PSGL-1 engagement by E-selectin signals through Src kinase Fgr and ITAM adapters DAP12 and FcRγ to induce slow leukocyte rolling

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E-selectin binding to P-selectin glycoprotein ligand-1 (PSGL-1) can activate the β_2 integrin lymphocyte function–associated antigen-1 by signaling through spleen tyrosine kinase (Syk). This signaling is independent of $G\alpha_i$–protein–coupled receptors, results in slow rolling, and promotes neutrophil recruitment to sites of inflammation. However, the signaling pathways linking E-selectin engagement of PSGL-1 to Syk activation are unknown. To test the role of Src family kinases and immunoreceptor tyrosine-based activating motif (ITAM)–containing adaptor proteins, we used different gene-deficient mice in flow chamber, intravital microscopy, and peritonitis studies. E-selectin–mediated phosphorylation of Syk and slow rolling was abolished in neutrophils from *fgr*$^{-/-}$ or *hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$ mice. Neutrophils from *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice lacking both DAP12 and FcRγ were incapable of sustaining slow neutrophil rolling on E-selectin and intercellular adhesion molecule-1 and were unable to phosphorylate Syk and p38 MAPK. This defect was confirmed in vivo by using mixed chimeric mice. $G\alpha_i$-independent neutrophil recruitment into the inflamed peritoneal cavity was sharply suppressed in *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice. Our data demonstrate that an ITAM-dependent pathway involving the Src-family kinase Fgr and the ITAM-containing adaptor proteins DAP12 and FcRγ is involved in the initial signaling events downstream of PSGL-1 that are required to initiate neutrophil slow rolling.

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Abbreviations used: GST, glutathione S-transferase; ICAM-1, intercellular adhesion molecule-1; ITAM, immunoreceptor tyrosine-based activating motif; PSGL-1, P-selectin glycoprotein ligand-1; Syk, spleen tyrosine kinase; TREM, triggering receptor expressed on myeloid cells.

The first three steps of the neutrophil adhesion cascade—capture, rolling, and slow rolling—require selectins and their counter receptors (1–3). These steps are mediated by endothelial P- and E-selectin, which both bind to P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils. In venules of the cremaster muscle, the rolling velocity of leukocytes on P-selectin is 40 μm/s (4), whereas under inflammatory conditions (e.g., when endothelium is activated by TNF-α), E-selectin mediates slower rolling (3–7 μm/s) (5, 6). Recently, it has been shown that P-selectin binding to PSGL-1 can induce Nef-associated factor 1–dependent downstream signaling (7). Disruption of this signal transduction pathway leads to reduced leukocyte adhesion in vivo and reduced leukocyte recruitment into the peritoneal cavity (7). E-selectin engagement of PSGL-1 can also induce downstream signaling, inducing tyrosine phosphorylation of p38 MAP kinase (8, 9), spleen tyrosine kinase (Syk) phosphorylation, and slow rolling (6). Antibody cross-linking of PSGL-1 has been shown to induce association of its cytoplasmic tail with ezrin and moesin and activation of Syk and transcription factors (10).

Neutrophils express the β_2 integrins LFA-1 ($\alpha_L\beta_2$) and macrophage antigen (Mac)-1 ($\alpha_M\beta_2$), which regulate the rolling velocity of leukocytes

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in inflamed microvessels (11). Neutrophils perfused through flow chambers coated with E-selectin plus intercellular adhesion molecule-1 (ICAM-1) roll much more slowly than on E-selectin alone, and this slow rolling is LFA-1 dependent (6). LFA-1 can undergo partial activation to an intermediate affinity state or full activation to a high affinity state (12). Allosteric inhibitors that stabilize LFA-1 in the extended conformation associated with intermediate affinity (12, 13) still allow LFA-1–dependent slow rolling on E-selectin plus ICAM-1, but block chemokine-triggered firm adhesion (6). The physiological behavior of LFA-1 in the extended conformation is similar to that of its isolated I domain, which also supports rolling (14).

Stimulation of immunoreceptors (TCR, B cell receptor, and FcR) (15), growth factor receptors (16), cytokine receptors (17), G-protein–linked receptors (18), and integrins (19) can activate Src-family kinases. Binding of P-selectin to PSGL-1 on isolated human neutrophils cells can also activate Src-family kinases (7). These Src tyrosine kinases share a high degree of structural homology and possess three major domains: an Src homology 3 (SH3) domain, an SH2 domain, and the tyrosine kinase (SH1) domain (20). The Src-family kinases Lyn, Hck, and Fgr are expressed in neutrophils and are involved in several signaling pathways by promoting phosphorylation of downstream effectors (19, 20).

The cytoplasmic tyrosine kinase Syk is expressed in hematopoietic cells and contains tandem SH2 domains and a C-terminal kinase domain (21). Syk is absolutely required in myeloid cells and platelets for downstream signaling after activation of receptors involved in innate immunity, such as Fc receptors and platelet glycoprotein (GP) VI (22). The related kinase Zap-70 is required for adaptive immune responses triggered by B cell and T cell receptors (22). After engagement of the respective cell surface receptors, Syk is activated by docking to phosphorylated tyrosine residues within immunoreceptor tyrosine-based activation motifs (ITAMs) that contain the characteristic amino acid sequence $YxxL/Ix_{6-8}YxxL/I$, where x represents any amino acid. Neutrophils express several ITAM-containing adaptor molecules, including ezrin, moesin, radixin, DAP12, and FcRγ (23). Ezrin and moesin have previously been shown to directly interact with the cytoplasmic tail of PSGL-1 and act as linkers between Syk and PSGL-1 cross-linked by antibodies in the human promyeloid cell line HL-60 (10). After stimulation of human neutrophils with chemoattractants, moesin and PSGL-1 are colocalized in the uropod of migrating cells (24).

