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Cross-Talk between Shp1 and PIPKIγ Controls Leukocyte Recruitment

Anika Stadtmann,*† Helena Block,*† Stephanie Volmering,*† Clare Abram,‡ Charlotte Sohlbach,*€ Mark Boras,*€ Clifford A. Lowell,§ and Alexander Zarbock*†

Neutrophil recruitment to the site of inflammation plays a pivotal role in host defense. However, overwhelming activation and accumulation of neutrophils in the tissue may cause tissue damage and autoimmunity due to the release of cytokines, oxidants, and proteases. Neutrophil adhesion in acute inflammation is initiated by activation of α4β1 (LFA-1), which can be induced by rolling on E-selectin (slowly) or by exposure to the chemokine CXCL1 (rapidly). Despite the clinical importance, cell-intrinsic molecular mechanisms of negative regulation of integrin adhesiveness and neutrophil recruitment are poorly understood. Mice deficient in the tyrosine phosphatase Src homology 2 domain-containing protein tyrosine phosphatase 1 (Shp1) show increased leukocyte adhesion, but the interpretation of these data is limited by the severe global phenotype of these mice. In this study, we used mice with global and myeloid-restricted deletion of Shp1 to study neutrophil arrest, adhesion, crawling, and transendothelial migration in vitro and in vivo. Shp1 deficiency results in increased neutrophil adhesion in vivo; however, neutrophil crawling, transmigration, and chemotaxis were reduced in these mice. Mechanistically, Shp1 binds and controls PIPKIγ activity and thereby, modulates phosphatidylinositol (4,5)-bisphosphate levels and adhesion. Thus, Shp1 is involved in the deactivation of integrins and regulation of neutrophil recruitment into inflamed tissue. The Journal of Immunology, 2015, 195: 1152–1161.

Inflammatory responses require efficient recruitment of leukocytes (1). Neutrophils are the first line of cellular defense against infecting microorganisms and play a central role in innate immunity and inflammatory processes (2). They rapidly migrate to sites of inflammation and release potent oxidants, proteases, and cationic peptides, which may induce collateral tissue damage (1). Thus, uncontrolled accumulation and activity of neutrophils can lead to profound tissue damage (2).

Neutrophil recruitment occurs in a cascade-like fashion (3, 4). Selectins and chemokines activate signaling pathways that cooperate to amplify neutrophil recruitment during inflammation (5, 6). The signaling pathway, triggered by selectin engagement, forces

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A.S. designed and performed most of the experiments, analyzed the results, and prepared the manuscript. H.B. performed biochemistry experiments, S.V. performed some flow chamber and intravital microscopy experiments. C.A. provided the Ptpn6m1/m1S100a8-cre and Ptpn6m1/m1S100a8+cre mice. C.S. performed biochemistry assays. M.B. performed chemotaxis assays. C.A.L. and A.Z. provided overall supervision, helped design all of the experiments, and prepared the manuscript.

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Abbreviations used in this article: FA, focal adhesion; GPCR, G protein–coupled receptor; PIPKIγ, phosphatidylinositol-phosphate kinase Iγ; PMN, polymorphonuclear leukocyte; Ptdln(3,4,5)P3, phosphatidylinositol (3,4,5)-trisphosphate; Ptdln(4,5)P2, phosphatidylinositol (4,5)-bisphosphate; Shp1, Src homology 2 domain-containing protein tyrosine phosphatase 1; shRNA, short hairpin RNA; WT, wild-type.

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LFA-1 in the extended conformation (7), which mediates slow leukocyte rolling and neutrophil recruitment (3). During rolling, neutrophils are exposed to chemokines, which are presented on inflamed endothelium and cause integrin activation, resulting in a change from a rolling behavior to firm endothelial adhesion (3). Following arrest, neutrophils undergo actin-dependent polarization and lateral migration or crawling on endothelial cells in search for permissive sites, before final transendothelial migration out of blood vessels (6, 8). Several proteins and kinases were demonstrated to regulate proadhesive signaling pathways by selectins and chemokine receptors (9). Negative regulators of integrin activation have also been described; however, it is unknown how integrin activation is turned off, which would naturally restrict inflammatory cell recruitment. Clearly, integrin deactivation is required to prevent overwhelming neutrophil recruitment that may lead to tissue damage.

The protein tyrosine phosphatase Src homology 2 domain-containing protein tyrosine phosphatase 1 (Shp1; encoded by the Ptpn6 gene) is expressed in all hematopoietic cells (10), and substantial effort has been devoted to the study of its function in the immune system (11, 12). However, a detailed understanding of how Shp1 regulates inflammation remains unclear. The motheaten mouse has a spontaneous autosomal recessive mutation in Ptpn6, leading to inflammation and immune deficiency (13). The inflammatory phenotype caused by transfer of bone marrow cells from motheaten mice into wild-type (WT) animals is abolished by the use of anti-CD11b Abs (14), suggesting that Shp1 is involved in the tight regulation of β2-integrin activation.

