LAT Displacement from Lipid Rafts as a Molecular Mechanism for the Inhibition of T Cell Signaling by Polyunsaturated Fatty Acids*

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Polyunsaturated fatty acids (PUFAs) suppress immune responses and inhibit T cell activation through largely unknown mechanisms. The displacement of signaling proteins from membrane lipid rafts has recently been suggested as underlying PUFA-mediated T cell inhibition. We show here that PUFA treatment specifically interferes with T cell signal transduction by blocking tyrosine phosphorylation of LAT (linker for activation of T cells) and phospholipase Cγ1. A significant fraction of LAT was displaced from rafts by PUFA treatment along with other signaling proteins. However, retaining LAT alone in lipid rafts effectively restored calcium signaling in PUFA-treated T cells. These data reveal LAT displacement from lipid rafts as a molecular mechanism by which PUFAs inhibit T cell signaling and underline the predominant importance of LAT localization in rafts for efficient T cell activation.

Polyunsaturated fatty acids (PUFAs) suppress immune responses (1) and are clinically applied as adjuvant immunosuppressive agents in the treatment of inflammatory disorders (e.g. rheumatoid arthritis and inflammatory bowel disease) (2, 3) and following organ transplantation (4). PUFAs of the n-3 series have been shown to be particularly effective and are known to interfere with proinflammatory eicosanoid (prostaglandin/leukotriene) synthesis (1). However, PUFA-mediated inhibition of T lymphocyte activation and function has repeatedly been shown to be independent of eicosanoid synthesis (5–8). The molecular mechanism by which PUFAs inhibit T cell signal transduction has remained largely unknown. Because PUFA treatment changes the lipid composition of lymphocyte membranes (9) and functionally important membrane lipid rafts (10), alterations of rafts have been suggested as underlying the PUFA-mediated inhibition of T cell signaling (11).

Membrane lipid rafts or microdomains are specialized regions within the plane of the plasma membrane and have been proposed as playing an essential role in T cell signal transduction (12–16). Lipid rafts are characterized by a high concentration of cholesterol and sphingolipids such as sphingomyelin and glycolipids, and their polar lipids contain predominantly saturated fatty acyl residues (17, 18). Such lipids spontaneously form liquid-ordered membrane regions that are insoluble in non-ionic detergents, facilitating raft isolation as detergent-resistant membrane domains (18, 19). Cytoplasmic and transmembrane proteins are targeted to lipid rafts mostly by acylation with saturated fatty acyl moieties (palmitoyl and myristoyl) which link these proteins to the cytoplasmic lipid leaflet of microdomains (20). Several proteins involved in T cell signaling such as Src family protein-tyrosine kinases (PTKs) and LAT (linker for activation of T cells) are concentrated in rafts because of post-translational palmitoylation, emphasizing the role of lipid rafts in T cell signaling (14, 21).

The activation of T cells requires stimulation of the T cell antigen receptor (TCR)/CD3 complex. Triggering CD3 leads to the activation of Src-family PTKs and the phosphorylation of immunoreceptor tyrosine-based activation motifs in the cytoplasmic domains of the CD3 complex, thereby facilitating the binding of other signaling molecules such as the Syk-family PTKs ZAP-70 and Syk (21). ZAP-70 associates with phosphorylated CD3ζ and is subsequently activated by the Src family kinase Lck. Following phosphorylation by ZAP-70, the central adaptor protein LAT recruits phospholipase Cγ1 (PLCγ1) to the plasma membrane (22, 23). PLCγ1 is also activated by ZAP-70 to liberate inositol 1,4,5-trisphosphate from the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate, thereby eliciting an increase in cytoplasmic calcium concentration, which is a key event for promoting downstream activation (24).

