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The Adapter Protein LAT Enhances Fcγ Receptor-mediated Signal Transduction in Myeloid Cells*

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Susheela Tridandapani‡§, Timothy W. Lyden‡, James L. Smith¶, Jo Ellen Carter‡, K. Mark Coggeshall‖, and Clark L. Anderson‡ ‡‡**

From the ‡Department of Internal Medicine, The Ohio State University, Columbus, Ohio 43210, the ¶Laboratory of Pathology, NCI, National Institutes of Health, Bethesda, Maryland 20877, and the ‖Department of Immunobiology and Cancer, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

FcγR clustering in monocytes initiates a cascade of signaling events that culminate in biological responses such as phagocytosis, production of inflammatory cytokines, and generation of reactive oxygen species. We have identified and determined the function of the adapter protein linker of activation of T cell (LAT) in FcγR-mediated signaling and function. Clustering of FcγRs on the human monocytic cell line, THP-1, induces phosphorylation of a major 36-kDa protein which immunoreacts with anti-LAT antisera. Our data indicate that although both the 36-kDa and 38-kDa isoforms of LAT are expressed in THP-1 and U937 human monocytic cells, FcγR clustering induces phosphorylation of the 36-kDa isoform only. Co-immunoprecipitation experiments revealed a constitutive association of p36 LAT with both FcγRI and FcγRIIa immunoprecipitates, and an activation-induced association of LAT with PLCγ1, Grb2, and the p85 subunit of phosphatidylinositol 3-kinase. Transient transfection experiments in COS-7 cells indicated that overexpression of a wild type but not a dominant-negative LAT, that is incapable of binding to p85, enhances phagocytosis by FcγRI. Furthermore, bone marrow-derived macrophages from LAT-deficient mice displayed reduced phagocytic efficiency in comparison to the macrophages from wild-type mice. Thus, we conclude that p36 LAT serves to enhance FcγR-induced signal transduction in myeloid cells.

Clustering of the Fcγ receptors (FcγR)[1] on monocytes/macrophages initiates a series of intracellular biochemical events that are necessary for induction of the various biological outcomes, such as phagocytosis, production of inflammatory cytokines, and generation of reactive oxygen species. Receptor clustering is the result of FcγR engagement of IgG-coated soluble or particulate antigens and is distinct from FcγR occupancy, which does not promote signaling biochemistry or biology (reviewed in Ref. 1). Phagocytosis of IgG-coated particulate antigens is elicited by all IgG receptors, with the notable exception of FcγRIIb (reviewed in Ref. 2), and is an essential function of the innate immune system.

The biochemical pathways initiated by FcγRs leading to phagocytosis is highly analogous to that of other immunoreceptors. Thus, FcγR aggregation by IgG-coated particulate antigen induces the activity of Src kinases, which phosphorylate a conserved receptor-associated amino acid motif known as the immunoreceptor tyrosine-based activation motif (ITAM) (2–4). ITAMs of the FcγRs are found in the receptor-associated γ-subunit except in the case of FcγRIIa (4, 5), which uniquely expresses the ITAM within its cytoplasmic tail (5, 6). ITAM phosphorylation initiates ITAM recruitment of a variety of enzymes that propagate the antigenic signal, and lead to and are essential for phagocytosis (reviewed in Refs. 4 and 7). However, while reports over the past several years have elucidated some of the FcγR-triggered signaling pathways leading to phagocytosis, the proximal events induced by FcγR clustering are not fully understood.

Studies from our laboratory (8) and others (9, 10) have demonstrated that the tyrosine kinase Syk is directly recruited to the phosphorylated ITAM of the receptor. Syk recruitment is followed by the recruitment of other SH2 domain-containing enzymes such as PLCγ1, the Grb2-Sos complex and the p85-p110 complex of PI 3-kinase (11, 12). Translocation of PLCγ1 to the membrane brings it in contact with its lipid substrate phosphatidylinositol 4,5-bisphosphate, thus generating second messengers involved in the activation of protein kinase C and release of intracellular stored calcium (13, 14). Association of the Grb2-Sos complex with the membrane facilitates the activation of the Ras/ERK pathway leading to the activation of transcription factors and gene expression (15–17). Membrane localization of the p85 subunit of PI 3-kinase is essential for the generation of lipid second messengers that are involved in the activation of a number of enzymes including those that potentiate actin polymerization and cytoskeletal rearrangements (11, 18–20). Indeed our recent experiments in fibroblasts expressing a chimeric receptor composed of the extracellular domain of FcγRI and p85 reveals that membrane recruitment of PI 3-kinase is necessary and sufficient to induce actin polymerization and phagocytosis (21). Thus, recruitment of these enzymes to the membrane is critical for their functioning.

Although membrane recruitment is essential for stimulation of these enzymes in phagocytosis, it is unclear how membrane recruitment is elicited by FcγRs. Experiments using synthetic

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§ Fellow of the Leukemia and Lymphoma Society (formerly Leukemia Society of America).

** Scholar of the Leukemia Society of America.

‡‡ To whom correspondence should be addressed: The Ohio State University College of Medicine, 2054 Davis Research Center, 480 W. 9th Ave., Columbus, OH 43210. Tel.: 614-293-4819; Fax: 614-293-4811; E-mail: anderson.48@osu.edu.

[1] The abbreviations used are: FcγR, Fc γ-receptor; LAT, linker for activation of T cells; ITAM, immunoreceptor tyrosine based activation motif; SH2, Src homology domain 2; PI 3-kinase, phosphatidylinositol 3-kinase; ERK, extracellular signal-related kinase; PLCγ1, phospholipase C γ1; BMM, bone marrow-derived macrophage; TCR, T cell antigen receptor; PBM, peripheral blood monocytes; FITC, fluorescein isothiocyanate; RBC, red blood cell; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; SRBC, sheep red blood cell.

phosphopeptides corresponding to the phosphorylated ITAM of FcγRI and FcγRIIa indicated that some but not all of the above enzymes directly bind via their SH2 domains with the ITAM (8, 11, 22). Other signaling enzymes do not directly bind the phosphorylated ITAM, but might be recruited through a receptor-associated adapter protein. While immunoreceptors expressed on lymphocytes and mast cells utilize multiple ITAM-bearing subunits to efficiently transduce signals, the γ-subunit is the only ITAM-bearing molecule identified to date that associates with Fcγ receptors in monocytes (23). Other FcγR-associated molecules may be present and function as an adapter protein, but are unidentified.

