Recombinant Anti-CD4 Antibody 13B8.2 Blocks Membrane-Proximal Events by Excluding the Zap70 Molecule and Downstream Targets SLP-76, PLC γ1, and Vav-1 from the CD4-Segregated Brij 98 Detergent-Resistant Raft Domains

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Recombinant Anti-CD4 Antibody 13B8.2 Blocks Membrane-Proximal Events by Excluding the Zap70 Molecule and Downstream Targets SLP-76, PLCγ1, and Vav-1 from the CD4-Segregated Brij 98 Detergent-Resistant Raft Domains

Myriam Chentouf,* Soufiane Ghannam,* Cédric Bès,2* Samuel Troade,* Martine Cérutti,† and Thierry Chardès3*

The biological effects of rIgG1 13B8.2, directed against the CDR3-like loop on the D1 domain of CD4, are partly due to signals that prevent NF-κB nuclear translocation, but the precise mechanisms of action, particularly at the level of membrane proximal signaling, remain obscure. We support the hypothesis that rIgG1 13B8.2 acts by interfering with the spatiotemporal distribution of signaling or receptor molecules inside membrane rafts. Upon cross-linking of Jurkat T lymphocytes, rIgG1 13B8.2 was found to induce an accumulation/retention of the CD4 molecule inside polyoxyethylene-20 ether Brij 98 detergent-resistant membranes at 37°C, together with recruitment of TCR, CD3, and CD4 molecules. Analysis of key upstream events such as Zap70 phosphorylation showed that modulation of Tyr292 and Tyr319 phosphorylation occurred concomitantly with 13B8.2-induced Zap70 exclusion from the membrane rafts. 13B8.2-induced differential raft partitioning was epitope, cholesterol, and actin dependent but did not require Ab hyper-cross-linking. Fluorescence confocal imaging confirmed the spatiotemporal segregation of the CD4 complex inside rafts and concomitant Zap70 exclusion, which occurred within 10–30 s following rIgG1 13B8.2 ligation, reached a plateau at 1 min, and persisted until the end of the 1-h experiment. The differential spatiotemporal partitioning between the CD4 receptor and the Zap70-signaling kinase inside membrane rafts interrupts the proximal signal cross-talk leading to subsequent NF-κB nuclear translocation and explains how baculovirus-expressed CD4-CDR3-like-specific rIgG1 13B8.2 acts to induce its biological effects. The Journal of Immunology, 2007, 179: 409–420.

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effects of new biotechnological drugs such as therapeutic Abs are linked to their ability to quantitatively and qualitatively modulate the clustering of target membrane receptors, signaling kinases, apoptosis-, proliferation-, and cytotoxicity-involved molecules, and lipids into these raft domains. Concerning the therapeutic Ab anti-CD20 rituximab, but also other anti-CD20 Abs, there is a correlation between the ability of these Abs to elicit CD20 translocation into Triton DRM (4) and their capacity to initiate kinase-dependent calcium mobilization, apoptosis induction (5), and complement-dependent cytolysis (6) of B lymphoma cells. Rituximab also induces ceramide accumulation together with CD20 translocation into Triton DRM, leading to activation of the ceramide-triggered signaling pathway, which mediates proliferation inhibition of B lymphoma cells (7). Lipids from DRM such as ceramide or cholesterol play a major role in the modulation of apoptosis (8, 9) and cell growth (7) but also influence the outcome of HIV infection (10). In this case, the virus hijacks raft DRM for cell entry and further budding, and the CD4 molecule, which is the primary receptor for virus anchoring, is predominantly localized in the DRM.

CD4 is a 55-kDa glycoprotein expressed on ~60% of peripheral blood T lymphocytes; it activates T lymphocytes by binding to the nonpolymorphic region of the MHC class II Ags expressed on the surface of APCs. Engagement of CD4 by therapeutic mAbs triggers various effects actually exploited in cancerology (11), HIV infection (12), and tolerance induction (13). CD4 epitope recognition by Abs has not been taken into consideration in clinical studies, except in the case of HIV infection. Nevertheless, gene-activating potential such as NF-AT activation, Ras/protein kinase C (PKC) pathway inhibition, and proapoptotic potential of anti-CD4 mAbs has been associated with recognition of different CD4 epitopes (14, 15). Biological effects (6) and, above all, different abilities to relocate CD20 in DRM (16) are also shown to be related to epitope recognition of anti-CD20 Abs. We evaluated a recombinant Ab (17, 18) derived from the 13B8.2 murine anti-CD4 mAb, directed against the CDR3-like loop of CD4; this loop has not been fully exploited as a target by clinical Abs, mainly directed against other CD4 regions (11–13, 19, 20). This baculovirus-expressed recombinant Ab (rIGG1) inhibits HIV replication at a post-gpl20-binding step (17, 18, 21) and induces complement-mediated lysis, Ab-dependent cell cytotoxicity, and growth arrest of T lymphoma cells (22). The biological effects of rLG1 13B8.2 are partly due to signals that prevent NF-κB nuclear translocation (23); ERK activation (24); and NF-AT, NF-κB, and AP-1 binding to the IL-2 gene promoter (25). However, precise mechanisms of action at the early level of membrane proximal signaling to explain how baculovirus-expressed CD4-CDR3-like-specific rLG1 13B8.2 acts to induce its biological effects remain obscure. We support the hypothesis that rLG1 13B8.2 acts by interfering with the distribution of signaling or receptor molecules inside DRM platforms.

