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The MHC Class II Cofactor HLA-DM Interacts with Ig in B Cells

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B cells internalize extracellular Ag into endosomes using the Ig component of the BCR. In endosomes, Ag-derived peptides are loaded onto MHC class II proteins. How these pathways intersect remains unclear. We find that HLA-DM (DM), a catalyst for MHC class II peptide loading, co-precipitates with Ig in lysates from human tonsillar B cells and B cell lines. The molecules in the Ig/DM complexes have mature glycans, and the complexes colocalize with endosomal markers in intact cells. A larger fraction of Ig coprecipitates with DM after BCR crosslinking, implying that complexes can form when DM meets endocytosed Ig. In vitro, in the endosomal pH range, soluble DM binds the Ig Fab domain and increases levels of free Ag released from immune complexes. Taken together, these results argue that DM and Ig intersect in the endocytic pathway of B cells with potential functional consequences.

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The pathway that bridges Ag/Ig delivery to MHC compartments with peptide/MHC-II association remains unclear. It is known that Ag capture by the BCR and its trafficking to late endocytic compartments contribute to the significant enhancement of Ig-mediated Ag presentation compared with presentation after fluid-phase Ag uptake (16). We investigated whether DM, a known

Abbreviations used in this article: cRPMI, RPMI 1640 supplemented with 10% FBS and 2 mM l-glutamine; DC, dendritic cell; DM, HLA-DM; DO, HLA-DO; endo H, endoglycosidase H; ER, endoplasmic reticulum; gp120-b, biotinylated gp120; HEL, hen egg lysosome; Ii, invariant chain; MHC-I, MHC class I; MHC-II, MHC class II; MHC, MHC class II loading compartment; PAS, protein A–Sepharose; PGS, protein G–Sepharose; PLA, proximity ligation assay; RT, room temperature; RU, resonance unit; sDM, soluble DM; SPR, surface plasmon resonance.
catalyst of peptide/MHC-II association, directly interacts with Ig in B cells.

Materials and Methods

Cells

The non-Hodgkin’s human B cell lymphoma cell line DHL-4 (IgG-κ) (17) and the human B lymphoma cell line OCI-LY8 (IgM/κ-Dλ) (18) (provided by Dr. Ron Levy, Stanford University, Stanford, CA) were grown in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine (cRPMI). EBV-transformed human B cell lines DPA-2 (IgG1-κ) (19) (provided by Christiane Hampe, University of Washington, Seattle, WA) were grown in cRPMI. Epstein-Barr-transformed human B cell lines 9.5.3 (HLA-DMβ) (20), 5.2.4 (HLA-DMβ and HLA-DOβ) (21), and 3T3.LacZ (HLA-DRα) (22) were grown in cRPMI. HLA class II-negative lymphoma lines Daudi (b2-microglobulin-1) and Raji are available through the American Type Culture Collection. De-identified tonsil samples were obtained in compliance with Stanford University Institutional Review Board protocol 12312. Live tonsil cells were isolated by density gradient centrifugation with resulting viability >95%. Human monocytes were obtained under a Stanford University Institutional Review Board–approved protocol from buffy coats of normal adults and treated with IL-4 and GM-CSF to derive immature DCs, as described (23). The human macrophage-protocol from buffy coats of normal adults and treated with IL-4 and GM-CSF to derive immature DCs, as described (23). The human macrophage-protocol from buffy coats of normal adults and treated with IL-4 and GM-CSF to derive immature DCs, as described (23). The human macrophage-protocol from buffy coats of normal adults and treated with IL-4 and GM-CSF to derive immature DCs, as described (23). The human macrophage-protocol from buffy coats of normal adults and treated with IL-4 and GM-CSF to derive immature DCs, as described (23).

Abs and probes

For Western blots, primary Abs and probes included: anti–HLA-DMα mouse monoclonal SC1 (also called Tal18.1) (Santa Cruz Biotechnology), anti-human λ chain baitin-labeled goat F(ab)2’ (SouthernBiotech, Birmingham, AL), anti-human κ λ chain baitin-labeled goat F(ab)2’ (SouthernBiotech), anti-human Ig H+L rabbit polyclonal (Thermo Fisher Scientific, Waltham, MA), anti–HLA-DOβ mouse monoclonal DOB.L1 (Santa Cruz Biotechnology), anti–MHC class I (MHC-I) mouse monoclonal (anti-human IgG1, nonconjugated) (Jackson ImmunoResearch, West Grove, PA), and anti-rabbit IgG HRP-conjugated donkey Ab (GE Healthcare, Little Chalfont, U.K.). For proximity ligation assay (PLA) Abs, used as probes included: anti-DM dimer mouse monoclonal MaP.DM1, anti-human Ig λ and κ chain Abs (BioLegend, San Diego, CA), anti-HLA-DR dimer L243 (25) (provided by Genencor, Palo Alto, CA), and anti-Ki67 rabbit polyclonal (Abcam, Cambridge, U.K.). For immunofluorescence microscopy, Abs and probes included: anti–Lamp-1 rabbit Ab (Cell Signaling Technology, Danvers, MA), anti-EEA1 rabbit Ab (Santa Cruz Biotechnology), and anti-rabbit Ig Alexa Fluor 488-conjugated goat Ab (Life Technologies).