Earlier studies from our laboratory (24) revealed an undefined 36-kDa phosphoprotein associated with the FcγRI α-chain. Experiments presented here seeking to identify proteins phosphorylated very early upon receptor clustering also revealed a 36-kDa phosphoprotein. Hence we set out to determine the identity and function of pp36. Here we show that this protein is the recently cloned adapter molecule LAT (linker for activation of T cells).

LAT, a membrane-associated adapter molecule that exists as 36- and 38-kDa isoforms, was originally cloned from T cells (25). This protein lacks any intrinsic enzymatic activity but facilitates enzyme function by serving as an adapter that recruits SH2 domain-containing enzymes and enzyme-adapter complexes (25). Palmitoylation of LAT targets it to glycolipid-enriched domains in the membrane (26). Mutational analyses indicated that localization of LAT to the lipid rafts is crucial for its function (27). T cell receptor cross-linking leads to phosphorylation of LAT and its association with a number of signaling proteins. The importance of LAT in T cell signaling by the T cell antigen receptor (TCR) is demonstrated by the inability of LAT-deficient cell lines to respond to TCR cross-linking (28, 29). Overexpression of a dominant-negative mutant of LAT, that is incapable of associating with some key SH2 domain proteins, severely impairs TCR-induced calcium flux, ERK activation, and production of interleukin-2 in Jurkat cells (25). The expression of LAT also appears to be critical for T cell development, as LAT knockout mice display a lack of mature T cells in the periphery (30).

Here, we report that LAT is expressed in myeloid cells, co-immunoprecipitates with FcγRs, and is phosphorylated in response to FcγR clustering. Additional studies indicate that LAT associates with p85, Grb2, and PLCγ1 upon monocyte activation and LAT phosphorylation. COS-7 transfectants overexpressing a wild-type LAT displayed enhanced phagocytic efficiency, while in contrast, COS-7 transfectants overexpressing LAT Y171F/Y191F showed decreased phagocytic efficiency. Consistent with these findings indicating a supporting role for LAT in phagocytosis, bone marrow-derived macrophages from wild type mice were approximately twice as efficient at ingesting IgG-coated SRBCs as those derived from LAT-deficient animals. Thus, we conclude that LAT is a functional component of FcγR signaling in myeloid cells, serving to recruit SH2 domain-containing signaling proteins to the membrane.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Reagents—THP-1, U937, Raji, Jurkat, and COS-7 cells were obtained from ATCC. COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All other cells were maintained in RPMI, supplemented with 10% fetal bovine serum. Anti-FcγRI antibodies 197 and 32.2, and anti-FcγRIIa antibody IV.3 were obtained from Medarex. Rabbit polyclonal anti-LAT antibody and anti-phosphotyrosine antibody 4G10 were purchased from UBI. Anti-p85 antibody was a generous gift from Dr. K. Mark Coggeshall (Oklahoma Medical Research Foundation, Oklahoma City, OK).

Isolation of Peripheral Blood Monocytes (PBM)—Peripheral blood mononuclear cells were first isolated by density gradient centrifugation over Histopaque (Sigma). Monocytes were then purified from the peripheral blood mononuclear cells by negative selection using the MACs Monocyte Isolation Kit (Miltenyi Biotech). Briefly, peripheral blood mononuclear cells were first treated with FcR blocking Reagent (hIgG), followed by a Hapten-Antibody Mixture (mixture of monoclonal hapten-conjugated CD3, CD7, CD19, CD45RA, CD56, and anti-IgE antibodies). The labeled cells were further treated with MACS anti-hapten magnetic microbeads that were conjugated to a monoclonal anti-hapten antibody. The cells were then passed over a MACS column, and the effluent was collected as the negative fraction representing enriched monocytes. The monocytes thus purified were subsequently analyzed for purity by double labeling with CD14-PE and CD45-FITC antibodies followed by flow cytometry. Data from 10,000 cells indicated that the isolated monocytes were 100% CD14 positive.

Culture of Murine Bone Marrow-derived Macrophages—Strain-matched wild type and LAT-deficient mice were a kind gift from Dr. Paul E. Love (National Institutes of Health, Bethesda, MD). Bone marrow macrophages (BMM) were derived as described previously (31). Briefly, bone marrow cells were cultured in RPMI containing 5% fetal bovine serum and supplemented with 50 ng/ml CSF-1 for 5 days. The BMMs were dissociated from the plates with Cell Dissociation Buffer (Life Technologies, Inc.) and analyzed for Fc receptor expression by flow cytometry, expression of LAT by Western blotting, and for their ability to bind and ingest IgG-coated sheep RBCs.

Immunoprecipitation and Western Blotting—THP-1 cells and transfected COS-7 cells were activated by clustering FcγRI and or FcγRIIa with mAb 197 and IV.3 and goat anti-mouse Ig secondary antibody. Resting and activated cells were lysed in TN1 buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na_3VO_4, 10 μg/ml each aprotinin and leupeptin)), and postnuclear lysates were incubated overnight with the antibody of interest and protein G-agarose beads (Life Technologies, Inc.) or goat anti-mouse Ig covalently linked to Sepharose, depending on the antibody. Immune complexes bound to beads were washed in TN1 and boiled in SDS sample buffer (60 mM Tris, pH 6.8. 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 5% 2-mercaptoethanol) for 5 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, probed with the antibody of interest and developed by enhanced chemiluminescence.

Transfection—COS-7 cells were transfected as described previously (32). Briefly, cells were grown on culture dishes until they were 60–70% confluent. 2 μg of cDNA for FcγRI α chain in pCEXV3, kindly provided by Dr. J. Ravetch (Rockefeller University, New York), 2 μg of γ-subunit cDNA in pSVL, a gift from Dr. J.-P. Kinet (Harvard University, Boston, MA), and 4 μg of Myc-tagged, wild type LAT or LAT Y171F/Y191F in pEF/Bos, a generous gift from D. L. E. Samelson (National Institutes of Health, Bethesda, MD) were mixed in various combinations with LipofectAMINE 2000 reagent (Life Technologies, Inc.). The DNA mixture was added to cells in serum-free Dulbecco's modified Eagle's medium and incubated for 3 h at 37 °C in a CO_2 incubator. The media was then replaced by Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were harvested 48 h later and analyzed for expression of the transfected cDNAs by flow cytometry and Western blotting. Having ensured that the various transfectants expressed comparable levels of protein, we then examined their ability to bind and ingest Ig-coated sheep RBCs.