DAP12 and FcRγ have short extracellular domains of 21 and 5 amino acids, respectively, and no specific extracellular binding ligands are known. Cysteine residues in the extracellular regions of these adaptor proteins allow their expression as disulfide-linked homodimers (25). A charged aspartic acid residue present in the transmembrane domain of DAP12 and FcRγ mediates their association with ligand-binding receptors such as FcγRIII, FcεR, and GP VI. The association of DAP12 or FcRγ with specific ligand binding receptors is required for initiation of intracellular signaling.

DAP12 is expressed in neutrophils, mast cells, natural killer cells, monocytes, and dendritic cells (26). Triggering receptor expressed on myeloid cells (TREM)-1, TREM-2, myeloid DAP12-associating lectin-1, and signal-regulatory protein β are surface receptors associated with DAP12 in myeloid cells (27, 28). Engagement of the DAP12-associated receptor TREM-1 on neutrophils and monocytes leads to cell activation and subsequent secretion of proinflammatory cytokines and chemokines (29).

The γ chain (FcRγ) of immunoglobulin Fc receptors is an essential component of the high-affinity receptor for IgE (FcεRI), expressed by mast cells and basophils, the low-affinity receptor for IgG (FcγRIII, CD16), and is associated with the high-affinity receptor for IgG (FcγRI, CD64) and the TCR (30). FcRγ is essential for receptor assembly and provides the ITAM domain necessary for signal transduction. Elimination of FcRγ results in immunocompromised mice showing reduced phagocytosis, defects in macrophage and NK cell–mediated cytotoxicity, osteopetrosis, and defective mast cell–mediated allergic responses (31).

Engagement of β_2 integrins by ligands leads to outside-in signaling that promotes leukocyte adhesion, degranulation, and proliferation (21). A recent study showed that DAP12 and FcRγ are involved in outside-in signaling through β_2 integrins in neutrophils (32). In this signaling pathway, DAP12 and FcRγ have a partially redundant function, and elimination of both molecules is necessary to abolish signaling transduction and superoxide production after integrin engagement (32).

It is well established that Src-family kinases and ITAM-containing adaptor proteins are involved in Syk activation during integrin and immunoreceptor signaling. The present study was designed to test whether Src-family kinases and ITAM-containing adaptor proteins DAP12 and FcRγ are also required for Syk activation and slow rolling of neutrophils after E-selectin engagement of PSGL-1. Using in vitro phosphorylation assays in neutrophils, ex vivo flow chamber assays, and in vivo inflammation experiments with several gene-targeted mouse strains, we find that the Src-family kinase Fgr is involved with DAP12 and FcRγ in initiating the signaling events downstream of PSGL-1 that are required for activation of neutrophil slow rolling.

RESULTS

Src family kinases are involved in E-selectin–mediated rolling

Binding of P-selectin to PSGL-1 induces Src kinase–dependent phosphorylation of downstream molecules (7). To test whether Src kinases are involved in E-selectin–mediated slow rolling, we investigated the rolling velocity of neutrophils from mice pretreated with the Src-family kinase inhibitor (PP2) or an inactive control (PP3) in an autoperfused flow chamber. In this system, neutrophils can be investigated in native whole blood, without any isolation procedure that might activate the cells and alter their ability to regulate rolling velocity. As previously shown (6), the rolling velocity of WT neutrophils rolling in autoperfused flow chambers coated with

E-selectin plus ICAM-1 is significantly reduced compared with E-selectin alone (6). Pretreatment of mice with PP2 did not influence the rolling velocity of neutrophils on E-selectin, but the decrease of rolling velocity on E-selectin plus ICAM-1 was almost completely abolished after Src-family kinase blockade (Fig. 1 A).

To confirm our flow chamber data in vivo, we conducted intravital microscopy in mixed chimeric mice. *hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$ triple knockout mice lack all three neutrophil Src-family kinases (33). Lethally irradiated WT mice received bone marrow cells from WT LysM-GFP^{+} mice (34) and GFP-negative *hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$ mice mixed in a ratio of 1:1. Leukocyte rolling was analyzed in TNF-α–treated venules of the cremaster muscle after blocking P-selectin (to limit our observations to E-selectin) and $G\alpha_i$-signaling (to block chemokine signaling). In this system, gene-targeted leukocytes can be compared side-by-side with leukocytes from WT mice under the same hemodynamic conditions in the same venules. The measured mean blood flow velocity and the calculated wall shear rates in these venules were 3.2 ± 0.2 mm/s and 2,000 ± 200 s^{-1}, respectively. Under these conditions, mean rolling velocity (V_{avg}) of *hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$ leukocytes in vivo was 7.3 ± 0.3 μm/s (Fig. 1 B), almost twice that of LysM-GFP^{+} control cells (V_{avg} = 4.4 ± 0.1 μm/s; $P < 0.05$). The high rolling velocity seen in *hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$ leukocytes is equal to the rolling velocity of leukocytes seen in WT mice after antibody blockade of both β_2 integrins, Mac-1 and LFA-1 (6). These findings suggest that Src-family kinases participate in β_2 integrin activation and subsequent slow rolling after E-selectin engagement.

