Distinct MHC Class II Molecules Are Associated on the Dendritic Cell Surface in Cholesterol-dependent Membrane Microdomains*§

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Very small amounts of MHC class II-peptide complexes expressed on the surface of antigen-presenting cells (APCs) are capable of stimulating antigen-specific CD4 T cells. There is intense interest to elucidate the molecular mechanisms by which these small amounts of MHC-II can cluster, cross-link T cell receptors, and promote T cell proliferation. We now demonstrate that a significant fraction of the total pool of MHC-II molecules on the surface of dendritic cells is physically associated in macromolecular aggregates. These MHC-II/MHC-II interactions have been probed by co-immunoprecipitation analysis of the MHC-II I-A molecule with the related I-E molecule. These molecular associations are maintained in gentle detergents but are disrupted in harsh detergents such as Triton X-100. MHC-II I-A/I-E interactions are disrupted when plasma membrane cholesterol is extracted using methyl β-cyclodextrin, suggesting that lipid raft microdomains are important mediators of these MHC-II interactions. Although it has been proposed that tetraspan proteins regulate molecular clustering, aggregation, and co-immunoprecipitation in APCs, genetic deletion of the tetraspan family members CD9 or CD81 had no effect on MHC-II I-A/I-E binding. These data demonstrate that the presence of distinct forms of MHC-II with plasma membrane lipid rafts is required for MHC-II aggregation in APCs and provides a molecular mechanism allowing dendritic cells expressing small amounts of MHC-II-peptide complexes to cross-link and stimulate CD4 T cells.

MHC class II molecules expressed on the surface of specialized antigen presenting cells (APCs) function by presenting peptides derived from foreign protein antigens to specific CD4 T cells, a process that initiates and propagates immune responses (1). The interaction of MHC-II-peptide complexes on APCs with T cell receptors for antigen (TCR) on T cells cross-links the TCR, thereby initiating a signaling cascade that leads to cytokine production and T cell proliferation. It is well known that ligation of less than 500 MHC-II-peptide complexes is sufficient for T cell activation (2, 3), and therefore it is widely believed that APCs possess a mechanism to cluster MHC-II on the cell surface. Immunologically relevant MHC-II-peptide complexes concentrate at the immunological synapse formed between CD4 T cells and APCs, thereby enhancing the local concentration of these MHC-II-peptide complexes and increasing the probability of TCR cross-linking (4). Curiously, we found that immunologically irrelevant MHC-II molecules are also recruited to the immunological synapse (4), demonstrating that either relevant MHC-II-peptide complex engagement “signals” MHC-II movement to the synapse or that distinct MHC-II molecules are constitutively associated prior to engagement with antigen-specific T cells. Indeed, a variety of microscopic studies has revealed that MHC-II is not uniformly expressed but rather is organized in small molecular patches on the surface of APCs (5, 6).

Eukaryotic cells possess a variety of distinct types of membrane microdomains that could potentially restrict protein lateral mobility in the plasma membrane and lead to protein clustering. One highly studied type of lipid-based membrane domain has been termed lipid rafts (7, 8). Lipid rafts are dynamic membrane microdomains enriched in cholesterol, glycosphingolipids, and GPI-linked proteins (8–10). Association of proteins with cholesterol-rich raft microdomains facilitates signal transduction, protein transport, and membrane fusion, and it has been proposed that these microdomains serve as platforms (or rafts), thereby concentrating the molecules involved in these diverse processes (11–14). MHC-II has also been shown to associate with cholesterol-dependent lipid raft microdomains (15–18), and lipid raft association is important for MHC-II-dependent activation of CD4 T cells by B cells (15) and dendritic cells (DCs) (18) under conditions of limiting antigen dose.

In addition to associating with lipid raft membrane microdomains, MHC-II binds to a class of proteins termed tetraspanins (19–25). Tetraspanins form a distinct type of membrane microdomain termed the tetraspan web (26). Tetraspanins form lateral associations with many different proteins, and tetraspan microdomains are important in a wide variety of processes, including signal transduction, cell proliferation, cell adhesion, cell migration, cell fusion, and host parasite interactions (26–28). Most importantly, these proteins play a key role in the function of many immune cells. For example, the tetraspanins CD9 and CD81 can provide a “co-stimulatory-like” function.
signal for T cells (29, 30), a signal that enhances the efficiency of TCR-dependent T cell activation.

