Immunity, Vol. 16, 547–558, April, 2002, Copyright ©2002 by Cell Press

Syk Is Required for Integrin Signaling in Neutrophils

Attila Mócsai,[1] Meijuan Zhou,[1,4] Fanying Meng,[1,5] Victor L. Tybulewicz,[2] and Clifford A. Lowell[1,3]

[1]Department of Laboratory Medicine
University of California
San Francisco, California 94143

[2]National Institute for Medical Research
London
United Kingdom

Summary

The Syk tyrosine kinase plays a critical role in the signaling machinery of various receptors of the adaptive immune system. Here we show that Syk is also an essential component of integrin signaling in neutrophils. *syk*$^{-/-}$ neutrophils failed to undergo respiratory burst, degranulation, or spreading in response to proinflammatory stimuli while adherent to immobilized integrin ligands or when stimulated by direct cross-linking of integrins. Signaling from the β_1, β_2, or β_3 integrins was defective in *syk*$^{-/-}$ cells. Syk colocalized with CD18 during cell spreading and initiated downstream signaling events leading to actin polymerization. Surprisingly, these defects in integrin-mediated activation did not impair the integrin-dependent in vitro or in vivo migration of *syk*$^{-/-}$ neutrophils or of cells deficient in Src-family kinases. Thus, integrins use different signaling mechanisms to support migration and adherent activation.

Introduction

Neutrophils are key players of the innate immune response against microbial infection. At sites of inflammation, cellular and humoral factors induce the transition of circulating neutrophils from a quiescent, poorly adhesive state to a primed, strongly adhesive phenotype (Lowell and Berton, 1999). Concomitant firm adhesion to the endothelium leads to local arrest of neutrophils, which then migrate through the vessel wall and the extravascular tissue to the site of microbial invasion. Through the release of reactive oxygen radicals, digestive enzymes, and antimicrobial peptides, neutrophils then eliminate the invading microorganisms. Integrins play a critical role in adhesive interactions during neutrophil migration and activation. However, the intracellular signaling pathways linking integrins to the various effector mechanisms of neutrophils are poorly understood.

Syk, together with the related kinase ZAP-70, plays a central role in immunoreceptor (B cell- and T cell-receptor and F_C-receptor) signaling (Turner et al., 2000; Chu et al., 1998). Targeted disruption of Syk leads to a blockade of antigen-receptor-dependent maturation of B cells (Turner et al., 1995; Cheng et al., 1995), impaired T cell receptor function (Turner et al., 1995; Cheng et al., 1995; Colucci et al., 2000), and defects in responses mediated by $F_C\gamma$ and $F_C\epsilon$ receptors (Crowley et al., 1997; Kiefer et al., 1998; Costello et al., 1996). On the other hand, little is known about the role of Syk in nonadaptive immune mechanisms.

Besides being indispensable for immunoreceptor function, Syk also becomes activated upon ligation of cell surface integrins in neutrophils (Yan et al., 1997), monocytic cell lines (Lin et al., 1995), and through the platelet integrin $\alpha_{IIb}\beta_3$ (Clark et al., 1994; Gao et al., 1997). However, demonstration of a physiological role of such a pathway in primary cells has been hindered by the lethality of the *syk*$^{-/-}$ mice and the nonspecific nature of the available pharmacological inhibitors (Fernandez and Suchard, 1998; Law et al., 1999; Mócsai et al., 2000; Miura et al., 2001).

In this work, we directly tested the role of Syk in integrin signaling of neutrophils using chimeric mice with a genetic deficiency of Syk in the hematopoietic compartment. We conclude that Syk plays a crucial role in multiple integrin-mediated effector functions of the innate immune system, but it is dispensable for integrin-dependent neutrophil migration both in vitro and in vivo.

Results

Generation of Bone Marrow Chimeras with *syk*$^{-/-}$ Hematopoietic System

To overcome the perinatal lethality of *syk*$^{-/-}$ mice, we generated bone marrow chimeras by injecting *syk*$^{-/-}$ fetal liver cells into lethally irradiated recipients. Donor and recipient cells were distinguished based on allelic differences between CD45 epitopes of donor (CD45.2 from the C57BL/6 background) and recipient (CD45.1) mice. Purified bone marrow neutrophils of *syk*$^{-/-}$ chimeras consisted virtually exclusively of donor (CD45.2$^+$) cells (Figure 1B) which were devoid of Syk protein (Figure 1C). No differences in neutrophil yield (data not shown) or expression of the Gr1 maturation marker (Figure 1D) were observed between the wild-type and *syk*$^{-/-}$ genotypes, indicating that Syk is not required for normal proliferation or differentiation in the granulocytic lineage. *syk*$^{-/-}$ neutrophils expressed normal levels of all integrins tested (Figure 1D).

Defective Integrin-Dependent Functions in *syk*$^{-/-}$ Neutrophils

At sites of inflammation, neutrophils are activated by proinflammatory stimuli while adherent to intercellular adhesion molecules or extracellular matrix proteins. These conditions can be mimicked by in vitro stimulation of neutrophils in the presence of an adhesion surface (Nathan, 1987), leading to spreading, respiratory burst, and degranulation of the cells (jointly referred to as adherent activation). These adhesion-dependent responses require the ligation of β_2 integrins (Nathan et al., 1989; Richter et al., 1990). As shown in Figure 2A, *syk*$^{-/-}$ neu-

[3]Correspondence: clowell@cgl.ucsf.edu
[4]Present address: Bayer Corp., Berkeley, California 94710.
[5]Present address: Telik Inc., South San Francisco, California 94080.

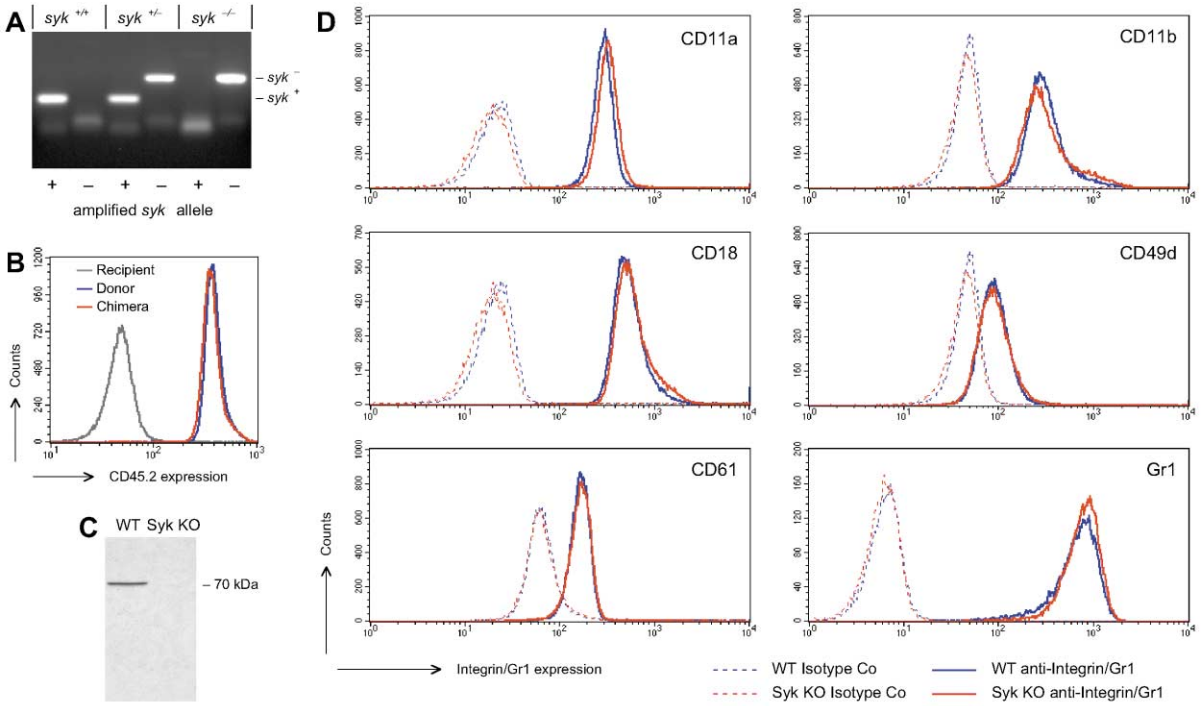

Figure 1. Generation of *syk*$^{-/-}$ Chimeras and Normal Expression of Integrins on *syk*$^{-/-}$ Neutrophils

(A) PCR analysis of *syk*$^{+/+}$, *syk*$^{+/-}$ and *syk*$^{-/-}$ embryos.

(B) Flow cytometry analysis of CD45 alleles on neutrophils purified from bone marrow chimeras. For controls, cells from a pretransplantation recipient (CD45.1 congenic on B6 background) and from a B6 mouse (CD45.2; same as the background of the *syk*$^{-/-}$ donor) were used.

(C) Immunoblot analysis of Syk in neutrophils prepared from chimeras with wild-type or *syk*$^{-/-}$ hematopoietic system.

(D) Flow cytometry analysis of integrin and Gr1 expression on *syk*$^{-/-}$ neutrophils.

trophils are completely defective in respiratory burst (superoxide production) when plated on a fibrinogen-coated surface in the presence of TNF-α (TNF). Under identical conditions, neutrophils deficient in the β_2 integrin chain CD18 show a similar defect, confirming the integrin dependence of the response (Figure 2A). In addition to the respiratory burst defect, *syk*$^{-/-}$ neutrophils also failed to undergo adhesion-dependent degranulation, as determined by the release of the secondary granule marker lactoferrin (Figure 2A). Other inflammatory mediators, such as the bacterial tripeptide formyl-MetLeuPhe (fMLP), bacterial lipopolysaccharide (LPS), or the chemokine MIP-2, failed to restore the defective integrin-dependent activation of *syk*$^{-/-}$ neutrophils (Figure 2B). Syk-deficient neutrophils also failed to respond to TNF when plated on surfaces coated with collagen or FCS (Figure 2B) or with purified ICAM-1, the major integrin ligand involved in the local arrest of circulating leukocytes at the vessel wall (Figure 2C).