With this perspective in mind, we treated target Jurkat lymphoma cells with rLG1 13B8.2 and demonstrated for the first time that rLG1 13B8.2 acts by inducing an accumulation/retention of the CD4 molecule inside polyoxyethylene-20 ether Brij 98 DRM extracted at 37°C together with exclusion of the Zap70 kinase and its downstream targets Src homology 2-domain-containing leukocyte protein of 76 kDa (SLP-76), phospholipase Cγ1 (PLCγ1), and p95/Vav-1. These results suggest that analysis of the lipid-protein rheostat inside Brij 98 DRM following treatment with biotechnological drugs could explain how these molecules induce their biological effects.

<table>
<thead>
<tr>
<th>Cells</th>
<th>rLG1</th>
<th>13B8.2 treatment</th>
<th>Raft</th>
<th>Non-Raft</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>A2.01 CD4⁺</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Jurkat</td>
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<td>Jurkat</td>
<td>A2.01 CD4⁺</td>
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FIGURE 1. Dot-blot characterization of Brij 98 DRM fractions from lymphocytes untreated or previously cross-linked by the baculovirus-expressed rLG1, anti-CD4 Ab 13B8.2. A2.01 CD4⁺ cells and Jurkat and A2.01 CD4⁺ cells were lysed with Brij 98 detergent at 37°C and segregated into fractions by sucrose density gradient. Fraction 12 represents the bottom liquid fraction of the tube. Membrane rafts are in fractions 4–6 as they contain ganglioside GM1. Nonraft fractions 11 and 12 were identified by binding of the transferrin receptor CD71. The CD4 receptor was detected by a goat polyclonal anti-human CD4 Ab. Results are representative of at least three independent experiments.

Materials and Methods

Cells

Jurkat, Sup-T1, and A2.01-CD4⁺ and -CD4⁻ T cells were grown in RPMI 1640 (Cambrex) supplemented with 10% heat-inactivated FCS (PAAL laboratories), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Sigma-Aldrich), and 2 mM glucose. Cells were provided by L. Briant (Centre National de la Recherche Scientifique (CNRS) Unité Mixte de Recherche (UMR) 5236, Montpellier, France). Primary T lymphocytes were purified by Histopaque (Sigma-Aldrich) density centrifugation of blood samples from healthy donors obtained at the Etablissement Français du Sang (Montpellier, France).

Abs and reagents

Anti-CDR3-like rLG1, and Fab (Fab) 13B8.2 Abs were expressed in the baculovirus/insect cell system and purified from culture supernatant by protein A and G immunoaffinity chromatography, respectively, as previously described (17, 18, 26). Other anti-CD4 mAbs used were directed against the CDR2-like loop (ST4; Sanofi-Aventis), the CDR3-like loop (ST40; Sanofi-Aventis), the D2 domain (BF5; Diaclone), and the D4 domain (OKT4; Ortho Biotech). Chinese hamster ovary-expressed anti-carcinoembryonic Ag recombinant control IgG1 was a gift from C. Germain (Institut National de la Santé et de la Recherche Médicale Unité 860, Montpellier, France). Goat polyclonal anti-CD4 Ab was obtained from R&D Systems. Anti-human TCR mAb (IP26) was purchased from BD Biosciences. The Alexa Fluor 488- and the peroxidase-conjugated cholera toxin B subunits were purchased from Molecular Probes; Cy3- and Cy5-conjugated Abs came from Jackson ImmunoResearch Laboratories. Rabbit polyclonal Abs to CD71, Syk p70, p56 Lck, Lyn, Zap70, and mAbs directed...
against nonphosphorylated p16 (6B10.2) and the phosphorylated p21/p23 CD3ζ chain (CA415.9A) were obtained from Santa Cruz Biotechnology. Rabbit polyclonal Abs directed against Tyr319- and Tyr493-phosphorylated Zap70, Vav-1, PLCγ1, and PKCζ were purchased from Cell Signaling; rabbit polyclonal Abs to Tyr292-phosphorylated Zap70 and to SLP-76 were obtained from BD Biosciences. Rabbit anti-linker for activation of T cells (LAT) and anti-LIME Abs were obtained from Upstate and Abcam, respectively. Csk-binding protein/phosphoprotein associated with glycosphingolipid (Cbp/PAG)-specific mAb was purchased from Alexis Biochemicals. Anti-phosphotyrosine mAb 4G10 was a gift from C. Gongora (Centre Régional de Lutte contre le Cancer Val d’Aurelle, Montpellier, France). Peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG (Sigma-Aldrich) were used as secondary Abs when necessary. Unconjugated polyclonal anti-human IgG Ab, polyoxyethylene-20 ether detergent Brij 98, methyl-β-cyclodextrin (MβCD), and cytochalasin D were purchased from Sigma-Aldrich. Soluble PP2 4-amino-5(4-chlorophenyl)-7-(tert-butyl)pyrazolo[3,4-d]pyrimidine was from Merck Biosciences. Baculovirus-expressed soluble recombinant gp120 from HIV was a gift from F. Veas (Institut de Recherche pour le Développement, Unité de Recherche 34, Montpellier, France) (27).

