

The lipid-modulating effects of a CD4-specific recombinant antibody correlate with ZAP-70 segregation outside membrane rafts

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Abbreviations

ADAP = adhesion- and degranulation-promoting adapter protein, Carma1 = caspase-recruitment domain [CARD]-membrane-associated guanylate kinase [MAGUK] protein 1, CDR3 = complementary determining region 3, GM1 = ganglioside M1, GC = gas chromatography, HER2 = human epidermal growth factor receptor-2, HP-TLC = high performance thin-layer chromatography, LAT = linker for activation of T cells, Lck = leukocyte-specific protein tyrosine kinase, NF- κ B = nuclear factor-kappa B, PDK-1 = 3-phosphoinositide-dependent protein kinase 1, PI3K = phosphoinositide 3-kinase, PKC = protein kinase C, PLC γ 1 = phospholipase C γ 1, rIgG₁ = recombinant IgG₁, SLP-76 = SH2-domain-containing leukocyte protein of 76 kDa, SMase = sphingomyelinase, TNF- α = Tumor necrosis Factor α , Vav-1 = p95^{vav}, ZAP-70 = Zeta-chain-associated protein kinase of 70 kDa

1. Summary

We previously reported that the anti-tumoral effects of the recombinant IgG₁ antibody 13B8.2, which is directed against the CDR3-like loop on the D1 domain of CD4, are linked to accumulation/retention of CD4 inside membrane rafts, recruitment of signaling molecules of the TCR/CD3 pathway and raft exclusion of the ZAP-70 kinase and its downstream targets Vav-1, PLC γ 1 and SLP-76. We thus wanted to assess whether this compartmentalization could be related to a possible effect of 13B8.2 on the lipid composition of rafts. Here we show that 13B8.2 treatment of Jurkat T cells did not affect neutral lipids and particularly cholesterol content in GM1-positive membrane rafts, but decreased phosphatidylserine synthesis. C18:0 saturated fatty acid level in GM1-positive membrane rafts and ceramide release were concomitantly increased following treatment with 13B8.2. Antibody-induced ceramide release in membrane rafts occurred through enhanced acid sphingomyelinase activity and was blocked by the acid sphingomyelinase inhibitor imipramine, but was not affected by inhibitors of *de novo* ceramide synthesis, myriocin and fumonisin B1. Similarly to 13B8.2, addition of bacterial sphingomyelinase increased ceramide release and segregated ZAP-70 outside GM1-positive membrane rafts from Jurkat T cells. Besides CD4/ZAP-70 modulation in membrane rafts, the 13B8.2-induced activation of the acid sphingomyelinase/ceramide pathway is an important event for structuring raft platforms and transducing CD4-related intracellular signals, which can further fine-tune antibody-triggered tumoral effects.

2. Introduction

The classical concept of plasma membrane, proposed by Singer and Nicolson [1], wherein proteins diffuse freely in two-dimensional homogeneous bi-layers, has been drastically modified during the last decade. Indeed, membrane rafts, which are “discrete” domains with a mean diameter of 10–200 nm, can be distinguished from the rest of the membrane due to their protein and lipid composition [2]. The ability of membrane rafts to segregate proteins in a defined lipid environment provides a mechanism for signaling compartmentalization in the plasma membrane by concentrating some components in membrane rafts and excluding others. Cholesterol/sphingolipids confer organization of membrane rafts through self-assembly but could also be incorporated in the quaternary structure of raft-located protein complexes (“lubrication” concept) [2], thus favoring their inclusion into and the assembly of functionalized membrane rafts. They play a major role in the modulation of apoptosis [3] and cell growth [4], and their targeting represents an attractive strategy of raft-based therapeutics [5, 6]. Indeed, cholesterol sequestrants and inhibitors of cholesterol synthesis as well as sphingolipid-modulating drugs, which mainly act on enzymes involved in ceramide metabolism [5], regulate cell growth, differentiation, stress response and apoptosis [7]. Ceramide accumulation occurs through two main pathways: hydrolysis from sphingomyelin through sphingomyelinase (SMase) stimulation and *de novo* synthesis by ceramide synthase activation. Interestingly, the anti-CD20 antibody rituximab [4] as well as CD40- [8] and CD95-specific [3, 9, 10] antibodies increase *in vitro* ceramide release, thus rendering cells sensitive to apoptosis or inhibition of proliferation. *In vivo* therapeutic synergy between rituximab and lipids modulators, such as fenretinide [11, 12] and aplidine [13], has been demonstrated, thus leading to the idea of combining lipid modulators and antibodies, as proposed in a combinatorial phase I/II clinical trial in B cell lymphoma (trial NCT00288067).