Preparation of IgG-coated Sheep RBCs—Sheep RBCs (Colorado Serum, Denver, CO) were washed in phosphate-buffered saline, and labeled overnight with 0.1 mg/ml FITC in phosphate-buffered saline at 4 °C. FITC-labeled cells were then washed in phosphate-buffered saline and incubated with a subagglutinating dose of rabbit anti-sheep RBC IgG (Diamedix, Miami, FL) at 37 °C for 1 h. Unbound IgG was removed by washing the cells with phosphate-buffered saline.

Phagocytosis Assay—IgG-coated SRBCs described above were added to COS-7 transfectants in suspension, and the cells were pelleted by low speed centrifugation to increase contact between SRBCs and phagocytes. The samples were prepared in duplicate and incubated for 1 h at either 4 °C to study binding, or 37 °C to study phagocytosis. All cells were fixed in 1% paraformaldehyde and mounted on slides to be viewed under a fluorescence microscope. For the phagocytosis assay, cells were subjected to brief hypotonic lysis with water to remove externally bound RBCs prior to fixation in paraformaldehyde. The ability of the transfected COS-7 cells to bind IgG-coated targets was expressed as the percentage of cells that each bound three or more SRBCs ("Rosetting Activity," Table I). That the binding was via the transfected Fc receptors was confirmed by the lack of binding observed in untransfected COS-7 cells. As an additional control, all cells were also incubated with fluoresceinated RBCs that were not opsonized with IgG. No binding or

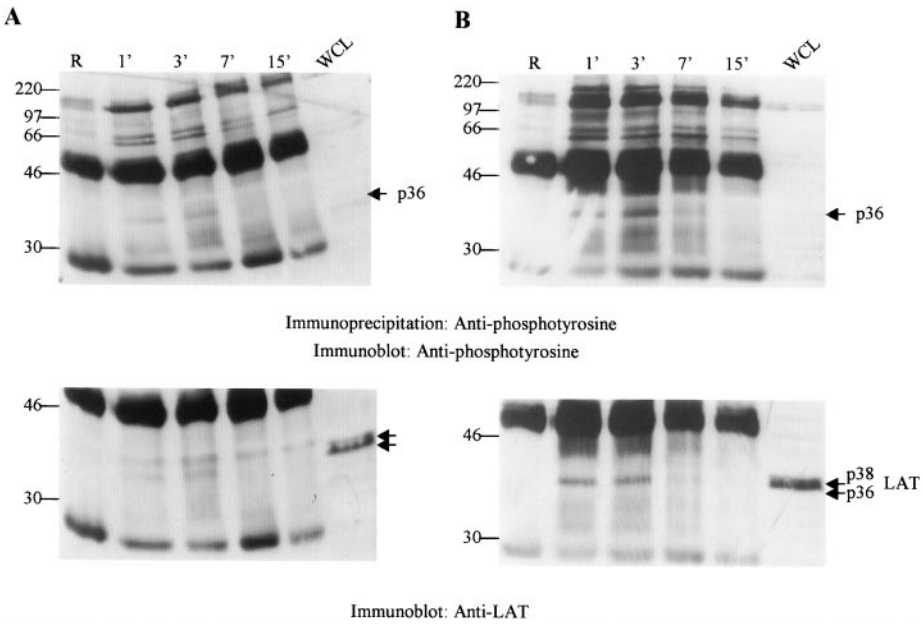

FIG. 1. **FcγR-clustering induces phosphorylation of a 36-kDa protein.** THP-1 cells were activated for the times indicated in the figure by clustering either FcγRI with mAb 197 (*A*) or FcγRIIa with mAb IV.3 (*B*), followed by goat anti-mouse IgG secondary antibody. Tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibody, separated by SDS-PAGE, and analyzed by Western blotting with anti-phosphotyrosine antibody (*upper panels*). The same blots were stripped and reprobed with anti-LAT antibody (*lower panels*). A whole cell lysate (*WCL*) from 10^6 THP-1 cells was loaded in the last lane as a positive control for Western blotting with anti-LAT antibody. *Margin numbers* indicate molecular weight in kDa.

phagocytosis was seen in any of the samples treated with non-opsonized RBCs. Phagocytosis was measured by counting the total number of RBCs ingested by 200 transfected COS-7 cells ("Phagocytic Index," Table I). The experiment was repeated three times.

Murine bone-marrow macrophages from wild type and LAT deficient mice were dissociated from culture dishes and treated with IgG-coated SRBCs by methods described above. The total number of SRBCs bound by 500 macrophages was counted and expressed as the "Binding Index" (Fig. 6*A*). The number of SRBCs ingested by 500 macrophages is expressed as the "Phagocytic Index" (Fig. 6*A*). The experiment was repeated twice with macrophages derived from two sets of mice.

Measurement of Receptor Expression by Flow Cytometry—Murine BMMs were tested for expression of FcγRs by incubating with anti-FcγRII/III mAb 2.4G2 (Pharmingen), at a concentration of 10 μg/ml for 30 min at 4 °C. The cells were washed and incubated with FITC-labeled goat anti-rat Ig secondary antibody for 30 min at 4 °C. Cells were subsequently washed, fixed in 1% paraformaldehyde, and analyzed by flow cytometry on an Elite EPICS fluorescence-activated cell sorter (Coulter, Hialeah, FL). Data from 10,000 cells per condition were recorded to yield the percentage of cells expressing receptors (Fig. 6*B*).

Transfected COS-7 cells were analyzed for FcγRI expression by incubating them with anti-FcRI mAb 197, followed by FITC-labeled goat anti-mouse Ig secondary and subsequent flow cytometry as described above (Table I).

