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B Cell Lipid Rafts Regulate Both Peptide-Dependent and Peptide-Independent APC-T Cell Interaction

Niclas Setterblad, Stéphane Bécart, Dominique Charron, and Nuala Mooney

Formation of an immunological synapse (IS) between APCs and T CD4+ lymphocytes is a key event in the initiation and the termination of the cognate immune response. We have investigated the contribution of the APC to IS formation and report the implication of the actin cytoskeleton, the signaling proteins and the lipid rafts of B lymphocytes. Recruitment of MHC class II molecules to the IS is concomitant with actin cytoskeleton-dependent B cell raft recruitment. B cell actin cytoskeleton disruption abrogates both IS formation and T cell activation, whereas protein kinase C inhibition only impairs T cell activation. Pharmacological B cell lipid raft disruption inhibited peptide-dependent T lymphocyte activation and induced peptide-independent but HLA-DR-restricted APC-T cell conjugate formation. Such peptide-independent conjugates did not retain the ability to activate T cells. Thus, B cell lipid rafts are bifunctional by regulating T cell activation and imposing peptide stringency. The Journal of Immunology, 2004, 173: 1876–1886.

In addition to their well-established role in antigen presentation, MHC class II molecules have been widely documented as signal transducing molecules. The protein kinase C (PKC) family of isoenzymes is strongly implicated in MHC class II-mediated signal transduction (1–3), given that truncation of the MHC class II molecules leads to defects in both PKC activation (4) and peptide presentation (5, 6). Studies demonstrating the localization of immunoreceptors in discrete membrane microdomains have led to a revision of the notion of their random distribution throughout the plasma membrane (7). The site of interaction between T lymphocytes and APCs, termed the immunological synapse (IS), is specifically enriched in signaling proteins localized in lipid-rich membrane microdomains, also known as glycolipid-enriched membrane microdomains or lipid rafts (8, 9).

MHC class II molecules localize within lipid-rich microdomains in diverse APCs including primary B lymphocytes and dendritic cells (10–12) as well as in EBV-transformed B cell lines and cell lines established from solid tumors (3, 13). Lipid raft-localized MHC class II molecules mediate Src kinase activation in myelomonocytic cells (15). Moreover, the integrity of MHC class II-containing lipid rafts was essential for MHC class II-mediated actin recruitment to lipid rafts (13) and for the recruitment and activation of PKC-α (3). Further, lipid raft disruption profoundly decreased MHC class II-mediated presentation of low concentrations of peptide Ag (12, 16). Localization of MHC class II molecules in lipid rafts was also required for Th1 orientation, whereas dispersion from lipid rafts led to a Th2 profile (17). Taken together, there are strong arguments for MHC class II signaling pathways implicated in Ag presentation emanating from lipid rafts.

Formation of an IS between APCs and T CD4+ lymphocytes is a key event in both the initiation and the termination of the cognate immune response (18). The IS can be organized in distinct concentric zones constituted by a central supramolecular activation cluster within which the TCR localizes, surrounded by a peripheral supramolecular activation cluster containing adhesion molecules (19, 20), although multifocal and mobile immunological synapses have been reported (21, 22). Integrity of the APC actin cytoskeleton is necessary for peptide presentation (23) and also implicated in the formation of the peptide-dependent IS (24–26). Relocalization of MHC class II molecules toward the site of APC-T cell interaction has been described (27) and also agonist peptide-MHC class II complexes, specifically recognized by the interacting T lymphocyte, and irrelevant MHC class II molecules were relocalized to the zone of APC-T cell interaction where they served as accessory molecules for T lymphocyte activation. Relocalization of MHC class II molecules to lipid-rich microdomains at the IS has been implicated in optimal peptide presentation and T lymphocyte activation (12), although MHC class II molecules localized in tetraspan containing microdomains were also attributed a key role in peptide presentation (10).

Many studies examining MHC class II-restricted peptide-dependent T lymphocyte interactions have overlooked any potential contribution of the APC by using either fixed APCs or artificial MHC class II-containing lipid bilayers (16, 19). We identify an active role for the APC in IS formation and demonstrate that optimal T lymphocyte activation requires the integrity of the APC actin cytoskeleton and depends on APC signaling. Further, we reveal a novel role for MHC class II molecules in lipid rafts in imposing peptide stringency of APC-T cell interactions.

Materials and Methods
Cell lines

T8.1 is a murine T cell hybridoma expressing human CD4 and a chimeric human-mouse TCR specific for a tetanus toxin peptide (TT$_{830–843}$)
restricted by HLA-DRB1*1102 (28). T8.1/CD3-YFP is the T8.1 hybridoma transduced with a retroviral expression vector expressing a CD3-YFP fusion protein (kindly provided by A. Trautmann, Institut Cochin, Paris, France). T8.1 and T8.1/CD3-YFP were maintained in DMEM with 10% FCS, 2 mM L-glutamine, and antibiotics supplemented with 400 nM methotrexate, 1 mg/mL G418, and 50 µM 2-ME. The homozygous EBV-transformed B cell lines JVM and BM15, expressing HLA-DR11 (both are HLA-DRB1*1102), were maintained in RPMI with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics. The HLA-DR11-expressing and endogenous B7-1 and ICAM-2-expressing murine L cells (L625.7) (29), cultured in complete DMEM with 250 µg/mL G418, have been shown to be efficient APCs of T8.1, compared to T8.1 (30). The MHC class II B cell lymphoma RJJ2.2.5 and the non-HLA-DR11 MHC class II B cell lymphoma Raji B-EBV (HLA-DR3/DR10) were cultured in RPMI with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics.

