MHC Class II-Peptide Complexes and APC Lipid Rafts Accumulate at the Immunological Synapse

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Activation of CD4+ Th cells requires their cognate interaction with APCs bearing specific relevant MHC class II-peptide complexes. This cognate interaction culminates in the formation of an immunological synapse that contains the various proteins and lipids required for efficient T cell activation. We now show that APC lipid raft membrane microdomains contain specific class II-peptide complexes and serve as platforms that deliver these raft-associated class II molecules to the immunological synapse. APC rafts are required for T cell:APC conjugate formation and T cell activation at low densities of relevant class II-peptide complexes, a requirement that can be overcome at high class II-peptide density. Analysis of confocal microscopy images revealed that over time APC lipid rafts, raft-associated relevant class II-peptide complexes, and even immunologically irrelevant class II molecules accumulate at the immunological synapse. As the immunological synapse matures, relevant class II-peptide complexes are sorted to a central region of the interface, while irrelevant class II molecules are excluded from this site. We propose that T cell activation is facilitated by recruitment of MHC class II-peptide complexes to the immunological synapse by virtue of their constitutive association with lipid raft microdomains. The Journal of Immunology, 2003, 170: 1329–1338.

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D4+ Th cells are activated by specific antigenic peptides presented by class II MHC molecules on professional APCs. Although each T cell bears a clonotypic TCR specific for one particular MHC-peptide complex, this relevant MHC-peptide complex is likely to represent an extremely small fraction of the total pool of class II-peptide complexes on the surface of an APC. Although several studies have established that activation of T cells requires less than 500 specific MHC-peptide complexes per APC (1–3), the molecular mechanisms by which such a small number of class II molecules are able to stimulate T cells remain to be determined.

Activation of Ag-specific T cells by APCs requires the orchestration of a complex series of biochemical and morphological changes in T cells. Before interaction with an APC, the TCR is broadly distributed over the entire T cell surface. Encounter of an Ag-specific T cell with Ag-loaded APC halts its migration (4), results in polarization of the T cell cytoskeleton toward the APC (5–7), and initiates the movement of the TCR and CD3 to the T cell:APC interface (7, 8). This early and critical step in T cell activation ultimately leads to the formation of an immunological synapse, a tight, highly organized cognate interaction between the T cell and APC (7, 9, 10).

Although the presence of distinct membrane-anchored proteins at the immunological synapse is well documented (9), the partitioning of membrane lipids and membrane microdomains in T cell:APC conjugates has not been extensively investigated. Lipid rafts are dynamic membrane microdomains enriched in cholesterol, glycosphingolipids, and GPI-linked proteins (11–13). Association of proteins with cholesterol-rich raft microdomains is thought to facilitate signal transduction, protein transport, and membrane fusion by acting as platforms (or rafts), thereby effectively concentrating the molecules involved in these diverse processes (14–17). We have recently shown that MHC class II molecules constitutively reside in plasma membrane lipid raft microdomains on resting B cells (18), and have suggested that raft association increases the probability for TCR cross-linking by MHC class II-peptide complexes.

To evaluate the importance of APC lipid rafts during the initial events in T cell activation, we have now examined their role in the formation of conjugates between Ag-specific T cells and Ag-loaded APCs. We find that the integrity of APC lipid rafts is critical for T cell:APC conjugate formation and T cell activation, but only under conditions of low relevant class II-peptide density. We have also used confocal immunofluorescence microscopy to examine the distribution of APC lipid rafts and distinct MHC class II-peptide complexes at the immunological synapse. Lipid rafts and class II-peptide complexes accumulate at the immunological synapse in conjugates formed between T cells and APCs possessing limiting amounts of specific class II-peptide complexes. At the immunological synapse, class II molecules segregate from each other based on their relevance to the T cell. We propose that under physiologically relevant conditions, the association of class II molecules with lipid rafts results in the delivery of TCR-ligated and nonligated relevant class II-peptide complexes to the immunological synapse, thereby facilitating T cell activation.

Materials and Methods

Cell lines, Abs, and reagents

The mouse B lymphoma cell line, CH27 (H-2b), and the 3A9 T cell hybridoma (specific for hen egg lysozyme (HEL)3 (1) residues 46–61 presented by I-Ak) were gifts from S. Pierce (National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health). As indicated, splenic primary T cells from 3A9 TCR transgenic mice (The Jackson Laboratory, Bar Harbor, ME) were purified by negative selection and used immediately. B cells were maintained in DMEM containing 15% FCS, with 50 μM 2-ME and 50 μg/ml gentamicin. T cells were maintained in

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2 Abbreviations used in this paper: HEL, hen egg lysozyme; MCD, methyl-β-cyclo-dextrin; MFI, mean fluorescence intensity; DiIC16(3), 1,1’dihexadecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate.

