The Lyn Tyrosine Kinase Negatively Regulates Neutrophil Integrin Signaling

Shalini Pereira and Clifford Lowell

*J Immunol* 2003; 171:1319-1327; 
http://www.jimmunol.org/content/171/3/1319

References
This article cites 39 articles, 22 of which you can access for free at: 
http://www.jimmunol.org/content/171/3/1319.full#ref-list-1

Subscriptions
Information about subscribing to *The Journal of Immunology* is online at: 
http://jimmunol.org/subscriptions

Permissions
Submit copyright permission requests at: 
http://www.aai.org/ji/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: 
http://jimmunol.org/cgi/alerts/etoc
The Lyn Tyrosine Kinase Negatively Regulates Neutrophil Integrin Signaling¹

Shalini Pereira and Clifford Lowell²

The Src family kinase Lyn has been shown to play both stimulatory and inhibitory roles within several hemopoietic cell types. In this study, we investigated the role played by Lyn in neutrophil integrin signaling. Loss of Lyn resulted in a hyperresponsive phenotype on engagement of surface integrins at low valency. Lyn−/− neutrophils displayed enhanced respiratory burst, secondary granule release, and a hyperadhesive phenotype when adherent to surfaces coated with either cellular counterreceptors or extracellular matrix proteins. In contrast, Lyn-deficient and wild-type cells expressed similar levels of surface integrins and responded equivalently to activating agents in suspension, indicating that the enhanced responses of lyn−/− cells was specific to the integrin signaling pathways. Lyn−/− macrophages also displayed a hyperadhesive phenotype. Biochemical analysis of macrophages from lyn−/− mice revealed that Lyn plays an essential role in the adhesion-dependent phosphorylation of the immunoreceptor tyrosine-based inhibitory motif of the inhibitory receptors SIRPα and PIR-B, which in turn recruit the phosphatase SHP-1. These observations suggest that reduced mobilization of SHP-1 to the membrane in lyn−/− neutrophils results in a hyperadhesive and hyperactive phenotype. This hypothesis is further supported by the fact that neutrophils from mev/mev mice, which have significantly reduced SHP-1 activity, are also hyperresponsive following integrin engagement. This is the first direct evidence using primary leukocytes from lyn−/− mice that this kinase functions as a negative regulator in integrin signaling. The Journal of Immunology, 2003, 171: 1319–1327.

Neutrophils play an essential role in the innate immune system as the first line of defense against invading pathogens. Accumulation of polymorphonuclear leukocytes (PMNs) at sites of tissue invasion and injury requires migration through the vascular endothelium and basement membrane via a multistep process involving different classes of adhesion molecules, which include selectins, integrins, and Ig superfamily members. The initial adhesion of neutrophils to inflamed endothelium is mediated by selectins, which tether the PMNs, thus increasing their exposure to chemotactic and proinflammatory agents, leading to an enhancement in the avidity and affinity of integrins (inside-out signaling) for cellular counterreceptors. Firm adhesion by integrins leads to the arrest of PMNs on the endothelial surface, followed by migration into the subendothelial space, where they release reactive oxygen intermediates and granule components, leading to the elimination of pathogens (1–6). Although the central role of integrins in the adhesion and migration of neutrophils is well appreciated, the integrin-mediated intracellular signaling pathways that lead to cellular activation are not as well understood.

The Src family kinases have been shown to play an essential role in integrin signaling. In PMNs, there is increased activation of the kinases Lyn and Fgr on cross-linking of surface integrins (7). Mutant cells that lack all three myeloid Src family kinases, Hck, Fgr, and Lyn, are unable to spread on integrin-specific Ab-coated surfaces or perform downstream effector functions (8). In vivo experiments conducted in mice lacking Hck and Fgr revealed decreased accumulation of PMNs in tissues during endotoxemia as compared with wild-type cells (9), indicating the importance of these kinases in signaling through neutrophil integrins.

The intracellular protein tyrosine kinase Lyn participates both positively and negatively in B cell, mast cell, platelet, and myeloid cell signaling. In B lymphocytes, its positive role in phosphorylating the Igα and Igβ subunits of the B cell receptor (BCR) is probably redundant with other Src family kinases (10, 11). Its predominant and unique role appears to be negative, through phosphorylation of the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of the low affinity Ig receptor FcγRIIB1 and CD22, leading to recruitment of the protein tyrosine phosphatase SHP-1 and the inositol phosphatase SHIP, which in turn dampen BCR signaling events and subsequent B cell activation (12–14). In mast cells, Lyn is positively involved in signaling from the FcεRI receptor, as lyn−/− mice are unable to mediate passive cutaneous anaphylaxis triggered by IgG plus Ag (15, 16) and primary mast cells display delayed Ca2+ mobilization on cross-linking of the FcεRI receptor (17). However, FcεRI-induced secretion of TNF-α and IL-2 is enhanced in lyn−/− mast cells indicative of a role in negative regulation possibly via the FcγRIIB1 receptor expressed on mast cells (17, 18). In a megakaryocytic cell line, Lyn was found to be required for activation and spreading on fibronectin (19). However, lyn−/− platelets are hyperresponsive to shape change and magnitude of aggregation in response to collagen and thrombin, and although they display slower mobilization of Ca2+ as compared with wild-type cells at the initial time points, there is a slower decline in the plateau once established, again indicative of a role in negative regulation (20). In macrophages, Lyn has been

¹ Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; BCR, B cell receptor; BMDM, bone marrow-derived macrophage; CB, cytochalasin B; ECM, extracellular matrix; ITIM, immune-based tyrosine inhibitory motif; mICAM, murine ICAM.