Correlated with the above defects in adhesion-dependent activation, *syk*$^{-/-}$ neutrophils did not spread over a fibrinogen-coated surface (Figure 2D). This response was also absent in *CD18*$^{-/-}$ cells (not shown). Furthermore, *syk*$^{-/-}$ cells failed to manifest increased adhesion to fibrinogen- or FCS-coated surfaces following treatment with TNF (Figure 2E), likely due to their inability to signal actin cytoskeletal changes required for cell spreading. This adhesion response is also mediated by CD18, as shown by a defect in *CD18*$^{-/-}$ cells (Figure 2E).

Taken together, the *syk*$^{-/-}$ mutation renders neutrophils incapable of adhesion-dependent responses, irrespective of the type of response studied, the adhesion surface used, or the stimulus added.

Syk Is Not Required for Adhesion-Independent Neutrophil Functions

As shown in the above experiments, adherent activation of neutrophils requires two signals: a soluble proinflammatory stimulus (like TNF) and another signal generated during the ligation of integrins by the proteins immobilized onto the adhesion surface. According to the current paradigm, soluble stimuli provide intracellular signals that lead to an increase in affinity and avidity of cell surface integrins toward their ligands (inside-out signaling). Concomitant engagement of integrins generates a second signal, delivered from integrins to the interior of the cells, leading to actin cytoskeletal rearrangements and further cellular responses (outside-in signaling). We next determined whether the failure of Syk-deficient neutrophils to respond to adhesive stimuli was due to a defect in signaling of the soluble stimuli or in that triggered by engagement of integrins.

No difference was observed between wild-type, *syk*$^{-/-}$, or *CD18*$^{-/-}$ neutrophils in respiratory burst or degranulation induced by the phorbol-ester PMA, a known adhesion-independent stimulus (Figure 3A). Moreover, in both wild-type and *syk*$^{-/-}$ cells, responses to PMA stimulation in suspension were similar to those in adhesion (Mócsai et al., 1999 and data not shown),

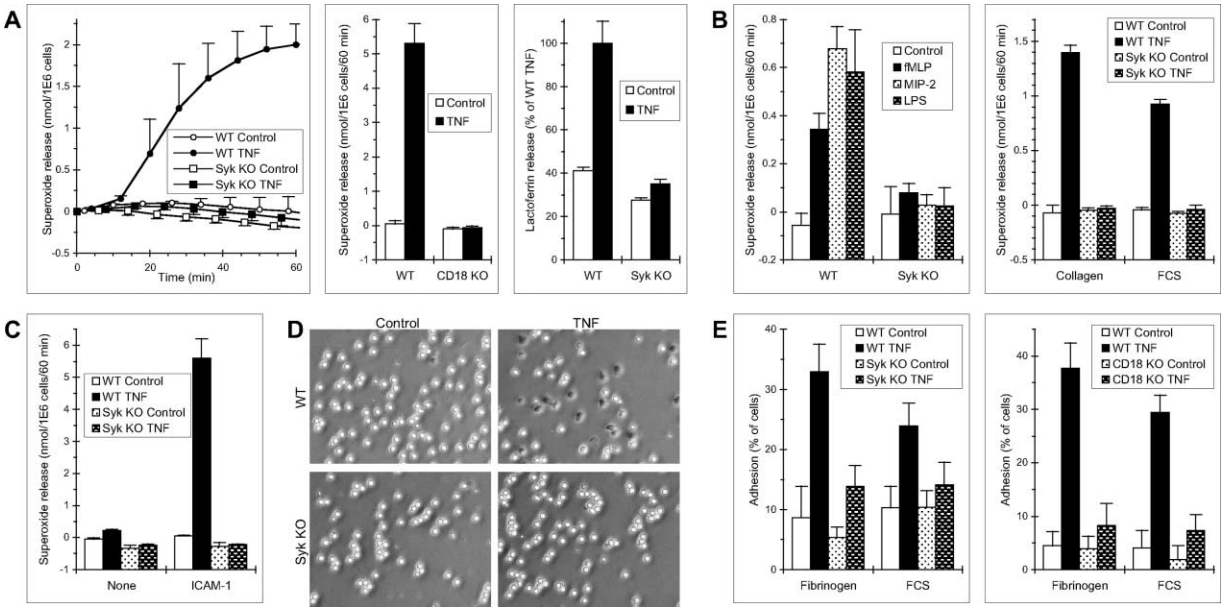

Figure 2. Defective Adhesion-Dependent Responses in *syk*$^{-/-}$ Neutrophils

(A), Respiratory burst and degranulation of *syk*$^{-/-}$ and *CD18*$^{-/-}$ neutrophils plated on fibrinogen; (B and C), respiratory burst of *syk*$^{-/-}$ neutrophils in response to fMLP, MIP-2, or LPS on fibrinogen, or to TNF on collagen, FCS, or murine ICAM-1; (D), spreading of *syk*$^{-/-}$ neutrophils on fibrinogen; (E), adhesion of *syk*$^{-/-}$ and *CD18*$^{-/-}$ neutrophils to fibrinogen and FCS.

confirming that these responses are in fact independent of integrin ligation. Deficiency of Syk did not interfere with initial signaling from TNF receptors, since the TNF-induced upregulation of CD18 (Figure 3B) and shedding of L-selectin (Figure 3C) were both normal in *syk*$^{-/-}$ neutrophils. Additionally, *syk*$^{-/-}$ neutrophils manifested no major defects in G-protein-coupled receptor signaling of suspended cells (data not shown). Taken together, adhesion-independent responses seem not to be affected by the *syk*$^{-/-}$ mutation. On the other hand, *syk*$^{-/-}$ neutrophils spread normally in response to PMA (Figure 3D), whereas *CD18*$^{-/-}$ cells were strongly defective in this response (data not shown). Thus, the integrins on *syk*$^{-/-}$ cells can still bind their ligands and support spreading when the Syk-dependent signaling step is bypassed by PMA.

Syk Is Required for Responses Initiated by Direct Crosslinking of Integrins

To test whether outside-in integrin signaling initiated in the absence of concomitant inside-out signals required Syk, cells were plated on surfaces coated with an engineered polypeptide containing multiple copies of the integrin binding RGD motif of human fibronectin (poly-RGD). Likely due to the high valency of this integrin ligand, wild-type neutrophils plated on poly-RGD-coated surfaces released superoxide without any additional proinflammatory stimulus (Figure 4A). The integrin-dependence of the response to poly-RGD was confirmed by the fact that *CD18*$^{-/-}$ neutrophils responded poorly to this ligand (Figure 4A). *syk*$^{-/-}$ neutrophils were also unable to respond to the direct crosslinking of integrins by poly-RGD surfaces (Figure 4A).

To more specifically address the responses of *syk*$^{-/-}$ neutrophils to integrin crosslinking, cells were plated in microwells coated with surface-immobilized anti-integrin antibodies. When plated onto anti-CD18-antibody-coated surfaces, wild-type but not *syk*$^{-/-}$ neutrophils responded with a strong release of superoxide (Figure 4B). Cells plated on isotype-matched control antibody did not release superoxide, and *CD18*$^{-/-}$ neutrophils failed to respond to plate-bound anti-CD18 stimulation (Figure 4B). Antibody-mediated crosslinking of CD18 also resulted in exocytosis of secondary granules and spreading of the cells over the antibody surface in wild-type but not *syk*$^{-/-}$ neutrophils (Figures 4B and 4C). These observations provide direct evidence that Syk is an essential component of signaling pathways from β_2 integrins to spreading, respiratory burst, and degranulation of neutrophils.

Syk Is Involved in Signaling from Multiple Integrins

The antibody-mediated crosslinking approach was also used to determine which integrins present in neutrophils signal through Syk. Plate-bound antibodies against the CD11a (α_L) or CD11b (α_M) subunits of β_2 integrins induced respiratory burst in wild-type but not in *syk*$^{-/-}$ neutrophils (Figure 4D). Superoxide release could also be induced by antibodies against CD29 and CD61 (the β chains of β_1 and β_3 integrins, respectively), and these responses were also defective in the *syk*$^{-/-}$ cells (Figure 4E). We have recently shown that murine neutrophils express functional CD49d (α_4) integrins, which probably pair with the β_1 or, possibly, the β_7 chain (Pereira et al., 2001). Respiratory burst triggered by immobilized anti-α_4 antibodies was also completely defective in *syk*$^{-/-}$ neutrophils (Figure 4E). Taken together, these data suggest that Syk is required for signaling from multiple integrins, including, but not necessarily limited to, members of the β_1, β_2, and β_3 families.