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RPMI containing 10% FCS, 50 µM 2-ME, and 50 µg/ml gentamicin. DCEK (I-E^d transfected) and RT7.eH3.B45 (I-A^d transfected) mouse fibroblast cell lines were a gift from R. Germain (NIAID, National Institutes of Health) and were maintained as previously described (19).

mAb 14-4-4S, a mouse IgG2a specific for I-Ed, was purchased from American Type Culture Collection (Manassas, VA), and C4H3, a rat IgG2b specific for the complex of I-A^d-HEL{(c–e)}, was a gift from R. Germain. Both Abs were used as culture supernatants from hybridoma cell lines. Transferrin receptor was detected using a mAb (clone C2D2); CD3e was detected using mAb 500A2; and B7.2 was detected using mAb GL1, all purchased from BD Pharmingen (San Diego, CA). Fluorescently labeled secondary Abs used included Alexa 488- and Alexa 633-labeled anti-rat and anti-mouse Abs purchased from Molecular Probes (Eugene, OR). Anti-mouse and anti-rat Cy5-labeled Abs were purchased from Jackson ImmunoResearch (West Grove, PA). HEL protein was purchased from Sigma-Aldrich (St. Louis, MO). A peptide encompassing HEL aa 41–61 was synthesized by the Experimental Immunology Branch Laboratory Resources section. Methyl-β-cyclodextrin (MCD) and cholera toxin B subunit conjugated to HRP were purchased from Sigma-Aldrich. Cholera toxin B subunit labeled with Alexa 488- and Alexa 633-labeled phalloidin were purchased from Molecular Probes.

Biochemical isolation of detergent-insoluble membranes

Lipid rafts were isolated as previously described (18). Briefly, 10^6 CH27 B cells were treated with 5 mM MCD at 37°C for 10 min (or mock treated), washed, and lysed by resuspending the cell lysate in the final volume of 1% Triton X-100 on ice for 1 h. The lysate was mixed with an equal volume of 90% sucrose, and overlaid with 6 ml of 1 ml of 1% Triton X-100 on ice for 1 h. The lysate was collected and analyzed immediately on a BD Biosciences (San Diego, CA) FACScan. For reversibility studies, after pretreatment with 5 mM MCD for 10 min at 37°C, stained B cells were pelleted, washed, and incubated in serum-containing medium for various times before interaction with T cells. The percentage of conjugates formed at each time point was expressed as a percentage of the amount secreted when T cells were incubated with MCD-treated APCs at each dose of Ag was expressed as a percentage of the amount secreted when T cells were incubated with APCs at each dose of Ag. The percentage of conjugates formed under low Ag conditions and twenty-five conjugates formed under high Ag dose conditions were examined quantitatively, and the relative recruitment of relevant APC lipid rafts, relevant I-E^d-I-E^k complexes, and irrelevant I-E^d molecules was calculated. The data are plotted with average +/- SEM for these conjugates, and statistical significance was calculated using a Student’s t test.

The overlap or colocalization in the fluorescent staining patterns for the indicated molecules was determined using fluorescence intensity profile analysis acquired from the Zeiss LSM 510 confocal image analysis software. The profile of fluorescence intensity (expressed in arbitrary units) was measured along a line taken through the mid-plane of the cell in two channels.

Cross-linking and immunofluorescence

CH27 B cells were incubated in the presence of 10 µg/ml primary Ab for 30 min on ice. Unbound primary Ab was removed with two washes in cold PBS. The B cells were resuspended in warm PBS containing 1 µg/ml fluorescently taggated cross-linking secondary Ab. The cells were cross-linked at 37°C for 10 min, pelleted, and resuspended in ice-cold PBS. An equal volume of 4% paraformaldehyde was then added to fix the cells for 30 min. Following fixation, the cells were washed twice in PBS, resuspended in PBS, and plated on poly(t-lysine)-coated coverslips. The cells were stained with the appropriate Abs or cholera toxin, mounted, and analyzed by confocal microscopy. To determine colocalization of cholera toxin and Dil_C5 staining patterns, Dil_C5-stained cells were incubated with Alexa 488-tagged cholera toxin (1 µg/ml) for 30 min on ice. The cells were washed and warmed to 37°C for 10 min to locally patch lipid rafts. The cells were then fixed, mounted on poly(t-lysine)-coated coverslips, and analyzed by confocal microscopy. The images shown are three-dimensional reconstructions of stacked (x,y) sections acquired from the top of the cell through the mid-plane.
Results
Class II I-A\(^k\)-HEL\(_{46-61}\) complexes reside in lipid raft membrane microdomains