² Address correspondence and reprint requests to Dr. Clifford Lowell, Department of Laboratory Medicine, University of California, 513 Parnassus Avenue, San Francisco, CA 94143. E-mail address: clowell@cgl.ucsf.edu

Received for publication February 19, 2003. Accepted for publication May 27, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported in part by a California Cancer Research Program Fellowship to S.P. and by National Institutes of Health Grant DK58066 to C.L., who is a Scholar of the Leukemia and Lymphoma Society.

Department of Laboratory Medicine, University of California, San Francisco, CA 94143

The Journal of Immunology.
implicated as a negative regulator of cytokine signaling based on the observation that Lyn-deficient macrophages are hyperresponsive to CSF-1 and GM-CSF stimulation (21).

Studies with integrin-mediated adhesion to date have not extensively explored the specific role played by Lyn. A positive role for Lyn in integrin signaling was postulated based on studies in which Lyn was found associated with a Triton X-100 insoluble fraction along with α-actinin and other effector proteins when surface integrins were cross-linked with specific Abs on human neutrophils (7) and on observing an association of Lyn with β1 integrins following adhesion of promonocytic cells to fibroconnectin or a β1-specific Ab (22). In this work, we directly tested the function of Lyn in integrin signaling in primary neutrophils using lyn−/− mice. We demonstrate that lyn−/− PMNs are hyperadhesive to surfaces coated with integrin ligands and manifest increased integrin-dependent functional responses. In contrast, adhesion-independent responses remain normal in Lyn-deficient PMNs. The mechanism of this hyperresponsiveness is most likely mediated through adhesion-induced phosphorylation of the inhibitory receptors SIRP1α and PIR-B, which is impaired in lyn−/− PMNs, resulting in reduced recruitment of the phosphatase SHP-1. These results provide the first direct evidence that Lyn plays a negative role in regulation of integrin-mediated signaling in primary myeloid cells.

Materials and Methods

Reagents

The following Abs were used in these experiments: biotinylated Abs against α5 integrin (P1S2), α5 integrin (SH10-27), α5 integrin (M1714), β1 integrin (Hb25), β1 integrin (C71/16), β1 integrin (2C9/G2), rat IgG2a (A95-1), and rat IgG2b (R35-95) (BD Pharmingen, San Diego, CA). The L-selectin-specific Ab (MEL-14) was conjugated to PE, and the anti-GrlAb (11-26c.2a) was conjugated to FITC (BD Pharmingen). Immunoprecipitation and Western blotting experiments used the anti-phosphotyrosine Ab 4G10 (Upstate Biotechnology, Lake Placid, NY), anti-PIR-B, and anti-SHP-1 Abs (Santa Cruz Biotechnology, Santa Cruz, CA), and the anti-SIRP1α Ab, gifted by B. Noel (Harvard Medical School, Boston, MA). Marine ICAM-1 (mICAM-1) was a kind gift from C. Vines (University of New Mexico School of Medicine, Albuquerque, NM). The extracellular matrix (ECM) proteins used were rat collagen type I, vitronectin from human plasma, fibrinogen from mouse plasma factor I, and fibronectin from human and bovine plasma (Sigma-Aldrich, St. Louis, MO). Stimulants used were murine TNF-α (Peprotech, Rocky Hill, NJ), PMA, and fMLP (Sigma-Aldrich). Cytochalasin B (CB) was used in degranulation studies conducted in suspension (Sigma-Aldrich).

Isolation of bone marrow neutrophils and culture of bone marrow-derived macrophages (BMDM)

Bone marrow neutrophils were isolated from C57Bl/6 mice (Charles River, Wilmington, MA), lyn−/− or mεε/− mice backcrossed to C57Bl/6 for 15 generations to ensure congenicity. The isolation procedure was essentially as described previously (8). Neutrophils used in the assays with ECM proteins as integrin ligands were preincubated in HBSS containing 10 mM HEPES, 0.5 mM CaCl2, and 1 mM MgCl2 before use in adhesion-mediated assays. When assaying PMNs in suspension, the cells were maintained and used in Mg2+-free medium. BMDM were obtained by culturing bone marrow macrophages in the same manner in DME H-16, 1 g/L glucose medium (University of California Cell Culture Facility, San Francisco, CA), supplemented with 10% FCS (Life Technologies, Grand Island, NY), 20 mM HEPES, and 10% M-CSF1-conditioned medium. Adherent primary macrophages were used between days 6 and 9 of culture.