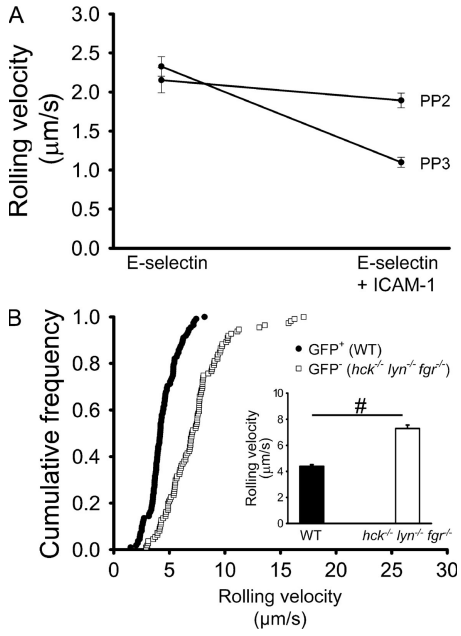

Figure 1. Src-family kinases are involved in slow rolling after E-selectin engagement. (A) Rolling velocities of neutrophils from WT mice treated with a specific Src-family kinase inhibitor (PP2) or inactive control (PP3) in E-selectin and E-selectin/ICAM-1–coated autoperfused flow chamber experiments ($n = 3$). The wall shear stress in all flow chamber experiments was 5–6 dynes/cm^2. (B) Mixed chimeric mice were generated by injecting bone marrow cells from LysM-GFP^{+} WT mice and *hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$ mice into lethally irradiated WT mice. Rolling velocities of 111 GFP^{+} (WT, filled circle) and 111 GFP^{-} (*hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$, open square) leukocytes in inflamed cremaster muscle venules of mixed chimeric mice ($n = 4$) treated with PTx and a monoclonal blocking antibody against P-selectin presented as cumulative histogram. (inset) Mean ± SEM. #, $P < 0.05$.

Elimination of the Src family kinase Fgr abolishes E-selectin–mediated slow rolling

It is known that the Src-family kinases have a considerable redundancy and that elimination of more than one kinase is often necessary to impair downstream signaling and subsequent biological activity (19, 20). To test whether such a redundancy exists in the signaling pathways triggered by PSGL-1 engagement with E-selectin, we investigated the rolling velocity of WT, *hck*$^{-/-}$, *lyn*$^{-/-}$, *fgr*$^{-/-}$, and *hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$ neutrophils using the autoperfused flow chamber

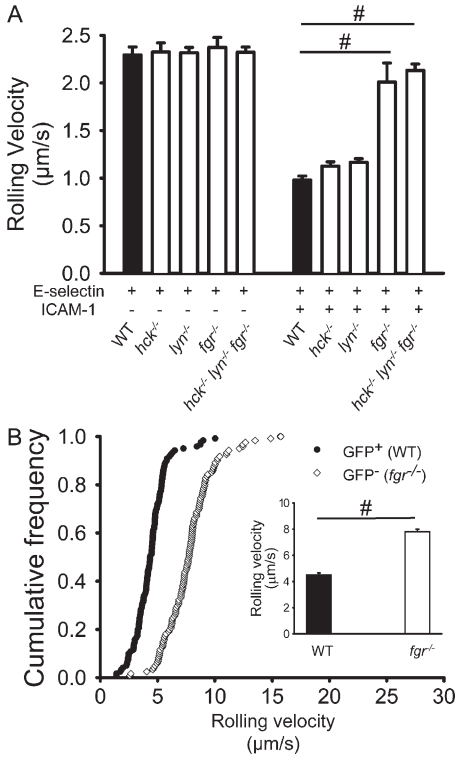

Figure 2. Fgr is required for E-selectin–mediated slow rolling. (A) Carotid cannulas were placed in WT mice ($n = 3$), *hck*$^{-/-}$ mice ($n = 3$), *lyn*$^{-/-}$ mice ($n = 3$), *fgr*$^{-/-}$ mice ($n = 3$), and *hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$ mice ($n = 3$) and connected to autoperfused flow chambers. The wall shear stress in all flow chamber experiments was 5–6 dynes/cm^2. Mean rolling velocity of neutrophils on E-selectin (left) and E-selectin/ICAM-1 (right) presented as mean ± SEM (B) Mixed chimeric mice were generated by injecting bone marrow cells from LysM-GFP^{+} WT mice and *fgr*$^{-/-}$ mice into lethally irradiated WT mice. Cumulative histogram of rolling velocity of 121 GFP^{+} (WT, filled circle) and 121 GFP^{-} (*fgr*$^{-/-}$, open diamond) leukocytes in inflamed cremaster muscle venules of mixed chimeric mice ($n = 4$) treated with PTx and a monoclonal blocking P-selectin antibody. (inset) Mean ± SEM. #, $P < 0.05$.

described int the previous section. The rolling velocity of neutrophils from WT mice and gene-deficient mice on E-selectin alone was similar (Fig. 2 A). As expected, neutrophils from WT mice showed a reduction of the rolling velocity on E-selectin plus ICAM-1 (Fig. 2 A). However, *fgr*$^{-/-}$ neutrophils failed to reduce their rolling velocity on E-selectin plus ICAM-1 compared with WT neutrophils (Fig. 2 A). This rolling velocity was not further increased in *hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$ triple knockout mice, and *hck*$^{-/-}$ or *lyn*$^{-/-}$ mice showed no rolling defect.

Mixed chimeric mice, generated with bone marrow from LysM-GFP^{+} mice and *fgr*$^{-/-}$ mice, showed that the rolling velocity of *fgr*$^{-/-}$ leukocytes was significantly increased (7.8 ± 0.2 μm/s, Fig. 2 B) compared with leukocytes from WT mice (4.5 ± 0.2 μm/s, Fig. 2 B). Collectively, these data show that Fgr, but not Hck or Lyn, is required for slow rolling triggered by PSGL-1 engagement.

DAP12 and FcRγ are involved in E-selectin–induced slow rolling

To address whether ITAM-containing adaptor proteins are involved in E-selectin–dependent rolling, we investigated the rolling velocity of neutrophils from DAP12-deficient mice (*Tyrobp*$^{-/-}$), FcRγ-deficient mice (*Fcrg*$^{-/-}$), double mutant mice (*Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$), and WT mice using the whole-blood autoperfused flow chamber. Elimination of either FcRγ or DAP12 alone slightly increased rolling velocity on E-selectin plus ICAM-1 compared with neutrophils from WT mice (Fig. 3 A). The rolling velocity on E-selectin plus ICAM-1 of neutrophils lacking both adaptor molecules was significantly increased compared with neutrophils from WT mice, and was similar to the rolling velocity on E-selectin alone (Fig. 3 A). These data suggest that the two ITAM-containing adaptor proteins, DAP12 and FcRγ, have a partially redundant function. When both are eliminated, E-selectin–dependent slow rolling is abolished.

