Cutting Edge: Quantitative Imaging of Raft Accumulation in the Immunological Synapse

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Although the accumulation of lipid rafts at the immunological synapse is now well accepted, the degree of the accumulation, the localization within the fine structure of the immunological synapse, and the region from which lipid rafts are recruited have not been defined. In this work we show that lipid rafts preferentially accumulate in the central zone of the immunological synapse, the central supramolecular activation complex (C-SMAC). However, quantitative analyses indicate that the level of recruitment of lipid rafts to the C-SMAC is relatively small and suggests that rearrangement of lipid rafts from the peripheral zone of the synapse into the C-SMAC can account for this accumulation. We also assessed the effects of CD28 deficiency on lipid raft recruitment to the immunological synapse. The accumulation of lipid occurred independently of the CD28/B7 system and was not measurably altered by CD28.

T he immunological synapse (IS) is the specialized contact surface that is formed between the T cell and the APC (1, 2). Functional and imaging experiments indicate that membrane microdomains termed detergent-insoluble glycoprotein-golgolipid-enriched domains (detergent-insoluble glycolipids (DIGs)), detergent-insoluble membranes, glycolipid-enriched membranes, or “rafts”) may play an important role in helping to form the structure of the IS (3–7). Furthermore, it has been suggested the recruitment of DIGs to the IS may be dependent on CD28 costimulation (8). The distinct microenvironment of the lipid raft might also serve as the structural basis for synapse stability, but it remains unclear whether “rafts” are necessary for the stable associations of relevant molecules such as TCR and linker for activation of T cells (9, 10).

The central and peripheral supramolecular activation complexes (C- and P-SMAC) are defined imaging the relative accumulations of various T cell components at the contact surface. These studies have not generally allowed quantitative analyses. Because the supported bilayer system produces an Ag-specific IS that is precisely oriented and because the ligand fluorophores are confined to the plane of the bilayer, this system produces high-resolution images that allow for quantification. In this work we localize “rafts” within the immune synapse. We also use quantitative approaches to assess the extent of “raft” recruitment to the T cell:APC contact, the relationship between “raft” accumulation and other parameters of synapse structure, and the role of CD28-mediated costimulation in IS formation.

Materials and Methods
Cells and reagents
2B4 and DO11.10 (wild-type (WT) and CD28-deficient) T cells were cultured as previously described (1, 11). The 2B4 T cells are used in the supported bilayer system, for which methods and analyses are as previously described (1). In this system, recombinant GPI-E binds a mohacryl c-derivative peptide. T cells were settled onto the bilayer, allowing them to become stably associated with the bilayer and within the focal plane before imaging. More than 90% of the T cells formed an IS within 5 min that remained stable for up to 2 h; unless otherwise noted, all images shown were obtained between 10 and 20 min. Conjugate formation using the DO11.10 T cells and the TA-3 APC line and immunofluorescence methods have been previously described (11). The APC line, TA-3, is a B cell line that by fortunate coincidence has relatively low levels of surface GM1. Our TA-3 cells have a surface density of GM1 that is at most 5% of that for DO11.10 T cells (as assessed by flow cytometry and grossly confirmed by imaging). Note that the presence of detectable cholera toxin staining in the TA-3 cells would cause an overestimate of the fraction of T cell GM1 at the interface. TA-3 cells loaded with OVA induce robust proliferation of DO11.10 T cells (11). Labeling with fluorescent cholera toxin was performed both with and without intentional trition permeabilization; there was no gross difference in the staining of the T cells. Native and fluorescein-labeled cholera toxin B subunit (CTx) was purchased from Sigma-Aldrich (St. Louis, MO).

For live cell labeling, ~5 × 10^6 cells were pelleted, chilled on ice, incubated for 1 min with 0.2 μg/ml FITC-CTx on ice, washed cold, and applied immediately to the 37°C bilayer. Labeling with CTx did not appreciably alter any parameter of IS formation. The mean fluorescence intensity varied linearly with the concentration of FITC-CTx over the range assessed (0.1–2 μg/ml), indicating that GM1 sites were not saturated. Cells were also simultaneously labeled with FITC-CTx and Cy5-CTx (prepared with monoacryl Cy5; Pharmacia, Peapack, NJ) and imaged under the conditions in the live experiments. There was a linear relationship between the pixel intensities over a 15-fold range of fluorescein intensities; there was no indication of a loss of linearity (see Fig. 2D). Bodipy-GM1 (Molecular Probes, Eugene, OR) was used in labeling reactions at 1 μg/ml.