Superoxide release assays

Purified bone marrow neutrophils (2–4 × 106 cells/well) were added to wells coated with varying concentrations of ECM proteins or mICAM-1 (8) of a 96-well Immulon-4 plate (Dynex Technologies, Chantilly, VA) to measure adhesion-dependent superoxide release. Where appropriate, 20 ng/ml TNF-α (Peprotech) or 100 mM PMA (Sigma-Aldrich) was added. Superoxide production under nonadherent conditions was estimated by adding cells to uncoated, 20% FCS-blocked 96-well tissue culture plates. A total of 3 mM fMLP (Sigma-Aldrich), 100 mM PMA (Sigma-Aldrich), and 10 μM CB (Sigma-Aldrich) was added, as indicated. Respiratory burst was measured using the cytochrome c reduction test, as described (8, 9). The data are presented as a cumulative assay, and all time points were performed in triplicate and the results were averaged.

Secondary granule release assays

Lactoferrin was used as a marker for secondary granule release, and the assays were performed essentially as described (8). A total of 100 μl of cells (2 × 106/ml) was incubated in either ECM- or mICAM-1-coated (integrin-mediated reactions) or 20% FCS-blocked (integrin-independent measurements) wells of a microtiter plate at 37°C for 60 min (adhesion-mediated granule release) or for 10 min (integrin-independent reactions). A total of 3 mM IMLP or 100 nM PMA was added, as indicated. CB (10 μM) was added where appropriate to cells that were assayed in suspension. After 10 min, the samples were transferred to a 15-mm V-bottom plate and centrifuged at 2000 rpm for 10 min. A total of 25 μl supernatant/sample was diluted 4-fold in carbonate buffer (pH 9.6) and incubated overnight at 4°C in an Immulon-4 microtiter plate. All subsequent steps were as described (23). Assays were performed in triplicate and the results were averaged.

Cell adhesion assays

A total of 100 μl of purified neutrophils at 4 × 106/ml was incubated with the indicated stimulants in ECM protein (blocked with 20% FCS) or mICAM-1-coated wells (not blocked with FCS) of a 96-well microtiter plate at 37°C for 60 min. The percentage of cells that had adhered was estimated by measuring the membrane acid phosphatase activity of cells that remained adherent to the coated surfaces following four washes with warm (37°C) PBS and two applications of a g force of 60. All wells were done in triplicate and results were averaged.

Adhesive responses of BMDMs were assayed as follows. Day 5–6 adherent cultured BMDM were removed from culture dishes and suspended in IMDM medium (University of California Cell Culture Facility) supplemented with 5% FCS, 20 mM HEPES at 4–5 × 106 cells/ml. The cells were rested in suspension at 37°C for 2–3 h before transfer to 60 × 15-mm bacterial plates (KORD/VARKMAL; Midwest Scientific, St. Louis, MO) and incubated at 37°C for a further 30–120 min. Adherent cells were visualized on a Nikon microscope under phase contrast at ×10 magnification, and digital images of at least 10 random fields were captured using the IPLab Spectrum P program. The average cell area occupied by adherent macrophages was quantified using NIH version Image software (v1.63). A minimum of 100–150 adherent cells was analyzed and the results were averaged. Data were plotted as a function of average cell area in square pixels per cell for each genotype.

FACS analysis of neutrophils

Bone marrow and peripheral leukocytes were incubated with the indicated biotinylated or directly conjugated Abs, followed by incubation with streptavidin conjugated to PE (BD Pharmingen). The labeled cells were analyzed by flow cytometry, with gating on the basis of forward and side scatter and Gr-1 staining being used to define the murine PMN population. Stimulation-dependent changes in expression levels of CD18 and L-selectin, and the anti-SIRP1α Ab, were assessed by flow cytometry using anti-human GR-1 and CD18 Abs. Data from triplicate and results were averaged.

Immunoprecipitation and Western blotting

Purified PMNs (1 × 107) were added to 60 × 15-mm tissue culture plates that were coated with 0.5 μg/ml mICAM-1 and subsequently blocked with 20% FCS. The plates were incubated at 37°C for 60 min with or without addition of 20 μg/ml TNF-α, following which all cells (adherent and nonadherent) were pooled and lysed in either RIPA buffer. Insoluble material was removed by centrifugation, and the lysates were boiled with sample buffer before loading on a gel.

Adherent cultured BMDMs were removed from culture dishes and suspended in IMDM medium (University of California Cell Culture Facility) supplemented with 3% FCS, 20 mM HEPES at 4–5 × 106 cells/ml. The cells were rested in suspension at 37°C for 2–3 h before transfer to 100 × 15-mm bacterial plates (KORD/VARKMAL) or tissue culture plates coated with fibrogen (10 μg/ml) and incubated for a further 45–60 min. Adherent and nonadherent cells were pooled and lysed in either RIPA (30 mM HEPES [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 20 mM NaF, 5 mM Na-EGTA, 10 mM benzamidine, 0.1% SDS, 0.005 mg/ml pepstatin, 0.005 mg/ml leupeptin, 1 mM PMSF, and 0.002 mg/ml aprotinin) buffer or, when coimmunoprecipitating proteins, buffer containing 1% Nonidet P-40, 1% sodium deoxycholate, 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2 mM sodium vanadate, and protease inhibitors, and the lysates were cleared of insoluble material.
For immunoprecipitation studies, the lysates were precleared using recombinant protein G-agarose (Life Technologies) and incubated with anti-SIRP1α, anti-PIR-B, or anti-SHP-1 Abs. Immune complexes were captured with recombinant protein G-agarose, washed, and resuspended in sample buffer. Samples were run on an SDS-PAGE gel, transferred to nitrocellulose membranes, and blotted with 4G10, anti-SIRP1α, anti-PIR-B, and anti-SHP-1 Abs, followed by HRP-conjugated secondary Ab (Amersham, Piscataway, NJ, or Jackson ImmunoResearch, West Grove, PA). Immunoblots were developed using the ECL system (Amersham). All immunoprecipitation experiments were repeated to ensure reproducibility, and the data shown are representative of results obtained.