RESULTS

FcγR Clustering in THP-1 Cells Induces Phosphorylation of a Major 36-kDa Protein That Immunoreacts with Anti-LAT Antibody—To identify the initial events that ensue upon FcγR clustering in monocytes, we assessed tyrosine kinase activity in resting and FcγR-stimulated THP-1 cells. THP-1 cells were incubated with either anti-FcγRI mAb 197 or with anti-FcγRIIa mAb IV.3 and the mAb-bound receptors were subsequently clustered with goat anti-mouse Ig secondary antibody. Proteins were immunoprecipitated and analyzed by Western blotting with anti-phosphotyrosine antibody (Fig. 1, *A* and *B, upper panels*). The results indicated a number of proteins phosphorylated upon FcγR clustering, notable among them was a 36-kDa protein that appeared as early as 1 min after receptor clustering. To identify the 36-kDa protein, the blots were stripped and reprobed sequentially with several antibodies against known 36-kDa proteins such as Lnk, the Fcε receptor-associated β-chain and LAT. Of the antibodies used only anti-LAT immunoreacted with the 36-kDa phosphoprotein in the lysates of THP-1 cells activated by FcγR clustering (Fig. 1, *A* and *B, lower panels*).

LAT Is Expressed in Monocytes and Is Phosphorylated in Response to FcγR Clustering—Previous studies of tissue distribution of LAT indicated that myeloid cells did not express LAT mRNA (25). In order to ensure that the immunoreactivity of anti-LAT antibody observed in Fig. 1 was not an observation limited to the THP-1 cell line, lysates of several different cell lines and primary macrophages were analyzed by Western blotting with anti-LAT antibody. As indicated in Fig. 2*A*, the two isoforms of LAT, p36 and p38, were detected in THP-1 and U937 human monocytoid cell lines, and 30 μg of Jurkat T cell lysate positive control (UBI), whereas Raji B cells were negative. In parallel experiments, lysates from murine BMM (Fig. 2*B*) and PBM (Fig. 2*C*) were probed with anti-LAT antibody. The results indicate the presence of LAT in both murine BMMs, and in human PBMs. However, to our surprise we found that the amount of LAT present in monocytes is lower than that in a comparable number of Jurkat T cells. In order to obtain an estimate of the amount of LAT expressed in monocytes *versus* T cells, we used lysates from decreasing numbers of Jurkat T cells (Fig. 2*D*). Results indicated that LAT expression in monocytes is approximately 5–10% of that in Jurkat T cells. Thus, the LAT adapter protein is expressed in T cells and monocytes but not in B cells.

To further characterize the kinetics of phosphorylation of the two isoforms of LAT upon FcγR stimulation in monocytes, we immunoprecipitated LAT from monocytes stimulated with anti-FcγR mAb as above, or with IgG-opsonized SRBCs, the natural ligand for monocyte FcγRs. Tyrosine phosphorylation of LAT was seen as early as 30 s in both cases, peaked at 1 min and lasted until 5 min (Fig. 2, *E–G, upper panels*). Furthermore, while the 36-kDa isoform of LAT displayed robust phosphorylation, there was no detectable phosphorylation of the 38-kDa isoform. The blots were subsequently probed with anti-LAT antibody to ensure equal loading of protein in all lanes (Fig. 2, *E–G, lower panels*).

LAT Co-immunoprecipitates with FcγRs in THP-1 Cells—Our earlier studies on the FcγRI receptor complex had revealed a 36-kDa phosphoprotein associated with the FcγRI α chain (24). To address whether this 36-kDa protein was LAT, lysates from resting and activated THP-1 cells were immunoprecipitated with either anti-FcγRI mAb 32.2 or anti-FcγRIIa mAb IV.3. The immune complexes were separated by SDS-PAGE and analyzed by Western blotting with anti-LAT antibody (Figs. 3, *A* and *B, upper panels*). The 36-kDa isoform of LAT

