Leukocyte Ig-like Receptor B4 (LILRB4) Is a Potent Inhibitor of FcγRI-mediated Monocyte Activation via Dephosphorylation of Multiple Kinases

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The leukocyte immunoglobulin-like receptor (LILR) B4 belongs to a family of cell surface receptors that possesses cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). LILRB4 is believed to down-regulate activation signals mediated by non-receptor tyrosine kinase cascades through the recruitment of SHP-1. However, the exact mechanisms of LILRB4-mediated inhibition are not fully elucidated. In this study, we demonstrate high level surface expression of LILRB4 on THP-1 cells and primary peripheral blood monocytes, which profoundly inhibited production of a key pro-inflammatory cytokine (TNFα) induced by FcγRI (CD64). We also report that LILRB4 aggregated to sites of activation upon co-ligation with CD64 and that this may enhance its inhibitory effects. Co-linking of CD64 on THP-1 cells markedly increased phosphorylation of multiple proteins including tyrosine kinases and signaling molecules (Lck, Syk, LAT, and Erk), an adaptor protein that targets protein-tyrosine kinases for degradation (c-Cbl) and a protein involved in the formation of actin cytoskeletal rearrangement (α-actinin-4). Co-ligation of LILRB4 considerably reduced CD64-mediated phosphorylation of Lck, Syk, LAT, Erk, and c-Cbl but not α-actinin-4, suggesting selective inhibition of signaling molecules. Treatment of cells with a broad-spectrum phosphatase inhibitor, sodium pervanadate (SP), significantly reversed LILRB4-mediated inhibition of TNFα production and protein tyrosine phosphorylation. In comparison, treatment with an SHP-1 specific inhibitor, sodium stibogluconate (SS) has no effects indicating involvement of phosphatase(s) other than SHP-1 in LILRB4 signaling. Collectively, our data show LILRB4 is a potent inhibitor of monocytes activation. This may provide a new potential therapeutic strategy for inflammatory conditions characterized by excessive TNFα production.

Intracellular tyrosine phosphorylation is an important process in initiating and maintaining activating signals of mammalian cells, especially stimulation of immune receptors such as BCR, TCR, and KIR that are expressed on B, T, and NK cells, respectively (1). These receptors contain a consensus amino acid sequence present in their cytoplasmic domain, the immunoreceptor tyrosine-based activating motif (ITAM), which initiates the signaling cascade (1, 2). Upon receptor activation, Src family protein kinases (Lyn, Fyn, and Blk) phosphorylate tyrosine residues present in the ITAM (2). Phosphorylated ITAMs then serve as docking sites for the recruitment and activation of spleen tyrosine kinase (Syk) or Zeta chain-associated protein kinase-70 (ZAP-70) (3, 4), which then trigger several downstream signaling events including activation of phospholipase C-γ (PLC-γ), Grb2, and the p85 subunit of phosphatidylinositol-3-kinase (PI 3-kinase) (5, 6). Ultimately, activating signals propagate into the nucleus leading to effector functions such as Ca2+ mobilization, transcription of cytokine genes, and signals for cell differentiation and survival (4). Unregulated activation of cells may be deleterious to the host, leading to excessive inflammation in conditions such as rheumatoid arthritis (7) and anaphylaxis (8).

In recent years, a number of inhibitory receptors have been identified (reviewed in Ref. 9). These receptors contain unique consensus sequences (I/V/L/S)XYXX(L/V) in their cytoplasmic tail that are known as immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (10, 11). Activation of ITIMs recruits various phosphatases such as Src homology 2 domain containing phosphatases (SHP)-1, SHP-2, and SH2-containing inositol phosphatase (SHIP). These phosphatases are capable of dephosphorylating multiple signaling molecules, thus terminating the stimulatory signals induced by ITAM-containing receptors (4). Of particular interest is a family of activating and inhibitory receptors termed leukocyte immunoglobulin-like receptors (LILRs) that are increasingly shown to regulate cellular activation (9). These receptors contain highly homologous extracellular domains and hence are believed to control the threshold and amplitude of cellular activation via shared or similar ligands (9, 12). Activating LILRs contain a very short intracellular domain but upon activation, the receptor is phosphorylated and associates with the Fcγ chain that contains an ITAM, thus transduces activation signals (9). In contrast, inhibitory LILRs contain variable number of ITIMs in their intracel-

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

2 The abbreviations used are: ITAM, immunoreceptor tyrosine-based activating motif; LILRB4, leukocyte immunoglobulin-like receptor B4; ITIM, immunoreceptor tyrosine-based inhibitory motif; SHP-1, Src homology 2 domain-containing protein-tyrosine phosphatase 1; SS, sodium stibogluconate; SP, sodium pervanadate; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; TNF, tumor necrosis factor.
lular domain, which are capable of terminating activating signals initiated by various stimuli (4).