Results

Enhanced integrin-mediated responses by lyn−/− neutrophils

Engagement of PMN integrins by surface-bound ligands or counterreceptors such as ICAM-1 results in activation of cellular effector functions such as prolonged respiratory burst and degranulation (1). Under most circumstances, the cells need to be exposed to a proinflammatory stimulus (such as TNF-α) to activate the ability of the integrin to bind to ligand, after which the cells spread over the surface, assemble the subunits of the NADPH oxidase, and begin degranulation. To assess the role played by Lyn in these integrin-mediated signaling events, we used an in vitro assay to measure release of reactive oxygen intermediates by PMNs stimulated with TNF-α when their surface integrins are engaged (9). Bone marrow neutrophils from wild-type and lyn−/− mice were added to microtiter wells precoated with increasing concentrations of the ECM proteins fibrinogen, fibronectin, collagen, and vitronectin, or the cellular adhesion molecule mICAM-1 and the nanomoles of superoxide released by adherent PMNs over time were determined. Adhesion-mediated superoxide release by lyn−/− neutrophils was elevated in comparison with wild-type cells over a range of concentrations, with all the integrin ligands tested (Fig. 1, A and B). Although maximal superoxide release required stimulation with TNF-α when the cells were adherent to ECM proteins (Fig. 1B), at high concentrations of mICAM-1 (Fig. 1A) both wild-type and lyn−/− neutrophils were equally responsive in the absence of TNF-α, most likely indicative of the greater integrin cross-linking efficiency of the cellular adhesion molecule as compared with the ECM proteins.

As expected from the results obtained with high concentrations of mICAM-1, when signaling through surface receptors was bypassed by directly stimulating the wild-type and lyn−/− neutrophils with PMA, both cell types responded similarly (Fig. 1C), thus confirming that the lyn−/− neutrophils did not contain elevated levels of the NADPH oxidase complex.

To assess for alterations in the degranulation response of lyn−/− PMNs, we measured the release of the secondary granule marker lactoferrin in the medium after adhesion of cells to ECM- or mICAM-1-coated surfaces. In agreement with the superoxide release

FIGURE 1. Lyn-deficient neutrophils are hyperresponsive in adhesion-dependent respiratory burst activity. Wild-type and lyn−/− PMNs were added to wells coated with integrin ligands, and superoxide release was monitored over a 2-h period in the presence and absence of TNF-α stimulation. A, Wells coated with varying concentrations of mICAM-1 ranging from 0.1 to 10 μg/ml. B, Superoxide released at the 60-min time point when PMNs were adherent to varying concentrations of the indicated ECM proteins (0.1–10 μg/ml). C, Superoxide released when cells were stimulated with PMA. All assays were done in triplicate, and the results were displayed as an average ± SD. Data shown are representative of at least three independent experiments.
assay, there was enhanced adhesion-dependent secondary granule release by lyn<sup>−/−</sup> neutrophils as compared with wild-type cells when adherent to all the integrin ligands tested (Fig. 2, A and B). We further confirmed similar levels of secondary granule contents by stimulation of both lyn<sup>−/−</sup> and wild-type neutrophils with PMA, which activates maximal release of lactoferrin (Fig. 2C).

Cross-linking of PMN integrins by surface-bound ligands stimulates firm cell adhesion and spreading, leading to respiratory burst and degranulation, a phenomenon collectively known as adherent activation. To ascertain whether the elevated downstream responses of the lyn<sup>−/−</sup> neutrophils are due to an enhanced ability to tightly adhere to integrin ligand-coated surfaces, we measured the percentage of lyn<sup>−/−</sup> and wild-type cells that resisted washing and two applications of forces of 50–60 g after adhesion to ECM proteins or mICAM-1 and stimulation with TNF-α. In accordance with the superoxide and lactoferrin release assays, lyn<sup>−/−</sup> neutrophils displayed enhanced tight adhesion to all the integrin ligands tested (Fig. 3, A and B), indicative of a correlation between tight adhesion and downstream effector functions. Both lyn<sup>−/−</sup> and wild-type neutrophils adhered equally to ECM protein-coated surfaces when stimulated with PMA, confirming that the observed differences in adhesion between the two cell types following TNF-α stimulation were due to differences in membrane-proximal signaling events. In summary, the lyn<sup>−/−</sup> mutation enhances the adhesion-dependent activation of neutrophils, regardless of the responses studied or the integrin ligand used.