Lymphocyte treatment by Abs and Brij 98-DRM isolation

A total of $1 \times 10^6$ T cells was treated for 10 min with 20 μg/ml rIgG1 13B8.2 or rFab 13B8.2 or other anti-CD4 Abs diluted in complete medium.

### FIGURE 2.

rIgG1 anti-CD4 Ab 13B8.2 cross-linking of Jurkat T cells induced the redistribution of CD4 into the membrane rafts and the exclusion of the Zap70 and downstream targets SLP-76, Vav-1, and PLCγ1. A. Cells were cross-linked in solution with rIgG1 13B8.2 or with an irrelevant control IgG1 Ab, and sucrose gradient fractions were Western blotted with goat-polyclonal anti-CD4 Ab, anti-TCR mAb, anti-CD3ζ, and anti-phosphorylated CD3ζ mAbs; rabbit polyclonal Abs directed against CD71, Zap70, p56 Lck, Lyn, and Syk p70; and anti-LAT, -Cbp/PAG, -LIME, -SLP-76, -Vav-1, -PLCγ1, and -PKCζ Abs. Data are representative of three experiments. B. Raft fraction 4, nonraft fraction 12, and insoluble sediments from control IgG1- or 13B8.2-treated cells were immunoblotted with Abs directed to CD4, Zap70, SLP-76, Vav-1, and PLCγ1.

<table>
<thead>
<tr>
<th></th>
<th>Control IgG1 treatment</th>
<th>Anti-CD4 rIgG1, 13B8.2 treatment</th>
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<tr>
<td></td>
<td>4</td>
<td>12</td>
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<td>CD4</td>
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<td>Vav-1</td>
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In some experiments, rIgG1 13B8.2-pretreated cells were further stimulated with a mixture of anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) mAbs for 30 min. In the gp120 experiment, cells were preincubated for 30 min with recombinant gp120 (10 μg/ml) before anti-CD4 treatment. For the hyper-cross-linking experiment of rIgG1 13B8.2, the cells were further incubated for 20 min at room temperature with rabbit anti-human IgG polyclonal Ab. After washing in 160 mM PBS (pH 7.4), cell lysis was performed at 37°C for 30 min in 1% Brij 98 detergent diluted in TNE buffer (25 mM Tris-HCl (pH 7.5); 150 mM NaCl; and 5 mM EDTA) containing 1 mg/ml enzyme inhibitors (complete EDTA-free mixture of antiproteases; Roche). Cell lysates were mixed with an equal volume of 80% sucrose in TNE plus inhibitors, overlaid with 6.5 ml of 30% and 3.5 ml of 5% sucrose for 2 h at room temperature. The membranes were then washed three times with PBS-T and revelation was performed for 1 h at room temperature with secondary peroxidase-conjugated anti-rabbit Ab (1/3000 dilution), anti-mouse Ab (1/1000), or anti-goat Ab (1/1000), as appropriate. After three washes, all Western blots were developed using the ECL Western Blotting Detection kit (Amersham Pharmacia Biotech).

Confocal microscopy

A total of 1 to 5 × 10^5 cells, treated with 13B8.2 Ab for 10 min, was washed and incubated in PBS for various times before fixing in 3% paraformaldehyde in PBS for 15 min at room temperature and then permeabilized with 0.1% Triton X-100 for 5 min at room temperature. After washing in PBS containing 2% BSA (PBS-BSA), the cells were incubated for 1 h at room temperature with the Alexa Fluor 488-conjugated cholera toxin B subunit, anti-CD4 rIgG1 13B8.2, or polyclonal anti-Zap70 Ab diluted in PBS-BSA and then washed. For CD4 and Zap70 detection, the cells were incubated for 45 min at room temperature in the dark with secondary Cy5- and Cy3-conjugated Abs, respectively. After washing, bound cells were settled onto polylysine-coated slides and analyzed with a Zeiss LSM 510 laser scan confocal microscope (P. Travo, Montpellier RIO Imaging, CNRS UMR 5237, Montpellier, France) for visualization. Colocalization assays were analyzed by excitation of the corresponding fluorochromes on the same section of the permeabilized or nonpermeabilized samples. Negative controls lacking the primary Ab showed no staining. Percentage of colocalization was obtained by using Imaris software (Bitplane).

Results

Recombinant anti-CD4 Ab 13B8.2 cross-linking of Jurkat T cells redistributes CD4 into the membrane rafts but excludes the Zap70 molecule

Dot-blot analysis of ganglioside M1-enriched Brij 98 DRM

A nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech) was spotted with 2 μg from each gradient fraction. The membrane was blocked for 1 h at room temperature with 5% semiskimmed milk in PBS containing 0.1% Tween 20 (PBS-T). Ganglioside M1 (GM1) detection in Brij 98 DRM was performed by adding a 1/1000 solution of peroxidase-conjugated cholera toxin B subunit and incubating for 1 h at room temperature. Nonraft fractions were detected following a 1-h incubation of a 1/1000 solution of anti-CD71 monoclonal Ab. CD4 was probed by using a 1/3000 solution of polyclonal goat anti-CD4 Ab, and sucrose gradient fractions were immunoblotted (A) with a goat polyclonal anti-CD4 Ab or (B) with a rabbit polyclonal Ab directed against Zap70.