We have previously reported that a significant portion of surface MHC class II molecules resides in lipid raft microdomains and that the inclusion of class II-peptide complexes in APC lipid rafts is important for the activation of T cells (18). We now set out to examine the association of specific class II-peptide complexes with these lipid rafts and determine the importance of this association for the activation of Ag-specific T cells. For these studies, we examined the lipid raft association of I-A\(^k\)-HEL\(_{46-61}\) complexes on HEL-pulsed mouse CH27 B cells (H-2\(^k\)) using a class II-peptide complex-specific mAb while examining the role APC rafts play in the activation of I-A\(^k\)-HEL\(_{46-61}\)-restricted 3A9 T cells.

The kinetics of I-A\(^k\)-HEL\(_{46-61}\) complex formation on CH27 B cells was examined using mAb C4H3 (Fig. 1A), a reagent that specifically recognizes SDS-stable I-A\(^k\)-HEL\(_{46-61}\) complexes (20). To determine whether I-A\(^k\)-HEL\(_{46-61}\) complexes were present in lipid rafts, Triton X-100 lysates of HEL\(_{46-61}\)-pulsed B cells were analyzed by sucrose density gradient centrifugation and immunoblotting using mAb C4H3. Approximately 26% of all I-A\(^k\)-HEL\(_{46-61}\) complexes partitioned into the Triton X-100-insoluble, low buoyant density lipid raft fractions of the gradient (Fig. 1B). The positions of the raft and nonraft fractions in the gradient were revealed by probing the blot with HRP-conjugated cholera toxin B subunit (which binds to ganglioside GM1) and anti-B7.2 mAb, respectively. To confirm that the low buoyant density of these class II-peptide complexes was a consequence of their association with lipid raft microdomains, Ag-loaded B cells were pretreated with MCD, a reagent that extracts plasma membrane cholesterol and perturbs lipid raft integrity (22, 23). Pretreatment of the cells with MCD completely prevented I-A\(^k\)-HEL\(_{46-61}\) association with these microdomains (Fig. 1B). Quantitative analysis of the immunoblots confirmed that MCD did not alter the total amount of I-A\(^k\)-HEL\(_{46-61}\) complexes on the cells.

APC lipid rafts are required for T cell activation at limiting Ag dose

To determine whether raft association of I-A\(^k\)-HEL\(_{46-61}\) complexes was required for T cell activation, we examined the effect of APC raft disruption on the activation of I-A\(^k\)-HEL\(_{46-61}\)-specific 3A9 T cells. Treatment of peptide-loaded CH27 B cells with MCD inhibited 3A9 T cell activation when the cells were pulsed with a suboptimal dose of HEL\(_{46-61}\) peptide; however, raft disruption had little effect when the APCs were preloaded with a high dose of Ag (Fig. 1C). These results reveal that a significant fraction of I-A\(^k\)-HEL\(_{46-61}\) complexes constitutively resides in lipid raft microdomains and that B cell lipid rafts are important for 3A9 T cell activation when APCs possess suboptimal amounts of relevant I-A\(^k\)-HEL\(_{46-61}\) complexes.

APC lipid rafts are required for T:B cell conjugate formation at limiting Ag dose

Whereas APC rafts are predominantly important for T cell activation under conditions of limiting Ag dose, it remains to be determined at which point in the complex T cell activation cascade APC rafts exert their stimulatory effect. Because the formation of a T cell:APC conjugate is the earliest event in T cell activation, we have examined the requirement for APC rafts in the formation of T:B cell conjugates using a dynamic, live cell system. Conjugates between 3A9 T cells and CH27 B cells were examined using a flow cytometric assay in which cells were stained with distinct fluorescent tracers before interaction (21).