Abs and reagents

Ascites from mouse mAb anti-HLA-DR (L243), anti-HLA-DRα (DA6.147), and anti-HLA-1 (W6/32) Abs were purified on mAb trap columns (AP Biotech, Uppsala, Sweden). Mouse mAb anti-transferin receptor (TIR) Ab was from Zymed (San Francisco, CA). Mouse mAb anti-flotillin-1 was from BD Transduction Laboratories (Lexington, KY). Rabbit polyclonal Abs anti-PKC-δ (C-20; sc-937), anti-Lyn (sc-15), and anti-β₂-microglobulin (sc-15366) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAbs anti-talin (mAb 3264) and anti-actin (mAb 1501) were from Chemicon (Temecula, CA). HRP-conjugated anti-rabbit Ig and anti-mouse Ig were from DAKO (Glostrup, Denmark). FITC-conjugated cholera toxin B subunit (CT), HRP-conjugated CT, mouse mAb anti-β-tubulin (TUB 2.1), methyl β-cyclodextrin (MβCD), Triton X-100, poly-γ-L-lysine, streptavidin, cis-5,8,11,14,17-eicosapentaenoic acid (eicosapentaenoic acid) were from Sigma-Aldrich (St. Louis, MO).

1,1’-Dihexadecyl-3,3',3'-tetrathymethylindocarbocyanine perchlorate (DiIC18(3)), 1,1’-didecyl-3,3',3'-tetrathymethylindocarbocyanine perchlorate (DiIC12(3)), jasplakinolide, Alexa Fluor 568-conjugated goat anti-mouse Ig Alexa, Alexa Fluor 594-conjugated goat anti-rabbit Ig, and Alexa Fluor 488-conjugated phalloidin were from Molecular Probes (Eugene, OR). Rottlerin, safingol, calphostin C and cytochalasin D were from Calbiochem (Merck Eurolab, Fontenay-sous-Bois, France).

The Clontech pEGFP-actin vector was purchased from BD Biosciences (San Jose, CA).

The TT830-843 peptide (QYIKANSKFIGITE) was purchased from Neo- systems (Strasbourg, France) and was >90% pure. The CellTracker orange CMTPR (5(6)-carboxyfluorescein) and CellTracker green CMFDA (5-chloromethylfluorescence diacetate) probes are thiol-reactive cell fluorescent chloromethyl derivates reacting with intracellular components (Molecular Probes).

Cell treatments

Implication of the actin cytoskeleton was analyzed by pretreating the APCs or the T cells for 30 min with latrunculin A (0.1–1 µM), jasplakinolide (1–10 µM), or cytochalasin D (40 µM). Implication of PKC was analyzed by pretreating the APCs or the T cells for 45 min with either calphostin C at 50 nM (broad range PKC inhibitor), rottlerin at 6 µM (PKC-δ inhibitor), or safingol at 37.5 µM (PKC-α inhibitor). APCs and T cells were pretreated for 15 min with 10 µM MJβCD or as indicated. APCs were labeled for 10 min at room temperature with DiIC18(3) and DiIC12(3) (100 ng/mL). APCs were incubated for 90 min at 37°C with either streptavidin or eicosapentaenoic acid (50 or 200 µM). Each cell treatment was performed before APC-T cell coinoculation and terminated with three washes in PBS. None of the inhibitors used was either toxic, as evidenced by the absence of trypan blue and propidium iodide incorporation as well as the lack of

FIGURE 1. Characterization of peptide-specific APC-T cell conjugate formation by flow cytometry, T cell activation and immunological synapse imaging. A. The indicated CMFDA-labeled APCs, loaded or not with 10 µM TT830-843 peptide, were incubated for 1 h with CMTPR-labeled T8.1 cells before flow cytometry. APC-T cell conjugates are detected as CMFDA ‘CMTMR’ events. These data are representative of 10 independent experiments. B. FACS quantitation of APC-T conjugates calculated as the percentage ± SD of APCs forming a conjugate over the total number of APCs gated in R1 (defined in A); 10 independent experiments. C. The APCs were loaded or not with 10 µM TT830-843 peptide and cocultured with T8.1/CD3-YFP cells (a–d) or CMFDA-labeled T8.1 cells (e–h) for 1 h. HLA-DR-binding mAb (L243) was detected with an Alexa Fluor 568-labeled secondary Ab (a–d). Transferrin receptor (e and f) and HLA-1 (g and h) were stained with specific primary Abs detected with an Alexa Fluor-labeled secondary Abs (e–h). Bar, 5 µm; five independent experiments.
phosphatidylycerine externalization (annexin V-FTC; Boehringer Mannheim, Meylan, France), or modified cell surface expression of HLA-DR or CD3 at the selected concentrations.

**Cell transfection and cell sorting**

JVM B lymphocytes (2 × 10⁶) were transfected with 2 μg of pEGFP-actin plasmid DNA using an Amaxa nucleofector (Amaxa, Koeln, Germany). After overnight incubation in RPMI with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics, GFP-positive cells were sorted on a BD FACSVantage SE cell sorter (BD Biosciences); 10⁶ cells were isolated, allowed to recover overnight in complete RPMI, and subjected to conjugate formation for confocal analysis as described below.