Confocal microscopy

A total of 1 to 5 × 10^5 cells, treated with 13B8.2 Ab for 10 min, was washed and incubated in PBS for various times before fixing in 3% paraformaldehyde in PBS for 15 min at room temperature and then permeabilized with 0.1% Triton X-100 for 5 min at room temperature. The membranes were then washed three times with PBS-T and revelation was performed for 1 h at room temperature with secondary peroxidase-conjugated anti-rabbit Ab (1/3000 dilution), anti-mouse Ab (1/1000), or anti-goat Ab (1/1000), as appropriate. After three washes, all Western blots were developed using the ECL Western Blotting Detection kit (Amersham Pharmacia Biotech).

 SDS-PAGE and Western blot

Forty micrograms of protein lysate from each gradient fraction were separated by 12% SDS-PAGE under reducing conditions and electrophoretically transferred to Immobilon P (Bio-Rad). The membranes were blocked with 5% semiskimmed milk in PBS-T for 1 h at 37°C. After washing in PBS-T, membranes were incubated with an appropriate dilution of Abs directed against CD4, CD71, TCR, p16 CD3ζ chain and its phosphorylated forms; Syk p70, p56 Lck, and Lyn kinases; Zap70 and its phosphorylated forms; LAT, SLP-76, LIME, and Cbp/PAG adaptors; and Vav-1, PLCγ1, and PKCδ for 2 h at room temperature. The membranes were then washed with PBS-T and revelation was performed for 1 h at room temperature with secondary peroxidase-conjugated anti-rabbit Ab (1/3000 dilution), anti-mouse Ab (1/1000), or anti-goat Ab (1/1000), as appropriate. After three washes, all Western blots were developed using the ECL Western Blotting Detection kit (Amersham Pharmacia Biotech).

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Results

Recombinant anti-CD4 Ab 13B8.2 cross-linking of Jurkat T cells redistributes CD4 into the membrane rafts but excludes the Zap70 molecule

Dot-blot preliminary experiments were performed on fractions extracted at 37°C with the polyoxyethylene mild detergent Brij 98 from T cell lines A2.01 CD4−, Jurkat CD4+, and A2.01 CD4+. As shown in Fig. 1, GM1 detection, which identifies DRM raft fractions, was mainly observed in fractions 4–6, whereas CD71 binding was evidenced in fractions 11 and 12, identifying nonraft fractions. This pattern was obtained with the three cell lines tested, but with slightly different intensities. Without 13B8.2 Ab cross-linking, the CD4 molecule was demonstrated either inside the Brij 98 DRM or in the nonraft fraction from Jurkat CD4+ and A2.01 CD4+ cells but was not detected in fractions from A2.01 CD4− cells, thus demonstrating the binding specificity (Fig. 1). Baculovirus-expressed CD4-CDR3-like specific rIgG1 13B8.2 treatment completely redistributed the CD4 molecule inside the Brij 98
DRM, whereas no CD4 binding was observed in fractions extracted from A2.01 CD4$^+$ cells.

In an attempt to link 13B8.2-dependent CD4 translocation inside Brij 98 DRM with the downstream signaling pathways, we examined the distribution of CD4, TCR, CD3$^+$ chain, various kinases, and adaptor proteins among raft/nonraft fractions following Ab treatment (Fig. 2A). First, raft and nonraft fractions were similarly identified as shown in Fig. 1 by GM1 and CD71 detection, and raft compartmentalization of CD4 upon rIgG1 13B8.2 treatment was confirmed (Fig. 2A). Western blot analysis revealed that rIgG1 13B8.2 cross-linking of Jurkat T cells completely redistributed the Zap70 kinase and downstream targets SLP-76, PLC$^+$1, and Vav-1 in the nonraft fraction. These molecules were not detected in the insoluble sediments (Fig. 2B), neither following control IgG1 nor following anti-CD4 rIgG 1 13B8.2 treatment. In addition, CD4/Zap70 redistribution was also observed when A2.01 CD4$^+$ and Sup-T1 cell lines and when primary peripheral T lymphocytes from a healthy donor were treated with rlgG1 13B8.2 (Fig. 3).

In addition, TCR, CD3$^+$ chain p16, other kinases such as p56 Lck, Lyn, and Syk p70, and also adaptor proteins such as LAT, Cbp/PAG, and PKC$^+$ were located inside Brij 98 DRM upon rlgG1 13B8.2 treatment (Fig. 2A) as did CD4. In contrast, equal partitioning between raft/nonraft fractions was observed for these molecules upon treatment with a control IgG1 Ab, except for LIME, which remained in the raft fraction of cells either treated with the control Ab or with Ab 13B8.2. Phospho-CD3$^+$ chain, only detected in the nonraft fractions upon Jurkat treatment with control IgG1 Ab, was not evidenced following cell cross-linking by anti-CD4 rlgG1 13B8.2. Taken together, these results indicate that the baculovirus-expressed CDR3-like specific rlgG1 13B8.2 acts by excluding the Zap70 and downstream molecules SLP-76, PLC$^+$1, and Vav-1 from the CD4-segregated raft machinery.

