H2-O-specific Abs.

Assuming that H2-O functions as a modulator of H2-M-mediated class II peptide loading (18, 25, 29), H2-O expression levels correlated with the proposed functions of different DC subsets. Reduced H2-O levels and a high H2-M:H2-O ratio in myeloid DCs suggest that this DC subset has an optimally active class II Ag processing pathway, consistent with data suggesting that these DCs initiate immune responses (54, 60). Elevated H2-O levels and a low H2-M:H2-O ratio in lymphoid DCs correlate with the proposed role of this subset in tolerance induction (54, 61, 62). H2-O in these DCs may function to dampen the presentation of specific self-peptides that would otherwise generate autoimmune responses or may function to modulate the presentation of specific self-Ags to delete autoreactive T cells from the repertoire. Additional studies are required to determine whether H2-O expression in these DC subsets impacts class II Ag presentation and subsequent T cell responses in vivo.

Although DCs differ in many ways from B cells, it is intriguing to speculate that H2-O may play a similar role in both cell types. In addition to macropinocytosis, DCs also internalize Ags via receptor-mediated mechanisms (63). Furthermore, DCs have more receptors than B cells with which Ag-immune complexes can be captured (59, 64). Although fluid phase and receptor-mediated endocytosis both concentrate Ags into MIIICs, receptor-mediated endocytosis promotes up to 100-fold more efficient Ag presentation and CD4 T cell activation than fluid phase internalization (65–67). Additionally, specific DC receptors such as DEC-205, which is higher on sDCs than other DC receptors at delivering Ag to processing compartments (68–70), bypassing the route of fluid phase-internalized Ags, similar to BCR-mediated endocytosis. Interestingly, a recent study that characterized the expression of DO in human DC subsets determined that DO levels were highest in BDCA-3+ DCs, a DC subset of unknown function that expresses higher levels of DEC-205 compared with other populations (23). Additionally, Ag binding to receptors may mask certain Ag epitopes that are presented to T cells when Ags are internalized via nonreceptor-mediated pathways. This could either boost or suppress immune responses to Ags, depending on the mechanism of uptake. DCs and B cells...
modify the localization of class II pathway components when activated by Ag, further supporting a similar function of H2-O in these APCs. During DC maturation, class II-Ch1 complexes accumulate with H2-M and Ag in MHCs in immature DCs (71). BCR binding of Ag results in the concentration of class II, H2-M, BCR, and Ag in multivesicular MHCs, similar to the MHCs observed in DCs (72, 73). Human GC B cells also reorganize intracellular class II and DM to form MIICs (49). Therefore, similar mechanisms for promoting the presentation of receptor-internalized Ags may exist in DCs and could potentially be mediated by H2-O.

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References
18. Vanham, M. V., T. Kuwana, W. P. Fung-Leung, M. R. Jackson, P. A. Peterson, and L. Karlsson. 1996. HLA-DM modifies the localization of class II pathway components when activated by Ag, further supporting a similar function of H2-O in these APCs. During DC maturation, class II-Ch1 complexes accumulate with H2-M and Ag in MHCs in immature DCs (71). BCR binding of Ag results in the concentration of class II, H2-M, BCR, and Ag in multivesicular MHCs, similar to the MHCs observed in DCs (72, 73). Human GC B cells also reorganize intracellular class II and DM to form MIICs (49). Therefore, similar mechanisms for promoting the presentation of receptor-internalized Ags may exist in DCs and could potentially be mediated by H2-O.

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References