**FIGURE 2.** Lyn-deficient neutrophils are more efficient at adhesion-dependent degranulation. Wild-type and lyn<sup>−/−</sup> PMNs were incubated with A, fibrinogen, collagen, and fibronectin (10 μg/ml each); B, mICAM-1 at the indicated concentrations; or C, stimulated with PMA, and the release of the secondary granule marker lactoferrin was determined using an ELISA. All assays were done in triplicate, and the results were displayed as an average ± SD. Data shown are representative of at least three independent experiments.

**FIGURE 3.** Lyn<sup>−/−</sup> neutrophils are hyperadhesive to surfaces coated with integrin ligands. Wild-type and lyn<sup>−/−</sup> PMNs were incubated in wells coated with A, 10 μg/ml of fibrinogen, collagen, and fibronectin (+FCS block); B, 0.1 and 0.5 μg/ml mICAM-1 (+FCS block); or C, stimulated with PMA, and the percentage of input cells resistant to dislodgement by centrifugal forces of 60 g was determined. Data shown are averages ± SD of triplicate determinations and are representative of at least three independent experiments.

Lyn<sup>−/−</sup> neutrophils express normal levels of integrins on their surface

To determine whether increased expression of integrins on lyn<sup>−/−</sup> neutrophils was responsible for their enhanced integrin-mediated responses, we examined surface expression of several integrin subunits on wild-type and lyn<sup>−/−</sup> PMNs using flow cytometry. Surface expression of the major integrin subunits, which are known to adhere to the ECM proteins and mICAM-1, was found to be equivalent between wild-type and lyn<sup>−/−</sup> cells (Fig. 4). Hence, increased integrin expression was not the explanation for enhanced lyn<sup>−/−</sup> PMN responses.

Adhesion-independent neutrophil functions are not enhanced in lyn<sup>−/−</sup> neutrophils

When neutrophils are adherent to low valency integrin ligands, they require an additional soluble signal, such as TNF-α stimulation, for full functional responses to occur. Stimulation by the soluble inflammatory agonist acts to increase the avidity and affinity of the integrin molecules for their ligands (inside-out signaling), and the resultant increased cross-linking of integrins on the cell surface serves to set up a signaling cascade (outside-in signaling) that ultimately results in downstream responses. To determine whether the enhanced adhesion-dependent activation of the lyn<sup>−/−</sup> neutrophils was due to increased signaling through the soluble proinflammatory agonist receptor vs from the integrins themselves, we tested these responses on cells in suspension in which integrins were unengaged. Because PMNs do not mount a significant respiratory burst or degranulation response to TNF-α stimulation in suspension, we used another proinflammatory agent, the bacterial peptide fMLP, to test adhesion-independent responses. Bone marrow neutrophils were incubated in 20% FCS-blocked microtiter dishes, prepared with centrifugal forces of 60 g.

**FIGURE 4.**
wells in Mg$$^{2+}$$-free medium to prevent integrin binding, then stimulated with the chemoattractant fMLP and CB (24), and adhesion-activated supernoxide and lactoferrin release by wild-type and lyn$$^{-/-}$$ PMNs was quantified. Although CB treatment alone could not induce respiratory burst or lactoferrin release in either wild-type or lyn$$^{-/-}$$ neutrophils (Fig. 5, A and C), it strongly increased the fMLP-dependent activity of neutrophils in suspension (Fig. 5B). Both lyn$$^{-/-}$$ and wild-type cells mounted an equivalent and rapid respiratory burst to this agent (in an assay measured in seconds vs minutes, characteristic of adhesion-independent responses). Likewise, degranulation in response to fMLP stimulation was not significantly different between wild-type and lyn$$^{-/-}$$ PMNs (Fig. 5C).

To directly test potential differences in TNF-$$\alpha$$ signaling between wild-type and lyn$$^{-/-}$$ PMNs, we examined up-regulation of CD18 expression and shedding of L-selectin from cell surfaces following TNF-$$\alpha$$ stimulation using flow cytometry. The lyn$$^{-/-}$$ neutrophils did not demonstrate enhanced up-regulation of surface CD18 expression (Fig. 5D), nor was increased shedding of L-selectin observed (Fig. 5E) following TNF-$$\alpha$$ stimulation as compared with wild-type cells. Taken together, these data indicate that the hyperresponsiveness in adhesion-mediated activation displayed by lyn$$^{-/-}$$ cells relies on integrin-mediated signaling events and is not due to an enhancement in responses to the proinflammatory agonist.

Lyn$$^{-/-}$$ macrophages display increased adhesion and spreading responses

The above results indicated that Lyn operates primarily as a negative regulator of adhesion-dependent neutrophil activation. To ascertain whether Lyn plays a similar role in macrophages, we examined the adhesion/spreading responses of wild-type vs lyn$$^{-/-}$$ BMDMs following plating on nontissue culture plastic (VALMARK) plates, which have been shown to engage $$\beta_2$$ integrins, leading to firm adhesion and integrin-dependent signaling (25). To ensure that only integrin-mediated events were analyzed, the primary BMDMs were starved of growth factor in suspension for 3–4 h, the maximum length of time growth factor could be withdrawn before stress responses were activated, before plating on Valmark plates. As shown in Fig. 6A, while an equivalent proportion of wild-type and lyn$$^{-/-}$$ macrophages was attached to the $$\beta_2$$ integrin-specific surface within 60 min, the lyn$$^{-/-}$$ cells displayed markedly enhanced spreading as compared with the wild type. Quantitation of adherent cell areas of single cells revealed that the surface area occupied by adherent wild-type BMDMs was significantly less than that of lyn$$^{-/-}$$ BMDMs (Fig. 6B). Similar differences were observed at time points ranging from 30 to 120 min, but by 3–5 h of plating, the number of fully spread BMDMs became equivalent in wild-type vs lyn$$^{-/-}$$ cells, indicating the primary effect of the mutation was to increase the rate of macrophage spreading. These observations indicate that Lyn also serves as an inhibitor of integrin-dependent signaling events in macrophages.