Recent studies have shown that PUFA treatment leads to the displacement of Src family PTKs from lipid rafts in parallel with the inhibition of calcium response (11). However, the particular step in T cell signaling by which PUFA-mediated modification of T cell membrane rafts affects T cell activation remained unknown. Here we show that PUFA treatment of T cells specifically blocks signal transduction at the level of LAT and PLCγ1 phosphorylation by the partial displacement of LAT from lipid rafts. Moreover, the expression of a genetically modified LAT protein that is retained in the membrane rafts of PUFA-treated T cells effectively restores PLCγ1/calcium signaling in PUFA-enriched T cells. These results reveal that LAT displacement from lipid rafts is a crucial molecular mechanism for PUFA-mediated inhibition of T cell signaling.
**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies were obtained as follows: OKT3 (anti-CD3e) from Ortho-McNeil Pharmaceuticals (Raritan, NJ); SD3 (anti-CD3γ) and ID4 (anti-phospho-CD3δ) from BD Pharmingen (San Diego, CA); anti-Leak and anti-ZAP-70 from BD Transduction Laboratories (Lexington, KY); anti-ZAP-70, anti-Syk, and anti-PLCγ1 from Santa Cruz Biotechnology; anti-phospho-PLCγ1 (Tyr(P)-783) from BioSource International (Camarillo, CA); anti-phospho-ZAP-70 (Tyr(P)-493) from Cell Signaling Technology (Beverly, MA); anti-LAT, anti-PLCγ1, anti-Emt/Irk/Tsk and horseradish peroxidase-labeled 4G10 (anti-phosphotyrosine) from Upstate Biotechnology (Lake Placid, NY); F(ab')2-fractments of goat anti-mouse IgG from Sigma; horseradish peroxidase-labeled goat anti-mouse IgG from Bio-Rad; horseradish peroxidase-labeled goat anti-rabbit IgG from Accurate Chemical and Scientific (Webster, NY). Mouse monoclonal antibodies MEM-43, MEM-241 (anti-CD4), and MEM-255 (anti-PAG) were produced in the Prague laboratory.

**Cells and Lipid Modification**—Human Jurkat T cell lines E6-1 and J7.7 were purchased from ATCC (Manassas, VA), and ANJ3 (LAT-deficient; Ref. 25) was generously provided by L. E. Samelson, National Institutes of Health, Bethesda, MD. Peripheral blood T lymphocytes (PBTLs) were isolated by separating mononuclear cells from buffy coats via Ficol-Paque (Amersham Biosciences) density gradient centrifugation and by subsequent rosetting with neuramidase-treated sheep erythrocytes (Dade Behring, Marburg, Germany). The obtained population contained >90% CD3-positive cells (not shown). As described previously (11), Jurkat cells or PBTLs were enriched with fatty acids by incubation for 2 or 5 days, respectively, in serum-free Iscove's modified Dulbecco's medium (IMDM, containing 0.4% (v/v) bovine serum albumin (fraction V) and indicated concentrations of eicosapentaenoic acid (20:5(n-3)) or stearic acid (18:0; all from Sigma), which served as a control. Stearic acid was found previously to be indistinguishable from the vehicle alone (ethanol, ≤0.5% (v/v)) in regard to PUFA content, membrane subdomain distribution of proteins, and calcium signaling (Ref. 11 and data not shown).

**Quantitation of Calcium Response**—Cytoplasmic calcium concentration was determined by flow cytometry using the fluorescent Ca2++ indicator indo-1 (Molecular Probes, Eugene, OR) as described (26, 27). The ratio of the fluorescence intensities at 395 and 530 nm was computed as a direct estimate of the cytoplasmic calcium concentration. Maximal indo-1 fluorescence ratios achieved by stimulation were compared between PUFA and stearic acid-treated cells (26).

**Cell Stimulation and Immunoprecipitation**—Jurkat and peripheral blood T cells were washed with Hank's balanced salt solution (Invitrogen) including 10 mM Heps, pH 7.4, and stimulated via CD3 by incubation with 5 μg/ml OKT3 for 1 min followed by cross-linking with 10 μg/ml F(ab')2-fractments of goat anti-mouse IgG for 1 min at 37°C. After cessation by the addition of ice-cold washing buffer, cells (2 × 106) were lysed on ice for 30 min in Tris-buffered saline (pH 8.2) containing 1% Nonidet P-40, and protease inhibitors. After removing nuclei, indicated proteins were immunoprecipitated using GammaBind Plus beads (Amersham Biosciences) preincubated with specific antibodies.

**Analysis of Membrane Rafts**—Membrane lipid rafts were separated as detergent-resistant floating material by lyses of T cell membranes in Triton X-100 followed by isopycnic centrifugation in a 50%–90% sucrose gradient as described (11). Eleven fractions of equal volume were collected from the top. Standard Western blotting procedures were applied, and proteins were detected by chemiluminescence on a Lumi-Imager (Roche Molecular Biochemicals). This system allows linear collection from the top. Standard Western blotting procedures were applied, and proteins were detected by chemiluminescence on a Lumi-Imager (Roche Molecular Biochemicals). This system allows linear collection from the top.