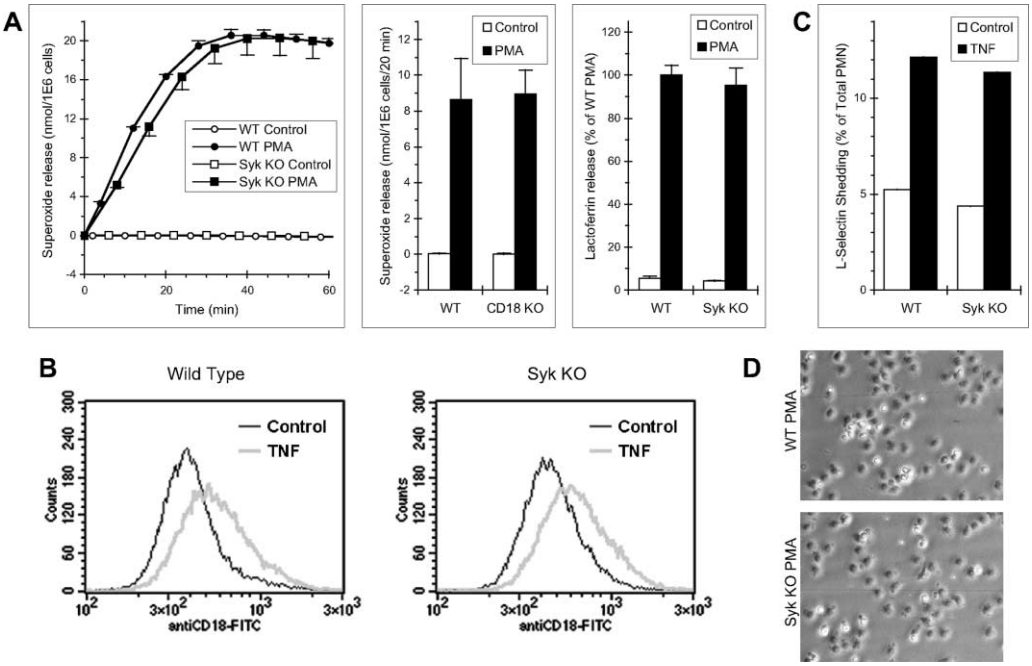

Figure 3. Normal Adhesion-Independent Responses in *syk*$^{-/-}$ Neutrophils

(A), PMA-induced respiratory burst and degranulation of *syk*$^{-/-}$ and *CD18*$^{-/-}$ neutrophils plated on fibrinogen; (B), upregulation of CD18 in suspension; (C), percentage of cells that have shed L-selectin in suspension; (D), PMA-induced spreading on fibrinogen surface.

Neutrophil Integrin Engagement Activates Syk and Induces Its Colocalization with CD18

The results described above directly implicate Syk in outside-in integrin signaling and would predict that Syk becomes activated in cells spreading on ECM-ligands. Plating of wild-type neutrophils on a fibrinogen-coated surface was sufficient to induce a modest tyrosine phosphorylation of Syk which was further enhanced by stimulation with TNF and was particularly elevated in cells firmly adherent to the fibrinogen surface (Figure 5A). Stimulation with TNF in suspension did not induce Syk phosphorylation. Plating cells on poly-RGD induced strong Syk phosphorylation without any additional stimulus, and this increase was defective in *CD18*$^{-/-}$ neutrophils (Figure 5B). Taken together, integrin-mediated activation of neutrophils leads to phosphorylation of Syk, which correlates with the CD18-dependent firm adhesion of the cells, rather than with stimulation by TNF.

The activation of Syk during β_2 integrin ligation suggests a close interaction between Syk and CD18. Indeed, coassociation of Syk and CD18 has been demonstrated by coimmunoprecipitation experiments using both human neutrophils and macrophage cell lines (Yan et al., 1997; Vines et al., 2001). To characterize this association in the context of cell shape changes during cell spreading, we followed the subcellular localization of Syk and CD18 by immunoflourescent staining and high-resolution digital microscopy. Following plating of wild-type neutrophils on poly-RGD, Syk and CD18 were enriched at the same submembraneous regions during the initial phase of cell spreading (Figure 5C, arrowheads). CD18 and Syk remained colocalized to the highly active edges of the cells throughout the spreading process (Figure 5C). However, less colocalization of the two proteins could be observed in fully spread cells (data not shown). Addition of inflammatory stimuli like TNF (data not shown) or fMLP (Figure 5D; see Figure 5E for staining controls) significantly increased the colocalization of Syk and CD18 in cells actively crawling over the poly-RGD surface. We conclude that Syk and CD18 temporarily colocalize at cell edges of neutrophils where the active protrusion of cell membranes over plate-bound integrin ligands occurs during cell spreading and crawling.

Biochemical Characterization of the Integrin Signaling Pathways Involving Syk

Next we examined the tyrosine phosphorylation of Syk and of other potential signaling molecules in neutrophils plated on poly-RGD surfaces. Similar to immunoreceptor signaling, activation of Syk by integrin ligation is dependent on Src-family kinases, since tyrosine phosphorylation of Syk was absent in cells deficient in Hck, Fgr, and Lyn, the neutrophil-specific members of the Src kinase family (Figure 6A). *syk*$^{-/-}$ neutrophils had normal level of protein tyrosine phosphorylation in resting state but failed to demonstrate increased phosphorylation upon direct crosslinking of integrins (Figure 6B). Similar results were obtained with *CD18*$^{-/-}$ cells, while both background and stimulated tyrosine phosphorylation were dramatically decreased in *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils (Figure 6B), pointing to a more general role for Src-family kinases in signaling tyrosine phosphorylation events in these cells.

Three of the major tyrosine-phosphorylated bands (Figure 6B, closed arrowheads) comigrated with the c-Cbl (Cbl), Pyk2, and Vav1 (Vav) proteins (data not shown). Integrin-mediated tyrosine phosphorylation of all three proteins could be detected in wild-type cells,

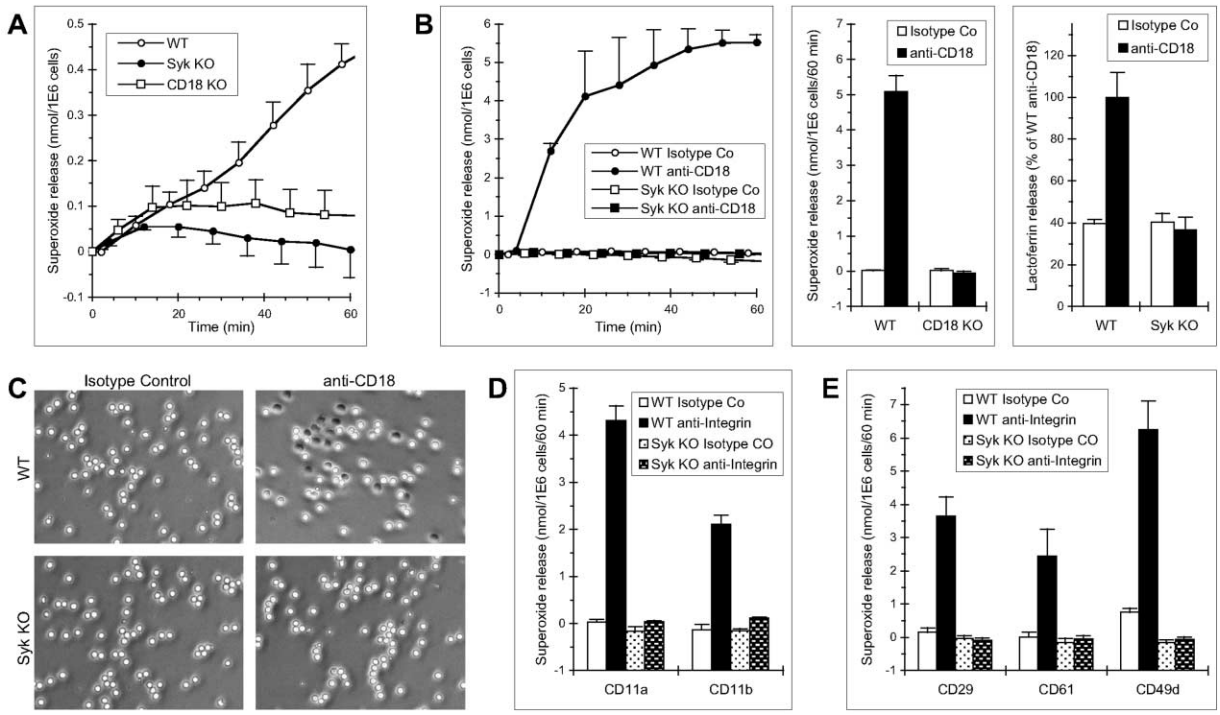

Figure 4. Defective Responses of *syk*$^{-/-}$ Neutrophils to Direct Crosslinking of Integrins

(A), Respiratory burst of *syk*$^{-/-}$ and *CD18*$^{-/-}$ neutrophils plated on a poly-RGD surface without any additional stimulus; (B), respiratory burst and degranulation of *syk*$^{-/-}$ and *CD18*$^{-/-}$ neutrophils plated on an anti-CD18 coated surface without any additional stimulus; (C), spreading of the cells on an anti-CD18 coated surface; (D), respiratory burst triggered by antibodies against the α chains of β_2 integrins; (E), respiratory burst triggered by antibodies against non-β_2 integrins.

but it was strongly impaired in the Syk-deficient cells (Figure 6C).

The well-known activation of the Rac small GTPase family by Vav and the effect of the Rac-related proteins on actin-based motility raise the possibility that these proteins are involved in the Syk-mediated integrin-signaling pathway in neutrophils. However, we were unable to observe an integrin-mediated activation of Rac family members (see supplemental data at http://www.immunity.com/cgi/content/full/16/4/547/DC1) or an immediate Rac-effector protein, Pak1 (data not shown). In contrast, activation of p38 MAP kinase, which is further downstream of Rac and suggested to be involved in adhesion-dependent neutrophil functions (Detmers et al., 1998), was clearly demonstrated in wild-type but not *syk*$^{-/-}$ neutrophils (Figure 6D). Activation of the MAP-kinase-activated protein kinase 2 (MAPKAPK2), a molecule directly downstream of p38 MAP kinase, was also defective in *syk*$^{-/-}$ neutrophils plated on poly-RGD (Figure 6E). The MAPKAPK2-dependent phosphorylation of the heat shock protein Hsp27 is thought to be involved in the polymerization of cellular actin, providing a possible link between the above integrin signaling pathways and the actin cytoskeleton. To directly assess the polymerization of actin in neutrophils, we determined the distribution of cellular actin between the Triton-soluble (cytosolic) and Triton-insoluble (cytoskeletal) fractions. As shown in Figure 6F, plating wild-type neutrophils on a poly-RGD surface leads to a partial decrease of actin in the Triton-soluble fraction with a concomitant increase in the Triton-insoluble fraction. This redistribution is absent in the *syk*$^{-/-}$ neutrophils (Figure 6F). Redistribution of the actin-bundling protein α-actinin to the Triton-insoluble fraction was also easily demonstrated in wild-type neutrophils plated on poly-RGD but was completely absent in *syk*$^{-/-}$ cells (Figure 6F). Thus, in neutrophil integrin signaling, Syk is required for an event prior to polymerization and cytoskeletal association of filamentous actin.