Purified proteins for interaction assays

Insect cell (S2)-derived soluble DM (sDM) was generated and purified as described (26) with the following modifications: filtered S2 supernatant was added directly to an M2 affinity column (Sigma-Aldrich, St. Louis, MO), eluted with 100 μM FLAG peptide (Sigma-Aldrich), and was purified using cation exchange chromatography on a SuperQ column (GE Healthcare). For biotinylation, an AviTag sequence (Avidine, Aurora, CO) was added to the C terminus of the sDM α-chain (27), and biotinylation was performed according to the manufacturer’s instructions. Human mAb b12 and mAb b6 (both IgG1-κ, anti-HIV-1 gp120) were obtained from Dr. Dennis Burton (Scripps Research Institute, La Jolla, CA) (28). mAbs b12 and b6 were expressed and purified as previously described (29). Human b12 Fab was generated using an Fab preparation kit (Thermo Fisher Scientific). Human IgG Fab (Jackson ImmunoResearch Laboratories) was also used. The following reagents were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health): anti-human β2-microglobulin, anti-DM dimer mouse monoclonal MaP.DM1, anti-human Ig G1κ, and anti-HIV-1 V3 IgG3-mAb (447-52D), no. 4475 from Dr. Susan Zolla-Pazner; anti-HIV-1 V3 IgG3-mAb (447-52D), no. 4030 from Dr. Susan Zolla-Pazner; anti-HIV-1 gp11 IgG1κ mAb (2F5), no. 1475 from Dr. Hermann Katinger; HIV-1 b12, gp120, no. 4961, expression vector constructed by Dr. Marvin Reitz; anti-HIV-1 gp141 IgG1κ mAb (4E10), no. 10091 from Dr. Hermann Katinger; HIV-1 gp141 IgG1κ mAb (F240), no. 7623 from Dr. Marshall Posner and Lisa Cavacini; HIV-1 gp11 MN (Escherichia coli), no. 12027; HIV-1 UG21 gp140, no. 12065 from Polynum Scientific; gp120, gp140, and gp41 were biotinylated using EZ-Link NHS-PEG4-biotin (Thermo Scientific) per the manufacturer’s instructions. For control proteins, human mAb DPA (IgG1-λ) was purified from supernatant of DPA cells. Purified pigeon cytochrome c, hen egg lysozyme (HEL), and BSA were purchased from Sigma-Aldrich.

Immunoprecipitation, endoglycosidase H digestion, and Western blot of cellular proteins

Cells (20 × 10⁶/ml) were lysed at 4˚C in buffer containing 5 mM MgCl₂, 150 mM NaCl, 1% CHAPS, and either 50 mM Tris (pH 7.2) or 50 mM MES (pH 6.5). Protein concentration in clarified lysates was measured by Bradford assay. To precipitate endogenous Ig, protein G-Sepharose (PSG; 4 Fast Flow; GE Healthcare), protein L–agarose (Thermo Fisher Scientific), or native protein A-Sepharose (PS; 4 Fast Flow; GE Healthcare) was incubated overnight with lysates titrated to provide comparable levels of relevant proteins for a given experiment. Washed precipitates were suspended in reducing or nonreducing sample buffer (Bio-Rad, Hercules, CA), incubated at 70–95˚C, and analyzed on mini-protein TGX gels (Bio-Rad). Proteins were transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA), which were blocked overnight, incubated with relevant Abs, washed (PBS/0.1% Tween 20), and incubated with HRP-conjugated goat F(ab)2’ (SouthernBiotech) for 0 or 3 h at 37˚C. Cells were lysed (10 × 10⁶ cells/0.25 ml) in 1% CHAPS at pH 7.2. Aliquots of lysates were incubated with PAS overnight. Precipitates were denatured in reducing sample buffer, analyzed by SDS-PAGE, and Western blotted as described above. Densitometry of selected Western blots was performed with Image J (http://rsbweb.nih.gov/ij/). The mean DM/Ig ratio normalized to noncrosslinked cells (set as 1.0) was calculated. A one-sample t test, using Prism 6 software (GraphPad Software, La Jolla, CA), was used to assess whether the mean DM/Ig ratio (in the presence of Ig crosslinker) statistically differed from 1.0.

Immunoprecipitation of purified proteins

To form Ig/resin complexes, polyvalent IgG, Fc, or mAb b12 was incubated with PSG 4 Fast Flow in reaction buffer for ≥2 h at 4˚C. Fab was incubated with protein L–agarose (Thermo Fisher Scientific). Polyvalent Ig or Fc reaction buffer contained 50 mM Tris (pH 7.2), 150 mM NaCl, and 1% CHAPS. mAb reaction buffer contained 50 mM MES (pH 6.0) and 150 mM NaCl. After washing, the resin was resuspended in reaction buffer containing 0.1–0.5% BSA and SDS and incubated overnight at 4˚C. Washed beads were suspended in reducing or nonreducing sample buffer and heated at ≥80˚C for 10 min. Proteins were analyzed by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and Western blotted, as described above.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde for 20 min at 37˚C and permeabilized with 1% Nonidet P-40 for 30 min at room temperature (RT). Cells were centrifuged onto microscope slides and slides were mounted in a Shandon Sequenza immunostaining rack (Thermo Fisher Scientific). Slides were blocked in buffer (PBS, 10% goat serum, 1% octylglucoside) for 30 min at 37˚C, followed by incubation with primary Ab in block buffer. Washed (PBS/1% octylglucoside) slides were incubated with fluorescently labeled secondary Ab for 1 h at 37˚C. Washed (PBS/1% octylglucoside) slides were air dried in the dark, and coverslips were mounted using mounting media with DAPI (Olink Bioscience, Uppsala, Sweden).