**Signaling events downstream of integrin cross-linking in lyn$$^{-/-}$$ neutrophils and BMDMs**

To begin to determine the mechanism by which Lyn deficiency results in exaggerated integrin signaling events, we examined overall protein tyrosine phosphorylation of wild-type and lyn$$^{-/-}$$ neutrophils following plating of cells on mICAM-1. Resting lyn$$^{-/-}$$ PMNs displayed a modestly increased overall level of protein tyrosine phosphorylation, which was further enhanced following TNF-$$\alpha$$ stimulation due to the ability of the cells to adhere and spread over the mICAM-1-coated surface (Fig. 7A). This exaggerated tyrosine phosphorylation response correlated very well with the observed hyperresponsive phenotype of the lyn$$^{-/-}$$ neutrophils in adhesion-mediated activation.

A potential mechanism by which Lyn kinase-deficient cells manifest hyperphosphorylation during adhesion may be due to inefficient recruitment of tyrosine phosphatases. To address this possibility, we examined the phosphorylation state of two well-known signal inhibitory receptors, PIR-B and SIRP1$$\alpha$$, both of which contain ITIMs in their cytoplasmic domains that, if phosphorylated, lead to recruitment of the phosphatase SHP-1 and subsequent down-regulation of signaling events. Owing to the difficulty in obtaining the large numbers of primary murine neutrophils required to carry out biochemical studies and due to the observation that the capture of stably associated protein complexes from neutrophils proved difficult, presumably due to the presence of large quantities of proteases and phosphatases in intracellular granules, we used BMDMs to extend our mechanistic studies. Because microscopic examination demonstrated an increased rate of spreading in lyn$$^{-/-}$$ BMDMs at early (45 min) times following plating on VALMARK surfaces, we performed our biochemical experiments at an equivalent time point. As shown in Fig. 7B, wild-type cells displayed increased phosphorylation of both PIR-B and SIRP1$$\alpha$$ in an adhesion-dependent manner following adhesion to VALMARK plates. However, both the basal as well as the stimulation-dependent phosphorylation of these two proteins was decreased in lyn$$^{-/-}$$ cells. Coimmunoprecipitation experiments revealed that the increased phosphorylation of both of these receptors following adhesion led to association of these molecules with SHP-1 in wild-type cells (Fig. 7C). However, in correlation with reduced basal and adhesion-activated phosphorylation of SIRP1$$\alpha$$ and PIR-B, there was significantly less recruitment of SHP-1 to these receptors in lyn$$^{-/-}$$ cells. These data suggest that impaired tyrosine phosphorylation of both SIRP1$$\alpha$$ and PIR-B following engagement of
integrins in lyn−/− leukocytes leads to reduced recruitment and activation of SHP-1, resulting in enhanced signaling reactions and the hyperadhesive/hyperresponsive phenotype.

A direct prediction from the above model would be that deficiency of SHP-1 itself should also result in hyperadhesive/hyperresponsive leukocytes. Indeed, BMDMs from mev/mev mice, which have a point mutation in the shp-1 gene that reduces expression of SHP-1 by ~90%, do display a hyperadhesive phenotype (26). To test whether PMNs from mev/mev mice displayed exaggerated functional responses analogous to lyn−/− cells, we tested them in the adhesion-induced respiratory burst assay. As shown in Fig. 8, deficiency of SHP-1 significantly increased the response of these PMNs to adhesion on fibronectin and on fibrinogen. Moreover, the mev/mev PMNs also manifested significant responses to ligands even in the absence of the TNF-α costimulation, similar to what was observed with lyn−/− cells plated on mICAM-1. Thus, deficiency of SHP-1 or impaired recruitment of SHP-1 to ITIM-containing receptors in lyn−/− PMNs results in hyperresponsiveness to integrin ligation.

Discussion

The Lyn kinase has been demonstrated to play a dual role in signaling through multiple receptors in hemopoietic cells. In this study, we demonstrate that Lyn plays a negative role in the regulation of adhesion-mediated signaling in neutrophils. Lyn−/− neutrophils manifest hyperresponsive integrin-mediated effector functions, including adhesion, respiratory burst, and secondary granule release. A specific role for Lyn in the regulation of integrin signaling is indicated by the normal responses of lyn−/− cells to soluble agonists. Adhesion-induced phosphorylation of tyrosine

FIGURE 5. Adhesion-independent responses of lyn−/− neutrophils. Purified bone marrow PMNs from wild-type and lyn−/− mice were suspended in buffer with or without CB, and the respiratory burst activity over a 10-min period was estimated without addition of stimulus (A) or after addition of fMLP (B). C, Adhesion-independent secondary granule release by wild-type and lyn−/− PMNs on treatment of cells without stimulus, with fMLP or PMA in the presence and absence of CB, as determined by ELISA. D, Up-regulation of CD18 expression, and E, increase in L-selectin shedding by bone marrow PMNs following TNF-α stimulation were analyzed by flow cytometry. Data shown are representative of at least three independent experiments.