Syk Is Not Required for In Vitro or In Vivo Migration of Neutrophils

One of the major functions of integrins in the immune system is to support directed migration of leukocytes to sites of inflammation. To determine whether Syk is required for integrin-mediated migration of neutrophils, we tested the directed migration of *syk*$^{-/-}$ neutrophils both in vitro and in vivo.

In vitro chemotaxis was determined by assessing migration through a fibrinogen-coated Transwell membrane in response to the bacterial peptide chemoattractant fMLP. Surprisingly, the overall transmigration of *syk*$^{-/-}$ neutrophils was similar to that of wild-type cells, with partial decrease at lower but slight increase at higher fMLP concentrations (Figure 7A). Similar results were observed utilizing MIP-1α or MIP-2 as chemoattractants (data not shown). The fact that *CD18*$^{-/-}$ neutrophils are strongly defective at all fMLP concentrations tested (Figure 7C) confirms the integrin dependence of this assay. Thus, in contrast to the CD18-dependent

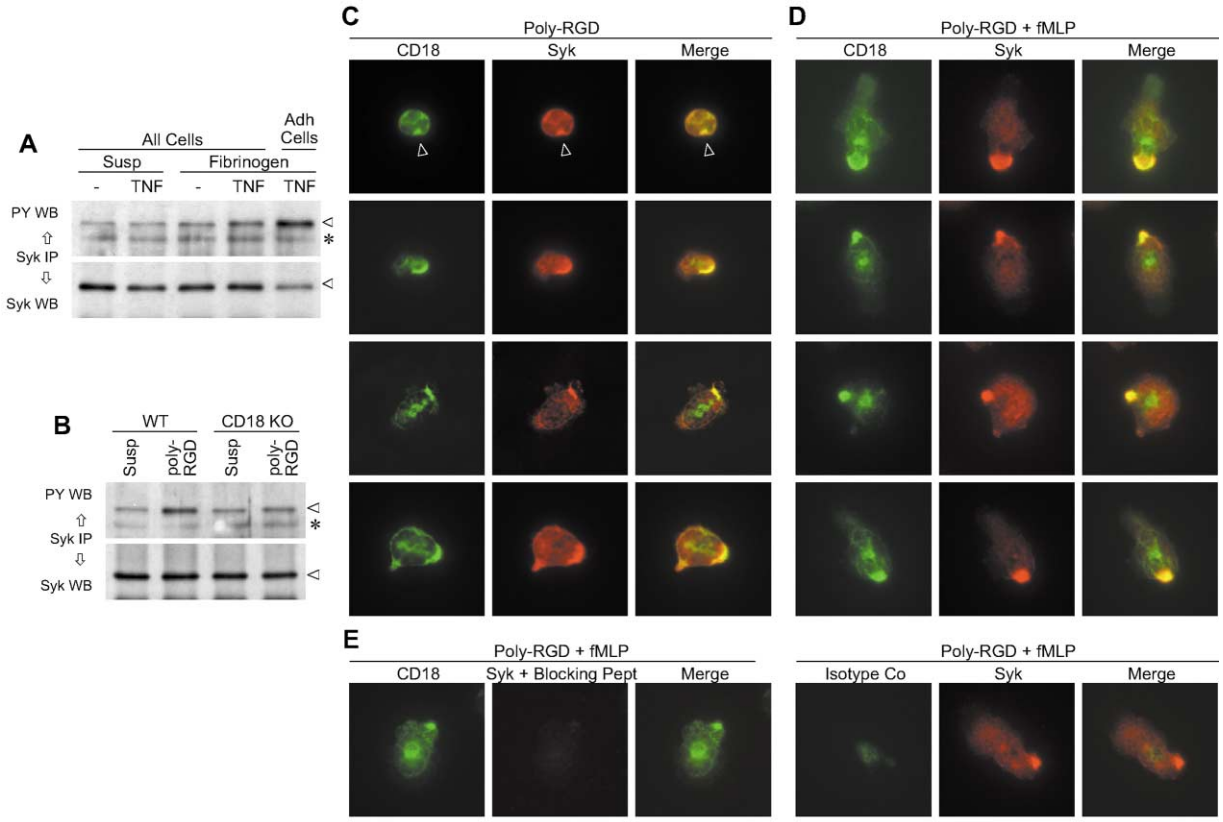

Figure 5. Activation of Syk and Colocalization of Syk and CD18

(A and B) (A) shows tyrosine phosphorylation of Syk in wild-type cells in response to TNF in suspension or on fibrinogen-coated surfaces. Syk was precipitated either from all (adherent and nonadherent) cells or only from firmly adherent cells resistant to a washing step. Syk immunoprecipitates were subjected to immunoblotting with the indicated antibody. (B) shows tyrosine phosphorylation of Syk in wild-type and *CD18*$^{-/-}$ neutrophils plated onto a poly-RGD surface. In (A) and (B), arrowheads indicate the location of Syk; asterisks indicate a nonspecific band, also present in immunoprecipitates from cell-free lysis buffer (data not shown).

(C–E) Localization of Syk and CD18 during spreading of wild-type neutrophils plated on poly-RGD with (D) or without (C) fMLP. (E) shows staining controls and is otherwise identical to (D). In (C), arrowheads indicate initial colocalization of Syk and CD18.

adherent activation events described in Figures 2–6, CD18-dependent in vitro migration of neutrophils does not require Syk.

To test the in vivo migration of neutrophils, we compared the peritoneal accumulation of wild-type and *syk*$^{-/-}$ cells during a thioglycollate-induced sterile peritonitis. Since bone marrow chimeras with pure *syk*$^{-/-}$ hematopoietic system lack B cells and develop chylous hemoperitoneum, subcutaneous bleeding, and gradual anemia, these mice were not suitable for our experiments, as these processes may have unpredictable secondary effects on neutrophil migration. Instead, we generated a series of mixed chimeric mice containing varying ratios of both wild-type and *syk*$^{-/-}$ hematopoietic cells, allowing us to study the migration of wild-type and *syk*$^{-/-}$ neutrophils under identical conditions within the same animal. To this end, lethally irradiated recipients were reconstituted with a mixture of wild-type and *syk*$^{-/-}$ bone marrow cells at a percentage of 25%, 50%, or 75% *syk*$^{-/-}$ bone marrow. Cells from the two genotypes were distinguished based on allelic differences in their CD45 epitopes. None of these chimeras developed any signs of bleeding or peritoneal leakage characteristic of pure *syk*$^{-/-}$ bone marrow chimeras. A sterile peritonitis was induced by intraperitoneal injection of the mixed chimeras with thioglycollate, and the percentage of *syk*$^{-/-}$ neutrophils was compared in the peritoneal lavage fluid versus blood samples taken at three different time points during the assay. As shown in Figure 7B, the percentage of *syk*$^{-/-}$ cells among peritoneal neutrophils was similar to or even slightly higher than their percentage among peripheral blood neutrophils in all mice. In contrast, when a series of wild-type/*CD18*$^{-/-}$ mixed bone marrow chimeras were tested in the same sterile peritonitis model, CD18-deficient cells were significantly retarded in their ability to migrate into the inflamed peritoneum (Figure 7D), thus confirming the integrin dependence of this assay. These findings show that the absence of Syk in neutrophils does not interfere with their capacity to migrate to the inflamed peritoneum in an integrin-dependent manner in vivo.

Since our previous data (Figure 6A) had demonstrated that Src-family kinases are required for integrin-dependent Syk activation, and since deficiency of these kinases results in impaired integrin-dependent spreading, respiratory burst, and degranulation (Lowell et al., 1996; Mócsai et al., 1999; Pereira et al., 2001; our unpublished data), we tested the ability of triple-mutant

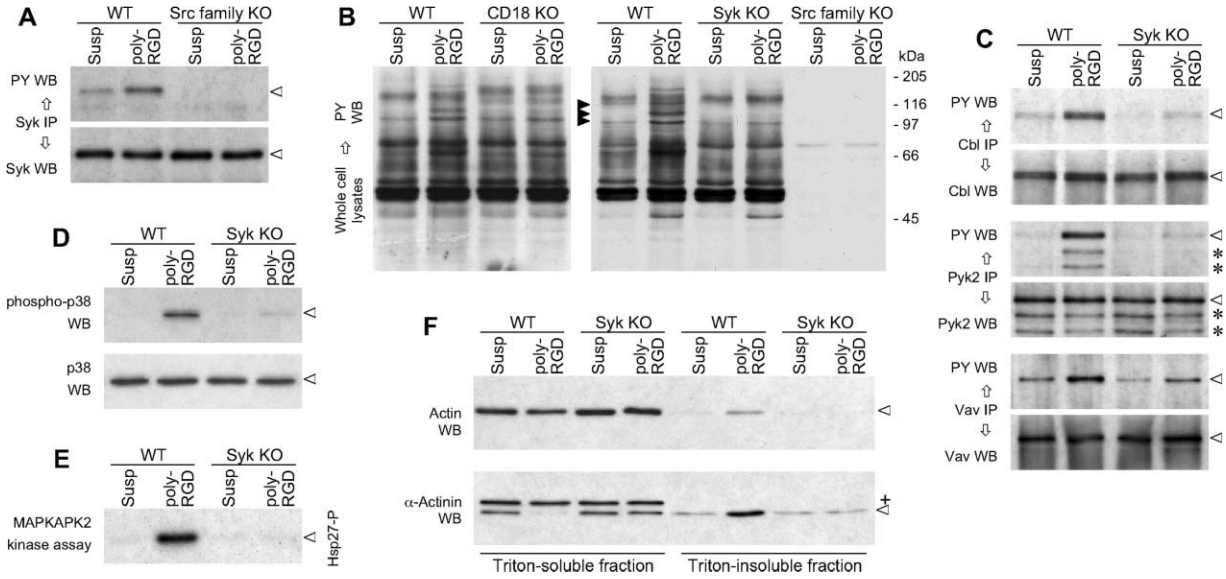

Figure 6. Identification of Signaling Molecules Participating in the Integrin Signaling through Syk