Proximity ligation assays

Abs were titrated for standard immunofluorescent stains; relevant immunofluorescent image are shown in Supplemental Fig. 1A and 1D. To prepare PLA probes, Abs were dialyzed against PBS, concentrated using Amicon Ultra centrifugal filters (EMD Millipore), and labeled directly using the Probesemaker kit (Olink Bioscience), per the manufacturers’ instructions. Cells were treated and slides prepared as for
immunofluorescent staining and then incubated with PLA probes and detection reagents, according to the manufacturers’ instructions. Coverslips were mounted with Duolink mounting media with DAPI. To colocalize PLA spots with Lamp-1 and EEA1 staining, PLA was performed followed by overnight incubation with anti-Lamp1 or anti-EEA1 Ab, diluted in blocking buffer. Washed slides were incubated with goat anti-rabbit Alexa Fluor 488, washed, and coverslips were mounted.

PLA image analysis

For PLA, images in a single z-plane were acquired using an Olympus FV1000 confocal microscope with a ×60 water immersion objective. For each sample, four images were acquired, and the number of spots/cell was calculated using CellProfiler (http://www.cellprofiler.org) (30). Only cell-associated spots were counted. For each experiment, data were analyzed using one-way ANOVA with a Bonferroni multiple comparison test using Prism 6 software. For aggregate data, specific spots (not from DM/Ab stain = background no. from DM stain alone) were calculated from replicate PLA assays of DM+, IgG1-λ′ cells, and IgG1-λ′ cells. Mean values were compared by a two-sample t test using Prism 6 software. For localization of PLA spots, colocalization of red spots (PLA) with green (Lamp-1 or EEA1) was assessed using Velocity software.

Octet

All experiments were performed using an Octet QK (Pall, Port Washington, NY) (31) at 1000 rpm in the indicated buffers containing 0.002% Tween 20 and 0.1 mg/ml BSA. BSA was omitted from the running buffer in experiments to assay for BSA/mAb interaction. Stock solutions of sDM, b12 Fab, mAb b12, and polyclonal Fab were purified with a Superdex 200 10/300 GL gel filtration column (GE Healthcare).

For sDM binding to Ag-bound Ab, the following buffers were used: 0.1 M MES (pH 6.0), 100 mM NaCl; for the gp120/b12/sDM experiment, and 50 mM MES (pH 6.5), 100 mM NaCl for the gp41/2F5/sDM experiment. Experiments were performed at 25°C. mAb was immobilized on protein G-coated sensors to a signal magnitude of ∼5.0 nm shift. Sensors were subsequently incubated with 500 nM gp120 Ag, 500 nM gp41 Ag, 500 nM lysozyme, or assay buffer alone until signals plateaued. To monitor DM association, 4.8 µM sDM was added to these reaction wells. To monitor protein dissociation, sensors were advanced to wells containing assay buffer. Plotted data reflect the net signal above reactions without sDM. For sDM versus BSA, experiments were performed in 50 mM MES (pH 6.0) and 100 mM NaCl at 30°C. mAb was immobilized on protein G-coated sensors to a signal magnitude of ∼4.5 nm shift. After 30 min incubation in assay buffer, sensors were incubated in solutions of sDM or BSA to monitor association and then advanced to assay buffer alone to monitor protein dissociation. For pH comparison, experiments were performed at 30°C using streptavidin-coated sensors. sDM-biotin was immobilized to a signal magnitude of ∼2.3 nm shift in PBS. Sensors were subsequently incubated in buffers of varying pH (4.7–7.2) for ≥25 min. Buffers contained 50 mM NaCl, 20 mM buffer (pH 4.7 and 5.2 for acetate buffer; pH 5.7, 6.2, and 6.7 for MES buffer; and pH 7.2 for sodium phosphate buffer). After incubation, 3 µl of sDM-FV1000 GL gel filtration column (GE Healthcare). Stock solutions of sDM and b12 Fab were pipetted into the reaction wells without advancing the sensor position. The resulting signal was compared with a reference reaction with sDM-biotin, but not b12 Fab. The equilibrium binding magnitude was defined as the net signal after both reactions had plateaued. Additional control reactions, with b12 Fab and no DM-biotin, gave negligible signals (not shown). For NaCl titration, experiments were performed at 25°C using streptavidin-coated sensors. sDM-biotin was immobilized to a signal magnitude of ∼6.0 nm shift in 20 mM sodium acetate buffer (pH 4.7), 100 mM NaCl. Sensors were subsequently incubated in buffers with varying NaCl concentrations (10, 40, and 150 mM) and 20 mM MES (pH 5.7) for 50 min. After incubation, 5 µl polyclonal Fab was pipetted into the reaction wells without advancing the sensor position. Sensors were incubated in buffer without Fab to monitor complex dissociation. The signal fluctuations during the association and dissociation phases were compared against a reference reaction of sDM-biotin, but not Fab. The net signal at each time point was plotted.