Shp1 is involved in the regulation of multiple signaling pathways (11, 15, 16). Previous in vitro work focused on investigating cells isolated from mice containing Ptpn6 mutations. Neutrophils from Ptpn6m1/me and Ptpn6m1/m1 mice are hyperadhesive in vitro assays (17). Ptpn6m1/me-mice macrophages are also hyperadhesive and show an increased responsiveness to different cytokines (18–20). Alterations in immune cell function may be a consequence of the indirect effects caused by the inflammatory milieu in gene-deficient mice (21). Cell-intrinsic abnormalities caused by loss of
Shp1 function may also cause abnormal leukocyte recruitment; however, it is unknown how Shp1 deficiency affects the different steps of the neutrophil recruitment cascade in vivo.

Shp1 interacts with and regulates the activity of different signaling molecules, including Vav, SFKs, and Slp76 (22–24). Phosphatidylinositol-phosphate kinase 1γ (PIPKy) also was found to associate with Shp1 (25). The group of PIPKiGs consists of PIPKic, β, and γ isoforms that are involved in the production of phosphatidylinositol (4,5)-biphosphate [PtdIns(4,5)P₂] (26). PtdIns(4,5)P₂ is subsequently converted into phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P₃] by PI3K (27). PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ participate in a variety of cell functions, including chemotaxis, superoxide production, and phagocytosis (27, 28). Several splice variants of PIPKγy are located at distinct subcellular localizations in hematopoietic cells, including a 90-kDa extended isoform that is able to bind talin-1 (29).

The present study was designed to identify how Shp1 in neutrophils regulates selectin- and chemokine-induced integrin activation and neutrophil recruitment. We demonstrate that Shp1 negatively regulates β₂-integrin activation, thereby controlling different steps of the leukocyte-recruitment cascade. We also show that PIPKγy is involved in neutrophil recruitment and that the regulation of PIPKγy and PI3K by Shp1 in neutrophils determines the production of PtdIns(4,5)P₂ and Akt and, thus, regulates chemotaxis. Thus, this study pinpoints the importance of Shp1 in neutrophils as a negative regulator of selectin- and chemokine-induced integrin activation and neutrophil recruitment.

Materials and Methods

**Animals**

Eight- to twelve-week-old C57BL/6 (JAXVIER) and Ptpn6-KO mice, expressing normal levels of a mutated form of Shp1 with very low catalytic activity, leading to a chronic inflammation of the skin, production of autoantibodies, and lethal pneumonia due to increased numbers of neutrophils and macrophages in the lungs after 9–12 wk (30), as well as Ptpn6+/+S100a8−cre (21), Ptpn6fl/flS100a8−cre (conditional knockout causing Shp1 deficiency in neutrophils, with an efficiency of an average of 80% Shp1-deficient neutrophils for the mice used in the described experiments) (21), and PIPKγty90−/− (lacking the 90-kDa isoform of PIPKγy) (29) were used throughout this study. Mice were housed in a specific pathogen–free facility. The Animal Care and Use Committee of the University of Munich approved all animal experiments.

Because of the phenotype of Ptpn6-KO mice, we used chimeric mice, derived from the transfer of bone marrow cells from Ptpn6−/− mice into lethally irradiated WT mice, for all in vivo experiments. Bone marrow chimeras were generated as described (31).

**Cell lines and constructs**

Stable knockdown of SHP1 in promyelocytic HL-60 cells was performed by lentiviral transduction of short hairpin RNA (shRNA), as described previously (32) (sequence: 5’-CCCGGGGACATGCACAAACGGGAATCTCGGTTCCCTGTCATGCTCCTCTTTTGTG-3’). The knockdown efficiency was confirmed by Western blot (Shp1 [C19]; Santa Cruz Biotechnology, Heidelberg, Germany).

During cell culture, Shp1 knockdown was maintained by puromycin selection.

**Intravital microscopy**

Mice were anesthetized using an i.p. injection of ketamine hydrochloride (125 mg/kg, Sanofi Winthrop Pharmaceuticals, Bridgewater, NJ) and xylazine (12.5 mg/kg, TranquilVet, Phoenix Scientific, St. Joseph, MO), and the cremaster muscle was prepared for intravital imaging as previously described (6, 31, 33). Some mice were treated with PBS or the Shp1/2 inhibitor NSC87877 (0.15 mg/mouse i.p.; EMD Millipore, Darmstadt, Germany) prior to the TNF-α injection (34). Measurements were performed in postcapillary venules with diameters of 20–40 μm. To determine leukocyte adhesion, 500 ng CXCL1 was injected via the carotid artery. The number of adherent cells prior to and following CXCL1 injection was analyzed. To determine selects-included-mediated slow rolling, adhesion, and transmigration in vivo, mice were injected intracranially with TNF-α (500 ng; R&D Systems, Minneapolis, MN) 2 h prior to preparation of the cremaster muscle. Intravital microscopy was performed on an upright microscope (Axioskop; Carl Zeiss, Jena, Germany) with a 40 x 0.75 NA saline-immersion objective. We determined leukocyte rolling velocity and adhesion by transillumination intravital microscopy. The number of extravasated leukocytes was investigated by reflected light oblique transillumination microscopy, as previously described (35–37). Emigrated cells were determined in an area (75 × 100 μm) to each side of a vessel (representing 1.5 × 10⁶ μm² tissue area).