**Quantitation of B-T cell conjugates by flow cytometry**

T8.1 cells were incubated with the CellTracker orange CMTMR probe at a final concentration of 5 μM for 30 min at 37°C in serum-free medium, incubated for 30 min in fresh medium, and washed twice in serum-free medium. The APCs (JVM, BM15, Raji, or R2.2.5), either preloaded or not for 2 h with the TT₃₃₀₋₄₄₃ peptide (10 μM), were labeled following the same procedure with the CellTracker green CMFDA. Where indicated, cells were treated as described above. APCs and T8.1 cells (APC:T cell ratio, 1:4) were then resuspended in serum-free medium and allowed to form conjugates for 1 h. In cholesterol recovery experiments, MJβCD-treated JVM cells were incubated for 4 h either in serum-free medium or in complete medium before APC:T cell coinoculation for 1 h. Conjugate formation was measured on a FACSCalibur using the FL-1 gate for the CMTMR probe and the FL-2 gate for the CMFDA probe and was characterized by a doubly stained population. The results are representative of at least 10 independent experiments.

**IL-2 secretion assay**

IL-2 secretion was measured by ELISA. Briefly, 2 × 10⁵ T8.1 lymphocytes were incubated in triplicate wells with 5 × 10⁶ peptide- or non-peptide-loaded APCs in flat-bottom 96-well plates at 37°C. Where indicated, cells were treated as described above. After 16 h, 70 μl of supernatants were harvested and analyzed for murine IL-2 production (R&D Systems, Minneapolis, MN). In cholesterol recovery experiments, JVM cells, MJβCD treated or not, were incubated either in serum-free medium or in complete medium with T8.1 cells for 16 h. Results are expressed as picograms per milliliter ± SD. The results are representative of at least five independent experiments.

**Analysis of conjugate formation by confocal microscopy**

JVM B cells, loaded or not with 10 μM TT₃₃₀₋₄₄₃ peptide for 2 h, and T8.1 cells were cocultured at an APC:T cell ratio of 1:1. Cells were pretreated as indicated with either MJβCD (10 mM, 10 min), jasplakinolide (10 μM, 30 min), calphostin C (50 nM, 30 min), or cytochalasin D (40 μM, 30 min). The JVM cells were then incubated with T8.1 cells (CMTMR labeled or not) for 30 min at 37°C and allowed to adhere on poly-l-lysine-coated cover slips. Cells were fixed in 2.5% paraformaldehyde-PBS, permeabilized with 0.1% Triton X-100, and washed with 50 mM NH₄Cl in PBS. Cells were incubated for 1 h in 1% BSA and labeled for 1 h with primary Abs as indicated, revealed with a secondary fluorescent Ab (Alexa Fluor 568 or Alexa Fluor 954) and/or with Alexa Fluor 488-labeled phalloidin. Slides were washed in PBS and mounted with Vectorshield (Vector Laboratories, Burlingame, CA). For confocal imaging of GFP-actin, transfected APCs, GFP-actin-positive JVM cells were loaded or not with TT₃₃₀₋₄₄₃ peptide, coincubated with CMTMR-labeled T8.1 cells, and adhered on slides as previously described before fixation with paraformaldehyde. Images were acquired with the LSM software on a Zeiss LSM-510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss Axiovert 100M (plan Apochromat ×63, 1.40NA oil immersion objective). Data are representative of at least 100 APC-T cell conjugates observed in 5 independent experiments, and the efficiency of the recruitment of a given protein was quantified by classifying the events in 3 distinct categories (no recruitment/partial recruitment/recruitment). Partial recruitment was scored when there was clear relocalization of one of the target protein to the IS, recruitment was scored when target protein was predominantly concentrated in the IS, and no recruitment indicates absence of relocalization to the IS.

**Biochemical isolation of rafts**

Rafts were isolated by sucrose gradient equilibrium centrifugation after nonionic detergent lysis as described by Xavier et al. (31). After peptide loading and pretreatment or not with MJβCD (as previously described) of the JVM, cells (15 × 10⁶ JVM cells and 15 × 10⁶ T8.1 cells) were cocultured for 1 h at 37°C. Cells were washed twice and lysed in 350 μl of 25 mM MES, 150 mM NaCl (pH 6.5), 1 mM Na₂VO₃, 1 mM PMSF, 10 mM NaN₃, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 4 μg/ml aprotinin (MBS), 0.5% Triton X-100 buffer for 30 min on ice. Lysates were ultracentrifuged after mixing with an equal volume of 85% sucrose (w/v) in MBS in polycarbonate ultracentrifuge tubes (Beckman Instruments, Palo Alto, CA) and overlaid with 2 ml of 35% sucrose and 1 ml of 5% sucrose in MBS. After centrifugation at 200,000 × g for 18 h at 4°C in a SW55Ti rotor (Beckman Instruments), nine fractions of 400 μl were collected from the bottom to the top of the tube with a peristaltic pump. Fractions 6–8 correspond to the raft-containing 5/35% sucrose interface. Each experimental condition was repeated at least four times. In the subsequent experiments, all fractions of each gradient were systematically included in the Western blot experiments to ensure that the data presented account for the total amount of a given protein present in the initial cell lysates.