In vitro co-igation of the inhibitory receptor, LILRB4 reduces monocytes activation after stimulation with CD11b, HLA-DR, FcγRIII (13), and LPS. Recent studies indicate that increased expression of LILRB4 by antigen-presenting cells may play a key role in immune tolerance in transplant recipients (14). Furthermore, disruption of a mouse ortholog of human LILRB4, known as gp49B1, caused increased susceptibility to collagen-induced arthritis, IgE-mediated anaphylaxis and LPS-induced septic shock (15, 16). However, the exact signaling pathways regulated by LILRB4 leading to its inhibitory functions are not fully elucidated. Interestingly, unlike other inhibitory LILRs that contain highly homologous four Ig-like domains in the extracellular region, LILRB4 contains a unique extracellular region of only two Ig-like domains, suggesting that it may have a unique ligand(s) and hence a distinct mechanism of inhibitory function (9, 13, 17).

This study is aimed at elucidating signaling pathways modulated by LILRB4 in monocytes activated through FcγR (CD64). CD64 is a high affinity IgG receptor and through its association to the ITAM-containing Fcγ chain is a potent activator of monocytes (18). Upon CD64 aggregation on the surface of monocyte by antibodies and monovalent antigens, CD64 induces phagocytosis of IgG-coated antigens, granule secretion, as well as generation of reactive oxygen species (19–22). We found that LILRB4 is a potent inhibitor of CD64-mediated pro-inflammatory cytokine (TNFα) production. We also report for the first time that LILRB4 aggregated to sites of activation upon co-igation with CD64 and that this may enhance its inhibitory effects. Cross-linking of CD64 on THP-1 cells markedly increased phosphorylation of multiple signaling proteins including protein-tyrosine kinases (Lck, Syk, LAT, and Erk), an adaptor protein that targets protein-tyrosine kinases for degradation (c-Cbl), and protein involved in the formation of actin bundles and cytoskeletal rearrangement (α-actinin-4). Co-igation of LILRB4 and CD64 considerably reduced CD64-mediated phosphorylation of Lck, Syk, LAT, Erk, and c-Cbl but not α-actinin-4, suggesting selective inhibition of signaling molecules involved in the secretory function of monocytes. Treatment of cells with SP prevented LILRB4-mediated inhibition of both TNFα production and protein-tyrosine dephosphorylation, indicating that the effects of LILRB4 are phosphatase dependent. Short term treatment of cells with SS (SHP-1 specific phosphatase inhibitor) did not affect LILRB4 signaling, indicating involvement of a phosphatase other than SHP-1. Interestingly, treatment of cells with SS for 72 h significantly reversed LILRB4-mediated inhibition of TNFα production, but had no effects on protein tyrosine dephosphorylation. Taken together, our data show that LILRB4 is a potent inhibitor of monocyte/macrophage activation by down-regulating phosphorylation of multiple protein-tyrosine kinases through the involvement of more than one phosphatase.

O. Huynh, H. K. Lu, L. Borges, and N. Tedla, unpublished data.

EXPERIMENTAL PROCEDURES

Cells

Human mononcytic leukemia THP-1 cells (ATCC clone TIB-202) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), 1 mM sodium pyruvate, 10 mM HEPES, and 0.05 mM 2-mercaptoethanol (GIBCO-Invitrogen, Carlsbad, CA) at 37 °C with 5% CO2. Peripheral blood mononuclear cells (PBMC) from five healthy donors were isolated by Ficoll gradient centrifugation, and monocytes were isolated by negative selection using a monocye isolation kit with >95% purity (Miltenyi Biotec) as described (23).

Antibodies

Mouse Monoclonal Antibodies—Mouse anti-LILRB1, B2, B3, and B4 (a gift from Amgen Inc, Seattle, WA), anti-CD64 (R&D Systems, Minneapolis, MN), anti-MHC class I (Pharmingen, Mountain View, CA), anti-β-actin, IgG, isotype control (Sigma Aldrich), anti-phosphorytosine (anti-pTyr) (clone 4G10) (Upstate Biotechnology, Lake Placid, NY), and anti-mouse IgG-coated polystyrene beads (Spherotech, Lake Forest, IL).