Biacore: sDM interaction with b12 Fab

Experiments with sDM immobilized were performed at 25°C using a Biacore 3000 (GE Healthcare). Experiments with Fab immobilized were performed using a Biacore T200 (GE Healthcare). Stock solutions of sDM and b12 Fab were monodisperse, after purification on a Superdex 200 10/300 GL gel filtration column (GE Healthcare). sDM-biotin was immobilized to ∼1000 resonance units (RUs) onto a streptavidin-coated sensor (GE Healthcare) by injection of ∼10 nM at 10 µl/min in HEPES-buffered saline with surfactant P20. b12 Fab was immobilized to ∼4000 RU by amine chemistry onto a CM5 sensor (GE Healthcare), injecting at 5 µl/min, 2.5 µg/ml in 10 mM sodium acetate buffer (pH 5). sDMFab interaction was measured in 20 mM MES (pH 5.7 or 6.7), 100 mM NaCl, and 0.002% p20. Where indicated, NaCl was elevated to 150 mM. Varying

FIGURE 1. DM coprecipitates with Ig in human B cell lysates. (A) Lysates of DM+IgG+ B cells (DHL-4), DM+Ig+ tonsil B cells, DM+Ig- macrophage-like cells (KG-1), or BSA in lysate buffer were incubated with PGS. Lysates or precipitated proteins, as indicated, were separated by SDS-PAGE and Western blotted for Ig and DM. Lysates were titrated so the amount of DM was comparable between cell lines for lysate analysis and coprecipitation experiments. (B) Western blots for Ig and DM of lysates or PGS-precipitated protein, as indicated, were stained with anti-IgM, anti-DCs or anti-DM+ B cells (DHL-4). (C) Western blots of DM+ IgM− DCs or DM+ B cells (DHL-4). (D) Western blots of DM+ IgM− DCs or DM+ B cells (DHL-4). (E) Western blots for Ig and DM of lysates or PGS-precipitated protein from DM+IgG1-κ′ B cells (DPA), DM+IgGl-λ′ B cells (DPA), or DM+ IgM− macrophage-like cells (KG-1). (F) Western blot of lysates or PGS-precipitated protein from DM+IgGl-κ′ B cells (DPA), DM+IgGl-λ′ B cells (DPA), or DM+ IgM− macrophage-like cells (KG-1). (G) Western blot of lysates or PGS-precipitated protein from DM+IgGl-κ′ B cells (DPA), DM+IgGl-λ′ B cells (DPA), or DM+ IgM− macrophage-like cells (KG-1). (H) Western blot of lysates or PGS-precipitated protein from DM+IgG1-κ′ B cells (DPA), DM+IgGl-λ′ B cells (DPA), or DM+ IgM− macrophage-like cells (KG-1). Data are representative of two to four experiments. Gels in (A) and (D) were run under reducing conditions, under which IgH (50 kDa) migrates separately from the Ig L chain (25 kDa). Gels in (B), (C), and (E) were run under nonreducing conditions, under which Ig migrates with 150- or 180-kDa markers.
concentrations of sDM, b12 Fab, or 40 or μM Fe were injected (20 μl/min) across a reference flow cell and the flow cell with immobilized ligand. To determine the apparent Kd, the reference signal was subtracted at each time point, and equilibrium data were fit to the “one site–specific” mass action model using Prism 6 software.

Detection of free Ag by Octet (Ag-capture assay)
mAb (20 μg/ml) was incubated for 2 h at 37°C in reaction buffer (50 mM MES [pH 6.0 or 6.5] or 50 mM acetate buffer [pH 4.7], 100 mM NaCl) in a black, high-binding microtiter plate (Greiner Bio-One, Frickenhausen, Germany). The plate was washed with reaction buffer, incubated (30 min) with blocking buffer (reaction buffer plus 0.25 mg/ml BSA, 0.05% Tween 20), washed, and incubated with 100 nM biotinylated gp120, 110 nM biotinylated gp140, or 440–500 nM biotinylated gp41 for ≥1.75 h at RT. Unbound Ag was removed, and reaction buffer solutions containing 300–1000 nM sDM, 40 nM unbiotinylated gp120, 700 nM unbiotinylated gp41, or control proteins were added. The plate was immediately transferred to an Octet QK instrument. During the run, streptavidin sensors (prehydrated in reaction buffer plus 0.1 mg/ml BSA, 0.002% Tween 20 for ≥15 min) were submerged into the reaction wells to detect biotinylated Ag in solution. Measurements occurred at 1,000 rpm, 37°C, for 15,000–16,000 s. In some experiments, the sensor surface then was probed for biotinylated gp120 (gp120-b) by submerging the sensors in 35 nM mAb b12 for 2000 s.

Detection of free Ag by ELISA
gp120-b/mAb b12 complexes were formed at pH 6.0 as described in the Ag-capture protocol, except 50 nM biotinylated gp120 was used. After 3 h incubation with sDM, HEL, unbiotinylated gp120, or buffer at 37°C, solutions were transferred to a Reacti-Bind streptavidin-coated microtiter plate (Thermo Fisher Scientific) and incubated 2 h at RT with orbital shaking. Reaction wells were washed three times (PBS, 0.1% BSA, 0.05% Tween 20), incubated 1 h with 0.4 μg/ml mAb b12 diluted in wash buffer, washed, incubated 1 h with anti-human Ig HRP-conjugated goat Ab diluted 1:15,000 in wash buffer, washed, and developed with tetramethylbenzidine substrate (BD Biosciences, San Jose, CA). The reaction was stopped with 1 M sulfuric acid. Absorbance at 570 nm was subtracted from thylbenzidine substrate (BD Biosciences, San Jose, CA). The reaction was washed, incubated 1 h with anti-human Ig HRP-conjugated goat Ab, and absorbance at 450 nm measured. Mean values were compared using a ratio-paired t test, using Prism 6 software.