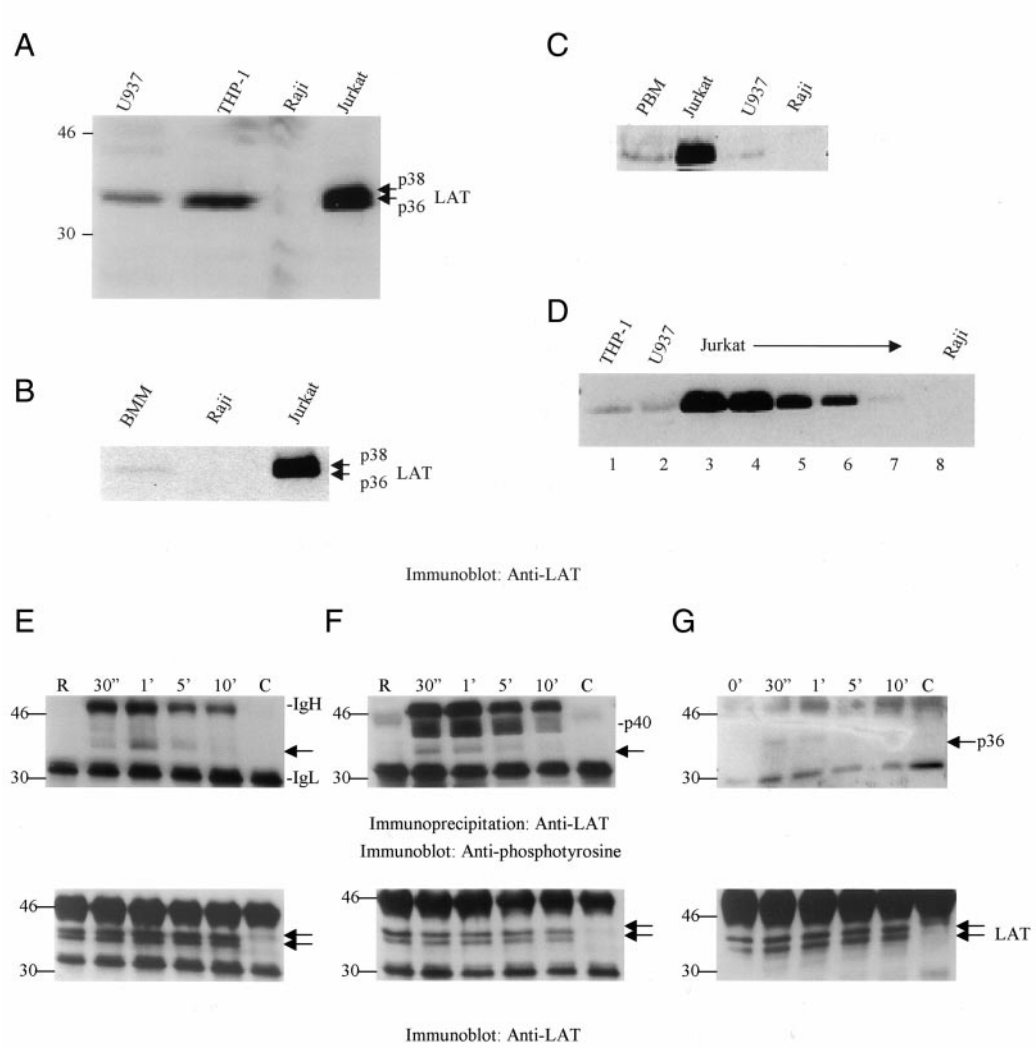

FIG. 2. **LAT is expressed in human monocytes and is tyrosine phosphorylated in response to FcγR clustering and target binding.** *A,* whole cell lysates from 2×10^6 THP-1, U937, Raji were probed with anti-LAT antibody. 30 μg of Jurkat lysate (UBI) was loaded in the last lane as a positive control. *B,* whole cell lysates from 10^6 murine BMMs, Raji, and Jurkat T cells were probed with anti-LAT antibody. *C,* whole cell lysates form 10^6 PBMs, Jurkat, U937, and Raji cells were probed with anti-LAT antibody. *D,* whole cell lysates from 10^6 THP-1, U937, Jurkat and Raji cells were loaded in *lanes 1, 2, 3,* and *8,* respectively. *Lanes 4–7* were loaded with whole cell lysates from 5×10^5, 2.5×10^5, 10^5, and 5×10^4 Jurkat T cells, respectively. The blot was then probed with anti-LAT antibody. *E–G,* THP-1 cells were activated for the indicated times by clustering either FcγRI (*E*) or FcγRIIa (*F*) with receptor-specific mAbs, or by incubating with IgG-coated SRBCs (*G*). Cell lysates were immunoprecipitated with anti-LAT antibody followed by Western blotting with anti-phosphotyrosine antibody (*upper panels*). The same blots were reprobed with anti-LAT antibody (*lower panels*) to ensure equal loading of protein in all lanes. Normal rabbit IgG was used in the last lane as a control for immunoprecipitation. *Arrows* indicate the position of LAT. The p40 in *F* is likely FcγRIIA. Also indicated in the figure are the heavy and light chains (IgH, IgL) of the activating and immunoprecipitating IgG antibodies.

was detected in both FcγRI and FcγRIIa immunoprecipitates. Interestingly, the presence of LAT in FcγR immunoprecipitates was detectable in resting cells and did not increase upon receptor clustering. No anti-LAT reactivity was seen in additional control samples of lanes loaded with the monoclonal antibodies without cell lysate; thus, the LAT immunoreactivity is not an artifact of the antibodies. These data suggest that LAT may be a component of resting, unphosphorylated FcγRI and FcγRIIa complexes, as is the ITAM-containing γ-subunit of FcγRI (23). However, it is conceivable that under the lysis conditions used in these experiments the lipid rafts are insufficiently solubilized, and that the co-immunoprecipitation simply indicates that the two molecules reside in the same rafts but are not physically associated. Studies to determine the exact nature of the interaction of LAT with the FcγRs are underway.

LAT Associates Inducibly with SH2-domain Containing Signaling Proteins—Having established that LAT is expressed in myeloid cells and is phosphorylated in response to FcγR activation, we next examined its part in FcγR-mediated function in myeloid cells. LAT contains several tyrosines that are potential phosphorylation sites and fit a Y*XXX* consensus motif for SH2-domain binding (33). Studies in T cells and NK cells indicated that phosphorylated LAT associated with several SH2 domain-containing proteins, thereby supporting their membrane translocation (25, 28, 29, 34). To test whether LAT phosphorylation induced by FcγR clustering also occurs on the tyrosine residues that promote binding of SH2 domain proteins, LAT immunoprecipitates from resting and FcγRI-activated THP-1 cells were analyzed by Western blotting with anti-p85 (Fig. 4*A, upper panel*), anti-Grb2 (Fig. 4*B, upper panel*), anti-PLCγ1 (Fig. 4*C*), and anti-SHIP antibodies. The data indicate that LAT inducibly associates with p85, Grb2, and PLCγ1 upon FcγR clustering in THP-1 cells, as it does in T cells (25). In contrast no association of LAT with SHIP was detected (data not shown). Thus, LAT may play a functional role in transducing signals from FcγR by associating with and promoting membrane translocation of these signaling proteins.

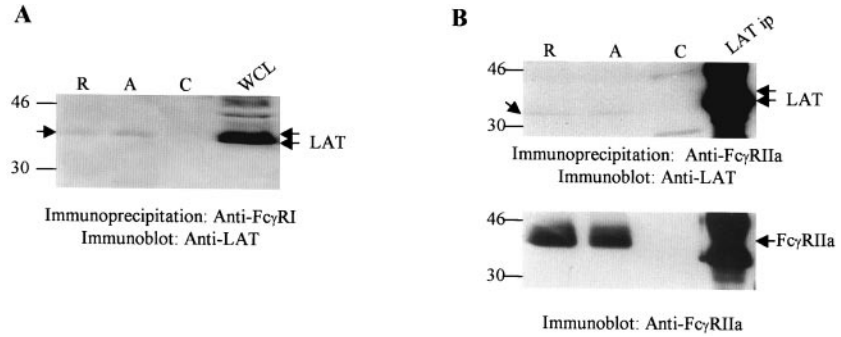

FIG. 3. **LAT co-immunoprecipitates with FcγRI α chain and with FcγRIIa in both resting and activated THP-1 cells.** THP-1 cells were activated for 3 min by either clustering FcγRI (*A*) or FcγRIIa (*B*) with mAb 197 and mAb IV.3, followed by goat anti-mouse Ig secondary antibody, respectively. The receptors were immunoprecipitated with mAb 32.2 and IV.3, respectively, and the immune complexes were separated by SDS-PAGE and analyzed by Western blotting with anti-LAT antibody. The lanes marked *C* were loaded with mAb alone in the absence of cell lysate. A whole cell lysate and a LAT immunoprecipitate were loaded as positive controls in the *last lanes* of *A* and *B*, respectively. The blot from *B* was reprobed with anti-FcγRIIa Ab 260 (*lower panel*). *Arrows* indicate the position of LAT. These results are representative of four separate experiments.