The quantitative analyses of the T cell:APC conjugates are based on single confocal images. To avoid selection bias, all conjugates in a random field were measured; because a single image was used for all conjugates, no attempt was made to find an “optimal” section that might best depict a canonical IS. Conjugates were operationally defined as a smaller cell expressing TCR abutting a large cell lacking TCR and CTx staining. Additionally, the pixel with the highest TCR fluorescence was within the region
of the T cell:APC contact. The region of the C-SMAC is generally <1–3 μm in diameter and is easily missed in confocal microscopy. Although we could make reconstructions of entire T cell:APC contacts, we feel that various technical issues make these reconstructions inadequate for quantification.

Some of the GM1 recruitment noted in the C-SMAC may represent internal membranous structures. The polarization of the microtubule organizing center, as expected in a cell forming a synapse, would bring the glycosphingolipid-rich Golgi membranes into the region of the synapse. However, we saw no appreciable differences in cholera toxin staining even when the T cells were intentionally permeabilized with weak detergent.

**Results**

*Rift lipid accumulates in the C-SMAC compared with the P-SMAC*

Wide-field images of T cells contacting the supported bilayer show that CTx and Eκ (marking TCR) accumulated specifically in the C-SMAC (Fig. 1A, *upper cell*). The accumulation required Ag (data not shown). Because the bilayer lacks B7, the accumulation did not require CD28 costimulation. We showed previously that C-SMAC (Fig. 1A, *lower cell*) or CTx, indicating that raft accumulation correlated specifically with C-SMAC formation. Similar results were obtained using a second raft marker, bodipy-GM1 (Fig. 1, *B* and *C*). Confocal images of Ag-specific T cell:APC conjugates also showed colocalization of TCR and CTx in the C-SMAC, confirming this localization in a more physiologic system (Fig. 1D).

*Local reorganization of membrane components is sufficient to account for the GM1 accumulated in the C-SMAC*

Among various parameters that we measured, raft lipid accumulation correlated best with the area of the P-SMAC. The correlation of CTx accumulation in the C-SMAC with P-SMAC area was striking (Fig. 2B; \( r = 0.86; p < 0.001; n = 10 \)), even in comparison to the correlation with Eκ (marking TCR, a raft resident complex) (Fig. 2A, \( r = 0.6; p = 0.002; n = 21 \)). The total contact area, defined by the interference reflectance microscopy (IRM) image (Fig. 2C; \( r = 0.23; p = 0.5; n = 10 \)) and the total cell size (data not shown) correlated poorly or not at all with cholera toxin accumulation. The strong correlation between P-SMAC area and the accumulation of cholera toxin in the C-SMAC suggested that raft depletion from the P-SMAC could account for the raft accumulation in the C-SMAC.

Previous work suggested that raft recruitment may involve substantial movement of lipid rafts from throughout the plasma membrane (8, 13). To assess the degree of recruitment from plasma membrane outside of the IS, we used confocal methods to image T cells as they formed an IS with the bilayer (Fig. 3A). As the synapse formed, cells were imaged approximately at their midplane. The average fluorescence intensity at the midplane did not change significantly during the time that the cells formed synapses, each with cholera toxin accumulated in the C-SMAC (Fig. 3B). Therefore, raft accumulation in the C-SMAC did not require detectable depletion of raft lipids from plasma membrane outside the IS.

To compare the concentration of cholera toxin in the C-SMAC to the remainder of the plasma membrane outside the IS, it is easiest to use images reconstructed from serial sections (Fig. 3C). The intensity of cholera toxin staining in the C-SMAC (as determined by the highest intensity pixel) was 2- to 3-fold the average membrane fluorescence. The lowest fluorescence values in the P-SMAC area were similar to background, which in this case was ~6-fold less than the average intensity in the C-SMAC. Therefore, GM1 appears to be both recruited to the C-SMAC (~2- to 3-fold compared with the plasma membrane outside of the IS, and ~8-fold relative to the P-SMAC) and depleted from the P-SMAC (~2-fold compared with the plasma membrane outside of the IS). The proportion of total GM1 accumulated in the C-SMAC was also calculated from these images, indicating that the C-SMAC contained only ~6~12% of the total GM1. This number is similar to the estimated proportion of the plasma membrane contact in the bilayer (10%). Thus, the concentration of cholera toxin within the entire contact (C-SMAC plus P-SMAC) was similar to that of the rest of the plasma membranes.