Rabbit Polyclonal Antibodies—Anti-LILRB4 (donated by A/Prof Jonathan Arm from Brigham and Women Hospital Boston, MA), anti-c-Cbl (Sigma), anti-pTyr (PY350) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-actinin-4 (Alexis, San Diego, CA), and anti-Syk[pY352] (Cell Signaling Technology, Danvers, MA).

Secondary Antibodies—Sepharose-conjugated goat anti-mouse antibody (Zymed Laboratories Inc., San Francisco, CA), unconjugated Fcγ-specific, F(ab')2 fragment Fc-specific, FITC-conjugated F(ab')2 fragment goat anti-mouse, and Cy2-conjugated donkey anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA), horseradish peroxidase (HRP)-conjugated rabbit anti-goat antibody (Dako, Glostrup, Denmark), HRP-conjugated goat anti-mouse, and HRP-conjugated goat anti-rabbit antibodies (Bio-Rad).

Flow Cytometric Analysis of CD64 and LILRBs Expression on THP-1 Cells and Monocytes

Cell surface expression of LILRB1 to 4 and CD64 were determined by flow cytometry as described previously (24). In brief, 2 x 10^5 THP-1 cells or PBMC were washed in PBS and incubated with 5 µg/ml anti-LILRB, anti-CD64 or isotype-matched control (IgG1) monoclonal antibodies followed by 3 µg/ml of FITC-conjugated F(ab')2 fragment goat anti-mouse secondary antibody. Samples were then analyzed using a FACSScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Co-staining of PBMC with PE-conjugated anti-CD14 monoclonal antibody was used to determine LILRB-positive peripheral blood monocytes.

Immunoprecipitation of LILRB4 and CD64

Surface proteins on THP-1 cells were biotinylated according to the manufacturer’s instructions (Pierce Biotechnology). Cells were lysed in PBS containing 1% Nonidet P-40, 10 mM EDTA, 20 mM iodoacetamide, 0.5 mM sodium orthovanadate, 5
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CD64 on the surface of THP-1 cells, negatively selected monocytes or PBMC were cross-linked using 96-well plate-immobilized anti-CD64 monoclonal antibody as described (28). In brief, 96-well flat-bottom tissue culture plates (Costar®) were coated with 50 μg/ml of goat anti-mouse F(ab')2 fragment (Fc-specific) in PBS overnight at 4 °C. Wells were then coated with 50 μl of anti-CD64 (0.01–10 μg/ml) or control IgG1 monoclonal antibodies in PBS + 2.5% bovine serum albumin for 2 h at 37 °C. THP-1 cells (2 × 10⁵), purified monocytes (5 × 10⁴) or PBMC containing 5 × 10⁴ monocytes in Cross-linking buffer (CLB: RPMI 1640 supplemented with 10 mM HEPES and 0.1% bovine serum albumin) were added to each well and incubated from 1 to 48 h at 37 °C. To determine whether LILRB4 will abrogate CD64-mediated TNFα production in monocytes, the two receptors were co-ligated using an optimal concentration of anti-CD64 and anti-LILRB4 antibodies (10 μg/ml) at various time points. Cell-free supernatants were collected and analyzed for TNFα (DuoSet ELISA kit, R&D Systems) (24).

Modulation of LILRB4 Signaling by Phosphatase Inhibitors

THP-1 cells (1 × 10⁶) were pretreated with 11 μM of freshly prepared specific SHP-1 inhibitor, sodium stibogluconate (SS) (30) or 100 μM of a broad-spectrum phosphatase inhibitor, sodium pervanadate (SP) from 10 min to 72 h at 37 °C, followed by CD64 cross-linking and/or CD64/LILRB4 co-ligation using 10 μg/ml of each antibody as described above. For TNFα detection in culture supernatants, inhibitor pretreated cells were incubated on plate-immobilized anti-CD64 and/or anti-LILRB4 for 6 h at 37 °C. For Western blot analysis, 5 × 10⁶ inhibitor pretreated THP-1 cells were co-ligated with 10 μg/ml of anti-CD64 and/or anti-LILRB4 antibodies in suspension for 2 min at 37 °C. Cells were lysed in Cell Signaling Universal Cell Lysis Buffer™ (Cell Signaling) containing 100 μM of sodium pervanadate and used for phosphorysine Western blotting and for plate-based multiplex assay of phosphorylated multiple signaling molecules.