It has been proposed that the ability of distinct forms of mouse MHC-II (I-A and I-E MHC-II molecules) to co-cluster is dependent on the direct association of MHC-II with the tetraspanin family member CD9 (25). The ability of GFP-tagged I-E to polarize toward anti-I-A-coated latex beads was reduced in CD9-deficient DCs, revealing a role for CD9 in this process (25). Surprisingly, it was also reported that I-A and I-E molecules co-immunoprecipitate when DCs were solubilized in gentle detergents and that overexpression of CD9 enhanced I-A/I-E co-immunoprecipitation in B cells (25). These data strongly suggest that most, if not all, MHC-II clustering behavior in APCs is due to MHC-II/CD9 interactions and brings into question the importance of lipid raft microdomains for MHC-II function in APCs.

In this study, we have explored in detail the importance of membrane microdomains in the physical association of MHC-II molecules with each other in DCs. By using genetically altered mice, we have directly asked whether CD9 or the related tetraspanin CD81 is important for MHC-II I-A/I-E interactions. Contrary to our expectations, deletion of either CD9 or CD81 did not have any effect on the interaction of I-A and I-E MHC-II molecules in DCs. By contrast, disruption of lipid raft microdomains profoundly inhibited I-A/I-E interactions and also inhibited the association of MHC-II molecules with tetraspanins, suggesting that lipid raft microdomains, and not the tetraspan web, are primarily responsible for the oligomeric assembly of MHC-II complexes in APCs.

**Experimental Procedures**

**Mice, Cells, and Reagents**—B10.BR (H-2b) mice were purchased from The Jackson Laboratory, and C57BL/6 (H-2b) mice were obtained from NCI-Frederick, MD. CD81 KO mice on C57BL/6 (H-2b) background (49) were from Raif Geha (Division of Immunology, Children’s Hospital, Harvard Medical School, Boston). CD9 KO on C57BL/6 (H-2b) background (50) were obtained from Gabriela Dveksler (Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda). CD9 or CD81 KO mice on the H-2k genetic background were generated by crossing of CD9 or CD81 KO mice with B10.BR mice. Dendritic cells were generated by culturing mouse bone marrow for 7 days in medium containing GM-CSF using standard protocols (31). The culture routinely contained >80% CD11c+ DCs that possessed a CD86 low, CD40 low, and MHC-II intermediate “immature” phenotype. Immature DCs were activated by incubation for 24 h with 1 μg/ml LPS (Sigma). In some experiments, DCs were incubated with 20 mM MCD in HBSS for 20 min at 37 °C, pelleted by centrifugation at 1200 rpm at 4 °C, and immediately incubated in lysis buffer for analysis by immunoprecipitation, SDS-PAGE, and immunoblotting. A20 B cells were maintained in RPMI 1640 medium containing 10% FBS, 10 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and penicillin/streptomycin antibiotics at 37 °C, 5% CO2. mCD9-pEGFP-N1 was a gift from Gabriela Dveksler. Brij-58, Triton X-100, protein A-Sepharose beads, streptavidin-agarose beads, and MCD were purchased from Sigma. CHAPS was obtained from Calbiochem. 3,3′-Dithiodis(bis(sulfosuccinimidylpropionate) (DTSSP) and EZ-link sulfo-NHS-biotin were from Pierce.

**Antibodies**—Mouse anti-mouse I-E α-chain mAb 14.4.4S, rabbit anti-mouse CD11c mAb HL3, rat anti-mouse CD9 mAb KMC8, and biotin-labeled hamster anti-mouse CD81 mAb EAT2 were from BD Biosciences. The mouse anti-mouse I-A β-chain mAb 10.3.6 was from BioLegend (San Diego). A rabbit anti-mouse I-A β-chain serum has been described previously (32); rabbit anti-mouse I-E α-chain serum was a gift from Ron Germain (Lymphocyte Biology Section, Laboratory of Immunology, NIAID, National Institutes of Health), and rabbit anti-mouse MHC I serum was a gift from Jon Yewdell (Laboratory of Viral Diseases, NIAID, National Institutes of Health). Mouse anti-mouse I-Aβ β-chain mAb 10.2.16 and mouse anti-mouse I-Aα mAb Y3P were obtained from the American Type Culture Collection (Manassas, VA). Rat anti-mouse CD81 mAb MT81 was a gift from Eric Rubinstein (Inserm, Villejuif, France). Goat anti-rat IgG Alexa-488 and goat anti-rat IgG Alexa-633 were from Invitrogen. HRP-conjugated goat anti-rabbit IgG, goat anti-rat IgG, and streptavidin were purchased from Southern Biotech.