**FIGURE 1.** The “raft” marker GM1 accumulates in the C-SMAC. *A*, Wide field images using cholera toxin as a raft marker. 2B4 T cells were labeled briefly at 4°C with fluorescein-CTx immediately before application to the bilayer. Synapses began to form within 2 min of application to a supported lipid bilayer containing Ag-loaded MHC (Eβ plus moth cytochrome c peptide) complexes and ICAM-1. Synapses were morphologically mature by ~5 min. Transmitted light images (TRANS) show the entire cell, while the IRM shows the region of close apposition between the T cell and the bilayer. Eγ and ICAM-1 mark the accumulation of TCR and LFA-1, respectively. The images of ICAM-1 (red) and CTx (green) are overlaid in the right image. Cells that form a C-SMAC (as noted by a clearing of ICAM-1 and an accumulation of TCR) also accumulated CTx (*upper cell*). The small number of cells that did not form a C-SMAC by these criteria also failed to accumulate TCR (*lower cell*). The halo of CTx staining at the cell periphery corresponds to the region where the plasma membrane curves away from the supported bilayer (scale bar = 10 μm). *B* and *C*. Wide field images using the fluorescent sphingolipid, bodipy-GM1, as a raft marker. The same field is shown in both panels, with ICAM-1 in red. Bodipy-GM1 (green) is overlaid in *B* and *B* (blue) in *C*. Confocal images of a canonical IS structure formed using Ag-specific conjugates of DO11.10 T cells with APCs. In this particular section there is clear accumulation of cholera toxin both in the C-SMAC and at the edge of the T cell:APC contact. This image uses CD28-deficient T cells (see below); similar structures are seen with the WT T cells.

**FIGURE 2.** The accumulation of GM1 correlates with the P-SMAC area and the accumulation of TCR. Using wide field images of the T cell: bilayer system, the CTX that accumulated in regions of excess Eβ was measured in 21 cells (*A*). CTX accumulation relative to the area of the P-SMAC (*B*) (area with excess ICAM), the contact area (*C*) (defined by IRM) was determined in 11 cells for which there was a well-defined circular P-SMAC with a central clearing containing excess TCR. Fitting the data to straight lines gave the following statistics: Eβ accumulation, \( r = 0.63 \) and \( p = 0.002; P-SMAC area, r = 0.86 \) and \( p < 0.001; IRM area, r = 0.23 \) and \( p = 0.5 \). Accumulations are in arbitrary fluorescence units; areas are in micrometers (2). *D*. Fluorescein intensity is linear with concentration. Jurkat T cells were labeled simultaneously with FITC-CTx (at 0.2 and 0.4 μg/ml) and Cy5-CTx. Each point is the intensity of the probes at a single pixel. The range of FITC intensities in these controls spans the range of values in the experiments. (\( r = 0.92 \) for both lines; slope = 0.11 ± 0.002 for low FITC-CTx and 0.22 ± 0.003 for high FITC-CTx).
membrane. Because the C-SMAC comprises only ~3–5% of the total cellular surface, we calculate that the movement of GM1 staining from the P-SMAC to the C-SMAC would result in a lipid raft concentration that is ~2–to 3-fold that of the plasma membrane outside of the contact area. This number correlates well with the directly measured value. Therefore, the degree of lipid raft accumulation in the C-SMAC does not require measurable amounts of recruitment from regions of the plasma membrane outside the IS.

