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Lipid Raft Heterogeneity in Human Peripheral Blood T Lymphoblasts: A Mechanism for Regulating the Initiation of TCR Signal Transduction

Andrew E. Schade* and Alan D. Levine2*†‡

Lateral mobility and spatial organization of proteins within the plasma membrane are likely to mediate the initial events coordinating T cell activation. Lipid rafts, distinct cholesterol/sphingolipid-rich membrane microdomains, provide a mechanism for this regulation by concentrating or excluding signaling proteins. We demonstrate in peripheral blood T cell lymphoblasts that immediate early phosphorylase signal transduction through the TCR complex is functionally dependent on a distinct population of lipid rafts. Specifically, cholesterol extraction destabilizes the membrane microdomains containing Lck, while the rafts containing the adapter protein linker for activation of T cells remain intact. Heterogeneity in the partitioning of these proteins in resting cells was confirmed by immunochemistry. After T cell activation, both Lck and the linker for activation of T cells colocalize to 50–100 nm microdomains in the plasma membrane, indicating that sequestration of these proteins into distinct lipid rafts may function to regulate the initiation of T cell signal transduction. The Journal of Immunology, 2002, 168: 2233–2239.

When T cells are stimulated through the TCR, a well-characterized sequence of biochemical events is initiated in which protein tyrosine phosphorylation is the earliest identifiable feature of T cell activation (1–5). Upon TCR binding to an MHC/peptide complex on the surface of an APC, signal transduction commences with the activation of the Src family protein tyrosine kinase (PTK) Lck, which phosphorylates the immunoreceptor tyrosine activation motifs (ITAM) of the CD3 complex of polypeptides and the TCR-ζ chain (TCR-ζ) homodimer. This results in the membrane targeting of another PTK, ZAP-70, to phospho-ITAMs via Src homology 2 domain interactions, and its subsequent activation by Lck. ZAP-70 then phosphorylates several substrates, including the linker for activation of T cells (LAT). Phospho-LAT acts as a docking molecule for a variety of enzymes and adapter proteins, such as Vav, phospholipase Cγ, phosphatidylinositol-3 kinase (85 kDa subunit), and Grb2 (6). This cascade is required for efficient activation of second messenger pathways, including calcium mobilization and the Ras/mitogen-activated protein kinase pathway (7).

In contrast, the spatial-temporal organization of these enzymatic events is less well understood. For instance, the lateral organization of membrane associated proteins such as the TCR/CD3 complex, PTKs, and adaptor molecules within the lipid bilayer is only beginning to be elucidated (8–16). The plasma membrane contains lipid assemblies enriched in cholesterol and glycosphingolipids, known as lipid rafts, which form distinct microenvironments for preferential enrichment and exclusion of certain molecules (17). Critical signaling molecules of the TCR/CD3 complex such as Src family PTKs (18), LAT (19), phosphoinositides (20), CD4 (21, 22), and the recently cloned phosphoprotein associated with glycosphingolipid-enriched microdomains/Cbp (23, 24) are targeted to lipid rafts. In the transformed Jurkat T cell line, disruption of rafts by cholesterol depletion reduces calcium mobilization (22, 25, 26). Therefore, it has been postulated that these microdomains are required for signal transduction in T cells.

Most of the studies in humans have been limited to the Jurkat T leukemia cell line. Therefore, we examined the peripheral blood T cell (PBT), which more closely reflects a physiological response. In this study, we report that the integrity of lipid rafts containing the kinase Lck is essential for the initiation of PBT signal transduction through the TCR. Furthermore, while both Lck and LAT are targeted to lipid rafts, we demonstrate heterogeneity in raft composition in the native plasma membrane of PBT, causing the physical sequestration of these critical signaling proteins in a resting T cell. However, after activation, both Lck and LAT coinhabit a localized region ~50–100 nm within the plasma membrane.