Clustering of surface adhesion molecules (LFA-1) was performed as previously described (35, 36). Recorded images were analyzed off-line using ImageJ and AxioVision (Carl Zeiss) software. The microcirculation was recorded using a digital camera (SenicaSM QM, PCO, Kelhheim, Germany). Blood flow centerline velocity was measured using a dual photodiode sensor system (Circusoft Instrumentation, Hockenheim, Germany). Centerline velocities were converted to mean blood flow velocities, as previously described.

**Intravascular crawling assay**

Leukocyte intravascular crawling behavior was determined using intravitral microscopy, as described previously (32). Briefly, the anti–Gr-1 Ab (750 ng; clone RB6-8C5), labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR), was injected directly prior to the experiment. Following preparation and exteriorization, the cremaster muscle was superfused with 5 nM CXCL2 (R&D Systems, Wiesbaden-Nordenstadt, Germany), and time-lapse microscopy was performed for 2 h.

**In vitro chemotaxis assay**

An in vitro chemotaxis assay was performed as described previously (38). Following isolation, bone marrow–derived murine neutrophils were seeded on fibronectin-coated (50 μg/ml) chemotaxis μ-slides (ibidi). Within the chemotaxis slide, a CXCL1 gradient (1 ng/ml) was applied. Cell movement was recorded over a period of 30 min using time-lapse microscopy (two frames/min). For analysis, cells were tracked with Manual Tracking (ImageJ) and analyzed with Chemotaxis plug-in (Ibidi). We analyzed the accumulated distance, velocity, and chemotaxis index of the cells (38).

**In vitro transmigration assay**

Polymorphonuclear leukocyte (PMN) transmigration experiments were performed as described previously (39). Briefly, bend.5 cells were activated for 16 h with 5 nM TNF-α on 6.5-mm-diameter Transwell filters with 5-μm pore size. Subsequently, cells were washed with DMEM containing 10% FCS and 25 nM HEPES. The upper reservoir was filled with 100 μl supplemented DMEM containing 5 × 10⁴ PMNs. After 30 min, the number of transmigrated PMNs in the lower reservoir containing 600 μl supplemented DMEM with 40 ng/ml CXCL1 was quantified using a CASY Cell Counter (Innovatis).

**ICAM-1 binding assay**

Murine bone marrow–derived neutrophils were isolated and suspended in HBSS containing 1 nM CaCl₂ and MgCl₂. Subsequently, neutrophils were left unstimulated or were stimulated with CXCL1 (100 ng/ml, 3 min, 37°C; PepTecBio, Rocky Hill, NJ) in the presence of ICAM-1/Fc (20 μg/ml; R&D Systems) and allophycocyanin-conjugated anti-human IgG1 (Fc specific; Southern Biotechnology). By using an anti-CD11b (clone M1/70; 10 μg/ml) blocking Ab, Mac-1–dependent ICAM-1 binding was prevented. Cells were fixed on ice, and neutrophils were stained with FITC-conjugated anti-Ly6G (BioLegend). ICAM-1 binding was measured using flow cytometry.

**Flow chamber systems**

To investigate the rolling velocity of murine neutrophils on E- or P-selectin, we used an autopercused flow chamber (6, 31, 33). Rectangular glass capillaries (20 × 200 μm) were filled with E-selectin- (2.5 μg/ml) or P-selectin- (20 μg/ml; both from R&D Systems) alone or in combination with ICAM-1 (2 μg/ml in combination with E-selectin, 5 μg/ml in combination with P-selectin; R&D Systems) for 2 h and then blocked for 2 h using casein (1%; Thermo Scientific). Cells were fixed on slide (Becton Dickinson, Sparks, MD) and used to control the wall shear stress in the capillary. To investigate chemokine-induced adhesion in vitro (33), glass capillaries were coated with P-selectin (50 μg/ml) and ICAM-1 (15 μg/ml) or P-selectin and ICAM-1 in combination with CXCL1 (25 μg/ml).