To address the importance of APC lipid rafts in conjugate formation, Ag-loaded B cells were pretreated with MCD, washed, and immediately cultured with T cells before FACS analysis. As shown in Fig. 1B, MCD treatment does not perturb pre-existing I-A\(^k\)-HEL\(_{46-61}\) complexes. In the absence of Ag, ~3% of T cells were present in T:B cell conjugates representing nonspecific or background adhesion (Fig. 2A). MCD-mediated extraction of plasma membrane cholesterol had no effect on background adhesion in this system, demonstrating that APC rafts are not involved in Ag-independent adhesive interactions between T and B cells (Fig. 2B). When APCs were pulsed with various doses of antigenic peptide overnight, we observed a dose-dependent increase in the percentage of T cells in conjugates up to a maximum of ~15%. As was observed in our studies examining IL-2 production, APC lipid raft disruption had a minimal effect on conjugate formation when
The effects of APC raft disruption are reversible

An obvious concern in studies using agents that perturb cellular cholesterol levels is that these treatments can irreparably damage cell integrity and function. To determine whether the effects of APC raft disruption were reversible, we pretreated peptide-pulsed B cells with MCD for 10 min, washed away the drug, and allowed the B cells to recover in cholesterol-containing medium for various times before the addition of T cells. Although the initial treatment with MCD almost completely prevented conjugate formation, the ability of T cells to form conjugates with these B cells was regained in a time-dependent manner, leading to a 90% recovery in a 2-h culture period (Fig. 2C). Similarly, when MCD-treated B cells were allowed to recover, they regained their capacity to elicit IL-2 production by Ag-specific T cells (data not shown). These data show that raft perturbation does not irreparably damage the B cells and does not selectively remove preformed I-Ak–HEL(46–61) complexes from the B cell membrane.

APC lipid rafts accumulate at the immunological synapse

We next set out to examine the distribution of class II molecules and APC lipid rafts at the site of contact between T and B cells by immunofluorescence microscopy. To restrict our analysis to productive Ag-dependent T:B cell interactions, we routinely costained conjugates with fluorescent phalloidin, a marker for filamentous actin that polarizes to the immunological synapse during T cell signaling (7). Analysis of lipid raft localization in T:B conjugates using fluorescent cholera toxin (which binds to ganglioside GM1) revealed lipid raft concentration at the immunological synapse (Fig. 3A). Unfortunately, due to the ability of cholera toxin to bind to GM1 on both cells and the close apposition of APC and T cell membranes in these conjugates, it was impossible to exclusively examine APC lipid rafts with this technique. Therefore, to specifically monitor the behavior of B cell lipid rafts at the immunological synapse, we chose to prelabel the B cell rafts with DiIC16, a fluorescent lipid analog that preferentially inserts into liquid-ordered (raft-like) domains on the plasma membrane (24–27). To confirm that this fluorescent lipid faithfully tagged lipid raft microdomains, we assessed the colocalization of DiIC16 with fluorescent cholera toxin on raft-aggregated B cells. We found that the two markers exhibited almost complete colocalization (Fig. 3A), demonstrating that they labeled the same microdomains.

Because APC lipid rafts are most important for T:B cell conjugate formation at low doses of Ag, we examined the distribution of B cell lipid rafts at the immune synapse using APCs loaded with either low or high doses of Ag. In the absence of Ag, there was no accumulation of B cell rafts at the synapse of nonspecific T:B cell conjugates (data not shown), while at low Ag doses B cell lipid rafts accumulated at the T:B cell interface (Fig. 3B). As expected, in the absence of Ag there was no accumulation of B cell rafts at the synapse of nonspecific T:B cell conjugates (data not shown). Interestingly, under high Ag dose conditions, we also observed no detectable enrichment of B cell lipid rafts at the synapse relative to the rest of the cell. To address the prevalence of this dose-dependent lipid raft recruitment in a population of individual conjugates, raft recruitment to the synapse was scored by a blinded observer. Whereas significant raft accumulation was observed in 88% of conjugates formed under low Ag dose conditions, raft accumulation was observed in less than 10% of conjugates formed under high dose Ag conditions (Fig. 3B). Similar results were obtained when the B cell lipid rafts were prelabeled with fluorescent cholera toxin.
toxin B subunit (data not shown), demonstrating that these results were not restricted to our use of DiIC₁₆ as a lipid raft marker.