**Cell Surface Biotinylation**—DCs were incubated with 0.5 mg/ml EZ-link sulfo-NHS-biotin in the HBSS (containing Ca2+ and Mg2+) for 25 min on ice. Free biotin was quenched with two washes with 50 mM glycine in HBSS. To study the interaction of surface MHC-II I-A and I-E molecules, biotin-labeled cells were lysed in 1% Brij-58 lysis buffer, and after preclearing the lysate, the lysate was depleted of either MHC-II I-A or I-E by immunoprecipitation. Using this I-A or I-E immunodepleted lysate, biotin-labeled residual proteins were precipitated from these immunodepleted lysates using streptavidin-agarose beads. I-A and I-E molecules bound to these streptavidin-agarose beads were detected by SDS-PAGE and immunoblotting.

**Chemical Cross-linking of Surface Proteins**—DCs were incubated with 1 mM DTSSP in Bicine buffer (150 mM NaCl, 10 mM Bicine, pH 8.2) for 2 h on ice. DTSSP was quenched with 20 mM glycine; the cells were extensively washed in ice-cold buffer and lysed in Triton X-100 lysis buffer, and I-A immunoprecipitation was performed. The immunoprecipitates were boiled in SDS-PAGE sample buffer under reducing conditions to liberate cross-linked MHC-II I-A and I-E molecules and analyzed by SDS-PAGE and immunoblotting.

**Immunoprecipitation and Immunoblotting**—Unless otherwise indicated, all cells were lysed at 10 × 10^6 cells/ml for 1 h on ice in a detergent solution of 1% Brij-58, 1% Triton X-100, or 1% CHAPS in a buffer of 10 mM Tris, 150 mM NaCl, pH 7.4, containing protease inhibitors (50 mM PMSF, 0.1 mM N-tosyl-L-lysine chloromethyl ketone, 5 mM iodoacetamide, 10 μg/ml aprotinin, and 5 μg/ml leupeptin) and 1 mg/ml BSA (lysis buffer). Cell lysates were cleared of nuclei by centrifugation at 13,000 × g and precleared using isotype control antibodies bound to protein A-Sepharose beads for 1 h at 4 °C. Pre-cleared lysates were incubated at 4 °C for 4 h with specific mAb pre-bound to protein A-Sepharose beads. Immunoprecipitates were washed twice in cell lysis buffer and twice in 1:10 dilute lysis buffer (diluted into 10 mM Tris, 150 mM NaCl, pH 7.4). Unless otherwise indicated, the immunoprecipitates (and aliquots of cell lysates) were boiled in SDS-PAGE sample buffer.
under nonreducing conditions, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Nonspecific protein-binding sites on polyvinylidene difluoride membranes were blocked overnight using 1% nonfat dry milk, 0.1% Tween 20 in PBS. Immunoblots were probed with primary Abs followed by specific HRP-conjugated secondary antibodies. Blots were developed by ECL using Western Lighting Plus-ECL reagent and developed using standard x-ray films. The band intensity on all immunoblots was quantitated using Image Quant TL software. In all experiments, multiple exposures of each immunoblot were quantitated; data analysis was performed, and the average value for all data analyses from different exposures of the same immunoblot was obtained. This value represented a single result from a single experiment.

Flow Cytometry—Cells were stained with isotype control, MHC-II, CD9, or CD81 mAb on ice for 40 min. After washing the cells three times with PBS containing 2% FBS, the cells were stained with the fluorescently tagged secondary antibodies and analyzed using a FACSCalibur (BD Biosciences). Expression of CD81 and MHC-II on DCs was determined by co-staining the cells with CD11c mAb and by gating on CD11c+ cells in the cell preparation.