(A), Poly-RGD-induced tyrosine phosphorylation of Syk in neutrophils deficient of the Src-family kinases Hck, Fgr, and Lyn; (B), total cellular tyrosine phosphorylation in *syk*$^{-/-}$, *CD18*$^{-/-}$, and Src-family-deficient neutrophils; (C), tyrosine phosphorylation of Cbl, Pyk2, and Vav in *syk*$^{-/-}$ neutrophils; (D), phosphorylation of the p38 MAP kinase in *syk*$^{-/-}$ neutrophils; (E), activation of MAPKAP kinase 2 in *syk*$^{-/-}$ neutrophils; (F), redistribution of actin and α-actinin between the Triton-soluble and Triton-insoluble fractions. Open arrowheads indicate migration of the relevant proteins. Closed arrowheads indicate (from top to bottom) migration of Cbl, Pyk2, and Vav. Bands indicated by asterisks are likely degradation products resulting from C-terminal proteolytic cleavage of Pyk2. The plus sign indicates a fully Triton-soluble protein of unknown identity, with a molecular mass (120 kDa) clearly higher than that of α-actinin (100 kDa).

hck$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils to migrate in the above assay systems. As shown in Figure 7E, no overall defect was observed in the in vitro migration of *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils through a fibrinogen-coated Transwell membrane, though their migration was decreased at lower but increased at higher doses of the chemoattractant fMLP. Similarly, no defect in migration of *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ neutrophils was seen in the thioglycollate peritonitis model using a series of mixed bone marrow chimeras. Like the *syk*$^{-/-}$ neutrophils, the percentage of Src-family-deficient cells in the peritoneal exudate was consistently close to or slightly higher than that in the peripheral blood (Figure 7F). The same conclusion was drawn from previous experiments comparing intact wild-type and *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice (data not shown). Thus, similar to Syk, Src-family kinases are not required for CD18-dependent migration of neutrophils either in vitro or in vivo. These results confirm, by an independent genetic approach, the different signaling requirements of CD18-dependent migration versus CD18-dependent adherent activation of neutrophils.

Discussion

In this paper, we have shown that Syk is indispensable for signaling events from β_1, β_2, and β_3 integrins, leading to activation of neutrophil effector functions (Figures 2, 3, 4, and 6). Previous studies of *syk*$^{-/-}$ mice concluded that Syk is primarily involved in signaling from receptors of the adaptive immune system (B cell receptors, T cell receptors, and F_C receptors). Our findings indicate that this kinase is also critically involved in signaling mechanisms of innate immunity. Integrins are widely expressed in all cells of the immune system and function as adhesion receptors in host-pathogen interaction, lymphocyte homing, antigen presentation, or target recognition by NK cells. Extrapolating our results to these mechanisms would suggest that the role of Syk is much broader than previously anticipated.

According to the current paradigm, integrin signaling occurs in two consecutive steps, designated as inside-out and outside-in signaling (see Results). In experiments using surface-bound molecules to directly cross-link integrins (Figure 4), the inside-out step is bypassed since the ligands are of sufficient valency (poly-RGD) or affinity (anti-integrin antibodies) to directly aggregate integrins and initiate outside-in signaling. The strong defect seen in *syk*$^{-/-}$ neutrophils in these experiments indicates that Syk is intimately involved in outside-in signaling. On the other hand, the fact that *syk*$^{-/-}$ neutrophils are also defective in stimulus-induced adhesion to integrin ligands (Figure 2E), a classical readout of inside-out signaling, suggests that Syk may also contribute to inside-out signaling. However, since this assay depends on cell spreading to achieve firm adhesion, the adhesion assay likely assesses both inside-out and outside-in signaling events. Moreover, interpretation of the adhesion data is complicated by the possibility that outside-in signals from one subset of integrins can trigger an inside-out signal, leading to increased adhesion through another subset of integrins (Disatnik and Rando, 1999; Kiosses et al., 2001). Therefore, our experiments do not formally prove that Syk is involved in inside-out signaling. A more direct measurement will be required to assess inside-out signaling independently of outside-in events in neutrophils.

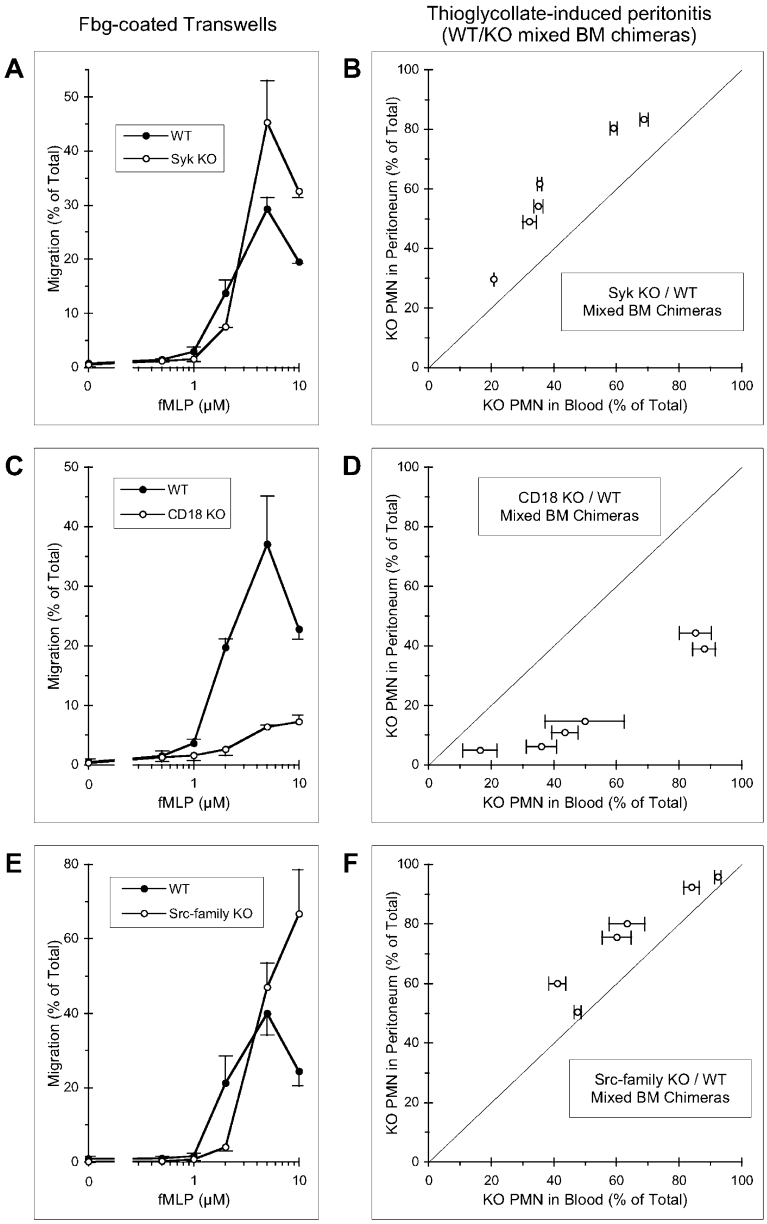

Figure 7. In Vitro and In Vivo Neutrophil Migration

Migration of *syk*$^{-/-}$ ([A] and [B]), *CD18*$^{-/-}$ ([C] and [D]), and Src-family-deficient ([E] and [F]) neutrophils. (A), (C), and (E) show in vitro migration through fibrinogen-coated Transwells; (B), (D), and (F) show the percentage of mutant neutrophils in peripheral blood and peritoneal lavage fluid of wild-type/mutant mixed bone marrow chimeras subjected to a thioglycollate-induced peritonitis. Values for the peritoneum represent the percentage of mutant cells in neutrophils from the peritoneal lavage fluid. Values for blood represent mean and SD of percentages of mutant cells among neutrophils in blood samples taken at 0, 2, and 4 hr time points. See the text for more details. Each data point represents one individual mouse.

In immunoreceptor signaling, Src family kinases phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) within the receptor complex, which then provide a docking site for the tandem SH2 domains of Syk and ZAP-70. The requirement for Src family kinases for the integrin-mediated activation of Syk (Figure 6A) as well as the physical association (Yan et al., 1997; Vines et al., 2001) and colocalization (Figures 5C and 5D) of Syk and CD18 would be consistent with a similar mechanism linking integrin ligation with activation of Syk. However, integrins lack ITAM motifs and, at least in heterologous expression systems, the integrin-mediated activation of Syk seemed to be independent of its tandem SH2-domains (Gao et al., 1997), suggesting that integrins and immunoreceptors use different mechanisms to activate Syk. Further experiments will be required to validate this hypothesis in primary cells such as neutrophils.