Materials and Methods

Cells and reagents

PBMC were isolated from healthy donors by Ficoll-Hypaque density separation. T lymphoblasts were prepared by PHA (0.5%) stimulation for 48 h in the presence of IL-2 (20 U/ml; Chiron, Emeryville, CA) in RPMI 1640, 10% heat-inactivated FCS, and 2.5% HEPES, and thereafter carried in IL-2 (20 U/ml) for >8 days to obtain a population of CD4+CD45RO+ peripheral blood T lymphoblasts. The following Abs were used: rabbit anti-LAT, rabbit anti-Lck, and 3A5 mouse anti-Lck mAb, mouse anti-ZAP-70 (Upstate Biotechnology, Lake Placid, NY), anti-phosphotyrosine (PY20; BD Transduction Laboratories, Lexington, KY), rabbit anti-CD4 and HRP-conjugated secondary Abs (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse TCR-ζ (Zymed Laboratories, San Francisco, CA), rabbit anti-phospho-ZAP-70 (Y319), and rabbit anti-phospho-Lck (Y394; Cell Signaling Technology, Beverly, MA). Methyl β cyclodextrin (MβCD) and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO). Lysol buffer for lipid raft preparation and immunosloation consisted of 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, protease and phosphatase inhibitor mixtures (Sigma-Aldrich), 1 mM PMSF, and 1% Brij 58 (Pierce, Chicago, IL).
Rockford, IL). Colloidal gold-conjugated secondary Abs, 12 nm anti-mouse, and 6 nm anti-rabbit were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

**T cell stimulation**

PBT, rested overnight in the absence of IL-2 (RPMI 1640, 10% FCS, 2.5% HEPES) at 37°C, were resuspended (5 × 10⁶ cells/ml) in RPMI 1640, 2.5% HEPES, and a protease inhibitor mixture with or without 10 mM MgCl₂ and incubated at 37°C for 5 min. Cells were then stimulated via OKT3 Ab (10 μg/ml; Ortho Diagnostics, Raritan, NJ) with cross-linking (sheep anti-mouse Fab(ab)₂, 10 μg/ml; Sigma-Aldrich) for 5 min at 37°C, immediately followed by the addition of 2× Laemmli sample buffer and boiled for 5 min before Western blot analysis. Unstimulated cells received only the sheep anti-mouse Fab(ab)₂.

**LAT immunosolubilization**

Rabbit anti-LAT was incubated for 5 h at 4°C with M-280 superparamagnetic polystyrene beads (Dynal Biotech, Lake Success, NY) to which sheep anti-rabbit Abs were covalently attached to the surface. PBT were stimulated as described above, except that reactions were quenched by immediately pelleting the cells at 4°C and resuspending them in 0.5 ml ice-cold lysis buffer. Cells were lysed at 4°C on a rotating wheel for 30 min, after which the postnuclear supernatant was added to the beads bearing rabbit anti-LAT and incubated at 4°C for 90 min with gentle rotation. The M-280 beads were then separated from the cell lysate by magnetic isolation on an MPC-5 magnet (Dynal Biotech), and washed four times in ice-cold lysis buffer. After the final wash, the beads were boiled in 50 μl of 1× Laemmli buffer.

**Lipid raft fractionation**

PBT were resuspended in 0.5 ml ice-cold lysis buffer and lysed at 4°C on a rotating wheel for 30 min. Postnuclear supernatants were gently mixed with an equal volume of cold 85% sucrose (w/v) in lysis buffer without detergent and placed in the bottom of a 4 ml ultracentrifuge tube (Sorvall TST-604, Newtown, CT). The sample was overlaid with 2 ml 35% sucrose and 1.2 ml 5% sucrose, all at 4°C in lysis buffer without detergent. Equilibrium centrifugation was performed at 200,000 × g, 4°C, for at least 3 h, as described (27). Fractions were collected as 0.4-ml samples from the top. A fixed volume from each fraction of the sucrose gradient (0.015 ml) was mixed with an equal volume of 2× Laemmli buffer and boiled for 8 min before Western blotting.