**MHC class II molecules and APC lipid rafts accumulate at the immunological synapse**

Because APC lipid rafts are critical to conjugate formation only at low Ag dose, and APC lipid rafts are differentially recruited to the immunological synapse under these same conditions, we set out to address the localization of class II-peptide complexes recognized by the T cell at the immunological synapse at high and low Ag doses. To specifically visualize the class II peptide complexes recognized by the 3A9 T cell, we used mAb C4H3, a reagent that specifically stains I-A<sub>K</sub>-HEL<sub>(46–61)</sub> complexes on living cells (20). Because several types of class II MHC molecules reside in lipid raft microdomains (18), we also wanted to determine whether other types of class II molecules (even those irrelevant class II molecules not recognized by the T cell) could also be recruited to the immunological synapse. To detect these irrelevant class II molecules, we used the mAb, 14-4-4S, which detects I-E<sub>K</sub> molecules. The exquisite specificity of these Abs was confirmed using class II-transfected mouse fibroblasts expressing only I-A<sub>K</sub> or I-E<sub>K</sub> molecules or using CH27 B cells in the absence or presence of HEL<sub>(46–61)</sub> peptide (Fig. 4).

Under conditions in which B cell lipid rafts were recruited to the immunological synapse (low Ag dose), we observed an enrichment of relevant I-A<sub>K</sub> HEL<sub>(46–61)</sub> complexes at the immunological synapse (Fig. 5A). This accumulation was not observed when B cells were not pulsed with HEL peptide (data not shown). Like lipid raft recruitment, the concentration of relevant class II-peptide complexes was not observed at high Ag dose. Surprisingly, under low Ag dose conditions, we also observed a moderate enrichment of irrelevant (I-E<sub>K</sub>) class II molecules at the T:B cell interface (Fig.
At either low or high Ag dose were stained independently with Abs for peptides to the immunological synapse was quantitated. Conjugates formed between 3A9 T cells and B cells loaded with a high dose of HEL peptide, there was no significant enrichment of either I-A\textsuperscript{k}–HEL\textsubscript{46–61} complexes as well as I-E\textsuperscript{k} and lipid rafts after I-E\textsuperscript{k} cross-linking. Similarly, patching of I-A\textsuperscript{k}–HEL\textsubscript{46–61} complexes resulted in copatching of some I-E\textsuperscript{k} molecules, but with less dramatic effects. This is most likely due to smaller numbers of molecules patched with the I-A\textsuperscript{k}–HEL\textsubscript{46–61} complex-specific mAb as compared with the I-E\textsuperscript{k}-specific mAb (which recognizes a large fraction of the total class II pool). As a control, we examined the distribution of the transferrin receptor, a nonraft protein, on I-E\textsuperscript{k} cross-linked cells. Patching of I-E\textsuperscript{k} did not alter the uniform distribution of transferrin receptor on these cells, an observation that was confirmed by the lack of correlation between the clustered intensity profile of the I-E\textsuperscript{k} patches with the relatively smooth intensity profile of the transferrin receptor (Fig. 6B). These data confirm that the I-E\textsuperscript{k} patches did not nonspecifically sequester all plasma membrane proteins.

To determine whether copatching of the two distinct class II molecules was dependent on the integrity of lipid rafts, we treated the B cells with MCD before Ab-mediated patching of I-E\textsuperscript{k}. Although patching of I-E\textsuperscript{k} molecules was still observed in MCD-treated B cells, copatching of both I-A\textsuperscript{k}–HEL\textsubscript{46–61} complexes and B cell lipid rafts with the cross-linked I-E\textsuperscript{k} molecules was completely prevented by raft disruption of the B cell (Fig. 6C, and data not shown). These results demonstrate that distinct types of class II molecules can occupy the same membrane microdomains and that their coaggregation is lipid raft dependent.