**Adhesion flow chamber**

Adhesion flow chamber experiments were carried out as described previously (7). Briefly, protein G-coated glass capillaries were coated with E-selectin (6.6 μg/ml) and IgG1 (25 μg/ml) or RIM127 (25 μg/ml) for 1 h and blocked with casein (Thermo Fisher Scientific, Bonn, Germany). In other experiments, capillaries were coated with P-selectin (20 μg/ml), IL-8 (50 μg/ml; PeproTech), and IgG1 (5 μg/ml), mAb24 (5 μg/ml; generous
Biochemical experiments

For biochemical assays, bone marrow–derived murine neutrophils were isolated and suspended in PBS (containing 1 mM each CaCl$_2$ and MgCl$_2$). Subsequently, the cells were incubated under rotating conditions (65 rpm) for 10 min on uncoated or E-selectin (3 µg/ml)-coated coverslips in multiwell plates or stimulated with CXCL1 (100 ng/ml, 37°C). Cells were lysed in RIPA buffer, and lysates were boiled with sample buffer (10 min, 95°C) or incubated with Sepharose A/G beads (Santa Cruz Biotechnology, Dallas, TX) and anti-Syk, anti-Shp1, or anti-PIPKI Ab (Santa Cruz Biotechnology) for 4 h at 4°C. Beads were washed four times, and bound proteins were eluted by adding boiling sample buffer. Cell lysates and immunoprecipitates were run on 10% SDS-PAGE and immunoblotted using Abs against phosphotyrosine (4G10; Millipore), Akt, phospho-Akt (Ser473), PLC$_gamma$1, PLC$_beta$1, and PLC$_beta$2 (all from Cell Signaling Technology, Danvers, MA), and Fgr, Hck, Lyn, and Syk (all from Santa Cruz Biotechnology). Immunoblots were developed using an ECL system (GE Healthcare). Densitometric quantification was performed using ImageJ software.

Statistics

Statistical analysis was performed with SPSS Statistics (version 21.0; IBM, Armonk, NY). Differences between the groups were evaluated by one-way ANOVA, the Student-Newman-Keuls test, or the t test, where appropriate. Data are presented as mean ± SEM, and p < 0.05 was considered statistically significant.

Results

Shp1 is involved in different steps of the leukocyte-recruitment cascade

To investigate the role of Shp1 in the different steps of the recruitment cascade in the systemic circulation, we performed intravital microscopy of the cremaster muscle. Two hours after TNF-α injection, leukocyte tethering and rolling along the endothelial surface is mediated by P- and E-selectin expressed on inflamed endothelial cells of the cremaster muscle (5, 6). The rolling velocity of Shp1-deficient neutrophils (Ptpn6$^{me-me}$/me-mecre$^{-/-}$) bone marrow chimeras, hereafter referred to as Ptpn6$^{-/-}$, and Ptpn6$^{fl/fl}$S100a8-cre$^{-/-}$) was reduced significantly compared with the rolling velocity of WT neutrophils (C57BL/6 and Ptpn6$^{+/+}$ S100a8-cre; Fig. 1A). Both E-selectin and G protein–coupled receptor (GPCR) signaling mediate leukocyte intravascular adhesion in venules of the cremaster muscle after TNF-α treatment (5, 6). The number of adherent cells was significantly higher in Shp1-deficient mice (Ptpn6$^{me-me}$ce-mecre$^{-/-}$ and Ptpn6$^{fl/fl}$S100a8-cre$^{-/-}$) compared with WT mice (C57BL/6 and Ptpn6$^{+/+}$ S100a8-cre; Fig. 1B). In contrast, the number of emigrated cells was significantly decreased in Shp1-deficient mice compared with WT mice (Fig. 1C). Representative videomicrographs of WT mice (C57BL/6 and Ptpn6$^{+/+}$ S100a8-cre) and Shp1-deficient mice (Ptpn6$^{me-me}$ce-mecre$^{-/-}$ and Ptpn6$^{fl/fl}$S100a8-cre$^{-/-}$) 2 h after TNF-α injection are shown in Fig. 1D. Microvascular parameters (vessel diameters, centerline velocities, wall shear rates) were similar among the groups (data not shown). These data suggest that Shp1 in neutrophils regulates different steps of the leukocyte-recruitment cascade. In this model, selectin-dependent slow leukocyte rolling, chemokine-induced adhesion, and transmigration of neutrophils are affected in the absence of Shp1.