For Western blot analysis, 20 μg of total cell lysate from each sample was resolved in 10% Tris-Tricine SDS-PAGE gels under reducing conditions. Proteins were transferred onto polyvinylidene difluoride membrane and probed with 1 μg/ml of mouse anti-pTyr antibody (clone 4G10) according to the manufacturer’s instructions. To confirm equal loading of protein, the same membrane was later stripped and blotted with anti-β-actin antibody according to the manufacturer’s instructions. The extent of LILRB4-mediated inhibition of protein phosphorylation was quantitated by densitometry using NIH ImageJ software (29). 5 μg of total cell lysate from each treatment were used for plate-based quantitative multiplex assay of key phosphorylated protein-tyrosine kinases (Lyn/Lck, Syk, LAT, and Erk) (Beadlyte® phosphoprotein receptor signaling kit, Upstate, Lake Placid, NY) (23).

Identification of Phosphorylated Signaling Molecules by Mass Spectrometry

Lysates from CD64 cross-linked THP-1 cells (5 × 10⁷) were immunoprecipitated using 6 μg/ml of anti-pTyr (clone 4G10) monoclonal antibody. Bead-bound proteins were washed, and immunoprecipitated proteins were then resolved on a 10% Tris-Tricine SDS-PAGE gels, silver-stained, and specific bands excised for nanoLC and mass spectrometry using LTQ-FT Ultra (25–27). The identity of these phosphorylated proteins was further confirmed by either Western blotting using specific antibodies (c-Cbl and actin-4) or by analysis of total cell lysates using plate based-multiplex assay as described.

Cross-linking of CD64 on THP-1 Cells and Monocytes with Anti-CD64 and Co-ligation with Anti-LILRB4 Monoclonal Antibodies

For Western blot, precipitated biotin-labeled proteins were transferred to polyvinylidene difluoride membranes (PerkinElmer Life Science, Boston, MA) and probed with streptavidin-conjugated HRP then developed using Western Lighting Plus chemiluminescent reagent (PerkinElmer). In a parallel experiment, silver-stained protein bands were excised and analyzed by nanoLC and mass spectrometry using LTQ-FT Ultra as previously described (25–27).

Western Blotting and Multiplex Assay for Intracellular Phosphorylated Signaling Proteins after CD64 and LILRB4 Co-ligation

THP-1 cells (5 × 10⁶) were incubated for 15 min with 5 μg/ml of anti-CD64, anti-LILRB4, anti-CD64 plus anti-LILRB4, or IgG1, control antibodies, and cross-linked for 2 min with 15 μg/ml goat anti-mouse (Fcγ-specific) secondary antibody. Cells were then lysed with Cell Signaling Universal Cell Lysis Buffer™ (Cell Signaling) containing 100 μM of sodium pervanadate and used for phosphorysine Western blotting and for plate-based multiplex assay of phosphorylated multiple signaling molecules.

For Western blot analysis, 20 μg of total cell lysate from each sample was resolved in 10% Tris-Tricine SDS-PAGE gels under reducing conditions. Proteins were transferred onto polyvinylidene difluoride membrane and probed with 1 μg/ml of mouse anti-pTyr antibody (clone 4G10) according to the manufacturer’s instructions. To confirm equal loading of protein, the same membrane was later stripped and blotted with anti-β-actin antibody according to the manufacturer’s instructions. The extent of LILRB4-mediated inhibition of protein phosphorylation was quantitated by densitometry using NIH ImageJ software (29). 5 μg of total cell lysate from each treatment were used for plate-based quantitative multiplex assay of key phosphorylated protein-tyrosine kinases (Lyn/Lck, Syk, LAT, and Erk) (Beadlyte® phosphoprotein receptor signaling kit, Upstate, Lake Placid, NY) (23).
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**Statistical Analysis**

Changes in TNFα production after cross-linking with various anti-CD64 and/or anti-LILRB4 concentrations at each time point were analyzed using one-way analysis of variance in conjunction with Dunnett post-test. Densitometry of protein phosphorylation and regulation of Lck, Syk, LAT, and Erk phosphorylation were analyzed using one-way analysis of variance with Bonferroni post-test. Values of $p < 0.05$ were considered significant.