To investigate the role of the ITAM-containing adaptor proteins DAP12 and FcRγ in E-selectin–dependent slow rolling in vivo, we generated mixed chimeric mice by injecting bone marrow cells from LysM-GFP^{+} mice and *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice into lethally irradiated WT mice and performed intravital microscopy. As before, we blocked P-selectin and $G\alpha_i$-coupled signaling to more clearly address the E-selectin pathway of neutrophil activation. In TNF-α–induced inflamed postcapillary venules, the rolling velocity of *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ leukocytes was 8.4 ± 0.4 μm/s (Fig. 3 B and Video 1, available at http://www.jem.org/cgi/content/full/jem.20072660/DC1), which is significantly higher than LysM-GFP^{+} control cells (4.3 ± 0.2 μm/s; $P < 0.05$). These findings show that β_2 integrin activation after E-selectin engagement is defective in *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ leukocytes.

$G\alpha_i$-independent neutrophil recruitment is defective in *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice

Neutrophil recruitment into the peritoneal cavity after thioglycollate injection is promoted by E-selectin– and chemokine-dependent pathways (6, 35). To investigate the physiological importance of DAP12 and FcRγ in a model of acute inflammation, neutrophil recruitment in thioglycollate-induced peritonitis was investigated in WT, *Fcrg*$^{-/-}$, *Tyrobp*$^{-/-}$, and *Tyrobp*$^{-/-}$ F*crg*$^{-/-}$mice with or without PTx treatment to block $G\alpha_i$-signaling. Consistent with previous data (6, 35), pretreatment with PTx reduced neutrophil recruitment to WT peritoneum 8 h after thioglycollate injection by ∼50% (Fig. 4). In the presence of intact GPCR signaling, *Fcrg*$^{-/-}$, *Tyrobp*$^{-/-}$, and *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice showed no major defect in neutrophil recruitment into the peritoneal cavity 8 h after thioglycollate injection. This is reminiscent of the phenotype of *Syk*$^{-/-}$ neutrophils in bone marrow chimeras after induction of thioglycollate peritonitis (36). Blocking of $G\alpha_i$ signaling by PTx reduced neutrophil recruitment in *Fcrg*$^{-/-}$ and *Tyrobp*$^{-/-}$ single knockout mice by ∼50%. However, treating *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ double knockout mice with PTx almost completely abolished neutrophil recruitment into the peritoneal cavity after thioglycollate injection.

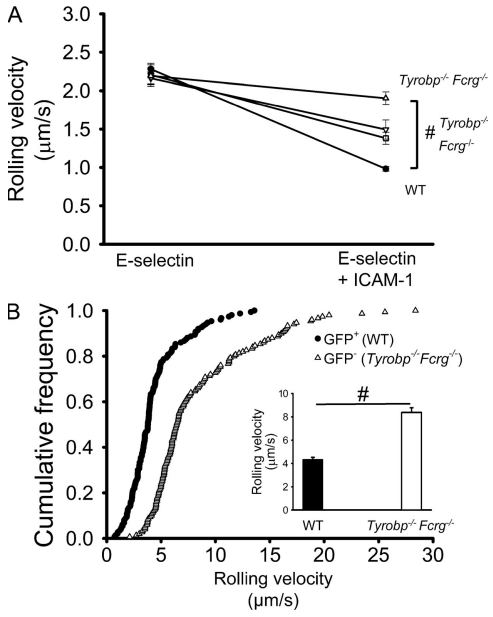

Figure 3. The ITAM domain-containing adapters DAP12 and FcRγ are involved in E-selectin–mediated slow rolling in vitro and in vivo. (A) Carotid cannulas were placed in *Tyrobp*$^{-/-}$ mice (upsidedown triangle, $n = 3$), *Fcrg*$^{-/-}$ mice (square, $n = 3$), *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice (triangle, $n = 3$), and WT mice (filled circle, $n = 5$) and connected to autoperfused flow chambers. The wall shear stress in all flow chamber experiments was 5–6 dynes/cm^2. Average rolling velocity of neutrophils on E-selectin (left) and E-selectin and ICAM-1 (right) presented as mean ± SEM. (B) Mixed chimeric mice were generated by injecting bone marrow cells from LysM-GFP^{+} WT mice and *Tyrob*$^{-/-}$ *Fcrg*$^{-/-}$ mice, mixed at a 1:1 ratio, into lethally irradiated WT mice. Cumulative histogram of rolling velocities of 150 GFP^{+} (WT, filled circle) and 150 GFP^{-} (*Tyrob*$^{-/-}$ *Fcrg*$^{-/-}$, open triangle) leukocytes in inflamed cremaster muscle venules of mixed chimeric mice ($n = 4$) treated with PTx and a monoclonal blocking P-selectin antibody (RB40.34; Video 1). (inset) Mean ± SEM. #, $P < 0.05$. Video 1 is available at http://www.jem.org/cgi/content/full/jem.20072660/DC1.