Transfection of A20 Cells—Plasmids containing EGFP alone or EGFP-tagged CD9 were introduced into A20 B cells growing in log phase by electroporation using a Bio-Rad GenePulser using a capacitance of 960 microfarads at 310 V. Cells were harvested after 72 h of transfection and analyzed for expression of CD9 by FACS and for association of MHC-II I-A with I-E by immunoprecipitation, SDS-PAGE, and immunoblotting as described above.

**RESULTS**

I-A and I-E Molecules Associate on the Plasma Membrane of DCs—Cells lysed in gentle detergents such as Brij-58 maintain membrane microdomain associations, whereas those lysed under more harsh detergent conditions, such as in Triton X-100, do not (33–35). To address whether co-immunoprecipitation of MHC-II I-A and I-E is membrane microdomain-dependent, we solubilized mature DCs in Brij-58 or Triton X-100 and studied the interaction of MHC-II I-A and I-E molecules. Although I-A and I-E mAb bound their respective target proteins in each detergent equally well, co-immunoprecipitation of I-A with I-E-specific mAb or I-E with I-A-specific mAb was only observed when cells were lysed in Brij-58 (Fig. 1A). By contrast, MHC-I molecules did not co-immunoprecipitate with MHC-II molecules when DCs were lysed in this detergent (Fig. 1B), confirming the specificity of this interaction.

To investigate whether co-immunoprecipitation of MHC-II I-A and I-E was not occurring after membrane solubilization, we biotinylated surface proteins on DCs isolated from C57BL/6 mice (that contain I-Ak but not I-E molecules) and mixed these cells with DCs isolated from B10.BR mice (that contain both I-Ak and I-Ek molecules). After cell lysis and immunoprecipitation using anti-I-Ek-specific mAb, we observed significant amounts of I-A in the I-E immunoprecipitate; however, these I-A molecules were derived exclusively from the B10.BR DCs as they were not biotinylated (Fig. 1C). In a similar way we did not observe co-immunoprecipitation of biotinylated I-Ak molecules in the I-Ak-specific immunoprecipitate. These data demonstrate that the co-immunoprecipitation of I-A and I-E molecules in detergent extracts is not a post-lysis artifact and requires that the I-A and I-E molecules be expressed on the same cells.

To confirm that the interaction of I-A and I-E molecules occurs on living cells and is not a detergent artifact, we treated
**MHC Class II Association with Lipid Raft Microdomains**

**A**

| IP | LPS | Control | I-A | I-E | Lysate |
|----|-----|---------|-----|-----|--------|
| IP | -   | +       | -   | -   | +      |
| LPS | -   | +       | -   | -   | +      |

![DIagram](image1)

**B**

- I-E α-chain remaining
- I-A β-chain remaining

![DIagram](image2)

**C**

| Immature | Mature |
|----------|--------|
| % total surface MHC-II |
| I-E bound to I-A | I-A bound to I-E |

![DIagram](image3)

**FIGURE 2. Developmental regulation of MHC-II I-A/I-E interactions in DCs.**

A, untreated or LPS-activated DCs were lysed in 1% Brij-58 lysis buffer, and I-A or I-E was immunoprecipitated (IP) using specific mAb, and the immunoprecipitates were analyzed by immunoblotting using the indicated antibodies. Aliquots of each lysate were also loaded on the gels. B, cell surface proteins of untreated or LPS-activated DCs were surface biotin-labeled on ice; the cells were washed and finally lysed in 1% Brij-58 lysis buffer. Lysates were depleted of I-A or I-E MHC-II molecules using 10.2.16 or 14.4.45 mAb-coated protein A-Sepharose beads. Residual surface proteins were isolated from these immunodepleted lysates using streptavidin-agarose beads and analyzed by immunoblotting by using the indicated antibodies. A representative gel indicating the amount of biotinylated (surface) I-E and I-A MHC-II remaining after the immunodepletion is shown. C, intensity of each band in B was determined by quantitative densitometry. The amount of surface I-E remaining in the I-A immunodepleted lysate (i.e. free) and I-A remaining in the I-E immunodepleted lysate (i.e. free) was expressed as a percentage of amount of surface I-A or I-E remaining in the control immunodepleted lysates. The percentage of total surface I-E bound to I-A or I-A bound to I-E was calculated as 100% - %free. The mean ± S.D. from three independent experiments is shown.