**Western blot analysis**

Sucrose gradient fractions (15 μl) were incubated in reducing Laemmli sample buffer and denatured at 95°C for 10 min before SDS-PAGE migration. Samples were loaded on a 10% SDS-PAGE gel. Proteins were transferred onto polyvinylidene difluoride membrane (AP Biotech) in Tris-glycine buffer. Membranes were blocked in 3% skim milk in PBS, incubated with the primary Abs for 2 h at room temperature in 1.5% skim milk.
in PBS, and then incubated with HRP-labeled secondary Ab before detection by ECL (AP Biotech). Ganglioside M1 (GM1) was detected with HRP-labeled CT by dot-blot. Each Western blot was performed at least three times for a given protein. Quantification of the bands was conducted using the National Institutes of Health Image-based Scion software (Scion, Frederick, MD).

Results

Characterization of B-T immunological synapse

We have taken a quantitative approach (32) to determine the number of APCs engaged in an APC-T cell interaction. APCs were labeled with CMFDA (green fluorescent vital dye), loaded or not with TT_{830–843} and incubated with T8.1 cells labeled with CMTMR (orange fluorescent vital dye), and conjugate formation was examined after 1 h of incubation. Coincubation of TT_{830–843} peptide-loaded HLA-DR11-expressing B cell lines (JVM and BM15) with the T lymphocytes leads to formation of peptide-specific APC-T lymphocyte conjugates, characterized as a CMFDA/CMTMR− cell population (Fig. 1A). In the absence of peptide, a basal level of conjugate formation is observed (~3%). Peptide-loaded HLA-DR− (RJ2.2.5) or non-DR11 HLA class II− B cell lines (Raji) did not form peptide-dependent conjugates with the T lymphocytes (~1%; Fig. 1, A and B). Optimal conjugate formation was observed after 1 h of APC-T cell interaction with a peptide concentration of 10 μM and an APC:T cell ratio of 1:4 (data not shown). Moreover, both DR11− APC cell lines (JVM and BM15) induce a peptide-specific and dose-dependent IL-2 secretion by the T lymphocytes (Fig. 1C and data not shown). Passive cell-cell diffusion of the vital dyes is ruled out by the lack of a doubly stained cell population in the absence of the specific peptide.

Confocal microscopy revealed the recruitment of MHC class II molecules and CD3 molecules to the immunological synapse formed between TT_{830–843} peptide-loaded APCs and T cells (Fig. 1D and Table I). In the absence of specific peptide, these molecules were evenly distributed at the cell surface of the APCs and the T cells. Two unrelated transmembrane molecules, the TfR and the human MHC class I (HLA-I), served as controls. The distribution of the transferrin receptor was unchanged by peptide-dependent APC-T cell interaction as detected with the anti-TfR mAb that detects both human and mouse isoforms (Fig. 1D). The distribution of HLA-I in the APC was also unchanged by peptide-dependent APC-T cell interaction (Fig. 1D).

Implication of APC cytoskeleton in IS formation and T cell activation

We have observed modifications of the actin cytoskeleton during artificial IS formation between B cells and Ab-coated latex beads (13). We therefore investigated the role of the B cell actin cytoskeleton in peptide-dependent B-T cell interaction. Confocal imaging of polymerized actin (F-actin) distribution, detected by phalloidin, revealed peptide-specific actin recruitment to the site of interaction paralleled by de novo localization of MHC class II molecules to the IS (Fig. 2A and Table I). Because phalloidin staining detects both T and APC F-actin, we took a second approach that allows us to discriminate between APC and T cell actin. JVM cells were transfected with EGFP-actin before conjugate formation followed by confocal imaging. EGFP-actin expressed only by the APC was recruited to the site of B-T cell interaction in a peptide-specific manner (Fig. 2B).

The effect of specific actin inhibitors on Ag-specific B-T cell conjugate formation was determined by flow cytometry. Latrunculin A and jasplakinolide alter actin dynamics by sequestering monomeric G-actin (latrunculin A) or by inhibiting the depolymerization of the F-actin (jasplakinolide). Pretreatment of APCs with either inhibitor before APC-T cell coincubation led to a dose-dependent inhibition of conjugate formation (Fig. 3A), similarly to the pretreatment of T cells with these drugs (data not shown). We next analyzed the role of the APC actin cytoskeleton in T cell activation by pretreating the B cells with latrunculin A, jasplakinolide, or cytochalasin D, which inhibits F-actin formation and elongation. Treatment of the Ag-loaded APCs with these drugs consistently led to a reduction of IL-2 secretion (21.9 up to 42.3% of stimulation). Treatment of the Ag-loaded APCs with these drugs consistently led to a reduction of IL-2 secretion (21.9 up to 42.3% of stimulation), similarly to the pretreatment of T cells with these drugs (data not shown). We next analyzed the role of the APC actin cytoskeleton in T cell activation by pretreating the B cells with latrunculin A, jasplakinolide, or cytochalasin D, which inhibits F-actin formation and elongation. Treatment of the Ag-loaded APCs with these drugs consistently led to a reduction of IL-2 secretion (21.9 up to 42.3% of stimulation). Thus, the integrity of the B cell actin cytoskeleton is essential for efficient IS formation and optimal T cell activation.