Signaling proteins downstream of the integrin-mediated activation of Syk include Cbl, Pyk2, and Vav (Figure 6C). Cbl has been suggested to be a positive regulator of integrin signaling, possibly via recruitment of PI3-kinase molecules to the plasma membrane (Meng and Lowell, 1998). The FAK-related kinase Pyk2 has been implicated in integrating multiple inputs into an effector function possibly similar to that of FAK. Vav is an exchange factor for the small GTP binding protein Rac, and thus it is suspected to mediate shape changes and related functions in response to cell activation. Although we could easily detect integrin-mediated activation of signaling responses both upstream and downstream of Rac (Figure 6), we were unable to directly demonstrate increased GTP loading of Rac itself (see supplemental data at http://www.immunity.com/cgi/content/full/16/4/547/DC1). It is possible that a low level of Rac activation at specific subcellular sites is all that is required to acti-

vate downstream responses. Likewise, an increase in Rac-GTP levels at one subcellular site may be balanced by a relative decrease at other locations, keeping the total pool of intracellular Rac-GTP constant. Alternatively, as is the case with several other small GTPases, the cycling between GDP- and GTP-bound forms might be functionally more important than the level of GTP-loaded Rac itself. Targeted genomic mutations and imaging methods to look for activated Rac in primary neutrophils will be required to sort out the contribution of this pathway to leukocyte integrin signaling.

The contribution of Syk to integrin signaling does not seem to be specific for neutrophils. Syk-deficient platelets show impaired signaling from $\alpha_{IIb}\beta_3$ integrins (Obergfell et al., 2002), and *syk*$^{-/-}$ macrophages also fail to spread on immobilized ICAM-1 (Vines et al., 2001). It is yet to be determined whether this mechanism is also shared with further cell types, including nonhematopoietic cells.

Similar to spreading and other adhesion-dependent responses, *CD18*$^{-/-}$ neutrophils were defective in directed migration both in the in vitro Transwell system and the in vivo thioglycollate peritonitis model (Figures 7C and 7D). There has been some controversy about the requirement for β_2 integrins in neutrophil migration during thioglycollate-induced peritonitis. While most studies concluded that this assay system is strongly dependent on the β_2 integrin LFA-1 (Lu et al., 1997; Schmits et al., 1996; Walzog et al., 1999), one report (Mizgerd et al., 1997) suggested that β_2 integrins are not required for this response. This apparent contradiction may stem from the 5- to 10-fold increase in circulating neutrophil count in *CD18*$^{-/-}$ animals, which may have led Mizgerd et al. to overestimate the peritoneal accumulation of *CD18*$^{-/-}$ neutrophils. Since our mixed bone marrow chimeric approach is intrinsically corrected for any changes in the circulating neutrophil count, the defective migration of *CD18*$^{-/-}$ neutrophils in our hands indicates a cell-autonomous requirement for β_2 integrins in neutrophil migration into the inflamed peritoneum.

Perhaps the most surprising finding in our study is that, unlike other integrin-dependent neutrophil responses, Syk appears not to be required for integrin-mediated neutrophil migration (compare Figures 7A–7D). Furthermore, Src-family deficient neutrophils, which are completely defective in the CD18-dependent adherent activation assays, also migrated normally in both systems tested (Figures 7E and 7F). In preliminary experiments, Syk-deficient neutrophils also demonstrated normal migration in a subcutaneous "air-pouch" model in vivo (data not shown); hence the normal migratory capacity of *syk*$^{-/-}$ neutrophils may not be restricted to specific inflammatory models. Thus, there seem to be different signaling requirements for the different CD18-dependent functions of neutrophils, i.e., adherent activation versus migration. At present, it is unclear whether leukocytes utilize two different integrin-signaling pathways, one leading to spreading, respiratory burst, and degranulation, and the other leading to migration. Alternatively, integrins may play a passive role in migration, i.e., their presence and/or ligand binding capability rather than their signaling capacity may be all that is required to support leukocyte migration. Furthermore, different integrins may have opposing roles in cell migration. If Syk and Src-family kinases were involved in signaling from both promigratory and antimigratory integrins, then the deficiency of these kinases would not have any major effect on the overall migration of the cells.

The inability of Src-family or Syk-deficient neutrophils to become activated by adhesive stimuli predicts that lack of these kinases would result in significantly reduced antimicrobial function and tissue damage during inflammatory responses. While this has been observed in the Src-family mutants (Lowell et al., 1994; Lowell and Berton, 1998; our unpublished results), the defects in multiple hematopoietic lineages in, and the eventual lethality of, the *syk*$^{-/-}$ bone marrow chimeras makes assessing inflammatory processes in Syk-deficient chimeras a challenging task.

Both this paper and the majority of previous reports study the function of Syk in the context of the immune system or of the platelet collagen receptor GPVI, a molecule structurally and mechanistically related to F_C receptors. However, there is growing evidence that Syk can also function in nonimmune cells through mechanisms supposedly unrelated to immunoreceptors. Syk has recently been shown to play a tumor-suppressive role in breast cancer (Coopman et al., 2000). In another report (Tsujimura et al., 2001), Syk was shown to be expressed in the central nervous system, and it was suggested to be involved in neural differentiation of embryonic carcinoma cells. While neither tumor invasion nor neural development is related to immunoreceptor function, integrins play important roles in both these mechanisms. Thus, it is tempting to speculate that the role of Syk in these processes is related to its function in integrin signaling. Furthermore, since a number of integrins are required for normal embryonic development, the possible role of Syk in integrin signaling could also contribute to the lethal phenotype of *syk*$^{-/-}$ mice. Further analysis of the Syk-mediated integrin-signaling pathway can lead to new paradigms of how integrins fulfill their wide spectrum of functional roles both inside and outside the immune system.

Experimental Procedures

Animals and Generation of Bone Marrow Chimeras

The mutation disrupting the *syk* gene (Turner et al., 1995) was maintained in heterozygous mice on C57BL/6 (B6) background (i.e., carrying the CD45.2 allele). Mice and embryos were genotyped by PCR (Figure 1A) using 5′-AGAGAAGCCCTGCCCATGGAC-3′ (*syk*$^{+}$) and 5′-CCTTGGGAAAAGCGCCTCCCCTACCC-3′ (*syk*$^{-}$) as forward primers in combination with the 5′-GTCCAGGTAGACCTCTTTGGGC-3′ reverse primer. The products (86 and 120 bp for *syk*$^{+}$ and *syk*$^{-}$, respectively) were resolved on a 2.5% agarose gel. *syk*$^{-/-}$ and littermate *syk*$^{+/+}$ fetal liver cells were obtained from E15-E17 embryos from timed matings of *syk*$^{+/-}$ carriers. Bone marrow chimeras were generated by intravenous injection of unfractionated fetal liver cells into lethally irradiated congenic recipients carrying the CD45.1 allele on the B6 background (Taconic Farms, Germantown, NY). The complete repopulation of the granulocyte compartment and the absence of the Syk protein in these cells was confirmed by flow cytometry and immunoblotting, respectively (see below). Chimeras were used 4–8 weeks after the bone marrow transplantation.

Mice lacking CD18 (Wilson et al., 1993) (provided by Dr. Arthur L. Beaudet, Baylor College of Medicine, Houston, TX) and triple-mutant mice deficient in the Src-family kinases Hck, Fgr, and Lyn (Meng and Lowell, 1997) were on the B6 background. Control B6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME). All animals were kept in a specific pathogen-free facility at UCSF

and used according to protocols approved by the UCSF Committee on Animal Research.

Isolation of Bone Marrow Neutrophils

Murine bone marrow neutrophils were prepared essentially as described (Mócsai et al., 1999). Purity of the preparation was assessed by flow cytometry using PE-labeled anti-Gr1 (RB6-8C5) and FITC-labeled anti-CD45.2 (clone 104) antibodies (both from Pharmingen, San Diego, CA) on a Beckton-Dickinson (Mountain View, CA) FACScan. Greater than 90% of the cells were mature granulocytes based on forward and side scatter characteristics and $Gr1^{hi}$ staining. The presence of Syk in neutrophil lysates was tested by immunoblotting.

Functional Responses

Functional tests were performed in HBSS supplemented with 20 mM HEPES (pH 7.4). All measurements were done at 37°C.

Coating 96-well tissue culture plates with extracellular matrix proteins or 20 μg/ml poly-RGD peptide (Sigma, St. Louis, MO) was performed as described (Lowell et al., 1996). Purified murine ICAM-1 (Pyszniak et al., 1994) (provided by Dr. Fumio Takei, University of British Columbia, Vancouver, BC and Dr. Charlotte M. Vines, University of New Mexico, Albuquerque, NM) was immobilized to 96-well Immulon 4 (Dynex, Chantilly, VA) plates at 1 μg/ml, followed by blocking with 10% FCS. For antibody-mediated crosslinking experiments, monoclonal antibodies against murine integrins or isotype-matched control antibodies were immobilized on 96-well Immulon 4 plates. The rat IgG2a-κ antibodies 121/7 (anti-CD11a), C71/16 (anti-CD18), 9EG7 (anti-CD29), and R35-95 (isotype control), and the rat IgG2b-κ antibodies R1-2 (anti-CD49d) and R35-38 (isotype control) were purchased unlabeled and bound directly to the plates at 20 μg/ml in carbonate buffer. The rat IgG2b-κ antibodies M1/70 (anti-CD11b) and A95-1 (isotype control) and the hamster IgG1-κ antibodies 2C9.G2 (anti-CD61) and A19-3 (isotype control) were purchased biotinylated and bound to streptavidin-precoated plates (Lowell et al., 1996) at 2 μg/ml. Except for the 121/7 antibody (from Endogen, Woburn, MA), all antibodies were from Pharmingen. Antibody-coated plates were blocked with 10% FCS. Except for the anti-CD29 antibody 9EG7, cell stimulation by immobilized antibodies was performed in the absence of Mg^{2+} salts.