**MHC Class II Association with Lipid Raft Microdomains**

Live DCs with the membrane-impermeable chemical cross-linker DTSSP prior to lysis in Triton X-100. I-E was only observed in anti-I-A immunoprecipitates when surface proteins on DCs were chemically cross-linked prior to lysis in Triton X-100 (Fig. 1D). These results demonstrate that I-A and I-E interact with each other in the DC surface and that this interaction can be maintained when cells are lysed in mild detergents or when surface proteins on living DCs are chemically cross-linked prior to lysis in even harsh detergents.

**MHC-II I-A and I-E Molecules Associate on the Membranes of Immature and Mature DCs—MHC-II expression is developmentally regulated in DCs (36), and we therefore examined whether the binding of MHC-II I-A and I-E molecules changed upon DC activation. Despite the fact that immature DCs expressed approximately four times less MHC-II than mature DCs, I-A and I-E molecules could co-immunoprecipitate in both immature and LPS-activated mature DCs (Fig. 2A). Cell surface biotinylation and immunodepletion from cell lysates was also used to quantitate the association of surface MHC-II I-A and I-E molecules. Immunodepletion of Brij-58 DC extracts with I-A mAb reduced the amount of biotinylated I-E remaining in the post-immunoprecipitate supernatant and vice versa (Fig. 2B). Quantitative analysis revealed that ~60% of all I-A and I-E molecules are bound to each other on the surface of immature and mature DCs (Fig. 2C), demonstrating that a significant fraction of the total pool of MHC-II I-A and I-E on the surface of DCs is associated with each other.

**Tetraspanin CD9 Is Not Essential for the Association of I-A and I-E Molecules—**A subset of MHC-II localizes to membrane microdomains together with tetraspanin proteins (19–25), and it has been proposed that the tetraspanin CD9 regulates MHC-II co-immunoprecipitation and the lateral association of MHC-II I-A and I-E on the DC plasma membrane (25). Like the co-immunoprecipitation of MHC-II I-A and I-E molecules, tetraspanin interactions with the tetraspanin web are also disrupted by Triton X-100 (26). To investigate whether MHC-II/tetraspanin interactions are also detergent-sensitive, we assayed MHC-II immunoprecipitates for the presence of the tetraspanin CD9. Like I-A/I-E interactions, co-immunoprecipitation of CD9 with I-A and I-E was only observed when DCs were lysed in Brij-58 and not in Triton X-100 (Fig. 3A), thus confirming that the tetraspanin CD9 binds to MHC-II in a detergent-dependent manner.

To directly address whether CD9 is essential for I-A/I-E interactions, we generated CD9-deficient mice on the H-2k genetic background. Analysis of cell lysates revealed that DCs isolated from CD9 heterozygous mice expressed approximately half as much CD9 as compared with wild-type DCs (Fig. 3B). FACS analysis revealed that MHC-II I-A and I-E expression is not altered in CD9-deficient DCs (supplemental Fig. 1). Despite this reduction in CD9 expression, we did not observe any alteration in MHC-II I-A/I-E co-immunoprecipitation in wild-type and CD9 heterozygous DCs (data not shown). Most importantly, MHC-II I-A/I-E co-immunoprecipitation was identical in DCs isolated from CD9-deficient mice and CD9 heterozygous littermates when cells were lysed in Brij-58 (Fig. 3C) or in CHAPS (data not shown), demonstrating that although both MHC-II I-A/I-E interactions and MHC-II/CD9 interactions are sensitive to disruption by the same detergents, CD9 itself is not essential for co-immunoprecipitation of MHC-II.