**RESULTS**

**LILRB4 Protein Is Highly Expressed on the Surface of Monocytes**—Flow cytometric analysis showed that THP-1 cells have expression of LILRB4 comparable to peripheral blood monocytes from healthy subjects (Figs. 1A and 2A). Moreover, a surface LILRB4 protein of ∼60kDa was immunoprecipitated from biotin-labeled THP-1 cells using anti-LILRB4 monoclonal antibody but not control IgG1 (Fig. 1A). The identity of LILRB4 on the surface of THP-1 cells was further confirmed by sequencing of the immunoprecipitate using nano-LC and data-dependent tandem mass spectrometry (data not shown) confirming specificity of our antibody. THP-1 cells also expressed high levels of surface FcγRI (CD64), comparable to peripheral blood monocytes (Figs. 1A and 2A). However unlike to peripheral blood monocytes, THP-1 cells do not express LILRB1, -B2, or -B3 (Fig. 2A), and hence provided an ideal model for investigating the functions of LILRB4. Interestingly, lymphocytes and natural killer cells that are the major constituents of the peripheral blood mononuclear cell fraction did not express LILRB4 (data not shown).

**Ligation of LILRB4 Significantly Inhibited FcγRI-mediated TNFα Production**—To determine whether LILRB4 modulates activation in response to CD64 activation, we first measured the amount of TNFα produced by cross-linking of CD64 on THP-1 cells. Cross-linking of CD64 showed significant dose-dependent up-regulation of TNFα production (50–300 pg/ml) when compared with cells treated with irrelevant mouse control IgG1 (2.5–10 pg/ml) (Fig. 1B). Time course studies showed time-dependent TNFα production in response to CD64 cross-linking with a peak effect at 6 h, leading to a ∼200-fold increase in TNFα production when compared with control IgG1 (Fig. 1C). More importantly, co-ligation of CD64 with LILRB4 significantly reduced CD64-mediated TNFα production by 50–65% irrespective of time (Fig. 1, C and D). Similarly, cross-linking of CD64 on the surface of magnetic bead purified peripheral blood monocytes (negative selection, >97% pure) caused a large increase in TNFα production that was substantially inhibited (by 51%) upon co-ligation with anti-LILRB4 (Fig. 2B). This was comparable to data obtained from corresponding peripheral blood mononuclear cells (Fig. 2B). Given LILRB4 and CD64 were only expressed by monocytes in the mononuclear fraction of the peripheral blood, and the responses between purified monocytes and non-purified mononuclear cells were comparable (Fig. 2B), subsequent experiments were performed on non-purified mononuclear cells from five independent donors (Fig. 2, B and C). Cross-linking of CD64 on mononuclear cells containing $5 \times 10^4$ monocytes causes significant up-regulation of TNFα production that was significantly inhibited by 40–70% (mean 53.8 ± 7.6%) upon co-ligation with anti-LILRB4 (Fig. 2, B and C).

**Co-ligation of LILRB4 with CD64 Reduced Tyrosine Phosphorylation of Multiple Proteins**—To understand the underlying mechanisms of LILRB4-mediated inhibition of monocytes activation, we examined the effects of LILRB4 ligation on protein tyrosine phosphorylation following CD64 cross-linking. We show that a brief (2 min) cross-linking of CD64 on the surface of THP-1 cells resulted in substantial tyrosine phosphorylation of multiple proteins as compared with the IgG1 control (Fig. 1E). The approximate molecular masses of these proteins were 160, 140, 100, 70, and 65 kDa, respectively. Co-ligation of LILRB4 with CD64 significantly reduced phosphorylation of all these proteins close to the basal level, with the exception of a 100-kDa protein band(s), where its phosphorylation was only partially reduced (Fig. 1E, arrow). Densitometry analysis of three independent experiments showed that ligation of LILRB4 reduced CD64-mediated protein tyrosine phosphorylation by ∼80–90% (Fig. 1F).

**LILRB4 Selectively Regulates Protein Tyrosine Dephosphorylation**—To determine the identity of the proteins phosphorylated in response to CD64 cross-linking, cell lysates from activated cells were immunoprecipitated using anti-pTyr monoclonal antibody, and then specific silver-stained gel bands were sequenced by mass spectrometry. Mascot Search output identified >15 high confidence candidate phosphorylated proteins (Mowse score > 50, p 0.05) that may be involved in CD64-mediated activation of monocytes, including a number of protein-tyrosine kinases, the adaptor protein c-Cbl, and cytoskeletal protein α-actinin-4 (supplemental Fig. S1).

Interestingly, immunoprecipitation with anti-pTyr monoclonal antibody followed by Western blot using anti-c-Cbl antibody showed a marked increase in phosphorylated c-Cbl in cells cross-linked with anti-CD64 (Fig. 3A). This was substantially down-regulated upon co-ligation with LILRB4 (Fig. 3A). In contrast, CD64-mediated induction of α-actinin-4 phosphorylation was not regulated by LILRB4 (Fig. 3B). The molecular mass of α-actinin-4 is 100kDa. This confirms that the major phosphoprotein that was not down-regulated upon co-ligation of CD64 and LILRB4 (Fig. 1E) is indeed α-actinin-4 and indicates selective regulation of tyrosine dephosphorylation by LILRB4.