**Western blotting**

Proteins were separated by SDS-PAGE on a 10% gel under reducing conditions and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA) in a transfer buffer consisting of 20 mM Tris-HCl, 150 mM glycine, and 20% methanol. Membranes were incubated at 4°C overnight in blocking buffer (5% nonfat milk, 0.1% Tween 20 in PBS). Primary and secondary Abs were diluted as recommended by the manufacturer in blocking buffer and incubated with the membranes for 1 h at room temperature with six washes in between. Detection of HRP-conjugated Abs was performed using SuperSignal (Pierce). Phosphotyrosine analysis was performed as follows. Membranes were blocked for 1 h at room temperature in blocking buffer (3% BSA, 10 mM Tris (pH 7.2), 100 mM NaCl, 1% Tween 20). HRP-conjugated PY20 Ab was diluted 1/2500 in blocking buffer and incubated with the membranes for 1 h at room temperature. Chemiluminescence of all membranes was detected using Hyperfilm ECL (Amersham, Piscataway, NJ).

**Transmission electron microscopy (TEM)**

PBT were either stimulated via OKT3 Ab (10 μg/ml) with cross-linking sheep anti-mouse (10 μg/ml) for 5 min at 37°C, as described above, or incubated with only the sheep anti-mouse Fab(ab)₂ at 37°C. Reactions were stopped by placing the samples on ice. The stimulating Abs were then washed away and cells were resuspended in RPMI 1640 at 4°C. Plasma membrane sheets were prepared as described by Sanan and Anderson (28) and Wilson et al. (29). PBT were settled onto poly-L-lysine-coated glass coverslips at 4°C for 45 min. The coverslips were then inverted onto nickel electron microscopy grids that had been previously floated on poly-L-lysine, washed in dH₂O, and air-dried. Light finger pressure was distributed over a 15-mm rubber cork placed on the coverslip for 10 s. The grid was plucked off, allowing the apical portion of the adherent PBT plasma membrane to remain attached to the grid and leaving the rest of the cell on the coverslip. The grid containing the membrane sheet was immediately washed in 25 mM HEPES (pH 7.2), 25 mM KCl, and 2.5 mM magnesium acetate for 5 s, to remove any cytosol and contaminants, before fixation in 2% paraformaldehyde for 10 min, followed by three 5-min washes in PBS. The grids were then blocked in 0.5% BSA-c (Electron Microscopy Sciences, Fort Washington, PA) in PBS for 30 min. To prevent cross-reactivity with any mouse OKT3 that may still be attached to the membrane, all samples (including controls) were incubated for 30 min in goat anti-mouse IgG Fab fragments (Jackson Immunoresearch Laboratories). Controls showed that this completely prevented secondary colloidal gold-conjugated Abs from binding any available OKT3 that may be present in the preparation. Samples were washed for 30 min in 0.5% BSA-c/PBS, with three exchanges between all Ab labeling steps. Primary Ab labeling of LAT (1:100 of rabbit anti-human LAT) and Lck (1:100 of mouse 3A5 anti-human Lck) were performed sequentially, with secondary colloidal gold-conjugated Abs immediately following their respective primary Ab. All Ab incubations were performed in the presence of 0.5% BSA-c/PBS at room temperature for 30 min. Following the final washing steps, samples were fixed overnight in 2% glutaraldehyde/PBS at 4°C. Samples were washed three times in PBS before a 10-min staining in 1% aqueous osmium tetroxide, followed by five 5-min washes in dH₂O. Samples were subsequently processed in 1% aqueous tannic acid for 10 min, followed by two 5-min washes in dH₂O, and then 10 min in 2% aqueous uranyl acetate, followed by two 1-min washes in dH₂O. Samples were air-dried before analysis on a JEOL 1200CX TEM (Peabody, MA). Gold particle distributions were quantified as previously described (29). Singlet and clusters of either 6 or 12 nm gold particles were counted over 40–100 μm² of membrane in both resting and activated T cells and scored for colocalization or not.