FIGURE 4. Similar distribution of GM1 in T cell:APC conjugates of CD28 knockout and WT DO11.10 T cells. To avoid selection bias in the quantitative studies, we used all conjugates in a randomly selected field that fulfilled the criteria listed in Materials and Methods. Quantification is based on a single optical section that is the same for all conjugates in one field. In addition to canonical synapses (shown in Fig. 1D), a range of IS structures was seen. Examples of these conjugates are shown in Fig. 4A (WT, 1–2; CD28-deficient, 3–4). The asterisks mark the T cells. B, A histogram showing the number of cells with a given fraction of cell surface-associated cholera toxin fluorescence confined to the region of the T cell:APC contact. These data are based on a single optical section of 25 WT and 25 CD28−/− cells.

FIGURE 3. Confocal imaging of bilayer contacts to quantify raft recruitment to the IS. A, Time course of Ag-specific IS formation. Cell: bilayer contacts were prepared as in Fig. 1. Three images show representative time points in the evolution of the IS (red, ICAM; green, CTx). B, Cholera toxin is not detectably depleted from the plasma membrane outside of the IS during IS formation. Five cells were imaged repeatedly approximately at their midplane. The total plasma membrane intensity was calculated and compared with the initial time (error bars = SD). C, Confocal reconstructions. The six images show sequential reconstructed sections of a single representative T cell. The arrow marks the position of the supported bilayer. The ICAM-1 accumulated in the P-SMAC (A) and is clearly in the reconstructions as a region depleted of CTx binding. The elongated regions of intensity at the base of the cell are due to an artifact associated with imaging at the cover slip surface. The boxes indicate the region selected as the C-SMAC and contents of all these regions were summed and compared with the total integrated intensity for the entire cell. Similar quantitative results were obtained using the original Z-sections without reconstruction (acquisition time, <1 min).

WT and CD28-deficient cells accumulate similar amounts of CTx at the T cell:APC contact

Because the lipid raft recruitment in the bilayer system occurred without CD28 costimulation, we directly assessed the role of CD28 on the degree of CTx accumulation in a T cell:APC system by using CD28-deficient T cells. No discernable differences between conjugates formed with WT or CD28-deficient T cells were seen. Consistent with previous data, we found no gross effect of CD28 deficiency on the efficiency of conjugate formation with APCs (14). Organized synapses with a central accumulation of TCR could be crisply imaged in ~5% of all conjugates (Fig. 1D). A larger number of conjugates with morphologies similar to those presented in other T cell:APC systems (15, 16) were also visualized with both WT and CD28-deficient T cells (Fig. 4, A and B).

Using three quantitative approaches, we could not reject the null hypothesis that CD28 has no effect on GM1 recruitment in the T cell:APC system. First, CD28 deficiency did not appreciably alter the fraction of conjugates in which the point of greatest GM1 accumulation was restricted to a point within the T cell:APC contact. Specifically, about half of the conjugates (31 of 50 for CD28−/− and 22 of 50 for WT) had the highest intensity pixel values for cholera toxin confined to the region of T cell:APC contact (with the contact defined by the differential interference contrast image). Second, CD28 deficiency did not appreciably alter the fraction of GM1 recruited to the T cell:APC contact (0.25 ± 0.1 (WT) vs 0.25 ± 0.2 (knockout)) or the fraction of the T cell perimeter contacting the APC (0.3 ± 0.1 (WT) vs 0.3 ± 0.2 (knockout)).
These measurements for each cell were used to calculate the relative concentration of GM1 in the contact compared with the remainder of the plasma membrane. Histograms of the results of this third approach (Fig. 4B) show that CD28 deficiency did not alter the fraction of conjugates that had a specific relative enrichment of GM1 at the contact. Thus, there does not appear to be an effect of CD28 costimulation on lipid raft enrichment in the C-SMAC.