FIGURE 6. Lyn-deficient bone marrow macrophages demonstrate enhanced adhesion and cell spreading. A, Phase-contrast microscopy of wild-type vs lyn−/− BMDMs following plating on VALMARK plates for 60 min. B, Quantitation of BMDM adhesion by assessing the average surface area occupied by a spread cell in square pixels. Data shown are for the 60-min time point and are representative of at least three independent experiments.
FIGURE 7. Integrin-mediated signaling by wild-type and lyn-/- cells. A. Total cellular tyrosine phosphorylation of wild-type and Lyn-deficient PMNs adherent to 0.5 μg/ml mICAM-1 with or without stimulation with TNF-α. B. Tyrosine phosphorylation of SIRP1α and PIR-B in adherent or nonadherent bone marrow macrophages derived from wild-type and lyn-/- mice. C. Association of SHP-1 with SIRP1α and PIR-B, as determined by coimmunoprecipitation, following plating of wild-type or lyn-/- bone marrow macrophages on VALMARK plates. Lysates from adherent or nonadherent wild-type and lyn-/- cells were immunoprecipitated with anti-SHP-1, subjected to PAGE, and sequentially probed with anti-PTY, anti-PIR-B, or anti-SHP-1 Abs. Tyrosine-phosphorylated proteins within the 120- to 130-kDa range are displayed.

residues within the ITIMs of two negative regulatory molecules, SIRP1α and PIR-B, was reduced in cells lacking Lyn, as was the association of these receptors with the tyrosine phosphatase SHP-1, indicating that Lyn is the primary kinase involved in activation of negative regulation following integrin cross-linking via these two receptors.

Of the three myeloid Src family kinases, Hck and Fgr have been demonstrated to play a positive role in integrin signaling (9). Cross-linking integrins on peritoneal exudate macrophages leads to an increase in activity of Hck and Fgr (27), and hck-/-fgr-/- double-mutant PMNs show a marked defect in spreading, superoxide release, and secondary granule release when plated on fibronectin-coated surfaces (27, 28). In the positive signaling pathway, one of the substrates that these kinases act on is the adapter protein c-Cbl, which in turn recruits lipid kinases to propagate downstream signals (28, 29). In contrast, the lyn-/- neutrophils were hyperresponsive relative to wild-type cells following adhesion to surfaces coated with low concentrations of mICAM-1 and ECM proteins. As was observed with mICAM-1 (Fig. 1A), at higher concentrations of ECM proteins, there is no difference in responses between the wild-type and lyn-/- cells (data not shown). This raises the possibility that Lyn plays a role in regulating the transition of integrins from a low affinity to a high affinity state (inside-out) signaling, so the threshold for adhesion-mediated activation of PMNs lacking Lyn could be lower than for wild-type cells. This would also explain the ability of Lyn-deficient (and SHP-1 mutant) PMNs to partially respond to integrin ligands even in the absence of costimuli from inflammatory mediators (such as TNF-α), a phenomena not seen in wild-type cells. Alternatively, these results could be explained by changes in postintegrin receptor signaling thresholds (outside-in). Impaired recruitment of SHP-1 to membrane-localized ITIM receptors may allow for triggering of PMN effector function following minimal engagement of integrin receptors, which would occur at lower concentrations of integrin ligands. Generation of activation-specific mAbs directed against murine integrin subunits will eventually allow us to distinguish between these two models.

Our data would support the model that the major effect of Lyn on neutrophils is ascribable to enhanced adhesion, because no major differences were observed between lyn-/- and wild-type cells in suspension activation assays. However, it remains a formal possibility that some of the increased capacity to produce superoxide by lyn-/- cells may be an intrinsic effect of the mutation. This could be addressed by examination of superoxide production on a per cell basis rather than using cell populations, as was done in this study. Additionally, we have performed the majority of our experiments using TNF-α as the main agonist to induce adhesion to ECM proteins. If the effect of the lyn-/- mutation is specific to the integrin pathway, then similar results should be observed with other proinflammatory agonists. Use of IMLP as an agonist for adhesion-dependent activation was complicated by the fact that this agent induces significant amount of superoxide independently of integrin ligation; hence, differences between wild-type and lyn-/- cells were difficult to interpret (data not shown). In other
assays, we have observed no major enhancements in PMN responses to chemokine agonists (H. Zhang and C. Lowell, unpublished). Hence, it is most likely that the major effect of Lyn on integrin-mediated neutrophil activation and macrophage spreading is through impaired phosphorylation of membrane-localized ITIM receptors, leading to reduced SHP-1 recruitment.