**Figure 5.** Class II-peptide complexes are corecruited to the immunological synapse under low Ag dose conditions. A, CH27 B cells were pulsed with either 100 μg/ml HEL\textsubscript{46–61} peptide (high dose) or 1 μg/ml HEL\textsubscript{46–61} peptide (low dose) overnight at 37°C, stained with DiIC\textsubscript{16} (red), and incubated with 3A9 T cells to form T:B cell conjugates. The cells were fixed and stained with mAb C4H3 to label relevant I-A\textsuperscript{k}–HEL\textsubscript{46–61} complexes (green) and mAb 14-4-4S to label irrelevant I-E\textsuperscript{k} molecules (blue). Because the T cells were not stained in these studies, the outline of the T cells (visualized by bright field imaging) is indicated by a dashed line. Scale bars represent 3 μm. B, Recruitment of lipid rafts and class II molecules to the immunological synapse was quantitated. Conjugates formed at either low or high Ag dose were stained independently with Abs for either I-A\textsuperscript{k}–HEL\textsubscript{46–61} or I-E\textsuperscript{k} along with the lipid raft dye DiIC\textsubscript{16} and fluorescent phalloidin. Relative recruitment index for 25 conjugates in each condition was calculated as described in Materials and Methods.
nological synapse (Fig. 7B). After 30 min of interaction, a period reported by others to be sufficient to form a mature synapse (29, 30), we observed the sorting of the relevant I-A^k-HEL_{46–61} complexes toward the center of the synapse and the exclusion of the I-E^k molecules from this region. The sorting of relevant and irrelevant class II molecules was observed in conjugates formed under both high and low Ag dose conditions (data not shown). Staining of conjugates with the pan I-A^k mAb 10.2.16 failed to reveal any partitioning at the immunological synapse (E. M. Hiltbold, unpublished observations), reflecting the fact that this mAb recognizes both relevant I-A^k-HEL_{46–61} complexes as well as irrelevant I-A^k-peptide complexes.

Because the distinction between relevant and irrelevant class II molecules is defined by the specificity of the TCR, we examined the distribution of CD3e with relevant and irrelevant class II molecules at the immunological synapse. In the mature synapse, there were dramatic concentration and marked colocalization of CD3e and relevant I-A^k-HEL_{46–61} complexes in a tight cluster near the center of the immunological synapse (Fig. 7C). By contrast, we consistently observed a complete exclusion of irrelevant I-E^k molecules from CD3 in the mature immunological synapse.

To determine the frequency of segregation of relevant and irrelevant class II molecules at a mature (30-min) immunological synapse, we scored the segregation of I-A^k-HEL_{46–61} complexes and irrelevant I-E^k molecules in 45 conjugates (Fig. 7C). We observed complete segregation of relevant and irrelevant class II molecules in 71% of the conjugates and partial segregation in an additional 15% of the conjugates, and no detectable segregation of these proteins at the immunological synapse was observed in only 14% of the conjugates examined. These results reveal that although a T cell may encounter a mixed population of relevant and irrelevant class II molecules upon initial interaction with an APC, these molecules, based on their relevance to the T cell, are sorted into either the center or the periphery of the interaction site over time.

FIGURE 6. Distinct MHC class II molecules coaggregate on the plasma membrane in a lipid raft-dependent manner. A, CH27 B cells were loaded with 1 mg/ml HEL protein overnight, washed, and incubated with the following Abs: rat IgG (Control), or mAb 14-4-4S (I-E^k X-linked) under cross-linking conditions (as described in Materials and Methods). The cells were then fixed and counterstained with the indicated Abs to stain bystander molecules (listed above pictures). Fluorescently tagged cholera toxin (blue) was used to label lipid rafts on the fixed cells. The fluorescence profile histogram for each image illustrates the fluorescence intensity in each channel along a line traversing the membrane along the mid-plane of the cell. B, I-E^k molecules on the B cells were cross-linked as above (red). The cells were then fixed and counterstained with mAb C2 to stain transferrin receptor (green). C, CH27 cells were treated with 5 mM MCD or mock treated for 10 min. The cells were then washed and cross-linked with 14-4-4S mAb, as above (red). The cells were then fixed and counterstained with the C4H3 Ab specific for I-A^k-HEL_{46–61} complexes, as above (green). Scale bars represent 3 μm.
Discussion

Although the precise physical nature of lipid raft microdomains is the topic of intense investigation and vigorous debate, there is little doubt that the concentration of specific membrane proteins within these microdomains on the plasma membrane of T cells is essential for TCR-dependent T cell activation (28, 31, 32). In this study, we have investigated the role of APC rafts in lymphocyte biology by examining the behavior of a particular class II-peptide complex involved in T cell activation. Approximately one-quarter of these I-A^k-HEL(46–61) complexes reside in lipid rafts on B cells and, most importantly, cholesterol depletion disrupted class II-raft associations and inhibited the activation of I-A^k-HEL(46–61)-restricted T cells when APCs possessed limiting amounts of relevant class II-peptide complexes. Because lipid raft isolation procedures are likely to perturb some protein-lipid interactions, our biochemical determination of class II association with lipid rafts may actually underestimate the in vivo association of I-A^k-HEL(46–61) complexes with these microdomains. This is supported by our