Deficiency of Shp1 increases chemokine-induced arrest and postadhesion strengthening

To dissect the role of Shp1 in these two signaling pathways leading to integrin activation, we performed chemokine-induced arrest in the cremaster muscle (33, 40). In this model, neutrophil rolling in the venules of the cremaster muscle is due to P-selectin expression on the endothelium. Injection of CXCL1 induces a shift in integrin from a closed to an open conformation that subsequently induces neutrophil arrest (41). The number of adherent cells after CXCL1 injection increased significantly in Shp1-deficient mice (Ptpn6$^{me-me}$ce-mecre$^{-/-}$ and Ptpn6$^{fl/fl}$S100a8-cre$^{-/-}$) compared with WT mice (C57BL/6 and Ptpn6$^{+/+}$ S100a8-cre; Fig. 2A, 2B). Although the number of adherent leukocytes in WT (C57BL/6 and Ptpn6$^{+/+}$ S100a8-cre) venules decreased over time, the number of adherent leukocytes in the venules of Shp1-deficient mice (Ptpn6$^{me-me}$ce-mecre$^{-/-}$ and Ptpn6$^{fl/fl}$S100a8-cre$^{-/-}$) remained significantly higher (Fig. 2A). These data suggest that Shp1 is involved in chemokine-induced arrest, which is dependent on the activation of the β2-integrin LFA-1, as well as in Mac-1–dependent postadhesion strengthening of neutrophils in the murine cremaster muscle.

Shp1 regulates selectin-dependent neutrophil slow rolling and selectin-mediated intracellular signaling

To further confirm the in vivo data, rolling velocity, integrin activation, and intracellular signaling were analyzed in vitro. To examine E-selectin–mediated rolling velocity, autopulsed flow chamber experiments were performed (6). In WT neutrophils, the rolling velocity on E-selectin was 2.3 ± 0.06 µm/s; it decreased to 1.42 ± 0.04 µm/s when neutrophils rolled on E-selectin and ICAM-1. This reduction in the rolling velocity is mediated by the binding of activated LFA-1 to ICAM-1 (7). Neutrophils from Shp1-deficient mice
Deficiency of Shp1 influences chemokine-dependent and selectin-dependent pathways. (A) Chemokine-induced arrest of neutrophils in postcapillary venules of WT (●), Ptpn6<sup>−/−</sup> (○), Ptpn6<sup>+/−</sup>S100a8-cre (■), and Ptpn6<sup>+/+</sup>S100a8-cre (△) mice before and after i.v. CXCL1 injection (500 ng). Intravital microscopy was performed on an upright microscope (Axioskop) with a 40×0.75 NA saline-immersion objective. (B) Representative images of postcapillary venules of WT, Ptpn6<sup>−/−</sup>, Ptpn6<sup>+/−</sup>S100a8-cre, and Ptpn6<sup>+/+</sup>S100a8-cre mice 1 min following CXCL1 injection. White dots represent adherent cells within the vessel. Scale bars, 20 μm. Rolling velocities on E-selectin and E-selectin plus ICAM-1 (C) or on P-selectin and P-selectin plus ICAM-1 (D) of neutrophils from WT (●), Ptpn6<sup>−/−</sup> (○), Ptpn6<sup>+/−</sup>S100a8-cre (■) and Ptpn6<sup>+/+</sup>S100a8-cre (△) mice. (E) Protein levels of Shp1 in HL-60 cells following transfection with three shRNAs in comparison with the protein level following transduction with scrambled shRNA. (F) HL-60 cells (transduced with scrambled shRNA or construct 2) were analyzed using a flow chamber adhesion assay with E-selectin and either an Ab specific for the intermediate confirmation of LFA-1 (KIM127) or an isotype control. Adherent cells/field of view were counted (mean ± SEM). (G) Bone marrow–derived neutrophils were plated on uncoated (unstim.) or E-selectin-coated wells for 10 min, and lysates were prepared. Representative Western blots of either immunoprecipitations or total lysates of neutrophils of WT and Ptpn6<sup>−/−</sup> mice showing the phosphorylation of Src kinases and the downstream molecules Syk, Akt, and PLCγ2. For the analysis of Syk phosphorylation, lysates were immunoprecipitated with a Syk (n = 3) Ab, followed by immunoblotting with a general phosphotyrosine (4G10) Ab or total Syk Ab. Total lysates were immunoblotted with Abs to phosphorylated Src-kinases (Tyr416; n = 3), total Src (n = 3), phosphorylated PLCγ2 (Tyr1217; n = 3), total PLCγ2 (n = 3), phosphorylated Akt (Ser473; n = 3), and total Akt (n = 3). *p < 0.05.
immunoprecipitations and Western blots (Fig. 2G). Phosphorylation of the different signaling molecules increased in WT neutrophils following stimulation with E-selectin. In Ptpn6−/− neutrophils, the phosphorylation level of these molecules was significantly elevated after E-selectin stimulation compared with phosphorylation levels in WT neutrophils (Fig. 2G, Western blot densitometry is shown in Supplemental Fig. 1A–D). Altogether, these data demonstrate that regulation of selectin-mediated intracellular signaling, which leads to integrin activation, is altered in Shp1-deficient neutrophils.