Altogether, these results clearly emphasized a dynamic crosstalk between sphingolipids/cholesterol and proteins (lipid-protein “rheostat”) in membrane rafts that can be modulated by antibodies [5].

We found that, in Jurkat T cells, rIgG₁ 13B8.2, a baculovirus-expressed recombinant IgG₁ (rIgG₁) anti-CD4 antibody [14, 15], induced accumulation/retention of CD4 inside membrane rafts, recruitment of TCR, CD3 ζ , kinases, adaptor proteins and PKC- θ , but excluded ZAP-70 and its downstream targets SLP-76, PLC γ 1, and Vav-1 [16]. Analysis of key upstream events showed that modulation of ZAP-70 Tyr²⁹² and Tyr³¹⁹ phosphorylation occurred concomitantly with rIgG₁ 13B8.2-induced ZAP-70 exclusion from the membrane rafts [16]. Such antibody-induced modulation of membrane raft signaling, which leads to inhibition both of NF- κ B nuclear translocation [17] and of binding to the IL2 gene promoter [18], partly explains the anti-proliferative effect of rIgG₁ 13B8.2 in T cell lymphomas [19]; however the effects of rIgG₁ 13B8.2 on lipid dynamics in membrane rafts remain unknown. Therefore, we decided to examine the effects of the anti-CD4 rIgG₁ 13B8.2 antibody on the lipid composition of membrane rafts in a T lymphoma cell line. Here we report that, besides CD4/ZAP-70 protein reorganization, rIgG₁ 13B8.2 affected the lipid rheostat by increasing ceramide release through acid SMase activation and decreasing phosphatidylserine synthesis without modifying the cholesterol content of GM1-positive membrane rafts. Finally, incubation of Jurkat T cells with exogenous SMase not only increased ceramide release, but also segregated ZAP-70 kinase outside GM1⁺ membrane rafts like following treatment with rIgG₁ 13B8.2.

2. Materials and methods

2.1. Cells

Jurkat T cells were grown in RPMI 1640 (Cambrex, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Sigma-Aldrich, St Louis, MO), and 2 mM glutamine (complete medium). Cells were provided by L. Briant (Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5236, Montpellier, France).

2.2. Lymphocyte treatment

1×10^8 Jurkat T cells were treated with 20 µg/ml rIgG₁ 13B8.2 diluted in complete medium for 10 min. A 10 min-treatment has been previously shown to be optimal for inducing CD4/ZAP-70 raft reorganization [16]. In other experiments, cells were only treated with 1 U/ml bacterial SMase from *Bacillus cereus* (Sigma) for 1 h, a procedure which has been previously determined to be optimal for ceramide release [20].

2.3. Brij 98-raft isolation

After washing in 160 mM PBS (pH 7.4), Jurkat T cells were lysed with 1% polyoxyethylene glycol Brij 98 detergent diluted in TNE buffer (25 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA) containing 1 mg/ml enzyme inhibitors (complete EDTA-free mixture of anti-proteases; Roche, Meylan, France) at 37°C for 30 min. Cell lysates were mixed with an equal volume of 80% sucrose in TNE with enzyme inhibitors, overlaid with 6.5 ml 30% and 3.5 ml 5% sucrose in TNE with enzyme inhibitors, and then centrifuged at 200,000g at 4°C for 20 h. From the top of the gradients, twelve 1-ml fractions were collected on ice and numbered from 1 to 12. The protein content of each fraction was quantified using

the micro Bradford Protein Assay kit (Pierce, Rockford, IL). GM1 and CD71 expression were used to discriminate between raft fractions and non-raft fractions, respectively (see below).

2.4. Cholesterol quantitation

Cholesterol levels were determined in GM1-positive (GM1⁺) raft fractions using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Karlsruhe, Germany) following the manufacturer's protocol. Briefly, 50 µl cell lysates or GM1⁺ raft fractions from antibody-treated Jurkat T cells were incubated with 50 µl Amplex Red working solution which contains 2 U/ml cholesterol oxidase, 0.2 U/ml cholesterol esterase, 2 U/ml horseradish peroxidase and 300 µM Amplex Red reagent. After 30 min incubation at 37°C in the dark, fluorescence was measured using a fluorescence microplate reader (Molecular Devices Corp., Sunnyvale, CA) at an excitation wavelength of 530 nm and emission wavelength of 590 nm. The fluorescence of lysis buffer alone (negative control) and of different concentrations of the cholesterol reference standard (Sigma) (positive control) was also assessed during each experiment. Cholesterol values were calculated based on the cholesterol standard curve obtained with the cholesterol reference standard and normalized to the protein content of each sample determined with the micro Bradford protein assay kit (Pierce).