**Results**

**Cholesterol extraction inhibits membrane proximal protein tyrosine phosphorylation in PBT**

We hypothesized that if lipid rafts were essential for signaling through the TCR in PBTs, disruption of raft integrity via cholesterol extraction with MβCD would inhibit the very earliest events in TCR-initiated signal transduction. Resting PBT exhibit a very low level of basal protein tyrosine phosphorylation (Fig. 1, lane 1). Upon addition of 10 mM MβCD to PBT for 10 min at 37°C, there is no induction of tyrosine phosphorylation (Fig. 1, lane 2). As expected, when PBT are stimulated through the TCR via CD3 cross-linking for 5 min at 37°C, there is a pronounced increase in

![Figure 1: Lipid raft disruption inhibits TCR-activated PBT protein tyrosine phosphorylation. PBT (5 × 10⁶) were left untreated (lanes 1 and 3) or treated with 10 mM MβCD (lanes 2 and 4) for 5 min at 37°C. Cells were stimulated via anti-CD3ε cross-linking (OKT3, 10 μg/ml; lanes 3 and 4) for 5 min at 37°C. Reactions were quenched by the addition of 2× Laemmli buffer and immediately boiled for 5 min. Samples were analyzed by SDS-PAGE and Western blotting for phosphotyrosine (PY20). Molecular weight markers are indicated. Immunoblot for LAT was used as a control for total protein loading. Results are representative of eight different donors.](http://www.jimmunol.org/)


total protein tyrosine phosphorylation (Fig. 1, lane 3). This robust induction of the membrane proximal signaling cascade is strongly inhibited when the PBT are treated with 10 mM MβCD for 5 min at 37°C, before the 5-min stimulation (Fig. 1, lane 4). To confirm that the heavily phosphorylated protein in the 36–38 kDa range was in fact LAT, we immunoprecipitated LAT from control and TCR-stimulated cell lysates. Conventional protein A-agarose was not a viable option for this experiment, because the stimulating OKT3 Ab is capable of binding protein A (A. E. Schade and A. D. Levine, unpublished results), leading to erroneous conclusions. Therefore, we used an immunomagnetic approach in which sheep anti-rabbit Abs are covalently attached to the surface of superparamagnetic polystyrene beads and then rabbit anti-LAT was incubated with the beads. Lysates from TCR-stimulated cells or controls were incubated with the immunomagnetic beads and the bound LAT proteins were extracted by magnetic isolation and analyzed via SDS-PAGE and Western blotting initially for total phosphotyrosine and subsequently for LAT (Fig. 2). When PBT are stimulated through the TCR via CD3 cross-linking for 5 min at 37°C, there is a pronounced increase in LAT tyrosine phosphorylation (Fig. 2, lane 2). However, phosphorylation of LAT is strongly inhibited when the PBT are treated with 10 mM MβCD for 5 min at 37°C, before the 5-min stimulation (Fig. 2, lane 4). These results demonstrate that disrupting lipid rafts in PBT inhibits membrane proximal signal transduction through the TCR, consistent with the reduction of calcium flux, a signaling event downstream from the tyrosine phosphorylation (22, 25, 26).

Cholesterol extraction preferentially disrupts lipid rafts containing Lck

Recognizing the crucial role of the PTK Lck in the initiation of TCR signaling and the essential function of LAT in linking the TCR proximal signaling with the more generalized secondary signaling cascades, we assessed the membrane partitioning of Lck and LAT via biochemical fractionation after lysing PBT in 1% Brij-58 (Fig. 3). Both Lck and LAT are enriched in detergent-resistant membranes (DRMs), which represent the microdomains of the native plasma membrane (rafts) exhibiting liquid-ordered phase behavior. This property is in large part responsible for rendering these lipid rafts resistant to nonionic detergent disruption and therefore, capable of being isolated as low density membrane vesicles. As shown in the leftmost panel of Fig. 3, in PBT, both Lck and LAT are indeed associated with lipid rafts, although not to the same extent. Although Lck is distributed between lipid rafts and the more prevalent nonraft plasma membrane, LAT is exclusively partitioned to lipid rafts. Upon TCR stimulation (Fig. 3, third panel), both proteins remain raft-associated. However, when PBT are treated with 10 mM MβCD under the same conditions that inhibit the induction of immediate early protein tyrosine phosphorylation, there is a prominent redistribution of Lck from lipid rafts to the nonraft plasma membrane. This change in the raft association for Lck is the same in both unstimulated and stimulated cells (Fig. 3, second and fourth panels). Approximately 80% of raft-associated Lck is displaced from rafts after cholesterol extraction (Table I). In contrast, >50% of LAT remains associated with lipid rafts in the presence of MβCD (Fig. 3, Table I). Importantly, to prevent any systematic error in quantitating the immunoblots, Ab staining for both proteins was performed on the same nitrocellulose membrane. The partitioning of both Lck and LAT to lipid rafts was previously proposed to be essential for successful activation and propagation of TCR signal transduction (19, 30–40). However, this is the first report to link the inhibition of membrane proximal TCR signaling with the concurrent loss of Lck from lipid rafts in PBT. Furthermore, the relative lack of LAT redistribution after cholesterol depletion, weighed against an almost complete loss of Lck from lipid rafts, suggests that these proteins associate with biochemically distinct lipid raft microdomains in the plasma membrane, a concept we call heterogeneity.