FIGURE 4. Displacement of Zap70 from the 13B8.2-induced CD4-segregated raft signaling platform from native Jurkat cells occurred concomitantly with its modulation of phosphorylation. A, Cells were treated with control IgG1 Ab or rlgG1 anti-CD4 Ab 13B8.2 alone. Sucrose gradient fractions were immunoblotted with rabbit polyclonal Abs directed against Zap70 and phosphorylated-Tyr$^{292}$, -Tyr$^{319}$, or -Tyr$^{493}$ Zap70. Data are representative of three experiments. B, Cells were pretreated with control IgG1 Ab or rlgG1 anti-CD4 Ab 13B8.2 and stimulated with CD3/CD28 mAbs. C, Whole cell lysates of untreated or Ab-treated cells were immunoblotted with anti-phosphotyrosine mAb 4G10.
Displacement of Zap70 from the 13B8.2-induced CD4-segregated raft signaling platform occurred concomitantly with its modulation of phosphorylation.

Early phosphorylation events on tyrosine residues in the Zap70 kinase are pivotal for its enzymatic activation and subsequent stimulation of downstream signaling pathway. Together with the Ab-induced Zap70 exclusion from the CD4-segregated Brij 98 DRM, we hypothesized that the phosphorylation profile of the kinase was modulated upon rIgG1 13B8.2 cross-linking. Because numerous tyrosine phosphorylation sites can be affected on Zap70, we focused on the three most critical residues, two of them phospho-Tyr319 and phospho-Tyr493, being known to favor CD3/TCR-mediated immune response, and the third, phospho-Tyr292, known to inhibit the immune response by targeting Zap70 to Cbl-mediated ubiquitination/degradation. As demonstrated in Fig. 4A, 13B8.2 cross-linking of Jurkat T cells led to phosphorylation of the Tyr292 residue in the Zap70 kinase redistributed in the nonraft fraction. In contrast, no Tyr292-specific phosphorylation was evidenced upon binding of a control IgG1 Ab. Simultaneously, activating phosphorylation of Tyr319 on the Zap70 kinase was inhibited upon rIgG1 13B8.2 cross-linking. Finally, no phosphorylation of activating Tyr493 occurred either following control IgG1 treatment or upon anti-CD4 13B8.2 binding (Fig. 4A). These results suggest that the baculovirus-expressed CDR3-like specific rIgG1 13B8.2 acts by favoring inhibiting phosphorylation of Tyr292 and blocking activating phosphorylation of Tyr319 together while excluding the Zap70 molecule from the CD4-segregated raft machinery of Jurkat T cells.

To assess whether Zap70 compartmentalization and its phosphorylation pattern were also affected when both Ag receptor and CDR3-like loop are engaged, we pretreated Jurkat T cells with rIgG1 13B8.2 before stimulation with the anti-CD3 and anti-CD28 mAbs. As shown in Fig. 4B, Zap70 was evidenced in the nonraft fraction upon CD3/CD28 stimulation alone together with the induction of activating phosphorylation of Tyr319 and Tyr493 whereas inhibiting phosphorylation of Tyr292 was not observed. Activating phosphorylation of Tyr319, observed upon CD3/CD28 stimulation, was affected by a pretreatment with 13B8.2 Ab (Fig. 4B). By separating whole lysates from CD3/CD28-stimulated cells pretreated or not with rIgG1 13B8.2 on 8% PAGE-SDS, we further assessed tyrosine phosphorylation of multiple proteins, essentially located in a molecular mass range for Zap70, SLP-76, and Vav-1.

FIGURE 5. rIgG1 13B8.2 treatment of gp120-cross-linked Jurkat T cells did not altered CD4/Zap70 differential partitioning but differently affects gp120-induced Zap70 phosphorylation. Cells were (A) left untreated or (B) incubated with gp120 or (C) preincubated with gp120 and treated by rIgG1 13B8.2. Cells were further lysed with Brij 98 detergent, and the fractions were separated by sucrose density centrifugation. Following PAGE-SDS and immunoblotting, CD4 binding was assessed by probing with a goat polyclonal anti-CD4 Ab, and Zap70 was visualized by a specific rabbit polyclonal Ab. Fractions from treated Jurkat cells were detected by rabbit polyclonal Abs directed against phosphorylated-Tyr292, -Tyr319, or -Tyr493 Zap70. Data are representative of three experiments.

FIGURE 6. rIgG1 13B8.2-induced CD4/Zap70 partitioning is epitope dependent but does not need Ab hyper-cross-linking. Before Brij 98 DRM preparation, Jurkat T cells were treated (A) with control IgG1 Ab or anti-CD4 Abs rIgG1, 13B8.2, ST40, ST4, BF5, and OKT4, which are directed against various epitopes on the CD4 molecule; or (B) with rIgG1 13B8.2 followed by a rabbit polyclonal anti-human IgG Ab for Ab hyper-cross-linking or rFab 13B8.2 alone. CD4 detection by Western blot was performed by using a goat polyclonal anti-CD4 Ab, and Zap70 binding was visualized by a specific rabbit polyclonal Ab.
13B8.2-induced CD4/Zap70 differential partitioning is epitope dependent but does not require Ab hyper-cross-linking

Therapeutic anti-CD20 Abs showed a marked difference in their ability to translocate CD20 inside Triton DRM, depending on epitope recognition (6, 16), such an observation being correlated with their capacity to induce biological effects (6, 16). We used five anti-CD4 Abs, directed against various epitopes on the CD4 molecule, to assess whether Ab-induced CD4/Zap70 segregation inside/outside Brij 98 DRM is epitope dependent (Fig. 6A). As demonstrated, cross-linking of Jurkat T cells by Abs 13B8.2 and ST40, directed against the CDR3-like loop on the D1 domain of CD4, induced CD4 partitioning inside raft fractions, whereas control IgG1 did not. In contrast, other anti-CD4 Abs tested, either directed against the CDR2-like loop on the D1 domain or which bound to the D2 or D4 domain of CD4, did not modify CD4 raft/nonraft distribution, similarly to the control Ab (Fig. 6A). Anti-CDR3-like Ab 13B8.2 translocated Zap70 inside the nonraft fraction, whereas neither of the anti-CD4 Abs, directed against other epitopes on the CD4 molecule, nor a control Ab affected Zap70 raft/nonraft distribution (Fig. 6A).