**LILRB4 Reduced Tyrosine Phosphorylation of Syk, Lck, LAT, and ERK Induced by CD64**—After identifying key kinases that were phosphorylated upon CD64-cross-linking using mass spectrometry, and showing that these were significantly down-regulated by LILRB4 (Fig. 1, E and F), we further confirmed their identity and modulation by LILRB4 using multiplex quantitative assay. A 2-min cross-linking with anti-CD64 caused significant phosphorylation of Lck and Syk, two important proximal signaling molecules in tyrosine kinase-mediated activation cascades (Fig. 3, C and D). The phosphorylation of these two molecules was significantly reduced upon co-ligation with anti-LILRB4 (Fig. 3, C and D), by 80 and 75%, respectively. Furthermore, co-ligation of LILRB4 substantially reduced phosphorylation of downstream signaling molecules, LAT and ERK, by 60 and 50%, respectively (Fig. 3, E and F).
FIGURE 1. Co-ligation of LILRB4 reduced CD64-mediated TNFα production and protein-tyrosine phosphorylation on THP-1 cells. A, representative data showing comparable surface expression of LILRB4 and CD64, as determined by flow cytometry and confirmed by immunoprecipitation. B, TNFα ELISA on culture supernatants from THP-1 cells cross-linked with anti-CD64 monoclonal antibody for 24 h (n = 4); *, p < 0.05; **, p < 0.01 as compared with IgG1 control. C, TNFα ELISA on culture supernatants from THP-1 cells co-ligated with saturation amounts of anti-CD64 and anti-LILRB4 monoclonal antibodies (10 μg/ml each) at various time points. D, shows a summary of four independent TNFα ELISA experiments at the 24-h time point; **, p < 0.01 compared with cells treated with anti-CD64 alone. E, representative anti-phosphotyrosine (anti-pTyr) Western blot of THP-1 cell lysates after a 2 min cross-linking with control IgG1 (lane 1), anti-LILRB4 (lane 2), anti-CD64 (lane 3), or a combination of anti-CD64 and anti-LILRB4 (lane 4) monoclonal antibodies (10 μg/ml each). The lower panel shows the same membrane blotted for β-actin. F, densitometry of three independent anti-pTyr Western blots; #, p < 0.05 compared with IgG1 control; *, p < 0.05 anti-CD64 versus anti-CD64 + anti-LILRB4.
Recruitment of Phosphorylated Proteins and LILRB4 Aggregation to Sites of Activation

Activation of cells with anti-CD64-coated polystyrene beads showed significant increase in total phosphorylated proteins (Fig. 4, A and B) and pSyk (Fig. 4, C and D) around the beads, indicating preferential recruitment of the activated signaling molecules to the activated receptor sites. These were significantly diminished around the beads coated with anti-CD64 and anti-LILRB4 confirming the potent inhibitory effects of LILRB4. Moreover, aggregation of LILRB4 receptors on the surface of THP-1 cells was observed only in LILRB4 and CD64-co-ligated (activated) cells but not in cells treated with anti-CD64, anti-MHC Class I or anti-LILRB4 only coated beads (Fig. 4, E and F), indicating LILRB4 may provide inhibitory signals primarily in the presence of an activation signal.

LILRB4-mediated Inhibition of Monocyte Activation

LILRB4 belongs to the inhibitory LILR family and is highly expressed on the surface of monocytes, macrophages, and dendritic cells (13). In vitro, co-ligation of LILRB4 using monoclonal antibodies, inhibited calcium flux mediated by CD11b and FcγRIII activation (13). It has been found that expression of LILRB4 was positively correlated with a poorer prognosis in pancreas cancer (31), but with a better outcome for transplant recipients (14), indicating an immune regulatory role of LILRB4. However, the underlying mechanisms on how LILRB4-mediated regulation of immune cell activation is not fully understood.
LILRB4-mediated Inhibition of Monocyte Activation