Lck and ZAP-70, two tyrosine kinases that activate TCR signal transduction, are under-phosphorylated after cholesterol extraction

Although we attribute the loss of proximal TCR signaling to dissolution of the Lck containing lipid rafts, the significant decline in total cellular protein and LAT phosphorylation upon MβCD treatment may also be due to an event downstream of Lck. Therefore.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Reduced phosphorylation of LAT after cholesterol extraction is confirmed by immunoprecipitation. PBT (35 × 10^6) were left untreated (lanes 1 and 2) or treated with 10 mM MβCD (lanes 3 and 4) for 5 min at 37°C. Cells were stimulated via anti-CD3 cross-linking for 5 min at 37°C (lanes 2 and 4). Reactions were quenched by immediately pelleting the cells at 4°C and resuspending them in 0.5 ml ice-cold lysis buffer. Immunomagnetic isolation of LAT from postnuclear supernatants was performed as described in Materials and Methods. Samples were analyzed by SDS-PAGE and membrane probed sequentially for phosphotyrosine (PY20) and then LAT.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Raft</th>
<th>Non Raft</th>
</tr>
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<tbody>
<tr>
<td>Unstimulated</td>
<td>-MβCD</td>
<td>+MβCD (loss)</td>
</tr>
<tr>
<td>Stimulated</td>
<td>-MβCD</td>
<td>+MβCD (loss)</td>
</tr>
<tr>
<td>Lck</td>
<td>91b (74%c)</td>
<td>220</td>
</tr>
<tr>
<td>LAT</td>
<td>266</td>
<td>152 (43%)</td>
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* Protein bands from the immunoblots in Fig. 3 were scanned on a BioRad Gel Doc 1000 (Hercules, CA) and densitometry was performed with Multi-Analyst 1.0.2. (Bio-Rad).

* Arbitrary units.

* Percentage loss from lipid raft fractions.
we examined the tyrosine phosphorylation profile of Lck and ZAP-70, key PTKs whose activation precedes, and is necessary for, LAT phosphorylation (Fig. 4). After TCR engagement, one of the earliest identifiable events of Lck activation is phosphorylation at tyrosine 394, leading to a conformational change in the catalytic domain that substantially increases kinase activity (41–43). Using an Ab that recognizes phospho-Y394 activated Lck, we demonstrate that Lck is prominently activated upon TCR cross-linking for 5 min at 37°C (Fig. 4A, lane 3). Dramatic inhibition (18-fold reduction normalized to total Lck protein) of stimulation-induced Lck kinase phosphorylation (Fig. 4A, lane 4) is observed after cholesterol extraction under the same conditions that lead to a global decrease in protein tyrosine phosphorylation, a decrease in LAT phosphorylation, and significant displacement of Lck from lipid rafts.

After TCR engagement, ZAP-70 is rapidly recruited to phosphorylated ITAM-bearing proteins of the TCR/CD3 complex, whereby it is subsequently activated by Lck initiating autophosphorylation on Y319. Phosphorylation of this residue is essential for positive regulation of ZAP-70 and its ability to phosphorylate LAT (44, 45). Using an Ab capable of recognizing only phospho-Y319 ZAP-70, we confirm that ZAP-70 is activated upon TCR cross-linking (Fig. 4B, lane 3). Pretreatment with MβCD produces a 9-fold reduction in phospho-ZAP-70 after stimulation via TCR cross-linking (Fig. 4B, lane 4). Because Lck activation is necessary for ZAP-70 activation, and ZAP-70 activation is proximal to and required for LAT phosphorylation, it is unlikely that the reduction in LAT phosphorylation results from a portion of that protein being redistributed to the nonraft membrane, and is more likely due to an inhibition in the upstream signaling cascade.