Results

DM coprecipitates with Ig in human B cell lysates

To determine whether DM interacts with Ig in B cells, we used PGS or PAS to directly precipitate Ig from lysates of tonsillar B cells (DM+, IgM+, and IgG+ cells) and from lysates of DM+ B cell lines expressing various Ig isotypes (IgG-κ [DHL-4], IgG1-κ [DPD], IgG1-κ [DPD], IgM-κ [OCI-LY8]; Fig. 1A–D). Immunoblotting showed that DM coprecipitated with Ig in all B cells tested, whereas no DM was detected using the same procedure with DM-expressing cells that lack Ig (e.g., macrophages [KG-1] or monocyte-derived DCs; Fig. 1A–D). Thus, DM/Ig interaction occurs with various Ig isotypes and both L chains. Neither DO nor MHC-I coprecipitated with IgG (Fig. 1E), indicating specificity of the DM/Ig coprecipitation and implying that free DM participates in this interaction.

DM and Ig are within interacting proximity in intact B cells

To investigate the DM/Ig interaction in intact cells, we performed PLA with directly labeled Abs, which detect and quantify in situ complexes of two interacting (within 28 nm) molecules (32–34) (see Materials and Methods). Unlike conventional fluorescence resonance energy transfer analysis, PLA detects interactions in untransfected cells with physiologic protein levels and does not rely on cytoplasmic tags, which may become dissociated from luminal domains by proteolysis (14). To establish PLA, we stained DM+, DR+ B cells (DPA) with heterodimer-specific Abs recognizing DM or DR proteins (Supplemental Fig. 1A). We detected DM/DR interaction (Supplemental Fig. 1B, 1C), known to occur in late endosomes (35). PLA of DM/DR in DM+, DR− (9.22.3) B cells showed negligible background signal, as expected (Supplemental Fig. 1B, 1C). To measure DM/Ig complexes, we used the anti-DM Ab with either anti-λ L chain (Supplemental Fig. 1D, 1G–I) or anti-κ L chain, in appropriate cells. PLA spots in DM+, IgG1-λ B cells (DPA) or in DM+, IgG1-κ B cells (DPD) assayed with appropriate Ab combinations, respectively, were detected at significant levels over background; background levels were measured using probes to DM and the alternate (nonexpressed) L chain together or using single probes (Figs. 2A–C). Fig. 2 shows representative cell images (Fig. 2A), quantitation of a representative experiment (Fig. 2B), and aggregate data from multiple experiments quantitating DM interaction with IgG1-λ in B cells (DPA) compared with λ− cells (Fig. 2C). In control

![FIGURE 2](http://www.jimmunol.org/)
reactions, PLA between DM (endosomal) and Ki67 (nuclear) proteins was negligible in DM+/IgG1− B cells (DPA) (Supplemental Fig. 1E, 1F). Taken together, these data argue that DM and Ig proteins are in close proximity in B cells, consistent with their being in complexes.

Endosomal localization of DM/Ig complexes in B cells

To localize DM/Ig complexes at steady-state, we analyzed the overlap of PLA spots with markers of endosomal compartments: anti-EEA1, early endosome marker, or anti-Lamp-1, late endosome/lysosome marker, was detected by immunofluorescence (Fig. 3A). Using Velocity software, we observed colocalization of PLA spots with EEA1 stain and with Lamp-1 stain, indicating the presence of DM/Ig complexes in both early and late endocytic compartments. Consistent with these results, PGS-precipitated complexes from two B cell lines (DPA, DHL-4) contain mature Ig molecules, with endo H–resistant N-linked glycans and mature DM molecules (Fig. 3B), with one of two DMα N-glycans being endo H–resistant, as expected (36). All detectable Ig in the precipitate is endo H–resistant, indicating that, in the lines tested, Ig is primarily located after Golgi at steady-state. The assay is not sensitive enough to detect the low levels of nascent Ig, so whether Ig initially associates with DM in the ER cannot be determined by this approach. BCR crosslinking drives the internalization of BCRs (Ig/Igα/Igβ) into endosomes (37). Treating tonsillar B cells with crosslinking Ab resulted in an increased ratio of DM/Ig in PAS precipitates (Fig. 3C, 3D). These data argue that DM/Ig complexes can form after ligand-triggered endocytosis of Ig.