PSGL-1 signals through Fgr, DAP12, FcRγ, and Syk

Engagement of PSGL-1 can activate Src-family kinases (7) and the tyrosine kinase Syk (10). However, because Syk cannot directly interact with the cytoplasmatic tail of PSGL-1, ITAM-containing adaptor molecules are necessary for this interaction (10). During immunoreceptor and integrin signaling, Src-family kinases phosphorylate ITAM-containing adaptor proteins (21, 22, 32). This leads to activation of Syk, which in turn induces further downstream signaling. Consequently, we tested whether downstream signaling of PSGL-1 after E-selectin engagement required Fgr, DAP12, and FcRγ. Stimulation of WT neutrophils with E-selectin under shear stress conditions induced phosphorylation of the tyrosine in the catalytic domain of Fgr in a time-dependent manner (Fig. 5 A). E-selectin engagement also activated Hck (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20072660/DC1), but did not induce tyrosine phosphorylation of Lyn (Fig. S1B). As *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice showed an abolished E-selectin–mediated slow rolling, we speculated that these ITAM-containing adaptor molecules are phosphorylated after E-selectin stimulation. To investigate DAP12 phosphorylation, we used a glutathione S-transferase (GST) fusion protein with the tandem SH2 domains of Syk (GST-Syk-[SH2]2) to pull down tyrosine-phosphorylated proteins. Bound proteins were immunoblotted with a phosphospecific DAP12 antibody. Stimulation of neutrophils with E-selectin for 10 min led to an increase in DAP12 phosphorylation (Fig. 5 B). To investigate whether Fgr is involved in phosphorylation of the tyrosine residues in the ITAM domains, we stimulated WT and *fgr*$^{-/-}$ neutrophils with E-selectin. Elimination of Fgr almost completely abolished DAP12 phosphorylation after stimulation with E-selectin (Fig. 5 C).

Phosphorylation of downstream effectors was abolished in neutrophils pretreated with an Src-family kinase inhibitor (7). To investigate whether only Fgr is responsible for signaling downstream of PSGL-1, we measured Syk phosphorylation in bone marrow–derived neutrophils from WT mice and *fgr*$^{-/-}$ mice. Plating neutrophils on immobilized recombinant E-selectin under shear stress conditions for 10 min induced phosphorylation of Syk in neutrophils from WT, *hck*$^{-/-}$, and *lyn*$^{-/-}$ (Fig. S1 C), but not *fgr*$^{-/-}$, neutrophils (Fig. 5 D). To directly demonstrate that DAP12 and FcRγ are also involved in this signaling pathway, we investigated Syk phosphorylation in unstimulated and stimulated neutrophils from WT mice and *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice. In contrast to

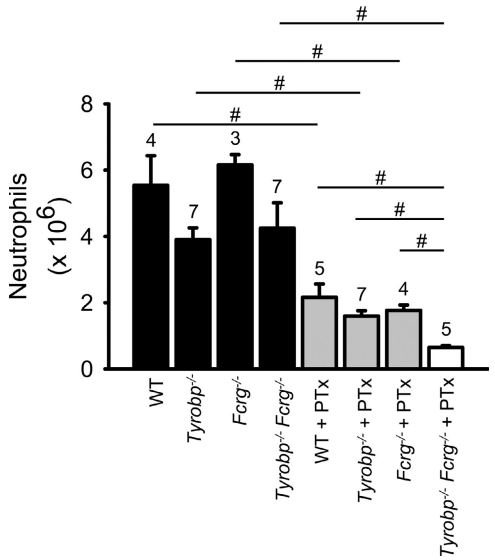

Figure 4. Gα$_i$-independent neutrophil recruitment drastically reduced in *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice. Neutrophil influx into the peritoneal cavity 8 h after 1 ml injection of 4% thioglycollate into WT mice ($n = 4$), *Fcrg*$^{-/-}$ mice ($n = 3$), *Tyrobp*$^{-/-}$ mice ($n = 7$), and *Tyrobp*$^{-/-}$ Fc*rg*$^{-/-}$ mice ($n = 7$). The same groups were also analyzed after pretreatment with 4 µg PTx i.v. (+PTx; WT mice + PTx [$n = 5$], *Fcrg*$^{-/-}$ mice + PTx [$n = 4$], *Tyrobp*$^{-/-}$ mice + PTx [$n = 7$], and *Tyrobp*$^{-/-}$ Fc*rg*$^{-/-}$ mice + PTx [$n = 5$]). Number of mice indicated above each bar. Total numbers of neutrophils in the peritoneal lavage fluid determined by flow cytometry and hemocytometer count. #, $P < 0.05$ from all groups by analysis of variance and Newman-Keuls post-hoc test. *, $P < 0.05$ compared with WT by Student's *t* test.