C-SMAC structure is stable to cholesterol depletion

The presumed aggregation of lipid rafts implies that lipid rafts may play a critical role in the stability of the C-SMAC. This is based on the idea that raft domains are dependent on cholesterol and are reported to dissipate when cholesterol is depleted (17). Therefore, the cholesterol-depleting agent β-methyl cyclodextrin (β-MCD) was used to assess the requirement of lipid rafts for C-SMAC structure and stability. Cells pretreated with β-MCD did not form close contacts with bilayers containing ICAM-1 and Ag/E conjugates, confirming that disruption of lipid rafts can profoundly inhibit T cell activation (data not shown). However, if the IS was first allowed to form, β-MCD treatment did not appreciably alter the amount of cholera toxin or E accumulation at the synapse (Fig. 5, B and C). Furthermore, the general shape of the C-SMAC and its area were preserved (Fig. 5A, ICAM-1 images, gray arrows). The efficacy of the β-MCD treatment was apparent from the decrease in the overall area of contact (Fig. 5A, IRM images; Fig. 5D, lower curves) and the shriveling of the entire cell (Fig. 5A, transmitted light images). These results indicate that regardless of the role “raft” membrane microdomains may have in the initial accumulation of molecules in the C-SMAC, the stability of the C-SMAC, once formed, is due to physical processes other than the cholesterol-driven lipid immiscibilities that are the basis of lipid rafts.

Discussion

Several experimental approaches suggest a critical role for DIGs in T cell activation. First, cross-linking glycosphingolipids activates lymphocytes (18–20). Second, drugs affecting DIGs block T cell signaling (7, 21). Third, Lck, linker for activation of T cells, protein kinase C-θ, and TCRζ co-cop with DIG constituents or partition into DIG fractions (3, 5–7, 10). Because this same set of essential signaling molecules is enriched in the C-SMAC, it has been widely hypothesized that the C-SMAC is highly enriched in “rafts” or might even represent a single large coalesced “raft” (4, 22–26). Published images have shown a relative accumulation of a lipidic raft component, GM1, at the T cell:APC contact (15, 16); however, these images did not have sufficient resolution to determine whether GM1 localized specifically to the C-SMAC. In addition, the level of accumulation was not measured. Here, we demonstrated for the first time that this marker of “rafts” is enriched specifically in the C-SMAC and that the concentration is only ~2- to 3-fold higher than the plasma membrane outside the IS.

Viola et al. (8) imaged the accumulation of a “raft” marker at the contact between human peripheral blood T cells and beads coated with anti-CD3 and anti-CD28 Abs. They found that beads containing anti-CD28 and anti-CD3, but not anti-CD3 alone, induced recruitment of GM1 to the bead:cell contact, suggesting that co-stimulation is required to stimulate lipid raft recruitment. While we demonstrate that CD28 costimulation is not required for lipid raft recruitment, other costimulatory signals may be active. In both the bilayer and APC systems, LFA-1 is present. LFA-1, a known co-stimulator, is able to stimulate the myosin-dependent movement system that may be responsible for lipid raft recruitment (13).

Theoretical considerations suggest that levels of raft recruitment greater than the ~2- to 3-fold we measured is unlikely. Maxfield et al. (27) have pointed out that “rafts” constitute a very large fraction of the plasma membrane, on the order of 40–70%. Assuming that in our T cells the “raft” fraction constitutes just 30% of the plasma membrane, then the maximum possible enrichment is 3-fold; if it is 50%, the maximum enrichment is only 2-fold. Greater degrees of GM1 concentration cannot occur unless one assumes that GM1 marks only a very small portion of the “raft” fraction or that the GM1 content of the “raft” fraction changes rapidly. We find it compelling that the measured accumulation of GM1 in the C-SMAC correlates fairly well with that predicted by a simple theory.

The stability of C-SMAC composition and shape to cholesterol extraction suggests that “rafts” as popularly understood might not be the best model of the mature C-SMAC. There are other models of membrane domain formation that do not invoke the cholesterol-induced lipid immiscibility (demixing) that is the physical basis of DIGs. A “corral” model for membrane domain structure seems to us to provide a better description of the forces that support the structure of the mature IS (28).

In summary, a lipid “raft” marker accumulates specifically in the C-SMAC, the site where proteins known to be DIG associated also accumulate. The Ag-dependent accumulation of GM1 in the C-SMAC involves local reappropriations within the IS rather than wholesale recruitment from distant regions of the plasma membrane. The results suggest that the composition of the C-SMAC could be determined by the specific exclusion of components from the P-SMAC rather than by active recruitment to the C-SMAC. In our system, CD28 does not play a unique role in supporting the accumulation of rafts in the C-SMAC. Last, while it seems likely that “rafts” composed of DIGs play a role in IS formation, other forces that can affect the lateral distribution of proteins within the plane of the bilayer may need to be considered to fully understand the IS structure.

References


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