Soluble DM directly interacts with the Ig Fab domain at endosomal pH

Detection of DM/Ig complexes led us to ask whether Ig and DM interact directly. We incubated an IgG1−k human mAb, mAb b12 (28), or purified, polycrystal human IgG with sDM. sDM precipitates with Ig (Fig. 4A), indicating direct DM/Ig binding and showing that transmembrane regions are not required for this interaction. To begin to map the DM binding site on Ig, we assayed sDM binding to purified polycrystal IgG Fab domains or Fc domains. Fab, but not Fc, domains coprecipitate with sDM (Fig. 4A). To determine whether this interaction is pH sensitive, we examined binding in real time using biolayer interferometry (Octet). We immobilized sDM on Octet sensors via DMα C-terminal biotin (27) and incubated the sensors with mAb b12 Fab at varying pH (4.7–7.2). The Fab/DM interaction rapidly achieved equilibrium, allowing comparison of equilibrium signal magnitude at each pH (Supplemental Fig. 2).
To examine the effect of bound Ag on DM/Ig interaction, mAb b12 or mAb 2F5, immobilized on Octet sensors, was incubated with saturating concentrations of Ag (gp120 or gp41, respectively, \(K_D\) of low nanomolar concentration) or HEL as a control and then with sDM. sDM interacted with Ag-free mAb but not with Ag-bound mAb (Fig. 5). This could reflect steric inhibition of DM binding by bound Ag or inhibition via an allosteric mechanism after Ag-induced conformational change. The slow association and dissociation kinetics of DM and Ag-free Ig, as opposed to the fast kinetics we observe on the Biacore (Fig. 4B, 4C), may reflect extensive re-binding of dissociated sDM to immobilized Ig or may reflect more stable interaction between sDM and intact Ig compared with Fab.

To further explore the competition between sDM and Ag for Ig interaction, we developed a sensitive, Octet-based assay to visualize competition in real time (Fig. 6A). Plate-bound complexes of gp120-b and mAb b12 were incubated with or without sDM, and the amount of released gp120-b was compared. Incubation with DM significantly increased signal intensity at pH 6.0 (Fig. 6B, top), Supplemental Fig. 3A, left). The signal reflected free gp120-b, not plate-desorbed gp120-b/mAb b12 complexes, as the mAb b12 epitope on gp120-b was accessible at the sensor tip (Fig. 6B, bottom, Supplemental Fig. 3A, right). The level of free gp120-b was significantly above the dissociation in buffer alone. Limited amounts of free gp120-b were detected with HEL, cytochrome c, IgG, and Fc as competitors (Supplemental Fig. 3A). BSA increased the free gp120-b signal to some degree, but no BSA/mAb b12 interaction was detected under the same conditions that generate sDM/mAb b12 complexes (Supplemental Fig. 3B), suggesting a nonspecific effect of BSA on immune complexes. sDM interacted effectively with gp120-b/mAb b12 Fab complexes (Supplemental Fig. 3C) and with gp120-b/mAb b12 complexes at pH 4.7 (Supplemental Fig. 3D), despite this being a pH where the measured binding affinity of sDM/b12 Fab is low (Supplemental Fig. 2). sDM increased levels of unbound gp120-b from complexes formed with mAb b6 or mAb 447-52D (Fig. 6C and 6D, respectively) and increased levels of another Ag, gp140-b, from complexes with mAb 126-7 (Fig. 6E). No effect was observed after incubation of DM with gp41-b/mAb 2F5 under similar
conditions, likely due to lack of dissociation of Ag (see below and Supplemental Fig. 3E). The DM effects on abundance of free Ag were not specific to the detection system, as we detected free gp120-b by ELISA with sDM, but not with HEL (Fig. 7).

To further characterize sDM effects on Ag/mAb complexes, we compared incubation with unbiotinylated gp120 to incubation with sDM. gp120-b accumulation on the sensor was 2-fold higher in the presence of excess gp120 compared with sDM (Supplemental Fig. 3F, 3G). Excess unbiotinylated Ag inhibits Ag/biotin rebinding to the mAb layer, and the corresponding curves define the fastest possible detection of free Ag/biotin for an uncatalyzed process. To determine whether sDM increases the dissociation rate \( k_{off} \) of Ag, we incubated gp120-b/mAb b12 complexes with unbiotinylated gp120 plus sDM or with unbiotinylated gp120 alone. sDM did not increase the apparent dissociation rate of gp120-b from mAb b12 or mAb b6 (Supplemental Fig. 3F, 3G), indicating that sDM likely inhibits rebinding of Ag, but does not act as an Ag release catalyst in this in vitro system.

**Discussion**

The novel finding of this work is that DM interacts with Ig. Using purified molecules, we observe direct interaction of sDM with polyclonal IgG and with several different monoclonal IgGs. By coprecipitation, diverse Ig species interact with DM, including different Ig isotypes (IgG, IgM) and Ig with either L chain. The fact that the Fab portion of IgG is critical to the interaction with DM also supports the idea that multiple isotypes likely engage with DM. To date, we have not found a class of Ab that is resistant to DM interaction. Rather, our evidence suggests that tight association with Ag inhibits DM binding. We also observed DM/Ig interaction in activated B cell lines and primary B cells and saw enhanced DM/Ig association after BCR crosslinking. The implication is that this interaction will be of relevance across the range of mature B cell differentiation states.

![FIGURE 5](image)

Recombinant sDM interacts with Ig but does not bind to Ig/Ag. Biolayer interferometry experiments were performed using an Octet QK with an orbital flow rate of 1000 rpm, as described in Materials and Methods. sDM binding to Ag-bound or Ag-free mAb b12, pH 6 (top) or mAb 2F5, pH 6.5 (bottom) is shown. Association of sDM was measured to indicated sensor-immobilized Abs after incubation with cognate Ag or HEL. Sensors were advanced to buffer alone to measure dissociation. Data are representative of two experiments.

![FIGURE 6](image)

Recombinant sDM competes with the Ag/Ig interaction. (A) Schematic of assay used in (B)–(E) (see Materials and Methods for detail). In (B)–(E), all reaction conditions within a plot were monitored simultaneously, each with an individual sensor. The indicated Ab, but not others in the same experiment, recognizes the Ag. (B, top) Sensor capture of free gp120-b in real time after incubation of sDM or buffer with plate-immobilized gp120/mAb b12 complexes or control reactions at pH 6.0. (B, bottom) Selected sensors incubated with mAb b12 to probe for sensor-immobilized gp120-b. (C–E) Experiments as described for (B), with indicated reactants, at pH 6.0 (C and D) or 6.5 (E). Data are representative of two to five experiments.