2.5. Measurement of acid sphingomyelinase activity

Activity of acid SMase in Jurkat T cells was measured using the Amplex Red Sphingomyelinase Assay Kit (Molecular Probes). Protein-normalized GM1⁺ raft fractions or whole cell lysates were diluted to 100 µl in 50 mM sodium acetate buffer at pH 5.0, which is optimal for the detection of acid SMase activity, and they were incubated with 10 µl 5 mM sphingomyelin at 37°C for 1 h. The positive control contained 100 µl 0.4 U/ml *Bacillus cereus* SMase (supplied in the kit) in 50 mM sodium acetate solution at pH 5 and 10 µl 5 mM

sphingomyelin. All samples were then mixed with 100 μ l Amplex Red working solution (100 μ M Amplex Red reagent with 8 U/ml alkaline phosphatase, 0.2 U/ml choline oxidase, 2 U/ml horseradish peroxidase in 100 mM Tris-HCl pH 8.0) in a 96-well microtiter plate. For each test, a negative control (i.e., Amplex Red working solution without sphingomyelin) was also co-incubated with each sample. After 30 min incubation at 37°C in the dark, measurements were made using a fluorescence microplate reader (Molecular Devices Corp.). The excitation and emission wavelengths were set at 560 and 587 nm, respectively. The acid SMase activity of the experimental samples, positive and negative controls was calculated as a percentage of the acid SMase activity (100%) of *Bacillus cereus* SMase.

2.6. Radioactive quantification of acid sphingomyelinase activity

Radioactive acid SMase assay was performed as previously described by Wiegmann *et al.* [21] and adapted by Jaffrézou *et al.* [22] and Grazide *et al.* [23]. Jurkat T cells were first incubated with or without 20 μ g/ml rIgG₁ 13B8.2 antibody or rituximab for different lengths of time. Cells were then washed and lysed in 300 μ l 0.1% Triton X-100 for 10 minutes followed by sonication. Reactions were started by adding 100 μ l substrate solution to 100 μ l whole cell lysate. The substrate solution consisted of [choline-methyl-¹⁴C]-sphingomyelin (100,000 d.p.m./assay; Dupont NEN; Boston MA) in 1 mM EDTA and 250 mM sodium acetate (pH 5.0). Radioactive phosphocholine produced from [choline-methyl-¹⁴C]-sphingomyelin in the presence of acid SMase was extracted by adding 2.5 ml chloroform/methanol (2:1 v/v) [24]. Phases were separated by centrifugation and the amount of released phosphocholine in 750 μ l of the upper phase was determined using a scintillation counter. For calculation of the specific activity of the total cell homogenates, values were corrected for protein content, reaction time and specific activity of the substrate.

2.7. Lipid analysis and quantification by high performance thin-layer chromatography (HP-TLC) and gas chromatography (GC)

Lipids were extracted and purified from the different gradient fractions according to Bligh and Dyer [25]. Phospholipids, glycolipids and neutral lipids were separated by mono-dimensional HP-TLC using the solvent systems described by Vitiello and Zanetta [26] and Juguelin *et al.* [27], respectively. Lipids were detected by spraying the plates with a solution of 0.1% (w/v) primuline in 80% acetone, imaged under ultra-violet light and then quantified from HP-TLC plates by densitometric scanning [28] using three independent biological samples. The identities of lipids were determined using well characterized standards. Fatty acids were determined and quantified using GC after conversion to the corresponding methyl esters by hot methanolic sulfuric acid treatment according to Browse *et al.* [29]. The retention times of fatty acid methyl esters were determined by comparison with standards.

2.8. Flow cytometry measurement of ceramide

Ceramide levels in Jurkat T cells were assessed by flow cytometry analysis. Cells were treated or not with 20 µg/ml anti-CD4 rIgG₁ 13B8.2 at 37°C for various times. In some experiments, before the antibody treatment, ceramide synthesis was inhibited by incubating cells at 37°C with myriocin (100 nM; Sigma), imipramine (20 µM; Sigma) or fumonisin B1 (50 µM; Sigma) for 30 min. After washes cells were fixed in 3% paraformaldehyde /PBS (wt/vol) for 10 min and permeabilized or not with cold methanol for 30 minutes. After three washes in PBS buffer without Ca⁺⁺ and Mg⁺⁺ supplemented with 2% FCS (PBS-2% FCS), cells were incubated with the anti-ceramide 15B4 monoclonal antibody (1:50) (Alexis Biochemicals, Germany) at 37°C for 1 h. After three washes in PBS-2% FCS, cells were incubated with FITC-conjugated goat anti-mouse IgM (1:100) (Jackson ImmunoResearch,

Baltimore, PA) in the dark for 1 h. Cells were then washed three times and suspended in PBS for analysis using an EPICS flow cytometer (Beckman-Coulter, Fullerton, CA).