**RESULTS**

**PUFA Enrichment Blocks T Cell Signaling at the Level of LAT and PLCγ1 Phosphorylation**—Treatment with PUFAs but not with saturated or monounsaturated fatty acids inhibits CD3-induced calcium response in Jurkat T cells (11). As shown in Fig. 1A, treatment with polyunsaturated eicosapentaenoic acid (20:5) but not saturated stearic acid (18:0) significantly inhibited CD3-stimulated calcium response not only in Jurkat T cells but also in PBTLs. Calcium signaling depends on the release of inositol 1,4,5-trisphosphate from the membrane lipid rafts. Tyrosine phosphorylation of PLCγ1 by PKC was markedly decreased when Jurkat or peripheral blood T cells were stimulated with puromycin (Sigma). The expression of transactivated protein was detected by immunoblotting with anti-LAT antibody (Upstate Biotechnology), which is directed against amino acids 31–223.

**Miscellaneous**—Surface expression of CD3ε was assessed by immunofluorescence analysis. Data are expressed as means ± S.E. Statistical comparisons were performed by a two-tail unpaired Student’s t test, and a p < 0.05 was considered statistically significant.

**FIG. 1. Calcium response and PLCγ1 phosphorylation in PUFA-enriched T cells.** Jurkat cells (J7.7) or PBTLs were incubated in medium supplemented with 50 μM saturated (18:0) or polyunsaturated fatty acid (20:5) and stimulated by cross-linking of CD3. A, the CD3-induced rise of cytoplasmic calcium concentration was monitored by indo-1 fluorescence ratio. Maximal fluorescence ratios from five independent experiments are given and compared with values obtained from solvent-treated control cells (E0OH), which were set to 100%. B, PLCγ1 was immunoprecipitated from Jurkat cell lysates, and tyrosine phosphorylation (pY) of the immunoprecipitated protein (IP) was determined by Western blotting with a phospho-PLCγ1-specific antibody. The PLCγ1 protein was detected on stripped blots. Relative amounts of phospho-PLCγ1 following stimulation were compared between PUFA (20:5)- and control (18:0)-treated samples (n = 4 for each cell type) and are given as a percentage of stimulated control, which was set to 100%. Significant differences are indicated by asterisks (*, p < 0.05; **, p < 0.01; ***, p ≤ 0.001).

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lysates (rylation was detected as pp36/38 in phosphotyrosine blots of whole cell detected by immunoprecipitation (18:0; control) or polyunsaturated fatty acid (20:5) and stimulated by phosphotyrosine Western blotting (pY/H9256 not interfere with CD3-induced phosphorylation of CD3 treatment (Fig. 2

phosphorylation of LAT was significantly blocked by PUFA enriched Jurkat T cells (Fig. 2

PTKs was apparently not inhibited in CD3-stimulated, PUFA-saturated Jurkat J7.7 cells (Fig. 2

such as Itk/Emt/Tsk (21). Tyrosine phosphorylation of these family PTKs (ZAP-70 and Syk) but also of Tec family PTKs (28, 32). Because PAG/Cbp was exactly co-distributed with GPI-anchored cell surface proteins, exemplified by CD59, remained inside lipid rafts in PUFA-enriched T cells as reported previously and were hence used as raft markers (Fig. 3 and Ref. 11). Targeting to lipid rafts of intracellular proteins such as LAT, CD3, and PLC

Lysozyme, CD3ζ, ZAP-70, Syk, Itk, LAT, CD59, PAG, Lck, and PLC were inhibited by PUFA treatment and (ii) LAT was effectively displaced from rafts, the displacement of LAT from lipid rafts could be the primary cause for disturbed signal transduction in PUFA-enriched T cells. Strikingly, another palmitoylated protein, PAG/Cbp (28, 32), was found in lipid rafts irrespective of fatty acid treatment (Fig. 3).

PAG-LAT Chimeric Protein Is Retained in Rafts of PUFA-enriched T Cells—To test the hypothesis that displacement of LAT from lipid rafts is the underlying molecular mechanism of PUFA-mediated T cell inhibition, we expressed a genetically modified LAT protein with the potential to be localized in the membrane lipid rafts of PUFA-enriched T cells. The transmembrane adaptor protein PAG/Cbp is structurally similar to LAT but contains a longer (16 amino acids) extracellular domain (28, 32). Because PAG/Cbp was exactly co-distributed with GPI-anchored CD59 in sucrose density gradients, we assumed that the extracellular and/or transmembrane domain of PAG/Cbp could underlie its unique retention in rafts of PUFA-treated T cells. Therefore, we combined the functionally important intracellular part of LAT with the extracellular and transmembrane domains of PAG/Cbp, including its membrane-proximal palmitoylation site. The chimeric PAG-LAT and the wild type LAT were stably introduced into the LAT-deficient Jurkat subline ANJ3 (25). The clones obtained from each transfectant expressed comparable amounts of the transfected proteins PLC-Y1 and CD3ζ, although the expression of LAT or PAG-LAT, respectively, exceeded LAT expression in parental Jurkat T cells (Fig. 4A). As we anticipated, PAG-LAT but not endogenous Lck remained in
lipid rafts in PUFA-enriched cells comparable with wild type PAG/Cbp. In contrast, transfected wild type LAT was markedly displaced from rafts in parallel to Lck and similar to the manner of endogenous LAT in parental Jurkat T cells (Fig. 4B, cf. Fig. 3).