To determine whether the association of LAT with an MβCD-resistant raft is unique and to further explore lipid raft heterogeneity in the plasma membrane, we examined the sensitivity to MβCD treatment of two additional transmembrane proteins, CD4 and TCR-ζ, involved in the initial events of TCR signal transduction. Approximately 30% of CD4 is raft-associated in resting PBT (Fig. 5, upper panel), and upon treatment with 10 mM MβCD, there is a 70% loss of CD4 from rafts (Fig. 5, upper panel). TCR-ζ is an essential component on the TCR/CD3 signaling complex, yet its association with lipid rafts in PBT has not been reported. In this study, we show that slightly >30% of TCR-ζ associates with lipid rafts and upon cholesterol extraction is completely displaced (Fig. 5, lower panel). These findings confirm that several TCR signaling proteins reside in MβCD-sensitive rafts in the native plasma membrane, while LAT is sequestered into distinct membrane microdomains. The significance of raft-associated CD4 and TCR-ζ proteins, compared with the more pronounced nonraft populations of these molecules, is currently under investigation.

Identification of discrete clusters of lipid rafts in resting T cell by TEM

To directly demonstrate the presence of lipid raft heterogeneity within the native plasma membrane, we examined the inner leaflet of the PBT membrane using TEM at a resolution not approachable with conventional optical microscopy. Using immunogold-conjugated Abs to detect Lck (12 nm colloidal gold) and LAT (6 nm colloidal gold), we demonstrate for the first time that these proteins of the TCR signaling cascade associate with mutually exclusive microdomains in the plasma membrane of a resting PBT (Fig. 6). We consistently observe a strong agreement between the TEM analysis of native membranes and biochemical fractionation of DRMs, predicting the partitioning of Lck and LAT between the raft and nonraft domains of the T cell plasma membrane. For instance, Lck fractionates into both the detergent-resistant (raft) and detergent-soluble (nonraft) membrane domains after density gradient ultracentrifugation. Similarly, systematic analysis of transmission electron photomicrographs reveals that Lck (Fig. 6, squares) partitions both in a clustered distribution in osmophilic (lipid rich) regions of the native membrane (75% of 12 nm particles in 105.5 μm² of membrane) and to a lesser extent, in a more diffuse random pattern (25% of 12 nm particles in 105.5 μm² of membrane). In contrast, LAT (Fig. 6, circles) is predominately clustered in microdomains of the native plasma membrane (>92% of 6 nm particles in 85.5 μm² of membrane).

**Rafts containing CD4 and TCR-ζ are both sensitive to cholesterol extraction**

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After TCR engagement both Lck and LAT colocalize to discrete membrane domains

We hypothesize that the heterogeneity of lipid rafts regulates T cell activation and predict that after TCR engagement, relevant signaling molecules must colocalize. To document this redistribution, we analyzed membrane sheets from PBT that had been stimulated through the TCR/CD3 complex. Within 5 min of cross-linking the TCR, there is a pronounced shift in the membrane distribution of Lck and LAT relative to one another (Fig. 7). Upon stimulation, some of the dispersed heterogeneous clusters sequestering Lck or LAT converge into common domains (39% of 12-nm labeled Lck and 46% of 6-nm labeled LAT in 42.7 μm² of membrane), characterized as osmiophilic membrane patches from 50 to 100 nm, which are likely to facilitate initiation of signal transduction (Fig. 7, hexagons).