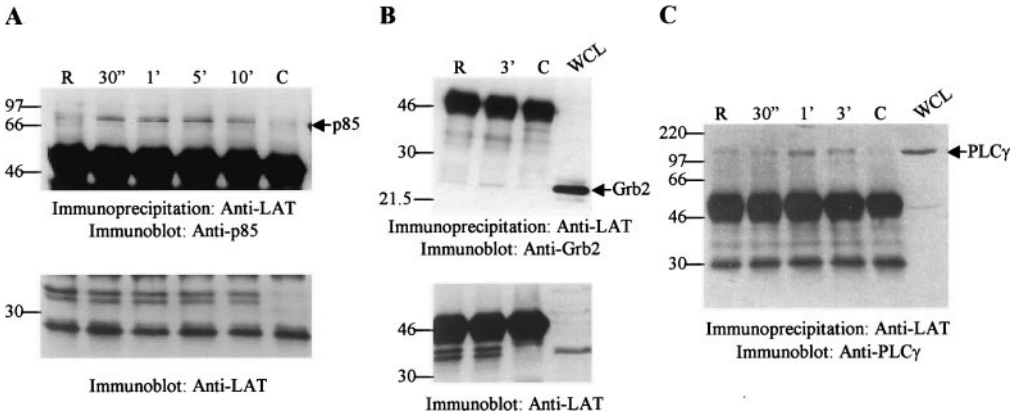

FIG. 4. **LAT associates with p85, Grb2, and PLCγ upon FcγR clustering.** THP-1 cells were activated for the indicated time points by clustering FcγRI. Cell lysates were immunoprecipitated with anti-LAT antibody and analyzed by Western blotting with: *A,* anti-p85; *B,* anti-Grb2; *C,* and anti-PLCγ1 antibodies. Control lanes analyze immunoprecipitations with normal rabbit IgG. A whole cell lysate was loaded in the last lanes as a positive control. The blots were subsequently reprobed with anti-LAT antibody (*lower panels*).

LAT Influences Phagocytic Efficiency of FcγRI- transfected COS-7 Cells—FcγRs bind to IgG-coated particles and mediate phagocytosis. The signaling process accompanying phagocytosis involves sequential activation of tyrosine kinases, and recruitment of PI 3-kinase to generate 3-phosphoinositides. These events promote actin polymerization and cytoskeletal rearrangements such that the phagocyte puts forth pseudopods to surround and engulf the particle (19, 35, 36). Recruitment of PI 3-kinase to the membrane has been shown to be both necessary and sufficient for phagocytosis (21). Since LAT associates with the p85 adapter subunit of PI 3-kinase, LAT could enhance phagocytic efficiency of FcγRs by promoting p85 recruitment and hence activation of PI 3-kinase. To test this possibility we took advantage of the COS cell model, which displays efficient phagocytosis upon transfection with cDNA encoding FcγRI plus the γ-subunit (32). We first assessed the ability of transfected LAT to become tyrosine phosphorylated upon FcγR clustering in the COS cell model. Thus, COS transfectants expressing FcγRI, the γ-subunit, and/or Myc-tagged wild-type or mutant (Y171F/Y191F) LAT, were activated for 3 min by clustering FcγRI receptors as described above. Phosphotyrosine proteins were immunoprecipitated, separated by SDS-PAGE, and analyzed by Western blotting with anti-LAT antibody (Fig. 5*A*). We observed that both wild-type and LAT Y171F/Y191F are tyrosine phosphorylated by FcγRI clustering in COS transfectants and that this phosphorylation required the presence of the γ-subunit. Tyrosine phosphorylation of the mutant LAT is reduced compared with that of wild-type LAT, most likely since two major tyrosines (Tyr^{171} and Tyr^{191}) are mutated to phenylalanine; other known phosphorylation sites are still present in this mutant. These findings indicate that LAT becomes tyrosine phosphorylated upon FcγRI clustering in the COS cell model in a γ-subunit dependent manner.

Earlier studies in T cells established that mutation of LAT at Tyr^{171} and Tyr^{191} completely abrogates association with p85 and Grb2, and partially reduces binding to PLCγ1 (25), indicating that Tyr^{171} and Tyr^{191} are the sites of SH2 engagement of LAT by these signaling enzymes. To measure the phagocytic capacity of COS-7 transfectants, FcγR and/or LAT-transfected COS-7 cells were incubated with IgG-coated SRBCs for 1 h at either 4 or 37 °C, respectively. Mock transfected COS cells were incubated with IgG-coated SRBCs to control for SRBC binding unrelated to FcγR expression, and all transfectants were also incubated with fluoresceinated SRBCs that were not opsonized with IgG. Binding efficiency was measured by counting the number of COS cells that bound 3 or more SRBCs and expressed as "% Rosetting Activity" (Table I). No binding of IgG-coated SRBCs was observed with mock transfected cells (data not shown). Similarly there was no binding of FcγRI-transfected COS cells with non-opsonized SRBCs, indicating that the binding observed was indeed between the transfected Fc receptors and IgG (data not shown). FcγRI-mediated binding to opsonized SRBCs was not influenced by the presence of any of the co-transfected molecules as indicated by the rosetting activity of the various transfectants (Table I).