It has been reported that MHC-II I-A/I-E molecules do not co-immunoprecipitate in B cells and that this is due to the lack of CD9 expression on B cells (25). Unlike DCs, the mouse B cell line A20 does not express detectable surface CD9 protein (Fig. 4A). We therefore overexpressed CD9 to ask whether CD9 could augment I-A/I-E interactions. Although the amount of CD9 expressed in the transfected A20 B cells was sufficient to augment both CD9/CD81 (Fig. 4B) as well as CD9/I-E interactions (Fig. 4C), CD9 overexpression had no effect on I-A/I-E co-immunoprecipitation in A20 B cells lysed either in either Brij-58 or CHAPS (Fig. 4C). It can be noted that MHC-II I-A/I-E interactions are more robust in Brij-58 than in CHAPS, a finding that highlights the gentle nature of the detergent Brij-58. Taken together with the results obtained above, these data...
demonstrate that CD9 does not regulate the association of MHC-II I-A and I-E molecules.

**Tetraspanin CD81 Is Not Essential for the Association of I-A and I-E Molecules**—CD81 is essential for the CD19/CD21 co-ligation-induced association of the B cell receptor with detergent-insoluble membrane microdomains in spleen B cells (37), and we therefore reasoned that CD81 was a particularly attractive candidate as a regulator of MHC-II microdomain associations and I-A/I-E co-immunoprecipitation. There is conflicting data, however, regarding the presence of CD81 on the DC surface (25, 38), a finding that can be partially explained by the fact that different CD81 mAb differentially react with either “free” or “tetraspan-web”-associated CD81 (35, 39). Using a mAb that reacts equally well with both free and microdomain-associated CD81 (mAb MT81 (35)), we find that this mAb does not recognize any surface proteins whatsoever on CD81-deficient DCs (crossed onto the H-2k genetic background) and that CD81 is

**FIGURE 3.** CD9 is not essential for the association of MHC-II I-A and I-E molecules. A, MHC-II molecules were immunoprecipitated (IP) from Brij-58 (B) or Triton X-100 (T) lysates of activated DCs using isotype control, I-A-, or I-E-specific mAb and analyzed by immunoblotting using I-E α-chain, I-A β-chain, or CD9 antibodies as indicated. An aliquot of the cell lysate was also analyzed. A representative gel is shown. B, LPS-activated DCs were prepared from wild-type (WT), CD9 heterozygous (Het), and CD9-deficient (KO) mice crossed onto the H-2k background (crossed onto H-2k). DCS were prepared from CD9 heterozygous (control), and CD9-deficient mice crossed onto the H-2k background. MHC-II molecules were immunoprecipitated from Brij-58 lysates of activated DCs using I-A or I-E-specific mAb and analyzed by immunoblotting as indicated. An aliquot of the cell lysate was also analyzed. The relative amount of I-A or I-E present in each immunoprecipitate was determined by densitometry and expressed as a fraction of the amount of I-A or I-E present in the cell lysate, and the binding of I-E to I-A and I-A to I-E in CD9 KO DCs was normalized to that obtained in the heterozygous controls. The mean ± S.D. from three independent experiments is shown.