**Ga_1-mediated integrin activation is regulated by Shp1**

Because the in vivo data suggest that Shpl is also involved in chemokine-induced integrin activation, we investigated Gαi signaling and integrin activation in vitro using different assays. To directly test whether Shp1 is involved in GPCR-mediated integrin activation under flow conditions, we used the Shp1-knockdown HL-60 cell line in an immobilized reporter Ab assay (7). We observed more adherent WT cells, in the presence of CXCL1, when the chambers were coated with a reporter Ab against activated LFA-1 (mAb24, high-affinity conformation, Fig. 3A) or Mac-1 (CBRM1/5, Fig. 3B) compared with IgG-control Ab. In control chambers, Shp1-knockdown cells showed the same number of adherent cells compared with WT cells (Fig. 3A, 3B). However, in the presence of the reporter Abs, the number of adherent Shp1-knockdown cells increased significantly (Fig. 3A, 3B). These data suggest that Shp1 in neutrophils modulates chemokine-induced LFA-1 and Mac-1 activation.

To investigate the adhesive state of LFA-1, an ICAM-1–binding assay was performed using Ptpn6−/− and WT neutrophils that were incubated with soluble murine ICAM-1 (32). Using flow cytometry, we investigated LFA-1–mediated ICAM-1 binding of unstimulated neutrophils in comparison with CXCL1-stimulated neutrophils. WT neutrophils showed a strong binding after CXCL1 stimulation, whereas Ptpn6−/− neutrophils after CXCL1 stimulation bound significantly more ICAM-1 compared with WT neutrophils (Fig. 3C).

To investigate the adhesion phenotype in vitro, adhesion flow chamber experiments were performed. P-selectin and ICAM-1 were coimmobilized in the presence or absence of the chemokine CXCL1, and the number of adherent neutrophils was determined. Although only few WT (Ptpn6+/+S100a8-cre) and Shp1-deficient (Ptpn6−/− and Ptpn6+/+S100a8-cre) neutrophils adhered in chambers coated with P-selectin and ICAM-1, the number of adherent neutrophils increased in the chambers where CXCL1 was coimmobilized with P-selectin and ICAM-1. Concurrent with the results of our in vivo experiments, the number of adherent neutrophils/field of view was increased significantly in Shp1-deficient mice (Ptpn6−/− and Ptpn6+/+S100a8-cre) compared with WT mice (C57BL/6 and Ptpn6+/+S100a8-cre, Fig. 3D).

Because LFA-1 adhesiveness is regulated by integrin affinity and avidity (clustering), we investigated LFA-1 clustering in the next step. To this end, a fluorescently labeled LFA-1 Ab was injected i.v., and avidity (clustering), we investigated LFA-1 clustering in the next step.

**Elimination of Shp1 in neutrophils abolishes intravascular crawling and migration**

The β2-integrin Mac-1 has a critical role in intravascular crawling of leukocytes (32). To investigate the role of Shpl in this step, we performed intravital microscopy of the cremaster muscle after stimulation with the chemokine CXCL2. During CXCL2 stimulation, leukocyte adhesion was observed in WT and Ptpn6−/− mice. In Ptpn6−/− mice, the percentage of adherent cells that crawled was reduced significantly compared with WT mice (Fig. 4A). Furthermore, Ptpn6−/− leukocytes that crawled had a reduced crawling velocity (Fig. 4B) and distance (Ptpn6−/−: 14.9 μm; WT, 26.8 μm, Fig. 4C, 4D) compared with WT leukocytes. Representative images of adherent neutrophils within postcapillary venules of WT (upper panels) and Ptpn6−/− mice (lower panels) during CXCL2 superfusion are shown in Fig. 4D.

To further examine the role of Shp1 in neutrophil migration, we investigated whether Shp1 is also involved in the transmigration step of the leukocyte-adhesion cascade. To assess this, we used an in vitro transmigration assay. In comparison with WT neutrophils, almost no Ptpn6−/− neutrophils transmigrated through the bend.5 cell layer toward CXCL1 (Fig. 4E).

Furthermore, we investigated whether Shp1 is involved in chemotaxis by using an in vitro assay (Fig. 4F–J). WT neutrophils, adherent on fibronectin, were able to migrate along a CXCL1 gradient (Fig. 4F). In contrast, Shp1-deficient neutrophils were unable to migrate along the chemotactic gradient and adhered at the starting point (Fig. 4G). Migration velocity (Fig. 4H), forward migration index (Fig. 4I), and migration distance (Fig. 4J) of Shp1-deficient neutrophils were reduced significantly compared with WT neutrophils (Fig. 4H–J). Together, these data indicate that Shp1 also plays a piv...
Crawling, migration, and transmigration are disturbed in \( \text{Ptn6}^{\text{-deficient}} \) neutrophils. Percentage of crawling cells (A), crawling velocity (B), crawled distance (C), and representative images (D) of WT or \( \text{Ptn6}^{\text{-/}} \) neutrophils in postcapillary venules of the cremaster muscle during CXCL2 superfusion (\( n = 4 \)). Data are mean ± SEM. Intravital microscopy to assess intravascular crawling was performed on an upright microscope (Axioskop) with a 40× 0.75 NA saline-immersion objective. (E) Transmigration of WT and \( \text{Ptn6}^{\text{-/}} \) neutrophils along a CXCL1 gradient through a TNF-\( \alpha \)-stimulated bend. Representative plots of an in vitro chemotaxis assay with WT (F) and \( \text{Ptn6}^{\text{-/}} \) (G) neutrophils on fibronectin along a CXCL1 gradient. Velocity (H), forward migration index (I), and distance (J) of neutrophils in migration experiments (\( n = 3 \)). Data are mean ± SEM. *\( p < 0.05 \).
Shp1 negatively regulates neutrophil recruitment