In addition, the need for Ab hyper-cross-linking for the efficiency of therapeutic Abs is a subject of debate (6, 16). rIgG1 13B8.2 hyper-cross-linking by secondary anti-human IgG did not enhance CD4/Zap70 raft segregation from T cells, as confirmed by the fact that rFab 13B8.2 similarly induced complete CD4 translocation inside Brij 98 DRM and subsequent Zap70 partitioning inside the nonraft fraction (Fig. 6B). These results indicate that the effect of the baculovirus-expressed CDR3-like specific rIgG1 13B8.2 on CD4 segregation into rafts is dependent on the CDR3-like epitope on the D1 domain of CD4 but does not require hyper-cross-linking, with formation of an Ab network.

Cholesterol integrity and actin polymerization are critical for 13B8.2-induced CD4 segregation inside Brij 98 DRM

Lipids from DRM such as cholesterol are critical for raft integrity and can mediate Ab-induced receptor translocation into rafts, leading to the modulation of various biological effects (7–10). We examined whether 13B8.2-induced CD4 raft partitioning is dependent on raft integrity by depleting cholesterol with MβCD. As evidenced in Fig. 7A, pretreatment of Jurkat T cells with MβCD before rIgG1 13B8.2 treatment, with presumed disruption of Brij 98 DRM, completely redistributed CD4 inside the nonraft fraction, whereas Ab cross-linking, without preincubation with MβCD, confirmed CD4 raft compartmentalization (Fig. 7A). The formation of an immunological synapse requires actin-dependent cytoskeleton restructuring, which can be stopped by an inhibitor of F-actin polymerization, cytochalasin D. As shown in Fig. 7A, cytochalasin D pretreatment affected 13B8.2-induced CD4 raft partitioning because CD4 was located in both the raft and the nonraft fractions, as observed in the absence of treatment (Fig. 5A) or upon treatment with an irrelevant control Ab (Figs. 2A and 3A)). In addition, an altered phosphorylation pattern induced by PP2 preincubation of Jurkat T cells did impact neither on the CD4/Zap70 distribution in raft vs nonraft fraction of untreated cells (Fig. 7B), nor on the CD4/Zap70 differential partitioning induced by 13B8.2 treatment (Fig. 7C), suggesting that phosphorylation events do not promote CD4/
Zap70 compartmentalization. In these experiments, GM1 and CD71 detection showed a similar binding pattern, in terms of intensity. Thus, we suggest that the CD4/Zap70 raft partitioning induced by the baculovirus-expressed CDR3-like specific rIgG1 13B8.2 requires raft integrity, is dependent on actin polymerization, but occurs independently of kinase activity.

**FIGURE 8.** Confocal microscopy confirmed the CD4/Zap70 differential partitioning into raft vs nonraft fractions upon cross-linking with rIgG1-13B8.2. Jurkat cells were treated with irrelevant IgG1 Ab or baculovirus-expressed rIgG1 13B8.2, fixed with paraformaldehyde, permeabilized, and then stained with Alexa Fluor 488-conjugated cholera toxin B subunit, which binds to GM1-enriched DRM (A and B), or with anti-CD4 (A), or anti-Zap70 polyclonal Abs (B), followed by appropriate Cy3- and Cy5-conjugates. C, The kinetics of CD4/Zap70 partitioning were observed by confocal microscopy using an experimental procedure similar to that described above and the GM1/CD4 and GM1/Zap70 clustering was quantified by using Imaris software.
Confocal microscopy visualization confirms CD4/Zap70 differential partitioning which occurs 10–30 s after rIgG1 13B8.2 cross-linking, reaches a plateau at 1 min, and persists up to the end of the 1-h experiment

Confocal microscopy confirmed the CD4/Zap70 differential partitioning into raft vs nonraft fractions upon cross-linking with rIgG1 13B8.2. Jurkat cells were treated with an irrelevant IgG1 Ab or the rIgG1 13B8.2, fixed with paraformaldehyde, permeabilized, and then stained with the Alexa Fluor 488-conjugated cholera toxin B subunit, which binds to GM1-enriched DRM, or with anti-CD4 or anti-Zap70 polyclonal Abs followed by appropriate Cy3 and Cy5 conjugates. CD4 (Fig. 8A) and Zap70 (Fig. 8B) were evenly distributed upon treatment of Jurkat cells with irrelevant control IgG1 Ab, colocalized with GM1 but also detected outside GM1-enriched regions. Preferential copatching of GM1 and CD4 was observed when cells were treated with rIgG1 13B8.2 (Fig. 8A); this was not the case for GM1 and Zap70 in 13B8.2-cross-linked cells (Fig. 8B). Zap70 stained at sites that are not coincident with GM1-enriched areas. We examined up to 1000 cells from several separate areas of 50–100 cells each, several times, in three independent experiments and obtained similar results. As shown and quantified in Fig. 8C, the kinetics of CD4/Zap70 differential partitioning occurred rapidly (10 s) upon Ab treatment. An increase in CD4/GM1 clustering, with ~70–80% colocalization, was observed 1 min after cross-linking, whereas a decrease in GM1/Zap70 clustering, with only 10–20% residual colocalization, was evidenced at the same time (Fig. 8C). This inversely different clustering was observed until the end of the experiment (1 h). These kinetics confirm the differential partitioning of CD4/Zap70 and demonstrate that these events occur rapidly but are not transient and persist for at least 1 h.