Adherent respiratory burst was measured by a cytochrome c reduction test essentially as described previously (Lowell et al., 1996). Where appropriate, 20 ng/ml murine TNF or 100 ng/ml murine MIP-2 (both from Peprotech, Rocky Hill, NJ) or 3 μM fMLP, 10 μg/ml LPS (from *E. coli* 0127:B8), or 100 nM PMA (all from Sigma) were used as stimulus.

Adherent degranulation of the secondary granule marker lactoferrin was determined by ELISA from supernatants of cells stimulated for 45 min, otherwise as described previously (Mócsai et al., 1999). Spreading of cells was assessed by phase contrast microscopy. Cell adhesion was determined by measuring the plate-bound acid phosphatase activity (Lowell et al., 1996) after washing the wells three times with ice-cold PBS supplemented with Ca^{2+} and Mg^{2+} salts, using a Labsystems (Helsinki, Finland) electronic multichannel Finnpipette at low speed setting.

Fluorescent Microscopy

Neutrophils were stimulated in 8-well glass chamber slides (Nalge Nunc, Rochester, NY) precoated with poly-RGD as described above. Cells were fixed with 2% paraformaldehyde, permeabilized by 0.2% Triton, and stained with a polyclonal anti-Syk antibody (N-19 from Santa Cruz Biotechnology, Santa Cruz, CA) followed by a goat anti-rabbit $F_{(ab')2}$ antibody conjugated to Alexa Fluor 594 (Molecular Probes, Eugene, OR) and a FITC-labeled anti-CD18 antibody (C71/16). For staining controls, a blocking peptide of the anti-Syk antibody (Santa Cruz) was added or the anti-CD18 antibody was replaced by a FITC-labeled isotype control antibody (R35-95). Samples were mounted in ProLong antifade reagent (Molecular Probes). High-resolution digital microscopy was performed on a Deltavision Restoration Microscopy System (Applied Precision, Issaquah, WA).

Expression of Cell Surface Integrins and of L-Selectin

Expression of cell surface integrins and of L-selectin was determined by flow cytometry. Cells were incubated with biotinylated anti-integrin and isotype control antibodies (same as the ones used for plate coating, except for the rat IgG2a-κ anti-CD11a antibody M17/4 [Pharmingen]) followed by labeling with FITC-conjugated streptavidin (Pharmingen). The rat IgG2a-κ antibodies MEL-14 (anti-L-selectin) and RM4-5 (isotype control; anti-CD4) were directly conjugated with PE and thus used in a single-step labeling procedure (both antibodies from Pharmingen). To assess changes in expression levels upon stimulation, cells were incubated with 20 ng/ml TNF for 30 min prior to antibody labeling.

Immunoprecipitation, Western Blotting, and Kinase Assay

For biochemical studies, cells were incubated in suspension or in fibrinogen- or poly-RGD-coated 6 cm tissue culture dishes for 20 min. All cells (i.e., after pooling adherent and nonadherent cells in experiments in adhesion) were then lysed with a Triton-based lysis buffer (Mócsai et al., 1997) supplemented with 0.1% SDS and 0.5% sodium deoxycholate (RIPA). After removal of insoluble material, lysates were either boiled with sample buffer or processed for immunoprecipitation. To test redistribution of actin and α-actinin, the Triton-soluble fraction was obtained by lysing cells with a cytoskeleton stabilizing buffer (Yan et al., 1997). The Triton-insoluble material was then solubilized in RIPA.

For immunoprecipitation studies, RIPA lysates were precleared with protein A Sepharose (Zymed, South San Francisco, CA) and incubated with rabbit anti-Syk (N-19), anti-Cbl (C-15), or anti-Vav (C-14), or goat anti-Pyk2 (N-19) antibodies (all from Santa Cruz). Immune complexes were captured by protein A Sepharose, washed three times, and boiled in sample buffer. In experiments determining tyrosine phosphorylation of Syk in adherent cells, samples were obtained by combining cells from four identical plates, but nonadherent cells were washed away before lysing the adherent cells.

Cell lysates and immunoprecipitates were run on SDS-PAGE and immunoblotted using antibodies against phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY), p38 MAP kinase (C-20; Santa Cruz), phospho-p38 MAP kinase (Cell Signaling Technology, Beverly, MA), actin (C-11; Santa Cruz), α-actinin (AT6/172; Upstate Biotechnology), or the precipitating antibodies followed by peroxidase-labeled secondary antibodies (Amersham, Little Chalfont, UK, or Jackson Immunoresearch, West Grove, PA). The signal was developed using Amersham's ECL system.

The activity of MAPKAPK2 was assessed by an immuncomplex kinase assay from RIPA lysates using agarose-conjugated anti-MAPKAPK2 antibodies for immunoprecipitation and recombinant human Hsp27 as kinase substrate (both reagents from Upstate Biotechnology), otherwise as described in Mócsai et al. (2000).

Rac/Cdc42 activation was tested by a GST-PBD pull-down assay (Benard et al., 1999) (see supplemental data at http://www.immunity.com/cgi/content/full/16/4/547/DC1).

In Vitro Migration

Transwell inserts (3 μm polycarbonate membrane; Corning, Acton, MA) were precoated with fibrinogen and filled with neutrophil suspensions. The inserts were placed into media containing indicated concentrations of fMLP in 24-well tissue culture plates. After 45 min, the plates were spun, the inserts were removed, and the number of neutrophils in the bottom of the wells was determined by an acid phosphatase assay (Lowell et al., 1996). Parallel samples were included to determine the signal intensity from the total cell number loaded into the Transwell inserts. Similar results were obtained when using a Neuro Probe (Gaithersburg, MD) 96-well chemotaxis chamber, which also allowed us to follow transmigrated cells still adherent to the bottom of the porous membrane (data not shown).

Thioglycollate-Induced Peritonitis

For these experiments, unfractionated bone marrow from a $syk^{-/-}$ bone marrow chimera or from intact $CD18^{-/-}$ or $hck^{-/-}fgr^{-/-}lyn^{-/-}$ mice were mixed with the bone marrow from a congenic CD45.1 mouse (as wild-type) at ratios of 1:3, 1:1, or 3:1 (25%, 50%, or 75% mutant bone marrow, respectively) and injected into lethally irradiated recipients as described above. Neutrophils from the different genotypes could be distinguished based on the expression of the CD45.1 epitope on wild-type and CD45.2 on the mutant cells.

Sterile peritonitis was induced by i.p. injection of the mixed bone

marrow chimeras with 1 ml of 3% thioglycollate broth (UCSF Cell Culture Facility). Blood samples were taken immediately before, as well as 2 and 4 hr after, the thioglycollate injection. Mice were sacrificed at 4 hr, and the peritoneum was washed with ice-cold PBS supplemented with 2% FCS. Blood and peritoneal lavage samples were then stained with PE-labeled anti-Gr1 and FITC-labeled anti-CD45.2 antibodies and analyzed by flow cytometry. Neutrophils were selected based on their forward- and side-scatter characteristics and $Gr1^{hi}$ staining, and the percentage of $CD45.2^{+}$ (mutant) cells within this gate was determined. In preliminary experiments with B6 mice, the number of peritoneal neutrophils ($Gr1^{hi}$ $CD45.2^{+}$ cells) dramatically increased during this assay from $2.0 \pm 1.1 \times 10^4$ to $9.2 \pm 4.0 \times 10^6$, while the number of nongranulocytic ($Gr1^{-}$ $CD45.2^{+}$) leukocytes increased only modestly from $1.9 \pm 0.3 \times 10^6$ to $2.9 \pm 1.0 \times 10^6$.

Presentation of Results

Quantitative data are presented as mean ± SD of triplicate measurements; in flow cytometry at least 10,000 individual events were counted. Unless otherwise stated, all results are representative of three or more independent experiments.

Acknowledgments

We thank Giorgio Berton for in part inspiring this project; Art Beaudet for the *CD18*$^{-/-}$ mice; Fumio Takei and Charlotte Vines for the murine ICAM-1; Gary Bokoch for reagents for Rac activation assays; Jeff Critchfield for help with initial bone marrow transfers; Hong Yu and Fang Liu for assistance with managing our mouse colony; Tony DeFranco and Henry Bourne for access to the Deltavision microscope; Neetu Gupta, Paul Herzmark, and Hiroshi Morisaki for help with microscopy; Niels Borregaard for suggestions about degranulation experiments; and Rick Brown, Hattie Gresham, Shalini Pereira, and Hua Chen for valuable suggestions. This work was supported by grants from the National Institutes of Health (HL-54476 and DK-58066) to C.A.L.

Received: May 25, 2001
Revised: February 22, 2002

References

Benard, V., Bohl, B.P., and Bokoch, G.M. (1999). Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. J. Biol. Chem. *274*, 13198–13204.

Cheng, A.M., Rowley, B., Pao, W., Hayday, A., Bolen, J.B., and Pawson, T. (1995). Syk tyrosine kinase required for mouse viability and B-cell development. Nature *378*, 303–306.

Chu, D.H., Morita, C.T., and Weiss, A. (1998). The Syk family of protein tyrosine kinases in T-cell activation and development. Immunol. Rev. *165*, 167–180.

Clark, E.A., Shattil, S.J., Ginsberg, M.H., Bolen, J., and Brugge, J.S. (1994). Regulation of the protein tyrosine kinase $pp72^{syk}$ by platelet agonists and the integrin $\alpha_{IIb}\beta_3$. J. Biol. Chem. *269*, 28859–28864.

Colucci, F., Guy-Grand, D., Wilson, A., Turner, M., Schweighoffer, E., Tybulewicz, V.L., and Di Santo, J.P. (2000). A new look at Syk in $\alpha\beta$ and $\gamma\delta$ T cell development using chimeric mice with a low competitive hematopoietic environment. J. Immunol. *164*, 5140–5145.