2.9. Dot blot analysis of GM1-enriched membrane rafts

Nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, UK, Buckingham) were spotted with 2 µg of each gradient fraction. Membranes were blocked with 5% semi-skimmed milk in PBS containing 0.1% Tween 20 (PBS-T) at room temperature for 1 h. GM1 was detected in Brij 98-extracted fractions by adding the peroxidase-conjugated cholera toxin B subunit (1:1000) (Molecular Probes) at room temperature for 1 h. CD71 was detected by incubating nitrocellulose membranes with an anti-CD71 rabbit antibody (1:1000) (Santa Cruz Biotech., Santa Cruz, CA) at 37°C for 1 h, followed by probing with a secondary peroxidase-conjugated anti-rabbit antibody (1:3000). After washing in PBS-T, binding was assessed using the ECL Western Blotting Detection Kit (Amersham Pharmacia Biotech).

2.10. SDS-polyacrylamide gel (SDS-PAGE) electrophoresis and western blot

Forty µg of protein lysates from each gradient fraction obtained after treatment with rIgG₁ 13B8.2 or *Bacillus cereus* SMase were separated on 12%-SDS-PAGE under reducing conditions and electrophoretically transferred to Immobilon P membranes (BioRad, Bedford, MA). Membranes were blocked with 5% semi-skimmed milk in PBS-T at 37°C for 1 h. After washing in PBS-T, membranes were incubated with the dilution of anti-CD71 (1:1000) (Santa Cruz Biotech.), -CD4 (1:3000) (R&D Systems, Minneapolis, MN) and -ZAP-70 (1:1000) antibodies (Santa Cruz Biotech.) at room temperature for 2 h. Membranes were then washed with PBS-T three times and incubated with a secondary peroxidase-conjugated anti-rabbit antibody (1:3000) or anti-goat antibody (1:1000) (Sigma), as appropriate, at room temperature for 1 h. After three washes in PBS-T, antibody binding was detected with the ECL Western

Blotting Detection Kit (Amersham Pharmacia Biotech). Neither the experiments performed for this paper, nor the dot-blot/western blots we previously published [16] showed GM1, CD4, CD71 or ZAP-70 reactivity in fractions 1—3 of the membrane raft gradient.

2.11. Statistical analysis

Results, when appropriate, were expressed as mean \pm SD and were the average of at least three different values per experiment. The Student's *t*-test was used to evaluate the statistical significance. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. The anti-CD4 antibody rIgG₁ 13B8.2 does not affect neutral lipid composition, and particularly cholesterol level, in GM1⁺ membrane rafts from Jurkat T cells, but decreases phosphatidylserine content.

To investigate whether treatment with rIgG₁ 13B8.2 could modify the lipid content in Jurkat T cells, cells were treated with 20 µg/ml rIgG₁ 13B8.2 for 10 minutes and then lipids were extracted with chloroform/methanol and further separated by HP-TLC with different solvent mixtures. Treatment with rIgG₁ 3B8.2 did not modify the proportion of neutral lipids (*e.g.* diacylglycerol 1-2 and 1-3, cholesterol, free fatty acids, triacylglycerol and cholesterol ester) in whole lipid extracts (data not shown). Similarly, cholesterol content was comparable in GM1⁺ membrane fractions 4-5 obtained from cells incubated or not with rIgG₁ 13B8.2, as demonstrated by Amplex Red fluorimetry (data not shown). Conversely, the content of polar lipids in whole lipid extracts was partially affected by treatment with rIgG₁ 13B8.2 (Fig. 1). Specifically, phosphatidylserine (PS) level showed a significant 0.81 fold-decrease (*P* value = 0.019), whereas sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidic acid and phosphatidylethanolamine content was not significantly modified and was comparable to that of a previous report [30].