**FIGURE 4.** Overexpression of CD9 does not enhance the association of I-A and I-E MHC-II molecules. A20 B cells were transfected with plasmids encoding EGFP alone or EGFP-tagged CD9. A, after 72 h, the expression of CD9 on the cells was determined by flow cytometry using anti-CD9 antibody. CD9 expression on EGFP-transfected cells (gray solid line) and CD9-EGFP-transfected cells (black solid line) is shown. Also shown are the isotype controls for EGFP-transfected cells (gray filled) and CD9-EGFP-transfected cells (black dotted line). B, transfected cells were lysed in 1% Brij-58 lysis buffer, and immunoprecipitation (IP) using control or CD9-specific mAb was performed, and each immunoprecipitate was analyzed using CD81 mAb or CD9 mAb (to detect CD9-EGFP). An aliquot of the cell lysate was also analyzed. A representative gel revealing the association of I-E with CD9 in EGFP (-) and CD9-EGFP (+) transfected cells is shown. C, upper panel, transfected cells were lysed either in 1% Brij-58 lysis buffer or 1% CHAPS lysis buffer, and immunoprecipitation using I-E-specific mAb was performed, and each immunoprecipitate was analyzed using the indicated antibody. An aliquot of the cell lysate was also analyzed. A representative gel revealing the association of I-A and CD9-EGFP with I-E in EGFP (-) and CD9-EGFP (+)-transfected cells is shown. Lower panel, amount of I-A present in the I-E immunoprecipitate shown above was determined by densitometry and was normalized to the amount of I-E present in the same I-E immunoprecipitate, and the binding of I-A to I-E in CD9-EGFP-transfected cells was expressed relative to that in EGFP-transfected cells. The mean ± S.D. from two independent experiments is shown.
expressed at comparable levels on the surface of immature and mature wild-type mouse DCs (Fig. 5A). Analysis of cell lysates revealed that DCs isolated from CD81 heterozygous mice expressed approximately half as much CD81 as compared with wild-type DCs (Fig. 5A). FACS analysis revealed that MHC-II I-A and I-E expression is not altered in CD81-deficient DCs (supplemental Fig. 1). Co-immunoprecipitation of CD81 with I-A and I-E was only observed when DCs were lysed in Brij-58 and not in Triton X-100 (Fig. 5B), demonstrating that the tetraspanins CD9 and CD81 each bind to MHC-II in a detergent-dependent manner. Co-immunoprecipitation studies carried out in Brij-58 detergent failed to reveal any differences in I-A/I-E interactions in wild-type DCs and their CD81 heterozygous littermates (data not shown) or in CD81-deficient DCs as compared with their heterozygous littermate controls when cells were lysed in Brij-58 (Fig. 5C) or CHAPS (data not shown), demonstrating that the tetraspanin family members CD9 or CD81 alone do not modulate MHC-II I-A/I-E interactions in DCs.

Lipid Rafts Regulate the Association of MHC-II I-A and I-E Molecules—Having failed to detect a role for the tetraspanins CD9 or CD81 in MHC-II interactions in DCs, we explored the possibility that co-immunoprecipitation of distinct forms of MHC-II was mediated by their mutual association with lipid raft membrane microdomains. The integrity of these domains is sensitive to cholesterol depletion, and the cholesterol-binding drug MCD disrupts lipid raft microdomains by reducing plasma membrane cholesterol levels (40, 41). FACS analysis confirmed that MCD treatment did not alter surface expression of MHC-II I-A or I-E in DCs (supplemental Fig. 1). Plasma membrane cholesterol extraction prior to cell lysis in Brij-58 significantly inhibited co-immunoprecipitation of I-A and I-E molecules (Fig. 6). Curiously, this treatment also inhibited, but did not prevent, the ability of the tetraspanins CD9 and CD81 to associate with MHC-II. Because prolonged incubation of cells with MCD can lead to cell death (presumably because of excessive extraction of plasma membrane cholesterol), it is likely that the results obtained here (in which DCs remain viable after MCD treatment) represent an underestimate of the importance of lipid raft domains in these interactions. These data therefore demonstrate both MHC-II I-A/I-E interactions, and the association of MHC-II with tetraspanins is cholesterol-dependent and is mediated by their mutual association with lipid raft membrane microdomains.

DISCUSSION

CD4 T cell activation requires the cross-linking of antigen-specific TCRs by relevant MHC-II-peptide complexes expressed on APCs (42). Because T cells are exquisitely sensitive to very small amounts of MHC-II-peptide complexes expressed on the APC surface (2, 3), it has been proposed that APCs are capable of locally increasing the concentration of MHC-II required for TCR cross-linking (15). MHC-II has been observed to exist in clusters on the plasma membrane of APCs (5, 6), and biochemical studies have revealed that mouse I-A and I-E MHC-II molecules can physically associate, demonstrating that at least dimers of MHC-II molecules can be isolated from APCs (25). The goal of this study was to characterize the molecular
basis for these MHC-II I-A/I-E interactions to better understand the importance of MHC-II oligomerization in APC biology.