The potential substrates on which SHP-1 may act to modulate integrin signaling events are unknown. Given previous observations that c-Cbl is underphosphorylated in hck−/−fgr−/−lyn−/− cells following adhesion and that antisense oligonucleotides (28) or c-crib−/− mutant macrophages have defects in cell adhesion and spreading (29), it is possible that c-Cbl is involved. Indeed, c-Cbl is markedly hyperphosphorylated and more strongly associated with phosphatidylinositol-3 lipid kinase activity in macrophages derived from me/me mice (R. Holmes and C. Lowell, unpublished), which may account for the increased membrane-associated phosphatidylinositol-3 kinase activity and increased adhesion-dependent signaling reactions reported in SHP-1-deficient leukocytes (26). Genetic crosses of Cbl-deficient mice with me/me animals will be required to formally test whether c-Cbl is a significant substrate of SHP-1 in the adhesion-dependent signaling pathway.

A further prediction based on the hyperadhesive phenotype of lyn−/− leukocytes would be that they might have reduced migratory capacity, analogous to the reduced chemotactic responses of hyperadhesive leukocytes from me/me mice (30). Indeed, preliminary data have indicated that Lyn-deficient PMNs have reduced chemotactic responses to endogenous chemokines (CCL3 and CXCL1) as well as reduced recruitment into the peritoneum during experimental peritonitis in vivo (H. Zhang and C. Lowell, unpublished). However, these migratory defects in Lyn-deficient cells may also be due to changes in chemokine signaling per se rather than hyperadhesion, hence complicating the interpretation of these observations. Changes in both these signaling pathways in Lyn-deficient leukocytes may alter their responses to inflammatory signaling in vivo. Indeed, in a more general sense, it remains to be determined how much of the overall phenotype of lyn−/− mice can be ascribed to the hyperresponsiveness we have observed in lyn−/− PMNs and BMDMs. The increased adhesive capacity of lyn−/− PMNs would logically contribute significantly to the tissue damage caused by the production of autoantibodies, which has been well documented in Lyn-deficient mice (12, 14, 15). Likewise, it is possible that increased macrophage adherence may lead to enhanced production of cytokines, which in turn could drive some of the autoimmunity in lyn−/− animals. Ultimately, these issues will have to be resolved through generation of myeloid lineage-specific Lyn-deficient mice.

Although the respiratory burst and lactoferrin release activity of PMNs adherent to mICAM-1 vs ECM protein-coated wells were similar (Fig. 1, A and B), in the adhesion assay the percentage of PMNs adherent to mICAM-1 was significantly higher (Fig. 3B). This result is most likely due to the differences in treatment of mICAM-1- and ECM-coated wells used in the adhesion vs activation assays. In the activation assays, both surface types were blocked with FCS. In the adhesion assay, ECM protein-coated wells were blocked, while ICAM-1-coated wells were not, as blocking abrogated adhesion of PMNs to ICAM-1 below the detection limit of the assay. The adhesion activity of cells to mICAM-1 in Fig. 3B is therefore likely to be a combinatorial effect of cells binding to the protein and to tissue culture plastic. We postulate that mICAM-1 is a very effective integrin cross-linking ligand capable of inducing respiratory burst/degranulation at very low levels of adhesion compared with ECM protein-coated surfaces. Regardless, lyn−/− cells maintain their hyperresponsive phenotype in all three assays, underscoring the global role played by Lyn in regulating integrin-mediated effector functions.

Inhibitory receptors modulate immune responses, which require both initiation and termination. We examined the role of two ITIM-containing receptors, SIRP1α and PIR-B, in the regulation of myeloid integrin-mediated signaling. SIRP1α has been implicated in the regulation of adhesion-mediated signaling in a variety of different cell types. Plating of BMDMs on fibronectin led to increased phosphorylation of SIRP1α and a concomitant increase in its association with the protein tyrosine phosphatase SHP-1 (31). In cultured fibroblasts and in Chinese hamster ovary cells, adhesion to ECM proteins induces tyrosine phosphorylation of SIRP1α and its association with SHP-2 (32–34). SIRP1α-deficient fibroblasts spread more quickly on fibronectin and manifest increased formation of actin stress fibers plus focal adhesions compared with wild-type cells (34). Although the prevailing view is that of SIRP1α playing an inhibitory role in cell adhesion, the observed association of SHP-2 with SIRP1α during integrin ligation may suggest that SIRP1α can have an activating function as well (35).

In contrast to SIRP1α, PIR-B has not been directly implicated in the modulation of adhesion-mediated signaling. PIR-B does play a role in the negative regulation of BCR signaling in B lymphocytes (36, 37), FcεR signaling in mast cells (38), and dendritic cell development (37), and a potential role in cytokine regulation (S. Pereira and C. Lowell, unpublished) (21, 39). Our studies are the first demonstration of a potential role for PIR-B in integrin-dependent negative regulation. Further studies with pir-B−/− mice will be required to sort out the individual contribution of this receptor to regulation of adhesion receptor signaling. It is likely that both these receptors may play many general inhibitory functions in leukocytes. However, it is clear that tyrosine phosphorylation of their C-terminal ITIM domains, which is required for their ability to recruit SHP-1 and down-modulate signaling reactions, is dependent to a large extent on Lyn. This would explain many of the diverse defects caused by the deficiency of this kinase.

**References**