3.2. rIgG₁ 13B8.2 increases C18:0 fatty acid level in GM1⁺ membrane rafts

To assess in detail the relative proportion of fatty acids, their length and degree of saturation, GM1⁺ membrane rafts from cells treated or not with rIgG₁ 13B8.2 were analyzed by GC (Fig. 2). As expected for liquid-ordered phases [30, 31], GM1⁺ membrane raft fractions mainly contained C16:0 (palmitic acid) and C18:0 (stearic acid) saturated fatty acids, which corresponded to approximately half of the whole fatty acids extracted from

untreated or 13B8.2-treated cells. C18:1 mono unsaturated fatty acid (oleic acid) was also mainly detected either in untreated or 13B8.2-treated cells. Treatment with rIgG₁ 13B8.2 induced a significant 1.28 fold-increase in C18:0 fatty acid level in GM1⁺ membrane rafts in comparison to untreated cells (Fig. 2, *P* value = 0.02), whereas C16:0 and C18:1 were not affected by antibody treatment.

3.3. rIgG₁ 13B8.2 treatment stimulates ceramide release in Jurkat T cells

Sphingolipids, particularly ceramides, which play a role in apoptosis and proliferation, are an important group of lipid mediators found in membrane rafts. Since C18:0 fatty acids, whose level was specifically increased following rIgG₁ 13B8.2 treatment, are constitutive elements of many types of lipids including ceramides, we then assessed by flow cytometry whether rIgG₁ 13B8.2 influenced ceramide release at the cell membrane. A 10-min treatment with rIgG₁ 13B8.2 induced a slight increase (11%) in ceramide concentration at the cell membrane (Fig. 3A, upper panels); this effect was time-dependent since a 4h-treatment increased ceramide accumulation up to 21%. Permeabilization of Jurkat T cells with methanol after antibody treatment further increased ceramide detection to 50% after 10-min antibody treatment and to 73% after 4-h antibody treatment (Fig. 3A, lower panels). No signal was detected when using fluorescein-conjugated secondary antibody alone (data not shown). These results suggest that ceramide accumulation following rIgG₁ 13B8.2 treatment could be due to *de novo* cytoplasmic synthesis or intracellular lysosomal SMase activation as well as to sphingomyelin hydrolysis through direct membrane SMase activation. Pre-treatment with imipramine, an acid SMase inhibitor which blocks membrane ceramide synthesis from sphingomyelin, reduced ceramide release in 13B8.2-treated and permeabilized cells, whereas neither fumonisins B1 nor myriocin (which inhibit *de novo* ceramide synthesis) did affect

ceramide production following a 10 min-antibody treatment (Fig. 3B). Taken together these findings indicate that rIgG₁ 13B8.2 treatment stimulates ceramide release in Jurkat T cells.

3.4. rIgG₁ 13B8.2-induced ceramide release is correlated with enhanced acid sphingomyelinase activity

Many stimuli including cytokines, G protein-coupled receptors and stress favor hydrolysis of sphingomyelin to ceramide and phosphorylcholine, a reaction catalyzed by SMases. We thus quantified acid SMase activity in whole cell lysates and GM1⁺ membrane raft fractions, from cells treated or not with rIgG₁ 13B8.2 using the Sphingomyelinase Amplex Red fluorimetry assay. As shown in Figure 4A, a significant 2-fold increase in acid SMase activity (P value = 0.015) was observed in 13B8.2-treated whole cell lysates in comparison to untreated cells, where the activity was comparable to that of the positive SMase control. This result was confirmed in GM1⁺ membrane rafts from 13B8.2-treated cells that showed a strong 5-fold increase in acid SMase activity (P value = 0.001) in comparison to GM1⁺-membrane rafts from untreated cells.

To confirm that acid SMase was activated by rIgG₁ 13B8.2, Jurkat T cells were treated with rIgG₁ 13B8.2 for 1 to 60 minutes, lysed in Triton X-100 and then incubated with radio-labeled sphingomyelin in pH 5.0 acetate buffer. A time-dependent, significant 1.62-fold increase in acid SMase activity was observed in 13B8.2-treated cells in comparison to untreated cells (medium) with values ranging from 1.599 ± 0.139 nmol/h.mg protein after 1 min-treatment (P value = 0.039) to 2.027 ± 0.107 nmol/h.mg protein after 60 min-treatment (P value = 0.012) (Fig. 4B). Finally, incubation with the irrelevant anti-CD20 rituximab antibody did not significantly increase the acid SMase activity in Jurkat T cells that are CD20-negative. These results indicate that 13B8.2-induced ceramide accumulation at the cell

membrane is linked to the increase of acid SMase activity in GM1⁺ membrane rafts following treatment with rIgG₁.