Our results provide some clues as to the topology of interaction between Ig and DM. The Fab portion of Ig is involved in binding to DM, and the Fc portion, if involved at all, likely contributes limited binding energy and only in context of Ig as a whole. The salt sensitivity of the interaction (Supplemental Fig. 4), together with the basic pl of IgG (38), implicates an acidic patch on DM as a binding surface. One acidic region of DM is involved in its interaction with DR and with DO; these two DM ligands bind the
same site on DM, based on mutagenesis and crystallography (9, 10, 27). It is possible that this region participates in the DM/Ig interaction, as protein/protein interaction faces are often reused. Alternatively, acidic patches on faces of DM distinct from the DR binding site (9) may mediate interactions with Ig. The binding of DM to Fab domains, despite their structural diversity, argues that DM primarily interacts with the framework and/or constant regions of Fab. Given the relative sizes of DM and Ig, we speculate that DM cannot “reach” the Fab domain of Ig when both DM and Ig are tethered to the same membrane plane. Instead, we speculate that DM and Ig/Fab interact across opposing membranes, which could be accommodated by the membrane structure of multivesicular bodies. Classical MHC family proteins MHC-II and MHC-I interact with Ig superfamily members on opposing membranes, such as CD4 and CD8, respectively. The neonatal Fc receptor, a nonclassical MHC molecule with structural homology to DM, including lack of a peptide binding groove (39), binds IgG in a pH-dependent manner (40).

The measured affinity of the interaction between soluble Fab and recombinant sDM by SPR is low. These in vitro results indicate weak binding, in apparent contrast to the stability of complexes of recombinant sDM by SPR is low. These in vitro results indicate weak binding, in apparent contrast to the stability of complexes of recombinant sDM by SPR. The measured affinity of the interaction between soluble Fab and recombinant sDM by SPR is low. These in vitro results indicate weak binding, in apparent contrast to the stability of complexes of recombinant sDM by SPR (40). The measured affinity of the interaction between soluble Fab and recombinant sDM by SPR is low. These in vitro results indicate weak binding, in apparent contrast to the stability of complexes of recombinant sDM by SPR (40).

A concerted set of changes that enhance Ag presentation occurs in a B cell upon Ag binding to its BCR, regardless of isotype (53). BCR ligation by Ag initiates intracellular signaling, leading to enhanced levels of free Ag in cells.

The hypothesis that DM effects on immune complexes are of particular importance for high-affinity Abs is compatible with several aspects of B cell selection in the GC reaction. The GC reaction is aimed at preferential expansion of clones expressing high-affinity Abs. GC B cells bearing high-affinity Ig receptors are first selected by follicular DCs, but they must then efficiently generate and present MHC-II/peptide ligands to follicular Th cells to complete GC formation where B cell expansion and Ig affinity maturation take place (47). A mechanism to increase free Ag from high-affinity Ig would be predicted to increase the number of MHC-II peptide complexes. Additionally, inhibition of rebinding would allow more extensive Ag proteolysis, producing a wider repertoire of potential T cell ligands (44, 48) and greater opportunities for T cell help. Notably, DO is downregulated in GC B cells (49), yielding more free DM, which our coprecipitation data suggest is the form that interacts with Ig.

Our in vitro functional assay did not detect significant DM enhancement of Ag off-rate from Ig. It is possible that this was missed because the interaction between the Octet sensor and free Ag was rate limiting. We do not yet know whether DM physically blocks Ag rebinding or stabilizes a confirmation of Ig that prevents rebinding. However, the second scenario has parallels to the mechanism by which DM facilitates peptide exchange on HLA-DR: it blocks rebinding of partially dissociated peptide to DR by stabilizing a conformation in which the P1 pocket is occupied by MHC-II residues, promoting full dissociation of prebound peptide (9, 50, 51). This process, in which a subset of attachment sites for ligand (peptide for MHC, Ag for Ig) becomes exposed during partial complex dissociation and can be engaged by another molecule (DM) to accelerate the intrinsic dissociation rate, has been termed “facilitated dissociation” (52).

A role for DM in facilitating Ag handoff from Ig to MHC-II was postulated by Moss et al. (42), who showed that exposure of fixed, Ag-coated B cells to protease resulted in stimulation of cognate T cells, and the T cell response was abrogated by Abs to surface DM. This study, however, could not differentiate DM activity as a chaperone/exchange catalyst for MHC-II from its possible action on Ig. Another published report that bears on our findings concerns Ig-mediated Ag presentation in DO knockout mice, which have unopposed DM function (46). When the T cell stimulation capacities of splenic B cells were compared, wild-type and DO-deficient B cells differed after BCR uptake of Ag. Increased presentation of Ag by DO-deficient B cells was observed at lower doses of Ag and for epitopes derived from Ag that remained stably bound to Ab down to pH 4; these two conditions may require DM/Ig interaction for efficient Ag release and presentation.