Many different investigators have analyzed MHC-II immunoprecipitates; however, to our knowledge, there is only one report in which MHC-II allele-specific mAb have been used to isolate mixed MHC-II I-A/I-E complexes (25). Riberdy and Cresswell (43) observed human MHC-II in anti-I-Ak immunoprecipitates in I-Ak-expressing human lymphoblastoid cell lysates; however, this result was attributed to the formation of “mixed nonamers” of human and mouse MHC-II with a core Ii trimer. The co-immunoprecipitation results obtained in this study are Ii-independent, as we find similar interactions of I-A with I-E in wild-type and Ii-deficient DCs (data not shown). We have found that the interaction of MHC-II I-A and I-E molecules is sensitive to disruption by the relatively harsh nonionic detergent Triton X-100 (conditions used by most investigators examining MHC-II immunoprecipitates) and that these interactions are maintained in the gentle detergent Brij-58. Mixing experiments confirmed that co-immunoprecipitation required that I-A and I-E molecules be co-expressed in the same cells, and cross-linking studies confirmed that these oligomers can be isolated from living DCs, demonstrating that the binding of I-A to I-E is not solely an in vitro phenomenon.

Our ability to co-immunoprecipitate I-A and I-E in gentle detergents but not in more harsh detergents suggested that hydrophobic interactions, potentially in the MHC-II transmembrane domains, are important for MHC-II I-A/I-E interactions. MHC-II is known to associate with cholesterol-dependent membrane microdomains termed lipid rafts (15–18). MHC-II association with lipid rafts lowers the dose of antigen required for T cell activation, a finding that has been attributed to local concentration of MHC-II in these domains (15). We have found that cholesterol depletion with MCD, which disrupts the integrity of lipid rafts, inhibits co-immunoprecipitation of I-A and I-E molecules. Our finding that co-immunoprecipitation of I-A with I-E requires raft integrity suggests that our co-immunoprecipitation protocol actually isolates intact membrane microdomains and that harsh detergents or cholesterol depletion inhibits these interactions by perturbing these microdomains.

It has been reported that overexpression of the tetraspan CD9 in B cells augments the co-immunoprecipitation of MHC-II I-A and I-E, suggesting that CD9 itself is a regulator of MHC-II aggregation and co-immunoprecipitation (25). Surprisingly, we failed to find any differences in MHC-II I-A/I-E interactions in control or CD9-deficient DCs. We also examined the role that CD81 could play in MHC-II aggregation, because we clearly find CD81 expressed on DCs. CD81 mediates CD19/CD21 co-aggregation-dependent BCR recruitment to detergent-insoluble membrane microdomains in B cells (44), a finding that suggested to us that CD81 could similarly regulate MHC-II clustering or oligomerization in DCs. Like CD9, we found no role for CD81 in co-immunoprecipitation of I-A and I-E by using CD81-deficient DCs, demonstrating that these tetraspanins are not required for MHC-II co-immunoprecipitation. Given the extensive redundancy in tetraspanin proteins that make up the tetraspan web, however, these data leave open the possibility that other tetraspanin family members, or the tetraspan web itself, is important for MHC-II aggregation.

Curiously, cholesterol depletion with MCD also partially inhibited the association of MHC-II with the tetraspanins CD9 and CD81, suggesting that lipid raft integrity is important for MHC-II/tetraspanin interactions. There is an extensive literature highlighting both the similarities and differences between the tetraspan web and lipid raft membrane microdomains (28, 29, 30)
MHC Class II Association with Lipid Raft Microdomains

In fact, many tetraspanins, including CD81, are extensively palmitoylated (a lipid modification found on many raft proteins (39, 47)), and palmitoylation is required for detergent insolubility, tetraspan web association, and CD81 function in B cells (39, 44). Our view is that tetraspan web microdomains and lipid raft microdomains are highly inter-related and that there is little, if any, distinction between these two “distinct” types of membrane microdomains. This idea is supported by data revealing a critical role of membrane cholesterol in regulating tetraspanin/tetraspanin interactions (35, 45).

It is likely that distinct forms of MHC-II are physically associated on the APC plasma membrane by virtue of their association with the same (or similar) membrane microdomains. Thus, MHC-II engagement with polyvalent antibodies or with the TCR on T cells would aggregate multiple MHC-II-peptide complexes. Whether these microdomains contain identical copies of MHC-II-peptide complexes that can engage identical TCRs or if they contain one specific MHC-II-peptide complex as well as one MHC-II-endogenous peptide complex as a “pseudo-dimer” (48) remains to be determined. It is important to note, however, that there might not be a functional distinction between these competing models as long as multiple MHC-II-peptide complexes are lipid raft-associated and that these rafts can concentrate at an immune synapse required for T cell activation.

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