3.5. Exogenous bacterial SMase, like rIgG₁ 13B8.2, increases ceramide release and excludes the ZAP-70 kinase from GM1⁺ membrane rafts

Bacterial SMase is a convenient tool to increase cell surface ceramide level because it readily hydrolyzes sphingomyelin to ceramide on the outer leaflet of the plasma membrane. Moreover, addition of exogenous SMase inhibits Ca⁺⁺ signals generated by CD3 cross-linking, thapsigargin and ionomycin in Jurkat T cells [32]. Since rIgG₁ 13B8.2 can inhibit Ca⁺⁺ signaling induced by CD3 stimulation [33], we questioned whether exogenous SMase could also modulate ZAP-70 reorganization outside membrane rafts. To this end, Jurkat T cells were incubated with bacterial SMase or rIgG₁ 13B8.2, lysed with Brij98 detergent and membrane rafts separated through sucrose gradient ultracentrifugation. Bacterial SMase treatment induced a 93.9% increase in ceramide release, as measured by the percentage of ceramide-positive cells (Fig. 5A), in comparison to untreated cells. No binding was observed when fluorescein-conjugated secondary antibody alone or the anti-ceramide antibody 15B4 alone was used (data not shown). Before rIgG₁ 13B8.2 treatment, CD4 and the ZAP-70 kinase were localized both in the raft and non-raft fractions from Jurkat T cells. rIgG₁ 13B8.2 treatment completely re-localized CD4 in the membrane raft fractions 4 and 5, whereas ZAP-70 was excluded from the raft fractions and concentrated in the non-raft fraction 12. Similarly, bacterial SMase treatment excluded the ZAP-70 kinase from membrane rafts (Fig. 5B), but did not affect CD4 localization inside and outside the membrane rafts. GM1 expression, which identifies Brij98-resistant raft fractions, was mainly observed in fractions 4 and 5 whereas CD71, a marker of non-raft fractions, was localized in fraction 12 as previously reported [16]. Taken together, these results indicate that exogenous SMase

increases ceramide content at the membrane and segregates ZAP-70 outside GM1⁺ membrane rafts like the rIgG₁ anti-CD4 antibody 13B8.2.

4. Discussion

Changes in lipid metabolism, which are closely connected to changes in lipid membrane composition, can dramatically affect the localization and function of membrane raft resident proteins [34]. For example, modifications of fatty acids in membrane rafts modulate ZAP-70 phosphorylation and consequently suppress signal transduction in T cells [35]. In a previous work we showed that, in Jurkat T cells, the anti-CD4 antibody rIgG₁ 13B8.2 induces accumulation of CD4 inside membrane rafts and exclusion of the ZAP-70 kinase and its downstream targets SLP-76, PLC γ 1 and Vav-1 from the raft machinery [16]. We now report that rIgG₁ 13B8.2 affects the lipid rheostat in GM1⁺ membrane rafts of Jurkat T cells by increasing ceramide release through acid sphingomyelinase activation and by decreasing the level of phosphatidylserine without modifying the cholesterol content. These effects are correlated with ZAP-70 exclusion from membrane rafts.

Cells undergo physiological turnover through induction of apoptosis and phagocytic removal, partly through externalization of phosphatidylserines from the cytosolic leaflet to the outer leaflet of the membrane. Phosphatidylserine externalization in cells undergoing death receptor-mediated apoptosis seems to be Ca⁺⁺-dependent [36]. Our finding that rIgG₁ 13B8.2 lowers phosphatidylserine level in Jurkat T cells explains why treatment with rIgG₁ 13B8.2 did not induce phosphatidylserine-dependent apoptosis in T cell lymphomas [19] and blocked CD3-induced Ca⁺⁺ increase and the subsequent signaling pathways [33]. Similarly, other CD4-specific antibodies have been shown to modulate phosphatidylserine level *in vitro* [37].