IS formation is not dependent on PKC activation in the APC

MHC class II-mediated signaling activates PKC-dependent pathways in both murine and human APCs (1, 2, 4, 33). The effects of a broad range PKC inhibitor (calphostin C) and of two isoform specific inhibitors (safingol and rottlerin) on conjugate formation were assessed by flow cytometry after treating the APCs. PKC activation was not necessary for conjugate formation (Fig. 3C), and pretreatment of the B cells with rottlerin, a PKC-δ specific inhibitor, actually led to a small increase in conjugate formation (Fig. 3C). We next analyzed the consequences of PKC inhibition in the APCs on IL-2 secretion by T cells. The APCs were treated...
with PKC inhibitors as before. IL-2 secretion was decreased by all of the PKC inhibitors, indicating that APC signaling regulates peptide-dependent T cell activation (Fig. 3D). Taken together, these data indicate that optimal T cell activation is regulated both by the B cell actin cytoskeleton and signaling.

APC lipid raft recruitment to the Ag-specific IS is actin dependent and PKC independent

Rafts have been described as cholesterol and glycosphingolipid-enriched regions within the plane of the plasma membrane that function as signaling platforms (34, 35). The APCs were stained with FITC-labeled CT (CT-FITC) which binds specifically to the raft-specific ganglioside GM1. Surface distribution of GM1 in APCs upon peptide-specific conjugate formation revealed that APC lipid rafts are recruited to the IS in a peptide-dependent manner (Fig. 4b and Table I). These data were confirmed by a fluorescent lipid analog (DiIC₆(3)) which preferentially inserts in liquid-ordered (raft-like) domains (12) and which was essentially detected at the APC-T cell interface (Fig. 4f). The fluorescent lipid analog showing affinity for liquid-disordered domains (DiIC₁₂(3)) was excluded from the IS (Fig. 4g). To further investigate the molecular events implicated in the APC lipid raft recruitment to the IS, CT-FITC-labeled APCs were pretreated either with actin inhibitors (jasplakinolide and cytochalasin D) or with a PKC inhibitor (calphostin C) before coincubation. Disruption of the actin cytoskeleton abrogated GM1 relocalization (Fig. 4, c and d, and Table I). In contrast, inhibition of PKC activation in the APC did not alter recruitment of lipid rafts to the APC-T cell interaction site (Fig. 4e and Table I). Altogether, these data revealed that peptide-specific APC raft recruitment to the IS is actin dependent but is independent of PKC activation in the APC.

Ag presentation leads to modification of lipid raft composition

Lipid rafts are generally assimilated to detergent insoluble glycolipid-enriched microdomains (DIGs or glycolipid-enriched membrane microdomains) which can be isolated by sucrose gradient equilibrium centrifugation after nonionic detergent lysis. Raft fractions were prepared from either APCs alone or APC-T cell coincubations, with or without specific peptide. The protein content of the nine collected fractions was analyzed by Western blotting and quantified by densitometry. Fractions 6–8, corresponding to the 5/35% sucrose interface, were enriched in lipid rafts based on their GM1 and flotillin-1 content (Fig. 5). In contrast, the transferrin receptor was typically excluded from rafts and was exclusively present in fractions 1 and 2 corresponding to detergent-soluble material. None of these markers was redistributed upon Ag presentation to T cells. HLA-DR molecules before and after peptide-dependent interaction with the T cells. HLA-DR is constitutively present in the lipid raft fractions in nonstimulated JVM cells, corresponding to 1/110±20% of total HLA-DR. APC-T cell coincubation led to a peptide-dependent enrichment of HLA-DR to the lipid raft fractions (Fig. 5; APC/H11001 T/H11011 26% vs
APC-TT$_{830-843}$ + T ~36%). In contrast, β$_2$-microglobulin was present only in the high density fractions (detergent-soluble material) and was not redistributed by peptide-specific APC-T cell interaction.

The gradients were also screened for cytoskeletal proteins, and signaling partners of the MHC class II molecules and their raft association was quantified (Fig. 5). The immunoblots revealed a

FIGURE 4. Characterization of APC lipid raft distribution by confocal microscopy. JVM cells, loaded or not with 10 μM TT$_{830-843}$ peptide, were labeled with fluorescent lipid raft-specific markers (cholera toxin-FITC, a–e; and DiIC$_{16}$, f) or with a specific fluorescent nonraft lipid analog (DiIC$_{12}$, g). APCs were then treated or not with the actin inhibitors jasplakinolide (Jaspak., c; 10 μM), cytochalasin D (Cyto. D, d; 40 μM) or with the PKC inhibitor calphostin C (Calph. C, e; 50 nM) before coincubation with untreated T8.1 cells and analyzed by confocal microscopy. Differential interference contrast (DIC) images show the interaction area between APCs and T cells. Bar, 10 μM. Data representative of at least five independent experiments.