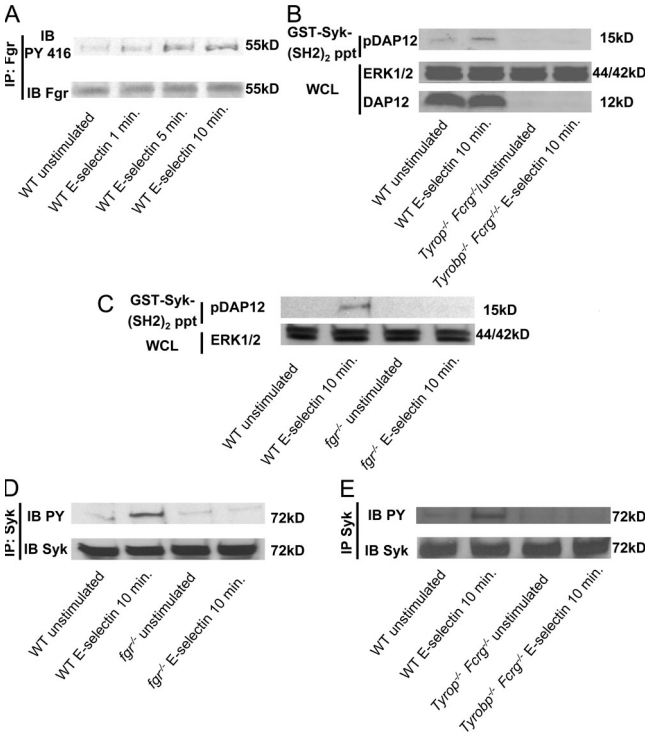

Figure 5. PSGL-1 signals through Fgr, DAP12, FcRγ, and Syk. Bone marrow–derived neutrophils were plated on uncoated (unstimulated) or E-selectin–coated wells for the indicated time, and then lysates were prepared. (A) Lysates were immunoprecipitated with anti-Fgr, followed by immunoblotting (IB) with a phosphospecific Src antibody that recognizes the tyrosine in the catalytic domain of Fgr ($n = 3$). (B and C) Lysates were incubated with GST–Syk–$(SH2)_2$ fusion protein (ppt), and bound proteins were immunoblotted with a phosphospecific DAP12 antibody ($n = 3$) or (D and E) immunoprecipitated with anti-Syk followed ($n = 3$) by immunoblotting (IB) for phosphotyrosine (PY) or Syk or ERK as a loading control.

stimulated WT neutrophils, no Syk phosphorylation was detectable in *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ neutrophils after stimulation (Fig. 5 E).

Elimination of DAP12 and FcRγ abolishes E-selectin–dependent downstream signaling in neutrophils

p38 MAPK is phosphorylated after E-selectin engagement and is involved in neutrophil slow rolling and adhesion (6, 8–10, 37). Pharmacological inhibition of p38 MAPK reduces slow rolling on E-selectin plus ICAM-1 through unknown mechanisms (6, 37). Consistent with this functional role, WT neutrophils incubated with immobilized E-selectin show significant induction of p38 MAPK phosphorylation that peaked at 10 min (Fig. 6, A and B). *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ neutrophils failed to phosphorylate p38 MAPK after stimulation with E-selectin (Fig. 6 C). In contrast, phosphorylation of p38 MAPK was normal in *Fcrg*$^{-/-}$ or *Tyrobp*$^{-/-}$ neutrophils (Fig. 6 C). These data suggest that ITAM domains provided either by DAP12 or FcRγ are sufficient for downstream signaling after E-selectin engagement.

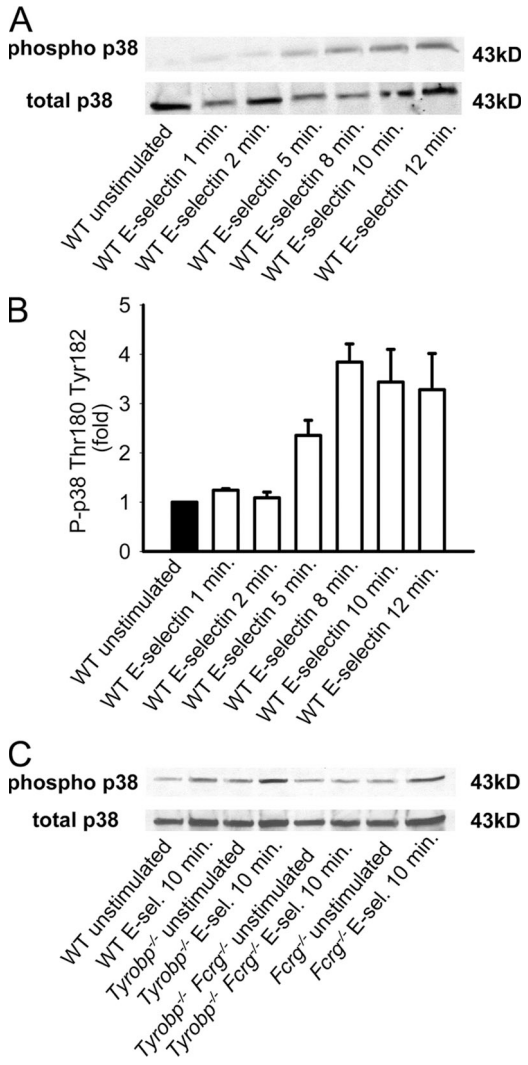

Figure 6. Elimination of DAP12 and FcRγ abolishes E-selectin–mediated downstream signaling in neutrophils. (A and B) Bone marrow–derived neutrophils from WT mice were plated on uncoated (unstimulated) or E-selectin–coated plates for the indicated times, after which lysates were prepared and immunoblotted with antibody to phosphorylated p38 MAPK (phospho-p38) or total p38 ($n = 2$). Data in B normalized to phospho-p38 in unstimulated WT neutrophils ($n = 2$). (C) Immunoblot analysis of total cell lysates generated from bone marrow–derived neutrophils of WT mice, *Fcrg*$^{-/-}$ mice, *Tyrobp*$^{-/-}$ mice, and *Tyrobp*$^{-/-}$ F*crg*$^{-/-}$ mice with antibody to phosphorylated p38 MAPK (phospho-p38) or total p38 (total p38). This was confirmed in four additional experiments.

DISCUSSION

Several prior studies have shown that engagement of selectin receptors on neutrophils can activate Src-family kinases (7, 38), induce Syk phosphorylation (10), integrin activation (6), slow rolling (6), and adhesion (9). However, the proximal signaling pathway between PSGL-1 and Syk was still unknown. In this study, we demonstrate that the Src-family kinase Fgr was indispensable for PSGL-1–dependent Syk activation and slow neutrophil rolling in vitro and in vivo. After E-selectin engagement, Fgr was activated and ITAM-containing adaptor proteins became phosphorylated and subsequently associated with Syk. Eliminating both DAP12 and FcRγ blocked this signaling pathway, and $G\alpha_i$-independent neutrophil recruitment into the peritoneal cavity was defective in *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice, confirming the physiological relevance of this signaling pathway.