Discussion

The critical cell that orchestrates an acquired immune response is the T lymphocyte. In this report, we demonstrate in peripheral blood T lymphoblasts that 1) heterogeneous lipid rafts are identifiable based on biophysical properties and electron microscopy, and 2) that initiation of PBT signal transduction is dependent on a distinct population of lipid rafts to which the kinase Lck is targeted. We show that raft disruption induced by cholesterol extraction, specifically those rafts enriched in Lck, leads to a strong inhibition of membrane proximal protein tyrosine phosphorylation after activation through the TCR/CD3 complex. In support of this interpretation, we observe significant inhibition of both Lck and ZAP-70 activation concurrent with Lck displacement from lipid rafts. This suggests that Lck association with rafts is required for effective induction of kinase activity upon TCR engagement. Therefore, we propose that the mechanism for the inhibition of TCR signal transduction in PBT after raft disruption is directly linked to the displacement of Lck from lipid rafts.

In contrast, raft-bound LAT is not affected by cholesterol extraction to the same extent as Lck in the same population of cells. One possible difference is that Lck is membrane-associated via posttranslational acyl modifications and LAT is a transmembrane protein that is palmitoylated on membrane proximal cytoplasmic cysteine residues. It is conceivable that a more stable association of the integral membrane protein LAT with the plasma membrane confers this greater resistance to the perturbing effects of cholesterol depletion. To address this possibility, we analyzed the membrane compartmentalization of two additional transmembrane proteins involved in the earliest aspects of signaling through the TCR/CD3 complex, CD4 and TCR-ζ. Both proteins associate with lipid rafts and both exhibit a sensitivity to cholesterol extraction similar to that of Lck, suggesting that the relative affinity of a protein for...
association with lipid rafts is not simply dependent on being either a peripheral or integral membrane protein. We consider the unique biophysical properties of the LAT-associated rafts (that is, the relative resistance to cholesterol extraction) to be evidence that distinct heterogeneous populations of lipid rafts exist in the plasma membrane of PBT, and this may be another mechanism by which the T cell regulates the initiation of signal transduction.

TEM is a powerful tool to characterize the distribution and composition of microdomains within the native plasma membrane of T cells. This is significant because optical techniques that lack the resolving power of electron microscopy, such as confocal microscopy, have failed to distinguish between spatially distinct regions for Lck and LAT compartmentalization, potentially leading to the erroneous conclusion that these proteins are localized to a common microdomain. The identification of distinct clusters of Lck and LAT proteins at nanometer resolution in resting PBT membranes not exposed to detergents provides a strong confirmation of the biochemical data that lipid raft heterogeneity exists in vivo. Similarly, studies in resting RBL-2H3 mast cells have reported that Thy-1 does not colocalize with Lyn or the FceRI (29) and that LAT rarely colocalizes with FceRI (46). Furthermore, TCR stimulation-induced reorganization of the membrane microdomains, resulting in Lck and LAT residing in common domains, suggests a functional role for these specialized membrane compartments in T cell activation.

The physiological role of lipid raft heterogeneity in T cells remains to be uncovered, but we hypothesize that maintaining signal molecules in distinct heterogeneous microdomains within the plasma membrane is a mechanism by which T cells orchestrate a rapid, controlled, and dynamic response to Ag exposure. In simple terms, lipid raft heterogeneity isolates an enzyme in lipid raft A from a substrate in lipid raft B in the nonactivated T cell. Upon TCR engagement, changes in the organization of the plasma membrane and its constituents, possibly mediated by the cytoskeleton, facilitate the rearrangement and redistribution of raft-associated proteins, thereby eliminating previous spatial constraints, thus catalyzing the initiation of the enzymatic cascade. In further support of this concept, Gomez-Mouton et al. (47) suggest that protein redistribution in polarized T cells involves raft partitioning. Heterogeneity in the composition, cholesterol-dependence, and localization of lipid rafts in the native membrane of the T cell may be a mechanism by which discrete signaling pathways can be differentially activated during an immune response, thereby modulating cytokine production patterns and T cell proliferation (48). Changes in the lateral mobility of enzymes, substrates, and adaptor proteins within the plasma membrane can regulate these functional differences within an activated T cell, eliciting dramatically different immunological effector functions.

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\textsuperscript{lck} through mutation of a regulatory carboxy-terminal tyrosine residue requires intact sites of autophosphorylation and myristylation. Mol. Cell Biol. 10:3197.