Phosphorylation of PIPKIγ in WT and Ptpn6−/− neutrophils. Stimulation of WT neutrophils with E-selectin or chemokine induced the tyrosine phosphorylation of PIPKIγ, which was enhanced significantly in Ptpn6−/− neutrophils (Fig. 6A, Supplemental Fig. 1I). The phosphorylation of PIPKIγ is not Src family kinase dependent (Fig. 6B, Supplemental Fig. 1J). In summary, the results suggest that Shp1 modulates PtdIns(4,5)P2 levels by regulating PIPKIγ activity in a Src family kinase–independent manner.

To further investigate the role of PIPKIγ in neutrophil recruitment, we performed intravital microscopy of the murine cremaster muscle in PIPKIγ90−/− mice, which lack the 90-kDa isoform of PIPKIγ (29). Two hours after TNF-α injection, the selectin-dependent neutrophil rolling velocity in PIPKIγ90−/− mice (12.0 μm/s) was increased significantly in comparison with WT mice (8.8 μm/s, Fig. 6C). The number of adherent (Fig. 6D) and transmigrated leukocytes (Fig. 6E) in PIPKIγ90−/− mice was decreased compared with WT mice. These data indicate an involvement of PIPKIγ in the selectin- and chemokine-triggered signaling pathways in neutrophils during the recruitment cascade.

Because we demonstrated that the activity of PIPKIγ is regulated by Shp1 following selectin and chemokine stimulation, we next investigated whether these two molecules are involved in the same signaling pathway regulating neutrophil recruitment in vivo. Therefore, we performed intravital microscopy of the murine cremaster muscle following TNF-α injection in PIPKIγ90−/− mice, as well as WT mice pretreated with a Shp inhibitor (NSC87877) (Fig. 6F–H). As expected, the rolling velocity of inhibitor-treated WT neutrophils was decreased in comparison with control neutrophils (Fig. 6F). Although the selectin-dependent rolling velocity was increased in PIPKIγ90−/− neutrophils, treatment with the Shp inhibitor had no effect on these cells. Similar results were seen with regard to the number of adherent and transmigrated cells: treatment of WT mice with the Shp inhibitor recapitulated the Ptpn6−/− phenotype of increased neutrophil adhesion and reduced transmigration, whereas treatment of PIPKIγ90−/− mice had no effect on the poor adhesion and transmigration of these neutrophils (Fig. 6G, 6H). Altogether, the data suggest that the phosphatase Shp1 is involved in the regulation of neutrophil recruitment and that Shp1 and PIPKIγ are located in the same signaling pathways.

Discussion

The function of Shp1 in the regulation of integrin adhesiveness and leukocyte recruitment has not previously been appreciated. We demonstrated that stimulation of neutrophils with selectins or chemokines deactivates the activity of Shp1, leading to integrin over-activation and an altered neutrophil recruitment. Our studies were initiated by the unexpected finding that neutrophil adhesion in gene-deficient mice is enhanced, whereas the number of extravasated neutrophils is reduced. This is in line with reduced intravascular crawling, transmigration capacity, and chemotaxis in Ptpn6-deficient neutrophils. A tight regulation of integrin adhesiveness is essential for the different steps of the leukocyte-recruitment cascade; however, in the absence of Shp1, β2-integrin adhesiveness was enhanced after stimulation with chemokines or selectins, explaining the observed phenotype. We demonstrated that Shp1 interacts with PIPKIγ and regulates the activity of PIPKIγ in a Src family kinase–independent fashion. The regulation of PIPKIγ by Shp1 led to an increased production of PtdIns(4,5)P2 in Ptpn6-deficient neutrophils.