FIGURE 7. Segregation of MHC class II molecules at the immunological synapse. A, CH27 B cells were pulsed with 100 μg/ml HEL(46–61) overnight at 37°C, washed, and cultured with 3A9 T cells for 10 min. Conjugates were fixed and stained with fluorescent phalloidin to visualize filamentous actin (green) and mAb 14-4-4S to visualize MHC class II molecules (red). A three-dimensional reconstruction of the conjugate is shown on the left panel, and a depiction of a typical xz plane used in confocal analysis of the immunological synapse is shown on the right panel. B, 3A9 T cells were cultured with Ag-loaded (100 μg/ml HEL(46–61)) CH27 B cells for either 10 or 30 min. The cells were then fixed and stained with mAb C4H3 to label relevant I-A^k HEL(46–61) complexes (red) or mAb 14-4-4S to label irrelevant I-E^k molecules (green). The organization of each type of class II molecule at the immunological synapse was analyzed by confocal analysis of z sections acquired through the T:B cell interface. Regions of colocalization are shown in yellow in the overlay. Scale bars represent 3 μm. C, 3A9 T cells and HEL-loaded (100 μg/ml HEL(46–61)) CH27 B cells were combined for 30 min at 37°C, fixed, and stained with a mAb recognizing mouse CD3ε (green), mAb C4H3 to label relevant I-A^k HEL(46–61) complexes (red), and mAb 14–4–4S to label irrelevant I-E^k molecules (blue). Confocal sections were collected in the x–y plane (two left panels) and through the z plane of T:B cell interface (three right panels). Scale bars represent 3 μm. D, Frequency of class II sorting was scored by a blinded observer. Forty-five conjugates like those shown above were examined in the xz plane, and the segregation of I-A^k-HEL(46–61) complexes from I-E^k class II molecules was scored as being either completely segregated, partially segregated, or not differentially sorted, as described in the text.
observation that class II clustering leads to a profound redistribution of rafts on live B cells. Overall, these data are in excellent agreement with our previous study using I-E\(^k\)-pigeon cytochrome c\(_{\text{4a1-103}}\) restricted T cells (18) and point to an important role of lipid rafts in concentrating specific class II-peptide complexes in membrane microdomains.

Because T cell activation (as measured by IL-2 release) is a relatively late indicator of Ag presentation, we set out to determine why disrupting APC rafts was detrimental to T cell activation only when APCs possessed limiting amounts of Ag. By examining the earliest event in T cell activation, T:B cell conjugate formation, we found that disrupting APC rafts also preferentially inhibited T:B cell conjugate formation under conditions of low Ag dose, thereby providing a mechanism to account for diminished T cell activation under these conditions. Because raft association is thought to increase the local density of plasma membrane proteins (17), it is likely that under conditions of high Ag dose the density of class II-peptide complexes is sufficiently great so that effective T:B conjugate formation and T cell activation occur even when APC rafts are perturbed.

The significance of the constitutive association of class II molecules with APC lipid rafts was revealed by our surprising discovery that even irrelevant or bystander class II I-E\(^k\) molecules were recruited to and retained at a synapse formed by an APC and an I-A\(^k\)-HEL\(_{40-61}\) restricted T cell. The corecruitment of relevant and irrelevant class II molecules to the immunological synapse could only occur if these two types of class II molecules were physically associated. By coaggregation analysis, we have shown that these distinct class II molecules can be associated with the same membrane microdomain and that perturbation of plasma membrane rafts prevented their association, suggesting that coassociation of relevant and irrelevant class II molecule with lipid rafts is responsible for their corecruitment to the immunological synapse. Unfortunately, because class II concentration at the synapse only occurs at low Ag dose conditions and because under these conditions raft perturbation prevents T:B cell conjugate formation, we are unable to test this hypothesis directly. Assuming that class II ligation by mAb is analogous to class II ligation by the TCR, the failure of distinct class II molecules to coaggregate in raft-depleted cells strongly supports this idea.

The concentration of relevant class II-peptide complexes to the immunological synapse is likely to be a TCR-driven process. During T cell activation, there is a profound reorganization of the T cell cytoskeleton to the immunological synapse (33), and although disruption of the T cell actin cytoskeleton prevents class II concentration and T cell activation, disruption of the APC actin cytoskeleton has no effect on either ICAM-I or class II concentration at the synapse (34, 35). Additional evidence in support of this hypothesis are data showing that lipid-anchored MHC-peptide complexes in artificial bilayers move to the center of the immune synapse upon encounter with an Ag-specific T cell (29). Based on these observations, we propose that the concentration of class II molecules and APC lipid rafts at the immunological synapse is due to TCR-mediated trapping of raft-associated relevant class II-peptide complexes that have passively diffused into the T:B cell interface. The rate of lateral diffusion of class II molecules on the B cell plasma membrane (\(\approx 1 \times 10^{-10} \text{ cm}^2 \text{s}^{-1}\)) (36) is sufficiently high so as to allow significant movement of class II molecules on the B cell to the T:B cell interface under the time frame of immunological synapse formation examined in this study; however, this does not necessarily rule out a novel active process of class II movement to the synapse.