A concerted set of changes that enhance Ag presentation occurs in a B cell upon Ag binding to its BCR, regardless of isotype (53). BCR ligation by Ag initiates intracellular signaling, leading to
reorganization of the B cell endocytic pathway and cytoskeleton. These changes allow convergence of incoming Ig/Ig complexes with MHC-II and the Ag processing and loading machinery, apparently through the de novo biosynthesis of an acidified MIC peptide loading compartment (54). BCR-mediated B cell activation also causes changes in protein expression important for Ag presentation, including increased expression of MHC-II (55) and costimulatory molecules (CD80/86, CD40). Expression of DM is reduced, but more significant reduction of DO increases the abundance of free DM (49, 56). In addition to increased trafficking of Ig to late endosomal compartments, reduction in DO may contribute to the enhanced interaction of DM and Ig that we observe after crossinglink of surface Ig. In turn, this enhanced DM/Ig interaction may facilitate MHC-II loading with Ag.

We have discovered that DM and Ig interact in B cells. In addition to a potential role in B cell Ag presentation, the DM/Ig interaction may also be of relevance in macrophages and DCs, which also express DM and internalize Ag/Ig complexes via Fc receptors.

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Disclosures

The authors have no financial conflicts of interest.

References

**Figure S1. Immunofluorescent stains and PLA analyses.** (A) Representative immunofluorescent stains on DM+/DR+ (DPA), DR- (9.22.3) DM-/DR- (BDC2.5) cells, with antibodies [anti-DM (Map.DM1), anti-DR (L243)] used for generating PLA probes. (B) Representative single cell images of DM+/DR+ (DPA) and DM+/DR- (9.22.3) B-LCL cells stained with anti-DM and anti-DR PLA probes, either individually or together, as indicated. PLA spots (red); DAPI-stained nuclei (blue). (C) Quantification of PLA spots from 40-60 cells per staining condition. Data are from one of two experiments with similar results. Mean spots/cell ± SD is shown; statistically significant differences are indicated. Inset: Representative single cell image showing phase contrast overlaid with PLA spots and nuclear stain. (D) Representative immunofluorescent stains on positive DM+/IgG-λ+ B cells (DPA) and negative control, DM+/ IgG-κ+ (DHL) cells and peripheral blood mononuclear cells, (PBMC) with antibodies [anti-lambda light chain (λ), anti-Ki67] used for generating PLA probes. (E) Representative single cell PLA images of DPA cells and unstimulated PBMC stained with anti-DM, anti-λ, anti-Ki67 probes, either individually or as pairs, as indicated. (F) Quantification of PLA spots, as described in (C). Data are from one of two experiments with similar results. (G) Representative single cell images of DM+/IgGλ+ (DPA) and DM+/IgGκ+ (DHL) cells, stained with anti-DM and anti-λ. PLA probes, either individually or together, as indicated. (H) Quantification of PLA spots, as described in (C). (I) Representative cell images of DM+/IgG-λ+ B cells (DPA) stained with anti-DM and anti-λ. PLA probes, either individually or together, as indicated. PLA spots (red); DAPI-stained nuclei (blue).
Figure S2. sDM/b12 Fab interaction at varying pH. *Left:* Representative Octet trace of b12 Fab binding to sensor-immobilized sDM-biotin, at pH 5.7. *Right:* Summary of binding data from various pH conditions. Equilibrium binding magnitudes at indicated pH were ranked: (+++) highest signal (+) some signal (-) zero signal. Data are representative of two experiments.
Figure S3. Control reactions. (A, left) Experimental design follows Fig. 6 in main text. Sensor capture of free gp120-b in real time after incubation of sDM or indicated control proteins [BSA, HEL (hen egg lysozyme), polyclonal IgG, Fc, or CytC] with plate-immobilized gp120/mAb b12 complexes at pH 6.0. (A, right) Selected sensors from panel A, incubated with mAb b12, to probe for sensor-immobilized gp120-b. Data are representative of two experiments. (B) Association profile of sDM/mAb b12 compared to BSA/mAb b12 at pH 6.0. Similar data were obtained with mAb 2F5 (not shown). (C, D) Experiments as described for (A), with indicated reactants, at pH 6.0 (panel C) or pH 4.7 (panel D). Data are representative of two experiments. * Curve heights reflect nonspecific binding between sensor and sDM. (E-G) Experimental design follows Fig. 6 in main text. (E) Incubation of indicated amounts of unbiotinylated gp41 or sDM with plate-bound gp41-b/mAb 2F5 complexes. Data are representative of two experiments. (F) Sensor capture of free gp120-b over time during incubation of indicated amounts of unbiotinylated gp120 +/- sDM and other indicated control reactants with plate-bound gp120-b/mAb b12 complexes. (G) As in panel F, except with plate-bound gp120-b/mAb b6 complexes.
Figure S4. NaCl sensitivity of sDM/Fab interaction.

(A) Association and dissociation profiles for the polyclonal Fab/sDM interaction at pH 5.7 at varying NaCl concentration were measured using the Octet. Data are from one of two experiments with similar results. (B) Non-specific binding between Octet streptavidin sensor and polyclonal Fab. Average signal intensity in the final 16 s of the association phase is shown. (C) Association and dissociation profiles for the b12 Fab/sDM interaction at pH 5.7 at varying sDM concentration were measured using a Biacore T200. Measurements were performed at 150 mM NaCl. The indicated concentration of sDM was injected (20 µl/min) across a reference flow cell and the flow cell containing immobilized b12 Fab. Complex dissociation was measured by injection of buffer.