FIGURE 5. Modification of rafts composition after Ag presentation. A, JVM cells (15·10$^6$), loaded or not with 10 μM TT$_{830-843}$ peptide, were either kept alone or incubated with T8.1 cells (15 × 10$^6$). Cell lysates were fractionated by sucrose gradient equilibrium centrifugation. Equal volumes (15 μl) from each of the nine fractions were immunoblotted to detect the following proteins: MHC class II (HLA-DRα-chain); MHC class I (β$_2$-microglobulin chain; β$_2$-m), the cytoskeletal proteins actin, talin, and tubulin; protein kinase C δ (PKC-δ); the nonraft marker transferrin receptor (TIR); the raft marker flotillin-1. The specific raft marker ganglioside GM1 was detected with HRP-labeled CT by dot-blot. Fractions 1–3 correspond to soluble material, and fractions 6–8 represent raft-enriched microdomains. Data are representative of at least four independent experiments. B, Western blots were scanned and analyzed by densitometry. The relative amount of a given protein localized in raft fractions was measured by calculating the ratio of the density of fractions 6–8 over the sum of densities and expressed as the percentage of raft-associated molecules ± SD (% raft-associated molecules).
peptide-independent recruitment of actin to the lipid raft fractions upon APC-T cell coinubation (from ~11% to ~16% of total actin), which was further increased in peptide-dependent interactions (~25% of total actin). Neither talin nor tubulin were lipid raft associated or redistributed upon APC-T cell interaction. PKC-δ, which has been implicated in MHC class II-mediated signaling (13, 33), was weakly present in lipid rafts of resting APCs (~10%), enriched after Ag-independent APC-T cell coinucbation (~20%) and further recruited by peptide-specific APC-T cell interaction (~26%). Actin and PKC-δ were detected in the top most gradient fractions. This could be due the heterogeneity of the lipid-rich microdomains collectively isolated on the basis of their insolubility in nonionic detergent. Such heterogeneity of lipid rafts has been documented in studies of T lymphocytes (36, 37).

These data indicate that peptide-dependent APC-T cell interaction modifies the composition of lipid rafts by corecruiting HLA-DR, cytoskeletal proteins and signaling proteins.

**APC raft disruption induce peptide-independent conjugate formation**

We next examined the role of APC rafts in IS formation and T cell activation. The cells were treated with MβCD, a cholesterol-sequestering molecule disrupting raft integrity. MβCD treatment of the APCs did not influence the formation of peptide-specific APC-T conjugates (Fig. 6A, d vs b), whereas MβCD treatment of the T cells completely abrogated peptide-specific conjugate formation (Fig. 6A). The lack of toxicity of the MβCD treatment was controlled in parallel by verifying that the cells did not incorporate either trypan blue or propidium iodide and did not externalize phosphatidylserine (data not shown). Moreover, flow cytometry analysis (see Materials and Methods) and confocal microscopy (Fig. 7A) confirmed that MHC class II expression and distribution was unaltered by MβCD treatment.

Remarkably, MβCD treatment of the APCs in the absence of TT<sub>830–843</sub> peptide induced the formation of peptide-independent APC-T conjugates, which we termed pseudoconjugates (Fig. 6A, c). Peptide dependency of conjugate formation was lost, and pseudoconjugates formed to the same extent as when peptide-loaded APCs were used (Fig. 6B, 20.2 ± 1.7% vs 24 ± 1.1%). Pseudoconjugate formation was strictly APC dependent given that they were not induced by cholesterol depletion of T cells (Fig. 6, Aa and B). Moreover, when both cell types were treated with MβCD, only basal level of conjugate formation was observed (~4%), indicating that the consequences of T cell lipid raft disruption override pseudoconjugation formation (Fig. 6B).

Non-HLA-DR11 APCs were subjected to MβCD treatment
and coincubated with T8.1 cells. Cholesterol depletion of either the RJ 2.2.5 cells or the Raji cells did not induce conjugate formation (Fig. 6B), demonstrating the HLA-DR11 restriction of pseudoconjugate formation. Reversibility of the MβCD-induced pseudoconjugate formation was assayed with a cholesterol recovery experiment. Peptide-independent conjugate formation, due to raft disruption (16.1 ± 1.9%), was abrogated by cholesterol repletion of MβCD-treated APCs (2.3 ± 0.7%) and peptide-dependency of conjugate formation was restored (16.8 ± 1.5%) (Fig. 6B).

Moreover, treatment of the JVM cells with increasing concentrations of MβCD revealed that the extent of peptide-independent conjugate formation correlated with the degree of cholesterol depletion (Fig. 6C). Similar results were obtained after MβCD treatment of the HLA-DR11-expressing murine fibroblast cell line L625.7 (Fig. 6D). Thus, this dose-dependent, reversible, and HLA-DR-restricted conjugation is the consequence of lipid raft disruption by cholesterol sequestration. We took a second approach based on the ability of polyunsaturated fatty acids (PUFAs) to

**FIGURE 7.** APC raft implication in T cell activation. A, JVM cells, loaded or not with 10 μM TTβ30–843 peptide, were either left untreated (−MβCD) or treated with 10 mM MβCD for 15 min (+MβCD) before incubation with T cells. Cells were colabeled with Alexa 488-phalloidin and with an anti-HLA-DR mAb (L243) revealed by a secondary Alexa 568 Ab. Bar, 5 μM. B and C, Consequences of MβCD treatment of JVM cells on T cell activation. B, JVM cells, loaded or not with 10 μM TTβ30–843 peptide, were treated with increasing doses of MβCD as indicated and incubated with T8.1 cells. IL-2 production ± SD was measured on cell supernatants. C, Cholesterol recovery assay was performed with JVM cells, prepared as in B, treated or not with 5 and 10 mM MβCD for 15 min, and incubated in either serum-free medium or complete medium (+cholesterol) with T8.1 cells. IL-2 production ± SD was measured on cell supernatants. Data are representative of at least five independent experiments.