Coopman, P.J., Do, M.T., Barth, M., Bowden, E.T., Hayes, A.J., Basyuk, E., Blancato, J.K., Vezza, P.R., McLeskey, S.W., Mangeat, P.H., and Mueller, S.C. (2000). The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. Nature *406*, 742–747.

Costello, P.S., Turner, M., Walters, A.E., Cunningham, C.N., Bauer, P.H., Downward, J., and Tybulewicz, V.L. (1996). Critical role for the tyrosine kinase Syk in signalling through the high affinity IgE receptor of mast cells. Oncogene *13*, 2595–2605.

Crowley, M.T., Costello, P.S., Fitzer-Attas, C.J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V.L., and DeFranco, A.L. (1997). A critical role for Syk in signal transduction and phagocytosis mediated by $F_c\gamma$ receptors on macrophages. J. Exp. Med. *186*, 1027–1039.

Detmers, P.A., Zhou, D., Polizzi, E., Thieringer, R., Hanlon, W.A., Vaidya, S., and Bansal, V. (1998). Role of stress-activated mitogen-activated protein kinase (p38) in β_2-integrin-dependent neutrophil adhesion and the adhesion-dependent oxidative burst. J. Immunol. *161*, 1921–1929.

Disatnik, M.H., and Rando, T.A. (1999). Integrin-mediated muscle cell spreading. The role of protein kinase C in outside-in and inside-out signaling and evidence of integrin cross- talk. J. Biol. Chem. *274*, 32486–32492.

Fernandez, R., and Suchard, S.J. (1998). Syk activation is required for spreading and H_2O_2 release in adherent human neutrophils. J. Immunol. *160*, 5154–5162.

Gao, J., Zoller, K.E., Ginsberg, M.H., Brugge, J.S., and Shattil, S.J. (1997). Regulation of the $pp72^{syk}$ protein tyrosine kinase by platelet integrin $\alpha_{IIb}\beta_3$. EMBO J. *16*, 6414–6425.

Kiefer, F., Brumell, J., Al-Alawi, N., Latour, S., Cheng, A., Veillette, A., Grinstein, S., and Pawson, T. (1998). The Syk protein tyrosine kinase is essential for $F_c\gamma$ receptor signaling in macrophages and neutrophils. Mol. Cell. Biol. *18*, 4209–4220.

Kiosses, W.B., Shattil, S.J., Pampori, N., and Schwartz, M.A. (2001). Rac recruits high-affinity integrin $\alpha_v\beta_3$ to lamellipodia in endothelial cell migration. Nat. Cell Biol. *3*, 316–320.

Law, D.A., Nannizzi-Alaimo, L., Ministri, K., Hughes, P.E., Forsyth, J., Turner, M., Shattil, S.J., Ginsberg, M.H., Tybulewicz, V.L., and Phillips, D.R. (1999). Genetic and pharmacological analyses of Syk function in $\alpha_{IIb}\beta_3$ signaling in platelets. Blood *93*, 2645–2652.

Lin, T.H., Rosales, C., Mondal, K., Bolen, J.B., Haskill, S., and Juliano, R.L. (1995). Integrin-mediated tyrosine phosphorylation and cytokine message induction in monocytic cells. A possible signaling role for the Syk tyrosine kinase. J. Biol. Chem. *270*, 16189–16197.

Lowell, C.A., and Berton, G. (1998). Resistance to endotoxic shock and reduced neutrophil migration in mice deficient for the Src-family kinases Hck and Fgr. Proc. Natl. Acad. Sci. USA *95*, 7580–7584.

Lowell, C.A., and Berton, G. (1999). Integrin signal transduction in myeloid leukocytes. J. Leukoc. Biol. *65*, 313–320.

Lowell, C.A., Soriano, P., and Varmus, H.E. (1994). Functional overlap in the *src* gene family: inactivation of *hck* and *fgr* impairs natural immunity. Genes Dev. *8*, 387–398.

Lowell, C.A., Fumagalli, L., and Berton, G. (1996). Deficiency of Src family kinases $p59/61^{hck}$ and $p58^{c\text{-}fgr}$ results in defective adhesion-dependent neutrophil functions. J. Cell Biol. *133*, 895–910.

Lu, H., Smith, C.W., Perrard, J., Bullard, D., Tang, L., Shappell, S.B., Entman, M.L., Beaudet, A.L., and Ballantyne, C.M. (1997). LFA-1 is sufficient in mediating neutrophil emigration in Mac-1-deficient mice. J. Clin. Invest. *99*, 1340–1350.

Meng, F., and Lowell, C.A. (1997). Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. J. Exp. Med. *185*, 1661–1670.

Meng, F., and Lowell, C.A. (1998). A β_1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. EMBO J. *17*, 4391–4403.

Miura, K., Lavens-Phillips, S., and MacGlashan, D.W., Jr. (2001). Piceatannol is an effective inhibitor of IgE-mediated secretion from human basophils but is neither selective for this receptor nor acts on syk kinase at concentrations where mediator release inhibition occurs. Clin. Exp. Allergy *31*, 1732–1739.

Mizgerd, J.P., Kubo, H., Kutkoski, G.J., Bhagwan, S.D., Scharffetter-Kochanek, K., Beaudet, A.L., and Doerschuk, C.M. (1997). Neutrophil emigration in the skin, lungs, and peritoneum: different requirements for CD11/CD18 revealed by CD18-deficient mice. J. Exp. Med. *186*, 1357–1364.

Mócsai, A., Bánfi, B., Kapus, A., Farkas, G., Geiszt, M., Buday, L., Faragó, A., and Ligeti, E. (1997). Differential effects of tyrosine kinase inhibitors and an inhibitor of the mitogen-activated protein kinase cascade on degranulation and superoxide production of human neutrophil granulocytes. Biochem. Pharmacol. *54*, 781–789.

Mócsai, A., Ligeti, E., Lowell, C.A., and Berton, G. (1999). Adhesion-

dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. J. Immunol. *162*, 1120–1126.

Mócsai, A., Jakus, Z., Vántus, T., Berton, G., Lowell, C.A., and Ligeti, E. (2000). Kinase pathways in chemoattractant-induced degranulation of neutrophils: the role of p38 mitogen-activated protein kinase activated by Src family kinases. J. Immunol. *164*, 4321–4331.

Nathan, C.F. (1987). Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. J. Clin. Invest. *80*, 1550–1560.

Nathan, C., Srimal, S., Farber, C., Sanchez, E., Kabbash, L., Asch, A., Gailit, J., and Wright, S.D. (1989). Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. J. Cell Biol. *109*, 1341–1349.

Obergfell, A., Eto, K., Mócsai, A., Buensuceso, C., Moores, S.L., Brugge, J.S., Lowell, C.A., and Shattil, S.J. (2002). Coordinate interactions of Csk, Src and Syk kinases with $\alpha_{IIb}\beta_3$ initiate integrin signaling to the cytoskeleton. J. Cell Biol., in press.

Pereira, S., Zhou, M., Mócsai, A., and Lowell, C. (2001). Resting murine neutrophils express functional α_4 integrins that signal through Src family kinases. J. Immunol. *166*, 4115–4123.

Pyszniak, A.M., Welder, C.A., and Takei, F. (1994). Cell surface distribution of high-avidity LFA-1 detected by soluble ICAM-1-coated microspheres. J. Immunol. *152*, 5241–5249.

Richter, J., Ng-Sikorski, J., Olsson, I., and Andersson, T. (1990). Tumor necrosis factor-induced degranulation in adherent human neutrophils is dependent on CD11b/CD18-integrin-triggered oscillations of cytosolic free Ca^{2+}. Proc. Natl. Acad. Sci. USA *87*, 9472–9476.

Schmits, R., Kundig, T.M., Baker, D.M., Shumaker, G., Simard, J.J., Duncan, G., Wakeham, A., Shahinian, A., van der Heiden, A., Bachmann, M.F., et al. (1996). LFA-1-deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. J. Exp. Med. *183*, 1415–1426.

Tsujimura, T., Yanagi, S., Inatome, R., Takano, T., Ishihara, I., Mitsui, N., Takahashi, S., and Yamamura, H. (2001). Syk protein-tyrosine kinase is involved in neuron-like differentiation of embryonal carcinoma P19 cells. FEBS Lett. *489*, 129–133.

Turner, M., Mee, P.J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L., and Tybulewicz, V.L. (1995). Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature *378*, 298–302.

Turner, M., Schweighoffer, E., Colucci, F., Di Santo, J.P., and Tybulewicz, V.L. (2000). Tyrosine kinase SYK: essential functions for immunoreceptor signalling. Immunol. Today *21*, 148–154.

Vines, C.M., Potter, J.W., Xu, Y., Geahlen, R.L., Costello, P.S., Tybulewicz, V.L., Lowell, C.A., Chang, P.W., Gresham, H.D., and Willman, C.L. (2001). Inhibition of β_2 integrin receptor and Syk kinase signaling in monocytes by the Src family kinase Fgr. Immunity *15*, 507–519.

Walzog, B., Scharffetter-Kochanek, K., and Gaehtgens, P. (1999). Impairment of neutrophil emigration in CD18-null mice. Am. J. Physiol. *276*, G1125–1130.

Wilson, R.W., Ballantyne, C.M., Smith, C.W., Montgomery, C., Bradley, A., O'Brien, W.E., and Beaudet, A.L. (1993). Gene targeting yields a CD18-mutant mouse for study of inflammation. J. Immunol. *151*, 1571–1578.

Yan, S.R., Huang, M., and Berton, G. (1997). Signaling by adhesion in human neutrophils: activation of the $p72^{syk}$ tyrosine kinase and formation of protein complexes containing $p72^{syk}$ and Src family kinases in neutrophils spreading over fibrinogen. J. Immunol. *158*, 1902–1910.