Discussion

Recombinant IgG1 Ab 13B8.2 (rIgG1 13B8.2) has been proposed as a potential therapeutic agent for the treatment of CD4+ malignant diseases because it induces complement-mediated lysis, Ab-dependent cell cytotoxicity, and growth arrest of T lymphoma cells (22). We demonstrated in this study that the baculovirus-expressed rIgG1 13B8.2 Ab acts by causing the accumulation of the CD4 molecule inside the Brij 98-extracted rafts and by excluding the raft domains by using this extraction protocol, which is not confirmed (28). Accordingly, we were able to identify TCRs inside the raft domains by using this extraction protocol, which is not possible when detergent with stronger solubilization strength such as Triton X-100 is used (1). Extraction at 37°C avoided the chilling step at 4°C used to obtain Triton X-100 DRM, which modifies the phase behavior of membranes (2). In addition, Brij 98 DRM showed less aggregation following detergent extraction (1), thus allowing easier raft characterization. We finally hypothesized that Brij 98 DRM extraction at 37°C is probably more effective to detect weak affinity protein associations in rafts, e.g., for the study of signaling or adaptor proteins, in contrast to Triton X-100 extraction at 4°C (29).

The different raft compartmentalization of CD4 vs Zap70 following treatment with anti-CD4 rIgG1 13B8.2 is important for subsequent development and longevity of signaling complexes and for the activation state of downstream molecules. To our knowledge, little data are available concerning a "physical" disconnection of the Ab-target CD4 molecule with membrane-proximal Zap70 kinase through dynamically oriented raft segregation. Other anti-CD4 Abs (30, 31–33) are known to block Zap70 phosphorylation in vitro and in vivo, but neither CD4/Zap70 differential patching by confocal microscopy nor CD4/Zap70 differential raft partitioning has been observed. Although no link between murine parental mAb 13B8.2 and Zap70 kinase has ever been established, down-regulation of LFA-1-dependent adhesion of Jurkat T cells to B cells, induced by murine parental mAb 13B8.2 cross-linking, requires colocalization of p56 Lck, LFA-1, and PI3K, but not Src homology 2 domain-containing phosphatase SHP-2, in Brij 58 GM3−membrane rafts together with CD4 (34, 35).

Most of the effects induced by 13B8.2 murine mAb, among which is inhibition of NF-kB and ERK activation, counteract the signaling pathways induced by TCR/CD3 engagement (23–25, 36–40). This engagement initiates the recruitment and activation of CD4 noncovalently attached p56 Lck kinase, which phosphorylates the Zap70 kinase (41). This enzymatically activated protein (42) further phosphorylates the adaptor molecules SLP-76, LAT, Vav-1, and Grb-2 (43), which form signaling complexes to further recruit and activate effector enzymes PLCγ1 and PKC (42). rIgG1 13B8.2 acts by decoupling Zap70 and also some of these downstream targets from the CD4-segregated raft machinery of native Jurkat cells and by modulating the Zap70 phosphorylation profile. However, the relationship between Ab-induced raft compartmentalization and modulation of phosphorylation remains to be clarified. In our hands, we did not observe Zap70 in raft membranes from CD3/CD28-stimulated Jurkat T cells, as was previously described following anti-CD3 activation alone (1) and reinforced when the CD28 costimulation signal was added (44). The low anti-CD3 concentration we used could in part explain our observation because Ahmed et al. (45), using the same anti-CD3 dosage, did not demonstrate Zap70 capping. In addition, the immune status of the cells, i.e., Th1 vs Th2 (46) or naive vs memory cells (47), also influences membrane reorganization of signaling complexes.

The anti-CDR3 like murine mAbs, including 13B8.2, inhibit HIV gene expression (48). The 13B8.2-induced effects (23–25, 38, 49, 50) require the cytoplasmic tail of CD4 (51) without the need of functional p56 Lck or CD45 regulation (49, 52, 53). Mouse 13B8.2 Ab inhibits soluble gp120 binding to soluble and membrane CD4, but fails to block either gp120-bearing virion binding to CD4 or HIV entry (54), thus suggesting that 13B8.2 and gp120 signaling could be closely related but not equivalent. This argument is correlated with the fact that gp120, like rIgG1 13B8.2, induces CD4 raft compartmentalization, as demonstrated by us and others (55), but preferentially triggers phosphorylation of signal-activating Zap70 Tyr319 and Tyr493 residues (41, 42, 56), rather than phosphorylating the signal-inhibiting Zap70 Tyr292 residue (57), which we observed upon rIgG1 13B8.2 binding. rIgG1 13B8.2 drives the gp120-induced phosphorylation pattern toward a Zap70-negative regulation pathway, explaining why anti-CD4 13B8.2 Ab treatment induces inhibition of NF-κB activation and transcription inhibition of the viral genome, whereas gp120 binding leads to NF-κB activation and productive transcription of the viral genome in the case of HIV infection (23). Of great interest is that Zap70 is required in infected donor cells for efficient cell-to-cell HIV transmission to recipients and for formation of the virological synapse (58).