**FIGURE 8.** Ag-dependent MHC class II recruitment to APC raft is abrogated by MβCD treatment. JVM cells, loaded or not with 10 μM TTβ30–843 peptide, were treated or not with 10 mM MβCD for 15 min and incubated with T8.1 cells. Cells were lysed and fractionated by sucrose gradient equilibrium centrifugation. The collected fractions were analyzed by Western blot for the proteins MHC class II (HLA-DRα), TIR, flotillin-1, and Lyn. GM1 was detected with HRP-labeled CT by dot-blot. Fractions 1–3 correspond to soluble material, and fractions 6–8 represent raft-enriched microdomains. Data are representative of at least four independent experiments.
disperse certain proteins from within the lipid rafts (38, 39). JVM cells were treated with increasing doses of eicosapentaenoic acid (PUFA) or with stearic, a saturated fatty acid serving as control (38). Similarly to the MβCD-treated APCs, JVM cells treated with eicosapentaenoic acid (200 μM) formed pseudoconjugates with T8.1 cells in the absence of TT83,84 peptide, whereas cells treated with stearic acid did not (Fig. 6f). Taken together, these results indicate that the formation of pseudoconjugates is the consequence of a reversible Ag-independent MHC class II-restricted APC-T cell interaction occurring exclusively on APC raft disruption.

**APC raft implication in T cell activation**

The role of APC rafts in pseudoconjugate formation was analyzed by confocal microscopy. Neither F-actin nor MHC class II relocalization to the IS was observed on pseudoconjugate formation (Fig. 7A, e and f, and Table I). However, peptide-loaded MβCD-treated APCs induced the recruitment of F-actin and MHC class II to the IS (Fig. 7A, g and h, and Table I). Next, we examined the consequences of APC lipid raft disruption on T cell activation. MβCD treatment of the APCs inhibited IL-2 production by T cells in a dose-dependent manner (up to 80.5% of inhibition at 10 mM MβCD) (Fig. 7B). Similarly to untreated and unloaded APCs, MβCD-treated unloaded APCs, which form pseudoconjugates (Fig. 6), did not induce T cell activation (Fig. 7B). Moreover, cholesterol recovery of raft-disrupted peptide-loaded APCs revealed that the inhibition of T cell activation was reversible (Fig. 7C). These data indicate that intact APC rafts are required for efficient T cell activation and that inhibition of T cell activation upon raft disruption is a reversible process. Moreover, the pseudoconjugates formed in the absence of peptide after raft dispersion were unable to activate T cells, which correlates with the inability of the APC to efficiently mobilize MHC class II and F-actin to the IS in the absence of specific peptide.

**MβCD treatment of the APC alters MHC class II recruitment to lipid rafts**

The consequences of MβCD treatment on MHC class II localization in lipid rafts were investigated by biochemical analysis in the context of peptide-independent and peptide-dependent conjugate formation. Detergent-insoluble low density fractions were isolated from APC-T cell coinuciations with or without pretreatment of the APCs with MβCD. Raft localization of flotillin-1 was unchanged after MβCD treatment (Fig. 8) as previously reported (40). Peptide-dependent recruitment of the raft-associated B cell-specific protein Lyn (41, 42) to the lipid raft fraction was quantified by densitometry and revealed a 1.9-fold increase of Lyn in the raft fractions. Upon MβCD treatment, the peptide-dependent recruitment of Lyn to the raft fractions was inhibited (Fig. 8). Under conditions of peptide-specific APC-T cell interaction, HLA-DR molecules are recruited to the lipid raft-enriched fractions 6 and 7, and densitometry analysis revealed an average 1.6-fold increase (n > 4; Fig. 8). MβCD pretreatment of peptide-loaded APCs completely inhibited Ag-dependent MHC class II recruitment to rafts. Moreover, MβCD treatment of unloaded APCs, which induce pseudoconjugate formation, did not modify MHC class II content of the raft fraction in comparison with untreated non-peptide-loaded APCs (Fig. 8). Thus, peptide-independent conjugate formation observed after lipid raft dispersion is intrinsically distinct from a bona fide IS as shown by the differences in lipid raft composition and the absence of T cell activation.

**Discussion**

MHC class II-mediated peptide presentation is tightly regulated by TCR/peptide-MHC affinity, costimulatory molecules as well as the activation state of the T lymphocyte. Recent studies have underlined the importance of IS formation for successful T lymphocyte activation (43–45). The data presented in this report demonstrate that the APC actively participates in controlling peptide-dependent T lymphocyte activation by the intervention of the actin cytoskeleton, PKC signaling, and MHC class II-containing lipid rafts. Disruption of the APC cytoskeleton leads to the inhibition of peptide presentation (23, 46), and the APC actin cytoskeleton is polarized upon peptide-dependent immune synapse formation (25, 47). We show that peptide-dependent relocalization of APC actin to the specific site of APC-T interaction is simultaneous to the relocalization of the MHC class II molecules to the IS. Inhibition of actin polymerization revealed the role of the APC actin cytoskeleton in both the early event of peptide-dependent APC-T cell conjugation and the late event of IL-2 secretion. Moreover, the assay used to determine conjugate formation does not take into account weak and/or transient cell-cell interactions. This could explain that the consequences of actin disruption on conjugate formation are not strictly matching the IL-2 secretion data. Peptide-dependent enrichment of lipid rafts in the APC-T cell interaction zone has been described (12), and we now show that this event is controlled by the APC cytoskeleton, in agreement with the recent data of Gordy et al. (26).