Monocytes, through their various receptors are critical effector cells in innate immunity and play a key role in linking innate to adaptive immune responses. One of these receptors is the FcαR1 or CD64 (32). CD64, through its association with the ITAM-containing FcyR chain, is a potent activating receptor of monocytes and macrophages leading to increased phagocytosis, production of inflammatory cytokines, and/or generation of reactive oxygen species (19–22, 33). CD64 delivers activation signals, *in vivo*, upon its aggregation on the surface of cells by antibodies and multivalent antigens (18), while *in vitro* cross-linking of the receptor by anti-CD64 antibody is sufficient to initiate the stimulatory cascade (33, 34). We have previously identified LILRB4 expression on human peripheral blood monocytes (28) and yet, the functional significance of this has remained poorly understood. Here, we show for the first time that unlike primary monocytes that express multiple inhibitory LILRs, THP-1 cells selectively co-expressed LILRB4 and CD64 on their surface (Figs. 1A and 2A). The latter provided an ideal experimental model to investigate whether LILRB4 can modulate CD64-mediated activation of monocytes. We showed that *in vitro* cross-linking of CD64 on the surface of THP-1 cells caused a significant increase in the production of TNFα in a dose- and time-dependent manner (Fig. 1). Similarly, CD64 cross-linking induced a significant amount of TNFα production in primary monocytes, albeit greater quantities (Fig. 2). TNFα is an important pro-inflammatory cytokine (35); its excessive (unregulated) production may be deleterious to the host, which is a characteristic of many chronic inflammatory diseases (36, 37). We demonstrate that co-ligation of CD64 with LILRB4 significantly abrogated TNFα production in primary monocytes and THP-1 cells (Figs. 1D and 2). Furthermore, the inhibitory effect of LILRB4 was consistently close to 50% regardless of the duration of monocytes activation (Fig. 1C). These results suggest that LILRB4 is a novel counter regulatory molecule that causes significant and sustained inhibition of monocytes activation via one of their key receptors. Interestingly, LILRB4 protein aggregation on the surface of THP-1 cells occurs only upon co-ligation with anti-LILRB4 and anti-CD64 but not with anti-LILRB4 alone (Fig. 4), suggesting that LILRB4 transduces inhibitory signals when paired with an activating receptor, likely through common or shared ligands. Moreover, aggregation of multiple LILRB4 molecules to activation sites might be essential for effective inhibitory function. Whether LILRB4 aggregates on cell surface in response to engagement by its natural ligand(s) remains to be investigated.

We showed that cross-linking of CD64 on the surface of monocytes caused phosphorylation of a number of proteins and demonstrated for the first time that most of these proteins were significantly dephosphorylated upon co-ligation with LILRB4 (Fig. 1E and F). As expected, some of these were key proximal protein kinases including the Src kinase Lck, and Syk (Fig. 3) (38). Most importantly, this is the first report to show that these kinases were significantly down-regulated by LILRB4. Furthermore, LILRB4 strongly abrogated phosphorylation of LAT and ERK, two important downstream signaling molecules involved in cytokine production (Fig. 3). These results indicate that LILRB4 inhibited CD64-mediated TNFα production by down-regulating signaling through protein-tyrosine kinase cascade.

Phosphorylation of the intracellular ITIMs and subsequent recruitment of SH2 domain-containing tyrosine phosphatases such as SHP-1, SHP-2, and SHIP are believed to be critical in terminating activation signals (4). Because the intracellular domain of LILRB4 contains three ITIMs (13), it is likely that the inhibitory effect of this receptor is through recruitment of
phosphatases. Pretreatment of THP-1 cells with the broad-spectrum phosphatase inhibitor, SP, significantly reversed LILRB4-mediated inhibition of TNFα production and markedly prevented LILRB4-mediated protein kinasedephosphorylation (Fig. 5, C and D). These results confirmed our postulate that LILRB4-mediated inhibition of monocyte activation, in response to CD64-cross-linking, is phosphatase dependent. It has previously been reported that SHP-1 associates with the intracellular domain of LILRB4 (13); however, the role of this phosphatase in LILRB4 signaling is yet to be elucidated. Here, we showed that inhibition of SHP-1, using specific pharmacological inhibitor (SS) for 10 min, 24 h, or 48 h, did not affect LILRB4-mediated inhibition of TNFα production (Fig. 5A); indicating that the contribution of SHP-1 to LILRB4 signaling in this setting is minimal. Interestingly, pretreatment of cells for 72 h with SS significantly reversed LILRB4-mediated inhibition of TNFα production by more than 90% (Fig. 5A), but did not prevent LILRB4-mediated dephosphorylation of protein-tyrosine kinases (Fig. 5B). This novel finding suggests the involvement of at least two phosphatases in LILRB4 signaling, one of which was inhibited by pretreatment with SS for 72 h. Collectively, our data suggest LILRB4 recruits phosphatases other than SHP-1 to dephosphorylate signaling kinases induced by CD64. Indeed, our preliminary data showed that immunoprecipitation using anti-LILRB4 monoclonal antibody caused co-precipitation of a SHP-1-like tyrosine phosphatase that was identified only by mass spectrometry, but not detected by SHP-1 Western blot (supplemental Fig. S2). This was further supported by our finding that the specific anti-SHP-1 monoclonal antibody immunoprecipitated the expected SHP-1, but not the SHP-1-like phosphatase and/or LILRB4 from THP-1 cells (supplemental Fig. S2). The identity of this novel SHP-1-like phosphatase, its structural homology to SHP-1 and its role in LILRB4 functions require further investigation. Overall, our results appear to indicate the involvement of more than one phosphatase: a non-SHP-1 phosphatase that regulates cytokine production and protein-tyrosine kinase phosphorylation; and a SHP-1 like phosphatase that dampens TNF signaling but allows LILRB4-mediated dephosphorylation to occur.