To measure phagocytic efficiency, similar transfected sam-

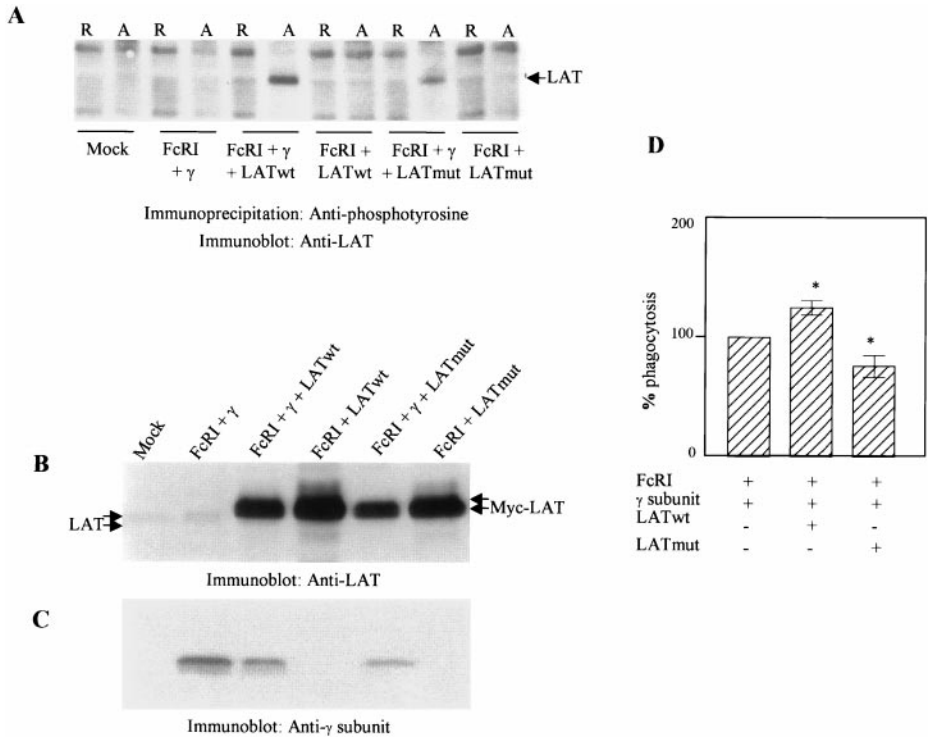

FIG. 5. **LAT enhances phagocytosis by FcγRI in transfected COS-7 cells.** COS-7 cells were transiently transfected to express the various proteins indicated in the figure. *A,* lysates from resting or FcγRI-activated COS cells were immunoprecipitated with anti-phosphotyrosine antibody and analyzed by Western blotting with anti-LAT antibody. *B,* whole cell lysates from transfected COS cells were separated by SDS-PAGE and analyzed by Western blotting with anti-LAT antibody; or *C,* anti-γ subunit antibody. *D,* COS-7 transfectants were tested for their ability to phagocytose fluoresceinated IgG-coated SRBCs. For this, COS-7 cells were incubated for 1 h with SRBCs, subjected to hypotonic lysis to remove external SRBCs, and subsequently fixed and mounted on slides to be analyzed by fluorescence microscopy. The number of SRBCs internalized by 100 phagocytic cells was counted for each transfected sample. The number of SRBCs ingested by COS cells transfected with FcγRI α chain and the γ-subunit was considered as 100%. The graph displays the mean and standard deviation of three separate experiments. *, p value < 0.001.

TABLE I
Influence of LAT on FcγRI-mediated phagocytosis in COS-7 transfectants

COS-7 cells were transiently transfected to express FcγRI α chain, γ-subunit ± LATwt or LATmut. FcγRI expression in transfectants was analyzed by flow cytometry, by first incubating the cells with mAb 197, followed by FITC-labeled goat anti-mouse IgG secondary antibody. Rosetting activity is the percentage of cells that bound 3 or more SRBCs. Phagocytic index represents the number of SRBCs ingested by 100 phagocytic cells. % Δ is the deviation from the "FcγRI + γ" transfected cells that were considered to display 100% phagocytic efficiency. The results of three separate experiments are shown.

Transfection	FcγRI expression	Rosetting activity	Phagocytic index
	%		% Δ
Experiment 1			
FcγRI + γ	50	33	388 (0)
FcγRI + γ + LATwt	39	33	468 (+20)
FcγRI + γ + LATmut	38	29	302 (−22)
Experiment 2			
FcγRI + γ	47	27	262 (0)
FcγRI + γ + LATwt	39	23	350 (+33)
FcγRI + γ + LATmut	42	26	174 (−34)
Experiment 3			
FcγRI + γ		24	284 (0)
FcγRI + γ + LATwt		21	354 (+25)
FcγRI + γ + LATmut		25	238 (−16)

ples were incubated at 37 °C and subsequently subjected to hypotonic lysis with dH_2O to remove unbound and externally bound SRBCs. The cells were subsequently fixed in 1% paraformaldehyde, mounted on slides, and analyzed by fluorescence microscopy. The number of SRBCs ingested by 100 phagocytic cells was counted and expressed as the phagocytic index (Table I); data from three separate experiments are shown. COS-7 cells overexpressing wild type LAT displayed ~25% greater phagocytic efficiency than those that overexpressed the Tyr to Phe LAT mutant or no transfected LAT (p value $<$ 0.001). Western blotting with anti-LAT antibody indicated almost equal levels of expression of the transfected wild type LAT and LAT Tyr to Phe mutant (Fig. 5*B*). Interestingly, the antibody also detected low levels of p36 and p38 LAT in COS-7 cells, migrating slightly faster than the Myc-tagged LAT. The possible presence of endogenously expressed LAT in COS-7 cells could explain the small influence of the transfected LAT proteins on phagocytosis. Expression of the γ-subunit and the FcγRI α chain was comparable in all transfectants, as determined by Western blotting and flow cytometry respectively (Fig. 5*C* and Table I).

BMMs from LAT-deficient Mice Display Reduced Phagocytic Efficiency—The functional studies using the COS-7 model suggest that LAT supports phagocytosis by FcγRI. To more rigorously address this issue, we obtained bone marrow macrophages from wild type and LAT-deficient mice by culturing bone marrow cells in the presence of colony stimulating factor-1 for 5 days. The resulting macrophages were harvested and analyzed for the expression of FcγRs. As seen in Fig. 6*B*, 99% of the BMMs from wild type and LAT-deficient mice expressed FcγRs as assessed by flow cytometry using antibodies directed against FcγRIII and FcγRII (2.4G2). We then tested the ability of wild type or LAT-deficient macrophages to bind and ingest IgG-coated SRBCs by methods described above. The results of two experiments performed with BMMs from separate sets of mice are presented in Fig. 6*A* and indicate that IgG binding is equivalent between wild type and LAT-deficient BMMs. Thus, consistent with results using the COS-7 model, LAT expression does not influence the affinity of FcγRI. However, despite comparable expression and affinity of FcγRI expressed on wild type and LAT-deficient BMMs, wild type BMMs were twice as efficient at phagocytosing SRBCs as were the LAT-deficient BMMs. These data strongly support a functional role for LAT in enhancing FcγR-mediated phagocytosis.