The PKC family of serine/threonine kinases are signaling partners of the MHC class II molecules (1, 2, 33). We demonstrate that PKC activation in the APC contributes to T lymphocyte activation, although early events monitored by APC-T cell conjugate formation were independent of PKC activation. The immunoblots show that PKC-δ was most strongly recruited to the lipid raft fraction by peptide-dependent APC-T cell interactions which is consistent with our previous observations demonstrating that MHC class II engagement by Ab-coated beads leads to recruitment and activation of PKC within the lipid raft fraction (3, 13). Taken together, peptide-dependent APC-T cell conjugate formation requires integrity of the APC actin cytoskeleton and precedes PKC-dependent signaling in the APC, which can ultimately regulate the T lymphocyte response. Thus, the lack of requirement for PKC signaling in APC-T conjugate formation would allow T cell sampling of MHC class II molecules without consequences for the APC until T cell recognition of specific peptide bound to MHC class II takes place.

A recent study proposed that the compartmentalization of the MHC class II molecules in lipid rafts is important for Ag presentation when limiting doses of peptide Ag are provided (16). However, because the APCs were systematically fixed before coculture with the responding T cells, this study excluded the potential contribution of dynamic events in the APC. The current study examines the dynamic role of the APC in T lymphocyte activation. MHC class II-enriched lipid microdomains mediate signaling as ligand-induced relocalization of MHC class II into lipid rafts is required for activation of Src kinases and for PKC recruitment and activation (3, 15).

Lipid rafts could serve as platforms for the delivery of peptide-specific MHC class II molecules to the IS (12). The peptide-dependent recruitment of lipid rafts and MHC class II molecules to the IS and the MHC class II enrichment in lipid raft fractions after peptide-specific APC-T cell interaction are consistent with this concept. Taken together, the data suggest concomitant relocalization of lipid rafts and MHC class II molecules to the peptide-dependent IS followed by PKC signaling in the APC leading to
optimal T lymphocyte activation. Our data support the role of MHC class II molecules as accessory ligands (27), because it is unlikely that the recruitment of MHC class II molecules into the lipid rafts exclusively results from relocalization of specific peptide-loaded molecules.

We demonstrate a novel function for the APC lipid rafts in preventing peptide-independent MHC class II-restricted APC-T cell interaction and reveal distinct roles for APC vs T cell lipid rafts. T cell lipid rafts are required for peptide-dependent conjugate formation, whereas APC rafts are dispensable. Therefore peptide-dependent APC-T interaction is independent of a lipid raft localization of MHC class II molecules. In contrast, the key role for APC lipid rafts was shown by the formation of HLA-DR-restricted peptide-independent APC-T cell conjugates after disruption of cholesterol-dependent APC lipid rafts. Cholesterol recovery restored peptide dependency for APC-T conjugate formation. Direct observation of pseudoconjugates showed that neither actin nor MHC peptide dependency for APC-T conjugate formation. Despite HLA-DR-restricted peptide-independent conjugation, IL-2 secretion was not induced and pseudoconjugates are therefore nonfunctional. A second approach to lipid raft dispersion was taken using PUFAs, which have been shown to disperse acylated proteins typically localized in the inner leaflet of lipid rafts (38, 39). Similar to the consequences of cholesterol depletion, PUFAs treatment led to the formation of pseudoconjugates.

How APC lipid rafts play a dual role in enhancing peptide-dependent T lymphocyte activation and in impeding peptide-independent APC-T cell interaction remains to be determined. The initial lipid raft localization of MHC class II molecules could be a crucial point under physiological conditions. Buatios et al. (17) showed that de novo raft-localized peptide-MHC class II molecules complexes form a signaling complex leading to a Th1 response, whereas dispersion of MHC class II molecules from the lipid rafts resulted in a predominantly Th2 response. In addition to the impact of the organization of the MHC class II molecules on T lymphocyte activation, APC lipid rafts could impose stringency by segregating MHC class II molecules. One hypothesis is that lipid raft disruption alters their proximity to adhesion molecules, resulting in the formation of peptide-independent conjugates in the course of T cell sampling of the peptides displayed by the APC. Such peptide-independent TCR/MHC class II interactions have been proposed to prime the T lymphocyte for activation with specific peptide (27). Finally, the control mechanism of MHC class II molecules localization to lipid rafts is unknown.

The pathological implications of peptide-independent conjugate formation remain to be considered. These could correspond to peptide-independent APC-T cell conjugates involving nonrafted MHC class II molecules associated with low affinity self peptides. Mature IS formation includes peptide-dependent interactions between costimulatory molecules which would be missing from the pseudoconjugate. Such aberrant interactions could result either in T cell tolerance or, given the data of Buatios et al. (17), in a predominantly Th2 response.

Finally, the clinical relevance of modifying lipid raft dynamics in immune cells should therefore be considered in approaches controlling cholesterol metabolism such as statin treatments or PUFAs which have been suggested to modulate the immune response (39, 48–50).

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References