In addition to the well-established signaling molecules (5, 6, 38), mass spectrometric analysis of proteins immunoprecipitated from CD64 cross-linked cells identified, for the first time, phosphorylated proteins that were not previously shown to be

**FIGURE 4.** Phosphorylated proteins are increased at sites of receptor activation; LILRB4 is preferentially aggregated at sites of CD64 and LILRB4 co-ligation and down-regulates tyrosine phosphorylation. A, C, and E, representative immuno-fluorescence staining with anti-pTyr (A), anti-pSyk (C), or anti-LILRB4 (E) antibodies on cells treated with anti-CD64, anti-CD64+anti-LILRB4, anti-LILRB4 or control anti-MHC-class I-coated beads. Arrows indicate bead-receptor contact sites. B, D, and F, integrated density of pTyr (B), pSyk (D), and LILRB4 (E) at bead-receptor contact sites. Each dot represents a contact site on an individual cell, and mean values are indicated by horizontal lines. **, p < 0.01; #, p < 0.05.
associated with CD64-mediated signaling. These include: a SH3 domain-containing protein of unknown function (hematopoietic cell-specific Lyn substrate (SPY75)) (39); adaptor proteins of the activating signaling cascade (LAT, lymphocyte cytosolic protein 2 (SLP76), and signal transducing adaptor molecule 2B (STAM-2)) (40–42); proteins involved in cellular cytoskeletal rearrangement, motility, and phagocytosis (α-actinin, hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), actin-related proteins, Clathrin, F-actin-capping protein and protein-tyrosine kinase 9) (43–45); and proteins of the ubiquitin ligase pathway (tripartite motif protein 21 (TRIM 21), and E3 ubiquitin-protein ligase Cbl (c-Cbl)) (46, 47) (supplemental Fig. S1).

Cbl is a key negative regulator of receptor activation (47, 48) that has been shown to be widely expressed in T cells, B cells, mast cells, and monocytes (49, 50). We show that cross-linking of CD64 on THP-1 cells caused strong phosphorylation of c-Cbl. Upon phosphorylation following cellular activation, c-Cbl increases ubiquitination of important signaling kinases such as Syk and/or ZAP-70, thus facilitating increased degradation of these molecules (51–53) and can be viewed as a negative feedback loop to terminate excessive or prolonged activation. Although it sounds counterintuitive, we found co-ligation of CD64 with an inhibitory receptor, LILRB4, strongly down-regulated phosphorylation of c-Cbl (Fig. 3A). This result may indicate a hierarchical inhibitory function by which LILRB4-medi-
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ated inhibition is proximal to the negative feedback loop initiated by c-Cbl. In contrast to the protein-tyrosine kinases and c-Cbl, phosphorylation of α-actinin-4 was not down-regulated by LILRB4 (Fig. 3B), indicating a degree of specificity in LILRB4 effects. Although its functions are not fully understood, α-actinin-4 plays a role as an actin cross-linking protein and may serve to anchor integral membrane proteins to the actin cytoskeleton (54). Furthermore, α-actinin-4 was found to be co-localized in the leading protrusion of the phagocytic cup (55) and thus may play a role in phagocytosis. Therefore, it is reasonable to postulate that LILRB4 may selectively inhibit secretory functions of monocytes by inhibiting signaling molecules involved in cytokine production, without affecting molecules that may be involved in phagocytosis and/or cytoskeletal rearrangements. In conclusion, our findings show that LILRB4 mediates inhibition of monocyte activation through recruitment of phosphatases, which profoundly down-regulate tyrosine phosphorylation of key signaling molecules, and hence may play an important role in immune regulation.

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