DISCUSSION

Here, we report that LAT is an additional and important component of FcγR-mediated signal transduction. Our results demonstrate that LAT is expressed in myeloid cells, that LAT co-immunoprecipitates with FcγRI and FcγRIIA in unactivated monocytes, and that LAT binds, upon its tyrosine phosphorylation, with SH2 domain-containing signaling proteins such as PLCγ1, Grb2, and p85. Furthermore, transient transfection

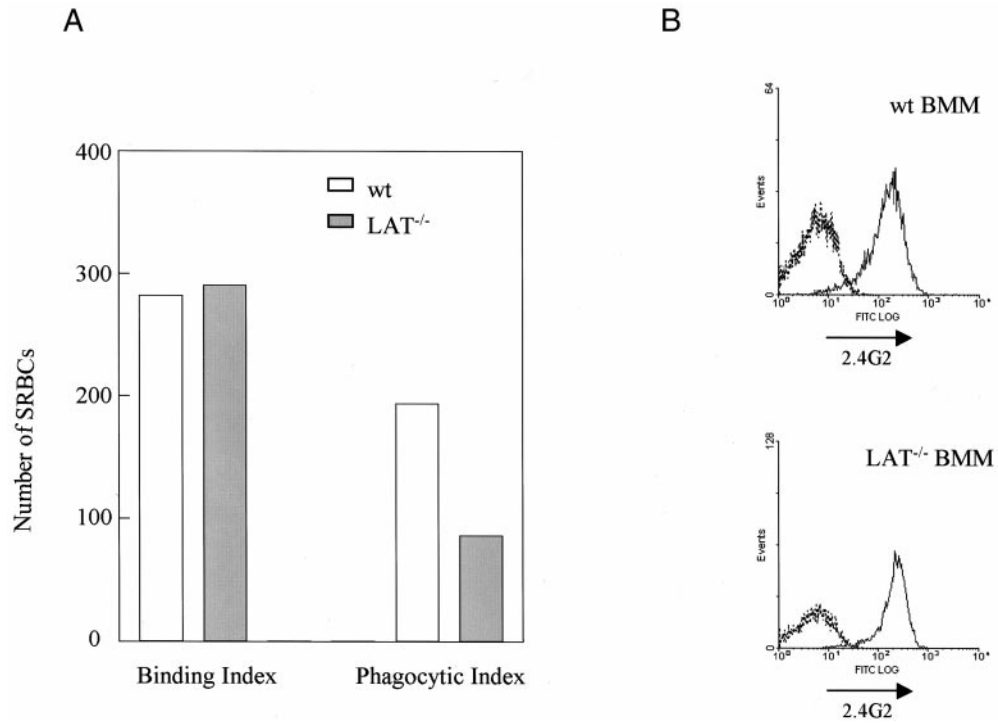

FIG. 6. **BMMs form LAT-deficient mice display reduced phagocytic efficiency.** *A,* BMMs from wild type and LAT-deficient mice were examined for their ability to bind and ingest IgG-coated SRBCs. *Binding Index* represents the total number of SRBCs bound to 500 macrophages. *Phagocytic Index* represents the number of SRBCs ingested by 500 macrophages. The graph displays the average of two experiments performed with BMMs derived from separate sets of mice. *B,* FcγR expression on the wild type and LAT-deficient BMMs was analyzed by flow cytometry. For this, the cells were labeled with anti-FcγRII/III mAb 2.4G2 followed by FITC-labeled goat anti-rat IgG secondary antibody (*solid line*). Cells were also labeled with secondary antibody alone (*dashed line*).

experiments in COS-7 cells demonstrated that co-transfection of wild type but not Y171F/Y191F mutated LAT with the FcR γ-subunit enhanced phagocytic efficiency. In addition, BMMs from wild type mice displayed a 2-fold greater phagocytic efficiency than those from LAT-deficient mice. Together, these findings suggest that LAT serves to recruit signaling molecules to the plasma membrane and thereby supports phagocytosis. While LAT is clearly not required for phagocytosis, LAT may function in a synergistic manner with the FcγR ITAMs to elicit signaling events and phagocytosis.

While Syk (8) and PI 3-kinase (22) appear to be directly recruited to FcγR ITAMs, there is no identified mechanism thus far that accounts for membrane recruitment of PLCγ1 or Grb2 in FcγR-stimulated myeloid cells. The association of phosphorylated LAT with PLCγ1 and Grb2 in THP-1 cells suggests a role for LAT in these signaling pathways. The Grb2 SH2 domain has been shown to associate directly with phosphotyrosines in cytokine receptors that conform to a consensus YVNV motif (37). Immunoreceptors do not bear a consensus motif for Grb2 SH2 binding and appear to associate with the Grb2-Sos complex via an additional adapter protein, Shc (38, 39). However, LAT has five potential tyrosine phosphorylation sites that fit a consensus binding motif for Grb2 SH2. Absence of LAT expression or mutation of the Grb2-binding sites in LAT completely abrogates the Ras/ERK pathway, calcium mobilization, and interleukin-2 production in T cells (25, 28, 29). Based on these observations, the influence of LAT on FcγR-induced activation of Ras pathway may be much more profound than the influence on PI 3-kinase-dependent biology. Studies are underway to determine the role of LAT in Ras-dependent biology of monocytes.

The importance of lipid rafts in signal transduction is becoming increasingly evident. Lipid rafts are biochemically distinct regions of the plasma membrane that are enriched in sphingolipids and cholesterol (40, 41). Recent studies in T cells indicate that several key signaling molecules are localized in lipid rafts, and that TCR clustering is followed by a rearrangement of the rafts in the plasma membrane (42). Studies in T cells indicate that LAT is palmitoylated, that it likewise resides in glycolipid-enriched lipid rafts (26), and that targeting of LAT to the lipid rafts is critical for its function (28). The TCR either moves into the rafts upon its clustering (43, 44), or is constitutively associated with lipid rafts (45). Similarly, the Fcε receptor in mast cells was reported to associate with lipid rafts upon FcεR clustering (46, 47). Our results showing that FcγRs co-immunoprecipitate with LAT in both resting and activated monocytes (Fig. 3) suggest that FcγRs may similarly exist in lipid rafts along with LAT and other important signaling molecules. Further analysis is required to determine the location of FcγRs in the plasma membrane of myeloid cells.

In conclusion, we have characterized the initial events that occur upon FcγR clustering and examined the role played by the adapter protein LAT in facilitating FcγR-induced signaling. The findings reveal an additional mode by which FcγRs relay signals to elicit biological responses that contribute to innate immunity.

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