We also report that rIgG₁ 13B8.2 treatment induces a time-dependent increase in ceramide production through acid SMase activation. Activation of the sphingomyelin/SMase/ceramide axis can be induced by radiations, chemotherapeutic agents or receptor ligands [3, 4, 38] and, in some cases, a biphasic ceramide release was observed

with a first short-term (1-10 minutes) phase [3, 4, 39, 40] followed by a later one [39]. Such biphasic cycle was also reported following neutral SMase activation [41]. We thus hypothesize that rIgG₁ 13B8.2, which triggers CD4/ZAP-70 raft reorganization mainly during the first 30-60 seconds post-treatment [16], induces ceramide release first from the membrane sphingomyelin pool through direct acid SMase activation in the outer leaflet of the membrane, and then stimulates a second ceramide burst either through *de novo* ceramide synthesis [42], or through PKC-mediated phosphorylation of lysosomal acid SMase leading to ceramide release [43]. The inhibition observed with the SMase inhibitor imipramine argues in favor of the implication of the sphingomyelin/SMase/ceramide axis, but additional experiments are needed to assess whether the imipramine-induced inhibition affects cell signaling. The inability of the myriocin and fumonisin B1 inhibitors to block ceramide release suggests that *de novo* ceramide synthesis is probably not activated by rIgG₁ 13B8.2 treatment, as previously reported for other antibodies [4, 38]. The role of antibody-mediated activation of intracellular lysosomal acid SMase remains to be clarified. Interestingly, antibody-triggered CD40 raft clustering [8] and ultra-violet radiations [40] also induce translocation of acid SMase from the intracellular pool leading to ceramide release in membrane rafts [8]. Ultra-violet radiations as well as chemotherapeutic agents [44] stimulate PKC- δ -mediated phosphorylation of intracellular acid SMase leading to remodeling of the cellular cytoskeleton and further acid SMase translocation to the membrane. Since rIgG₁ 13B8.2 modulates PKC- θ distribution together with CD4 [16], PKC- θ could participate in the activation/phosphorylation of lysosomal acid SMase in Jurkat T cells following treatment with rIgG₁ 13B8.2.

Exogenous SMase was reported to induce ceramide release in CD3-stimulated T cells concomitantly with inhibition of Ca⁺⁺ flux [32] and inhibition of IL2 production through inhibition of NF- κ B activity [45]. Ceramide also blocked IL2 production in T lymphoma cells

through PKC- θ -mediated, but not TNF- α -induced, NF- κ B activation [45]. Sphingomyelin appears to be a critical raft constituent that enables translocation of signaling molecules, such as ZAP-70 and PKC- θ , as demonstrated in *sphingomyelin synthase 1* knockdown cells [46]. Here we demonstrate that ZAP-70 is excluded from membrane rafts of Jurkat T cells following treatment with bacterial SMase, probably through sphingomyelin consumption. The use of sphingomyelin-deficient cells could probably clarify this point. Similarly, treatment with rIgG₁ 13B8.2 induces ceramide release (this work) and inhibits CD3-induced T cell activation and proliferation through blockade of intracellular Ca⁺⁺ flux, NF- κ B activation and IL2 secretion [15, 33]. These events have been associated with CD4/PKC- θ translocation inside rafts and ZAP-70 exclusion from membrane rafts [16]. All these findings indicate that the lipid-protein rheostat in membrane rafts can be modulated by therapeutic antibodies in order to physically structure signaling platforms through sphingomyelin consumption/ceramide-induced aggregation and to maximize and synergize their anti-tumoral effects through dynamic lipid/protein partitioning inside/outside rafts.

The signaling pathways, through which CD4/ZAP-70/PKC- θ modulation and SMase-induced ceramide release could synergize, remain to be elucidated in rIgG₁ 13B8.2-treated cells. Two upstream PKC- θ effectors (PDK1 [47] and ADAP [48]) and one downstream effector (the adapter protein Carma1 [49]) could play a role because they are involved in the PKC- θ -mediated NF- κ B pathway [47—49] and ceramide inhibits PKC- θ -mediated NF- κ B activity [45]. PDK1 recruits both PKC- θ and Carma1 to membrane rafts, phosphorylates PKC- θ , thereby regulating NF- κ B activation [47]. PDK1 also phosphorylates PKC- δ [50], which in turn activates lysosomal acid SMase through phosphorylation [43], leading to ceramide release. Moreover, sphingosine, a hydrolysis product of ceramide, can activate PDK1 by autophosphorylation [51]. ADAP binds to Carma1 and regulates NF- κ B activation [48] but also binds to PI3K-generated phosphoinositides [52] which activate PDK1 activity