**PAG-LAT Restores PLCγ1 Phosphorylation and Calcium Response in PUFA-enriched T Cells**—CD3 stimulation of the LAT-deficient Jurkat subline ANJ3 did not evoke PLCγ1 tyrosine phosphorylation or calcium response, but reconstitution with wild type LAT or PAG-LAT effectively rescued PLCγ1 and calcium signaling to levels comparable with wild type LAT or PAG-LAT in PUFA-enriched T cells. Accordingly, CD3-induced calcium response was diminished after PUFA treatment in ANJ3 clones transfected with wild type LAT or PAG-LAT, respectively. In contrast, transfected ANJ3 Jurkat T cells were incubated with 30 μM saturated (18:0) or polyunsaturated fatty acid (20:5). Membranes were solubilized by non-ionic detergent and fractionated on a density gradient with floating fractions 3–7 (fraction #) representing lipid rafts. Indicated proteins were detected by Western blotting (WB), whereby the anti-LAT antibody also binds to chimeric PAG-LAT. The proportion of each protein recovered from floating fractions of three independent experiments is given as a percentage.

**DISCUSSION**

A number of recent studies have shown that TCR signaling is dependent on lipid rafts and their stimulation-induced association (12–16). Membrane lipid rafts provide a platform where several molecules critically involved in the early phases of the TCR signaling cascade are concentrated, including Src family kinases (Lck and Fyn) and the adaptor protein LAT. The current model assumes that aggregates of TCR complexes induced by their natural or surrogate ligands fuse with rafts, and, thereby, activation motifs of the TCR-associated CD3 complex become phosphorylated by Lck and Fyn present in the rafts (21, 33). Phosphorylated CD3ζ chains then serve as docking sites for the cytoplasmic kinase ZAP-70, which subsequently phosphorylates its major substrate, the transmembrane adaptor protein LAT, which is constitutively present in rafts. Tyrosine-phosphorylated LAT then binds several other molecules involved in the initiation of further downstream pathways (15, 21).
LAT Displacement from Lipid Rafts by PUFAs

The integrity of membrane rafts mainly depends on the physical interactions of saturated fatty acyl residues, which are characteristic for their lipid and protein constituents (18). Consequently, the substitution of saturated residues in raft lipids and/or lipidated proteins by polyunsaturated acyl moieties could easily result in functional disturbances in TCR signaling. Previous data from our laboratory showed that Src family kinases, which are attached by acylation to the cytoplasmic membrane lipid leaflet, were largely displaced from rafts in PUFA-treated T cells, whereas markers of the raft exoplasmic leaflet such as GPI-anchored proteins or ganglioside GM1 remained in rafts of PUFA-enriched T cells (11). However, the results of the present functional analysis revealed that, rather than depletion of Src family kinases, the partial displacement of another raft component, the adaptor protein LAT, is of crucial importance for PUFA-induced suppression of TCR signaling.

As shown in Fig. 2, Lck-driven tyrosine phosphorylation of CD3ζ and ZAP-70 was not altered in PUFA-enriched T cells, indicating that the partial displacement of Lck from rafts by PUFAs does not significantly perturb its function in TCR signaling. A straightforward explanation of this observation is that the amount of Lck left in rafts after the PUFA treatment is still functionally sufficient, although the complete exclusion of Lck from rafts does disrupt TCR signaling (30). In line with this notion, the targeting of minute amounts of Lck to rafts effectively rescues the downstream activation of the nuclear factor of activated T cell (NFAT) (34). In addition, Lck was shown to phosphorylate its substrate CD3ζ independent of its raft localization (33, 35, 36). Thus, our present data showing that the PUFA-mediated partial displacement of Lck from lipid rafts is not per se sufficient to inhibit TCR signaling are in agreement with previous studies on this topic. Moreover, these data argue that the displacement of a significant fraction of a signaling protein from rafts does not necessarily disrupt its function.