Our study suggests that for APC lipid rafts to functionally concentrate class II molecules, there must be at least two of these molecules per raft at any given time. Whether this occurs through the fusion of pre-existing rafts, the preferential association of newly formed class II-peptide complexes in rafts during class II transports to the cell surface, or the association of class II molecules with pre-existing plasma membrane rafts at the cell surface remains to be determined. The delivery of class II molecules to the plasma membrane via tubules emanating from lysosomal Ag-processing compartments has recently been visualized in stimulated dendritic cells (37, 38), and it is intriguing to speculate that such a process could allow the delivery of large numbers of relevant class II-peptide complexes to plasma membrane microdomains. Furthermore, our demonstration that distinct class II molecules can reside in the same membrane microdomain provides a potential mechanism to retain not only TCR-engaged class II molecules, but also those potentially relevant class II-peptide complexes that have not yet engaged the TCR. It is possible that this is the physiological significance of class II association with lipid rafts.

The TCR-dependent segregation of immunologically relevant and irrelevant class II molecules at the synapse is perhaps the most striking finding of our study. Because distinct types of class II molecules can reside in the same lipid raft and because lipid rafts are thought to be less than 200 nm in diameter (12, 39), there must be some mechanism to remove a constraint to class II diffusion at the synapse. It is possible that fusion of many individual rafts at the synapse forms a giant raft that allows the diffusion of irrelevant class II molecules from the centrally located TCR in a raft-rich membrane, an idea that is supported by data showing that the immune synapse is lipid raft rich (38, and this study). It is also possible that at the synapse relevant and irrelevant class II molecules dissociate from rafts and that irrelevant class II molecules then diffuse from the centrally located TCR in a nonraft membrane. The idea that individual rafts exist only transiently is consistent with this latter possibility (40, 41); however, the precise mechanism behind class II segregation remains to be determined experimentally.

Our finding of differential recruitment and sorting of class II-peptide complexes may in part explain discrepancies in the literature regarding the extent of class II concentration at the immunological synapse (35, 42, 43). As we found by examining the distribution of relevant I-A\(^k\)-HEL\(_{40-61}\) complexes at the immune synapse, the amount of recruitment observed will depend primarily on the amount of relevant class II molecules bound to a given amount of TCR and, obviously, whether one is examining all class II molecules on the APC or only immunologically relevant class II-peptide complexes. It is interesting to note that when APCs possess extremely large amounts of relevant class II-peptide complexes, accumulation at the synapse is also not observed, a result that is most likely due to the fact that the number of relevant class II-peptide complexes far exceeds the number of TCRs available for ligation at the synapse.

It is interesting to note that like irrelevant class II molecules, CD4 sorts from a central to a peripheral location in the mature synapse (44). The possibility exists, therefore, that as CD4 molecules dissociate from the TCR and migrate to the periphery of the synapse, they bind class II molecules that are not engaged with TCR, resulting in their segregation into the periphery. The presence of these CD4-irrelevant class II complexes may serve to augment adhesive interactions between T cells and APCs that already exist in the ICAM-I/LFA-1-rich adhesive zone of the immunological synapse.

A recent study revealed the clustering of both agonist peptide-MHC complexes as well as nonstimulatory null class II-peptide complexes at the immune synapse (35). In contrast to our findings in which irrelevant class II molecules were excluded from the central region of the synapse, they observed these null complexes...
centrally concentrated at the immunological synapse. These null peptide-class II complexes are distinct from the immunologically irrelevant class II alleles examined in our study in that they consist of a version of the relevant agonist peptide (lacking two of three TCR contact residues) bound to a relevant class II allele. Wülling et al. (35) proposed that these complexes accumulate at the synapse in part due to their immeasurably low direct binding to the TCR. This hypothesis is entirely consistent with our data that demonstrate that class II-peptide complexes that are in any way relevant to the T cell sort into the center of the synapse along with the TCR.

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