[50]. Finally Carma1, upon phosphorylation mediated by PKC- θ , bridges membrane-proximal events and nuclear signaling leading to NF- κ B activation, and also organizes protein translocation in membrane rafts [53]. We can thus hypothesize that, upon CD4 targeting by rIgG₁ 13B8.2, ZAP-70 is physically excluded from membrane rafts, this exclusion being correlated with acid SMase-mediated sphingomyelin consumption for ceramide synthesis, thus inhibiting NF- κ B activity. ADAP/PDK1 proteins are concomitantly recruited to membrane rafts together with CD4 and PKC- θ [16], enabling PI3K activation, thereby producing phosphoinositides and further activating PDK1 and PKC- θ . PKC- θ -mediated phosphorylation of acid SMase increases ceramide release which acts as a negative feedback to control PKC activation, thus strengthening the inhibition of NF- κ B activity. These antibody-induced events lead to inhibition of cell proliferation [19]. In conclusion, in this work we show that besides modulating CD4/ZAP-70 in membrane rafts, rIgG₁ 13B8.2 activates the acid sphingomyelinase/ceramide pathway, an important event for structuring raft platforms and transducing CD4-related intracellular signals, which can fine-tune the rIgG₁ 13B8.2-triggered tumoral effects. These findings indicate that the assessment of the lipid-protein rheostat in membrane rafts following treatment with biotechnological drugs could open new avenues for raft-based therapeutics based on the combination of lipid modulators, such as fenretinide [11, 12] and aplidine [13] or the fatty acid synthase inhibitor C75 [54], and therapeutic antibodies.

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Figure legends

Fig. 1. Polar lipid quantification in rIgG₁ 13B8.2-treated cells. Jurkat T cells were treated with rIgG₁ 13B8.2 (■) or left untreated (□), and polar lipids were extracted from whole cell lysates with chloroform/methanol, separated by HP-TLC and quantified by densitometric scanning. SPM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; PE, phosphatidylethanolamine. Each experiment was done in triplicate from three independent biological samples and data are expressed as mean ± SD.

Fig. 2. Relative proportion, length and degree of saturation of fatty acids in rIgG₁ 13B8.2-treated Jurkat T cells. Lipids were extracted with chloroform/methanol from GM1⁺ raft fractions of untreated- (□) and rIgG₁ 13B8.2-treated (■) cells. The relative proportion of fatty acids, their length and degree of saturation were analyzed by GC after conversion to the corresponding methyl ester by hot methanolic sulfuric acid. These results were obtained from three independent experiments.

Fig. 3. Ceramide quantification in rIgG₁ 13B8.2-treated Jurkat T cells. (A) Jurkat T cells were treated with rIgG₁ 13B8.2 for 10 min or 4 h (grey line) or not (dark line), and ceramide release was analyzed by flow cytometry without (upper panels) or after methanol permeabilization (lower panels). (B) After 30-min pre-incubation or not with sphingomyelinase (SMase) inhibitors, Jurkat T cells were treated with rIgG₁ 13B8.2 for 10 minutes (■) or left untreated (□), permeabilized and analyzed by flow cytometry. The percentage of ceramide-positive

fluorescent Jurkat T cells was measured as in A. FB1, Fumonisin B1; Imi; Imipramine; Myr, Myriocin.

Fig. 4. Acid SMase activity is increased in rIgG₁ 13B8.2-treated cells. (A) Jurkat T cells were treated or not with rIgG₁ 13B8.2 for 10 minutes, and then lysed with Brij98 detergent. GM1⁺ membrane fractions were separated by sucrose gradient ultracentrifugation and identified by dot-blot with peroxidase-conjugated cholera toxin. Acid SMase activity in whole cell lysates and GM1⁺ raft fractions was quantified using the Amplex Red Sphingomyelinase Fluorimetry Kit. (B) Jurkat T cells were left in medium alone (□), or treated with rIgG₁ 13B8.2 (■) or the irrelevant antibody rituximab (▣) for different lengths of time, lysed and then mixed with radio-labeled sphingomyelin. Acid SMase (ASMase) activity was normalized to the amount of protein in each fraction. Each experiment was done in triplicate from two independent biological samples and data are expressed as mean ± SD.

Fig. 5. Effects of bacterial SMase treatment in Jurkat T cells. (A) Cells were treated with bacterial SMase for 1 h (dark line) or not (dotted line) and ceramide release was analyzed by flow cytometry as in Figure 4. (B) Analysis of ZAP-70 and CD4 distribution in membrane fractions (after Brij98 detergent lysis and sucrose gradient ultracentrifugation) from untreated, rIgG₁ 13B8.2-treated and bacterial SMase-treated Jurkat cells. Expression of GM1 and CD71 is used to discriminate between raft and non-raft fractions, respectively. Following treatment with rIgG₁ 13B8.2 or bacterial SMase ZAP-70 is excluded from the raft fractions, whereas only incubation with rIgG₁ 13B8.2 segregates CD4 in the raft fractions.