Activated ZAP-70 primarily phosphorylates LAT and subsequently LAT-associated PLCγ1 (37). In contrast to the preceding Lck-driven signaling events, the phosphorylation of LAT and PLCγ1 was markedly diminished following PUFA treatment (Figs. 1 and 2). Phosphorylation of LAT and PLCγ1 thus represents the most upstream CD3 signaling events that are inhibited in PUFA-enriched T cells. In addition, these findings indicate that, in contrast to Lck, the phosphorylation of ZAP-70 substrates requires them to be localized in membrane rafts. Under physiological conditions, the recruitment of phosphorylated CD3ζ to rafts may bring associated ZAP-70 into proximity with its substrates, particularly LAT and PLCγ1 (38). These protein-protein interactions are largely facilitated by spatial compartmentalization of unperturbed rafts in control T cells but appear to be effectively blocked in PUFA-enriched T cells.

LAT is a pivotal adaptor protein in T cell signal transduction, and its phosphorylation is a prerequisite for subsequent PLCγ1 activation and downstream signaling. LAT was recently shown (31, 39, 40) to depend on raft localization to function in T cell signaling. Moreover, LAT seems to be specifically located in the vicinity of the TCR, and this vicinity is lost by preventing the raft localization of LAT (41). When displaced from membrane rafts, LAT phosphorylation is either blocked as in PUFA-treated PBLTs or parental Jurkat T cells (Fig. 2), or when LAT is overexpressed, it may proceed in an uncontrolled manner possibly by overwhelming interactions with PTKs outside the rafts (40, 42). The overexpression of wild type LAT in transfected cells alone did not rescue PLCγ1/calcium signaling in PUFA-treated T cells, strongly indicating that the proportion of LAT located in rafts rather than the absolute amount of LAT present in rafts is functionally important. Because protein association with lipid rafts is a dynamic process (43, 44), the apparent PUFA-induced displacement of proteins from rafts in density gradients most probably represents an altered distribution equilibrium of proteins between raft and non-raft membrane subdomains.

The observation that LAT phosphorylation was the most upstream signaling event affected by PUFA treatment indicated that the displacement of LAT from rafts may be the key mechanism of the inhibitory effects of PUFAs on TCR signaling. It seemed likely that, in contrast to Lck, the correct amount of LAT in T cell rafts is a limiting factor, and its decrease, as induced by PUFAs, markedly affects the signaling process. This hypothesis was proven by introducing a chimeric LAT protein (PAG-LAT) that was retained in membrane rafts in PUFA-enriched T cells and restored PLCγ1/calcium signaling. PUFAs may affect T lymphocytes by a variety of mechanisms apart from modifying membrane and raft lipid composition (e.g. by activating nuclear receptors) (45, 46). However, the rescue of PLCγ1/calcium signaling in PUFA-enriched T cells by the chimeric LAT protein rules out the proposition that signaling steps upstream of LAT or other signaling events that contribute to CD3-induced calcium signaling are significantly altered by PUFA treatment.

The selective displacement of palmitoylated proteins such as Src family kinases and LAT from lipid rafts in PUFA-treated T cells is predominantly due to altered raft lipid composition (10), although altered protein acylation may contribute as well (47). However, the cause of the unique stable raft localization of PAG/Cbp has yet to be elucidated in detail. Because the distribution of PAG/Cbp in density gradients of membrane lysates exactly reflected that of the GPI-anchored protein CD59 in a variety of experiments (Fig. 3 and data not shown), interaction of the extracellular/transmembrane domains of PAG/Cbp with a glycolipid-anchored protein or a glycolipid may be a likely explanation. Notably, the intracellular domain of PAG/Cbp, which interacts with the cytoskeleton (48, 49), apparently does not contribute to its PUFA-insensitive raft targeting as evidenced by the chimeric PAG-LAT (Fig. 4B).

In conclusion, our results uncovered a molecular mechanism for the PUFA-mediated inhibition of T cell signaling by showing that the displacement of LAT from membrane lipid rafts is the primary cause for diminished PLCγ1/calcium signaling in PUFA-treated T cells. Similar disturbances of membrane rafts involving the displacement of Src family kinases and LAT apparently also contribute to the glucocorticoid-mediated inhibition of T cell signaling (50). Because dietary PUFAs are effectively incorporated into lymphocyte membrane lipids, altered LAT membrane subdomain distribution could also underlie the clinically apparent immunosuppressive action of PUFAs when these substances are applied to patients with inflammatory diseases. Moreover, understanding this principle of action may promote the development of more potent drugs for displacing LAT from lipid rafts, e.g. by interfering with its protein acylation.

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