rIgG1 13B8.2 cross-linking of Jurkat T cells does not induce Zap70 Tyr493 phosphorylation in the activation loop of Zap70, which is known to increase TCR/CD3-mediated immune responses (42), probably because DRM-located upstream of p56 Lck, known to specifically phosphorylate Tyr493 residue on the
Zap70 molecule (42), is physically disconnected from Zap70. Another possible means of regulation by 13B8.2 rlgG1 could involve the other autophosphorylation sites Tyr292 and Tyr319 in the interdomain B of Zap70. The Tyr292 residue is a binding site for the Src homology 2-like domain of the c-Cbl protooncogene and serves as a negative regulator of T cell function. Phosphorylated Tyr292 interaction with c-Cbl, which is a component of the ubiquitination machinery, may target phospho-Tyr292 Zap70 molecules for ubiquitination and degradation (57). Tyr319 residue is autophosphorylated in vitro and becomes phosphorylated in vivo upon TCR triggering (56), leading to positive regulation of TCR-mediated signaling. The fact that CD4-specific rlgG1, 13B8.2 induces Tyr292 phosphorylation of DRM-excluded Zap70, probably leading to increased kinase degradation, and inhibits Tyr319 phosphorylation, as do the anti-CD4 YHB.46 (30), is one further enhancing element for the switching-off-of signaling pathways leading to impaired immune responses or HIV infection. Decoupling of the Zap70 molecule from the raft machinery together with modulating its phosphorylation are among the “missing links” to explain early mechanisms of action of baculovirus-expressed anti-CD4 rlgG1 13B8.2. Are these mechanisms the only ones? Probably not, because 1) the disconnection of DRM-excluded Zap70 with adaptor protein LAT and LIME has been observed (59); 2) anti-CD4 Abs have been described to affect Zap70-induced phosphorylation of the downstream adaptor proteins SLP-76, LAT, and Vav-1 (30–32), and we observed the rlg1, 13B8.2-induced raft disconnection of Zap70-downstream targets SLP-76, Vav-1, and PLCγ1; and 3) other adaptor proteins such as Nck and Wiskott-Aldrich syndrome proteins (60) have been shown to localize with Zap70, linking the TCR to actin polymerization.

An important finding from our confocal studies was that Zap70/CD4 differential partitioning in Brij 98 DRM rapidly occurred within 10–30 s upon rlgG1, 13B8.2 ligation, increased until 1 min and persisted throughout the 1-h experiment. This observation must be taken into consideration with regard to the dynamics of signaling assemblies following TCR/CD3 ligation (61), which can be potentially inhibited by 13B8.2 treatment. These clusters quickly disassemble, reassemble, and change in composition as specific proteins associate and dissociate. Zap70 is rapidly localized in patches (15 s) whose intensity reaches a steady state at 3 min; Zap70 remains associated with these laterally immobile clusters for 30 min (59). In contrast, adaptor signaling proteins such as LAT, Grb2, Gads, and SLP-76 show similar initiation timing but persisted throughout the 1-h experiment. Although we cannot rule out the possibility of Ab aggregates, our observation emphasizes the fact that targeting of two adjacent epitopes by rlgG1 13B8.2 is not necessary for CD4 clustering inside rafts.

Ceramides and cholesterol are critical for the maintenance and the dynamics of membrane rafts and CD4 localization in raft domains is regulated by posttranslational lipid modifications (66). We demonstrated that cholesterol depletion before rlgG1, 13B8.2 cross-linking affects subsequent Zap70/CD4 differential partitioning into Brij 98 DRM. The integrity of Brij 58 or Triton DRM, together with CD20 translocation, Ca2+ mobilization, and apoptosis induced by anti-CD20 Abs, is compromised by cholesterol depletion (5). Raft distribution induced by other Abs has been shown to be impaired by cholesterol depletion (67). Similarly, cholesterol-lowering agents such as statins are known to inhibit HIV infection (68), probably by affecting membrane raft organization. It remains that the exact role of slow and fast pools of cholesterol (69) in raft stabilization should be taken into consideration for future studies. In addition, Abs have been described to modulate ceramide synthesis (7), together with enhanced Fas-mediated apoptosis and cell growth inhibition (7, 9). Ceramide and Abs liberate Ca2+ from the same intracellular pool (70). Agents able to trigger ceramide synthesis can induce apoptosis, which is interesting for anticancer therapy (71), but they have also been described to block HIV infection (72). These observed effects lead to the following question: are the effects of lipid modulators and Abs synergistic (70, 73)? If so, this could raise interesting avenues for investigation. It is worth noting that lipid homeostasis inside rafts is highly regulated (74). The tendency of cholesterol and ceramide to avoid exposure to water might be a driving force for the association of proteins with rafts (74), such dynamics being mutually regulated by the concentration of cholesterol and ceramide. For all these reasons, the analysis of the lipid-protein rheostat in 37°C-extracted Brij 98 DRM, either upon treatment with biotechnological drugs or through a pathological process, could open new strategies for raft-based therapies.