The PSGL-1–Fgr–DAP12–FcRγ pathway has striking similarities to the immunoreceptor and integrin outside-in (21) signaling pathways. However, there are also significant differences. Elimination of only one Src-family kinase does not affect β_2 integrin–dependent neutrophil functions (39) or the inflammatory response in an endotoxic shock model (40). At least two Src-family kinases must be eliminated to reduce β_2 integrin–dependent neutrophil adhesion and superoxide production (39). However, such a redundancy does not exist in the E-selectin–mediated signaling pathway. Elimination of Fgr abolishes downstream signaling, including Syk phosphorylation and slow neutrophil rolling. This study is one of the few examples demonstrating a specific and nonredundant function for a Src-family kinase. In most other signaling pathways studied, one Src-family kinase may be dominant (such as Src in β_3 integrin signaling in osteoclasts or Lyn in inhibitory signaling in B lymphocytes), or there may be no apparent kinase selectivity (such as in integrin signaling). The specific role of Fgr in neutrophil PSGL-1 signaling is unique and provides a potential explanation for the evolution of this individual member of the Src kinase family.

Another difference between PSGL-1 and immunoreceptor or integrin signaling is the strength of the signal. E-selectin engagement of PSGL-1 induces downstream signaling and leads to partial activation of β_2 integrins on the leukocyte surface, resulting in slow rolling on ICAM-1. In the autoperfused flow chamber, this activation is insufficient to cause arrest. This suggests that signaling through PSGL-1 is weak

compared with other immunoreceptor pathways such as TREM1, which can induce firm adhesion, and even activation, of effector functions like cytokine production and degranulation (29).

A study with isolated human neutrophils showed that P-selectin engagement of PSGL-1 induced Lyn and Hck activation (38). However, this study may be complicated by the ligands used. Piccardoni et al. stimulated neutrophils with either fixed platelets or a P-selectin–IgG chimera containing an Fc portion that does not rule out the possibility that the signal was induced by outside-in integrin signaling or engagement of Fc receptors. In our in vitro assay, we can exclude signaling through engagement of Fc receptors, because neutrophils from *Fcrg*$^{-/-}$ mice, which cannot signal through Fc receptors, show the same phosphorylation pattern of p38 as WT mice. Together with the present study, these data suggest that engagement of E- or P-selectin may activate different Src-family kinases.

Although Fgr is activated after E-selectin engagement of PSGL-1, the mechanism of this interaction remains unknown. It is tempting to speculate that phosphatases like CD45 (41) and/or kinases like Csk (42) may be involved in this process. Csk phosphorylates tyrosine 527 near the C-terminus of Fgr, which blocks Fgr activation. CD45 is a plausible candidate phosphatase that may dephosphorylate Y527 and activate Fgr (43, 44). Indeed, elimination of phosphatases is associated with a proinflammatory phenotype (42). Further detailed studies are needed to determine how Fgr is activated after E-selectin engagement of PSGL-1.

The tyrosine kinase Syk cannot directly interact with the cytoplasmatic tail of PSGL-1 (10). In HL-60 cells, the ITAM-containing adaptor proteins ezrin and moesin can act as linkers between the two molecules (10). In this study, we demonstrate that the ITAM-containing adaptor proteins DAP12 and FcRγ provide alternative linkers, and eliminating both adaptor proteins abolishes E-selectin–mediated downstream signaling and slow rolling. This suggests that the ITAM domains of ezrin and moesin cannot substitute for DAP12 and FcRγ. One likely explanation for this is that ITAM-containing adaptor molecules may be compartmentalized in the neutrophil, with one set (DAP12 and FcRγ) promoting slow rolling and another (ezrin and moesin) involved in directing PSGL-1 to the uropod of migrating cells (10). Compartmentalization of multimolecular signaling complexes is known to be important for linking different signals at the appropriate time and location to specific signaling cascades in the cell (45). Both FcRγ and DAP12 associate with many cell surface receptors in various hematopoietic cell types (22). However, PSGL-1, unlike TREM-1, myeloid DAP12-associating lectin-1, and signal-regulatory protein β, does not contain a basic amino acid in its transmembrane domain. Therefore, the interaction between DAP12 or FcRγ and PSGL-1 may be indirect. Other transmembrane proteins may be involved in linking PSGL-1 to DAP12 and FcRγ. The finding that PSGL-1 resides in lipid rafts (46) may bring it into close enough proximity to allow productive interactions with DAP12 and FcRγ.

Our data suggest that DAP12 and FcRγ are partially redundant in slow rolling after PSGL-1 engagement by E-selectin. However, they seem to be fully redundant for p38 MAPK phosphorylation, because the phosphorylation level is indistinguishable in *Tyrobp*$^{-/-}$ or *Fcrg*$^{-/-}$ neutrophils, but completely absent in double knockouts. This raises the possibility that more than one signaling pathway may be involved in LFA-1 activation after PSGL-1 binding to E-selectin. The p38 MAPK-dependent arm of the pathway may require only one ITAM domain-containing species, DAP12 or FcRγ, whereas another arm of the pathway may require both, resulting in partial loss of slow rolling function in *Tyrobp*$^{-/-}$ or *Fcrg*$^{-/-}$ mice.

The reduction of neutrophil recruitment in *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice after blocking GPCR signaling is quantitatively similar to the reduction seen in PTx-treated *syk*$^{-/-}$ chimeric mice (6). This suggests that eliminating DAP12 and FcRγ is as effective in blocking neutrophil recruitment as eliminating Syk. Like *syk*$^{-/-}$ chimeric mice (6) or E-selectin–deficient (*Sele*$^{-/-}$) mice (35), *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ mice show defective neutrophil recruitment to the inflamed peritoneum when chemokine receptor signaling is blocked. These data demonstrate the physiological relevance of the E-selectin–mediated PSGL-1 pathway in vivo. The new mechanistic insights we provide here suggest that Syk, DAP12, and FcRγ may be therapeutic targets for antiinflammatory therapy. The specific role of Fgr suggests that specific Fgr inhibitors that do not block Lyn or Hck could be devised that may specifically block E-selectin–dependent neutrophil activation, perhaps without compromising host defense.

MATERIALS AND METHODS

Animals and bone marrow chimeras. 8–12-wk-old C57BL/6 mice (The Jackson Laboratory), DAP12 (*Tyrobp*$^{-/-}$) (47), FcRγ (*Fcrg*$^{-/-}$) (31), *Tyrobp*$^{-/-}$ *Fcrg*$^{-/-}$ (32), Fgr (*fgr*$^{-/-}$) (48), Lyn (*lyn*$^{-/-}$) (49), Hck (*hck*$^{-/-}$) (48), Hck/Lyn/Fgr (*hck*$^{-/-}$ *lyn*$^{-/-}$ *fgr*$^{-/-}$) (33), and Syk (*syk*$^{+/-}$) (6) mice were housed in a specific pathogen–free facility. The Animal Care and Use Committees of the University of Virginia, the La Jolla Institute for Allergy and Immunology, and the University of California at San Francisco approved all animal experiments. Chimeric mice were generated by performing bone marrow transplantation, as previously described (50). In brief, bone marrow cells isolated from Lys-M-GFP^{+} (34) and gene-deficient mice were mixed 1:1, and 5×10^6 unfractionated cells were injected intravenously into lethally irradiated mice. Experiments were performed 6–8 wk after bone marrow transplantation.

Intravital microscopy. 2 h before cremaster muscle exteriorization, mice received 4 μg PTx i.v. (Sigma-Aldrich) and 500 ng TNF-α intrascrotally (R&D Systems). Mice were anesthetized with an i.p. injection of 125 mg/kg ketamine hydrochloride (Sanofi), 0.025 mg/kg atropine sulfate (Fujisawa), and 12.5 mg/kg xylazine (TranquiVed; Phoenix Scientific) and placed on a heating pad. The cremaster muscle was prepared as previously described (51). Postcapillary venules with a diameter between 20 and 40 μm were recorded using an intravital microscope (Axioskop, SW 40/0.75 objective; Carl Zeiss, Inc.) through a digital camera (sensicam qe; Cooke Corporation). Blood flow centerline velocity was measured using a dual-photodiode sensor system (CircuSoft Instrumentation).

Blood-perfused microflow chamber. Autoperfused flow chambers were used to investigate the rolling velocity of neutrophils, as previously described (6, 37, 52). Rectangular glass capillaries (20 × 200 μm) were coated either

with 30 μg/ml E-selectin (R&D Systems) alone or in combination with 15 μg/ml ICAM-1 (R&D Systems) for 2 h and blocked with 10% casein (Thermo Fisher Scientific). Each capillary was connected to a PE 10 catheter (Becton Dickinson) inserted into a mouse carotid artery. Wall shear stress (37) was controlled by hydrostatic pressure applied to the downstream side of the flow chamber. One representative field of view was recorded for 1 min using an SW40/0.75 objective and a charge-coupled device camera (VE-1000CD; Dage-MTI).

In some experiments, mice were pretreated with the specific Src-family kinase inhibitor PP2 (10 μg/kg, i.v., 30 min before the experiments, Calbiochem) or the inactive control (PP3).

Engagement of PSGL-1 with E-selectin. For biochemical assays, 15-mm Petri dishes were coated with 3 μg/ml E-selectin for 2 h and blocked with casein. Bone marrow neutrophils (53) were suspended in PBS (containing 1 mM each $CaCl_2$ and $MgCl_2$) and incubated at 65 rpm for 10 min at 37°C. Neutrophils were lysed with RIPA buffer (32). Lysates were boiled with sample buffer or incubated with protein A–coated magnetic beads (Miltenyi Biotec) and rabbit anti-Syk (N-19; Santa Cruz Biotechnology, Inc.) antibody for 30 min on ice and separated using a magnet (Miltenyi Biotec). Beads were washed four times and bound proteins were eluted by adding boiling sample buffer. To test DAP12 phosphorylation, GST-Syk-(SH2)2 was coupled to glutathione–Sepharose beads (GE Healthcare), as previously described (32).

Cell lysates and immunoprecipitates were run on 10% SDS-PAGE and immunoblotted using antibodies against phosphotyrosine (4G10; Millipore), p38 MAP kinase (3D7), and phospho-p38 MAP kinase (both from Cell Signaling Technology), developed using GE Healthcare's ECL system, and analyzed by ImageJ (National Institutes of Health).

Peritonitis model. Peritonitis was induced by injecting sterile 4% thioglycollate i.p. (Sigma-Aldrich) (6). Some mice received 4 μg PTx i.v. 2 h before thioglycollate injection. After 8 h, mice were killed, the peritoneal cavity was rinsed with 10 ml PBS (containing 2 mM EDTA), and the number of leukocytes was counted. Neutrophils were detected by flow cytometry (FACSCalibur; BD Biosciences) based on expression of CD45 (clone 30-F11), 7/4 (clone 7/4; both from BD Biosciences), and GR-1 (clone RB6-8C5).

Statistics. Statistical analysis was performed with SPSS (version 14.0, Chicago, IL) and included one-way analysis of variance, Student-Newman-Keuls test, and Student's *t* test where appropriate. All data are presented as mean ± SEM. $P < 0.05$ was considered significant.

Online supplemental material. Fig. S1 shows Src and Syk phosphorylation by E-selectin engagement in $hck^{-/-}$ and $lyn^{-/-}$ mice. Video 1 shows intravital microscopy of an inflamed cremaster muscle venule of a mixed chimeric mouse treated with PTx and a monoclonal blocking P-selectin antibody (GFP^+, WT leukocytes; GFP^-, $Tyrob^{-/-}$ $Fcg^{-/-}$ leukocytes). The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20072660/DC1.

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