Although inflammatory reactions are beneficial and necessary for host defense, they need to be balanced and controlled to prevent harmful consequences and tissue destruction. Different signaling pathways exist that ensure rapid and efficient integrin activation on leukocytes; however, different endogenous mechanisms counteract and balance integrin activation, thereby limiting leukocyte recruit-
ment and the extent of inflammation (40, 45–47). Different protein tyrosine kinases and phosphatases are required for initiating and limiting downstream signaling following cellular stimulation. In neutrophils, PIR-B was found to be constitutively phosphorylated and associated with Shp1 (48). Cellular stimulation results in transiently diminished phosphorylation of PIR-B and reduced association of the molecule with Shp1, allowing maximization of downstream signaling events (48). In this article, we show that Shp1 activity, assessed by Ser591 phosphorylation, is also transiently reduced following chemokine or selectin stimulation. The serine/threonine kinase that phosphorylates Shp1, allowing maximization of downstream signaling events (48). In this article, we show that Shp1 activity, assessed by Ser591 phosphorylation, is also transiently reduced following chemokine or selectin stimulation. The serine/threonine kinase that phosphorylates Shp1 on Ser591 is unknown, but it may be regulated by other signaling molecules involved in neutrophil activation. A recently published study suggests that the GTPase Rap1b is involved in the regulation of Shp1 activity. Kumar et al. (34) found that stimulation of WT neutrophils and Shp1-deficient neutrophils. The interaction of talin with Shp1, as well as the nature of this interaction, following phosphorylation of Tyr644 (50). Talin is required for inducing LFA-1 extension to the intermediate-affinity state, which is selectin dependent, as well as the high-affinity conformation, which is chemokine dependent (9). The increased phosphorylation of PIPKIγ leads to an increased interaction with talin, as well as the nature of this interaction, following phosphorylation of Tyr644 (50). Talin is required for inducing LFA-1 extension to the intermediate-affinity state, which is selectin dependent, as well as the high-affinity conformation, which is chemokine dependent (9).

The increased phosphorylation of PIPKIγ leads to an increased interaction with talin; this may explain the increased integrin activity in Shp1-deficient neutrophils. Additionally, the interaction between PIPKIγ and talin targets PIPKIγ to focal adhesions (FAs) (51), leading to an increased PtdIns(4,5)P2 concentration at FAs in Shp1-deficient neutrophils. The interaction of talin with β2-integrins in FAs is strengthened by PtdIns(4,5)P2 (52).

The increased PIPKIγ activity in Shp1-deficient neutrophils leads to increased accumulation of PtdIns(4,5)P2, which subsequently upregulates integrin affinity and avidity, explaining the hyperadhesive phenotype of Ptpn6−/− neutrophils. PtdIns(4,5)P2 accumulation on the plasma membrane promotes the detachment of α-actinin-1 from the cytosplasmic tail of the integrin, which allows attachment of PtdIns(4,5)P2-activated talin-1, leading to an increased integrin affinity

FIGURE 6. Increased PIPKIγ activity affects different steps of the leukocyte-recruitment cascade. Representative Western blots of immunoprecipitations of PIPKIγ in unstimulated and E-selectin− or CXCL1-stimulated WT and Ptpn6−/− neutrophils showing the phosphorylation of PIPKIγ in the absence (A and B) and presence (C-H) of a Src family kinase inhibitor. (C-H) Intravital microscopy of postcapillary venules in the murine cremaster muscle 2 h after intrascrotal TNF-α injection. Neutrophil rolling velocities (C and F), number of adherent cells (D and G), and emigrated cells (E and H) from WT mice (filled bars) and PIPKIγ−/− mice (open bars) that were left untreated (C–E) or pretreated with the Shp inhibitor (F–H) (n = 3). Intravital microscopy was performed on an upright microscope (Axioskop) with a 40× 0.75 NA saline-immersion objective. Data in the bar graph in (C) are mean ± SEM. Adherent cells/mm² (D and G) and the number of extravasated cells in 1.5 × 10⁴ mm² tissue area surrounding postcapillary venules (E and H) (n = 3). *p < 0.05.
and avidity. Moreover, PIPKIγ directly interacts with the head domain of talin-1 (53), which is an antiparallel homodimer (54), thus leaving one head domain free to bind integrins. Interestingly, PIPKIγ was implicated in conformer-specific regulation of LFA-1 affinity in lymphocytes. Thus, it is through these mechanisms that the increased PIPKIγ activity in Shp1-deficient neutrophils leads to an increased extended and high-affinity conformation of LFA-1 that is triggered by selectins and chemokines, respectively. P3K–PtdIns(3,4,5)P3–Akt signaling pathways are involved in selectin-mediated slow leukocyte rolling, postadhesion strengthening, and chemotaxis, because deletion of P3K in leukocytes abrogates these functions (55–57). Because Shp1-deficient neutrophils are hyperadhesive, it is tempting to speculate that PtdIns(3,4,5)P3 levels in these cells are elevated. The fact that either underactivation of PI3K–PtdIns(3,4,5)P3–Akt signaling, is increased and PtdIns(4,5)P2 levels [90] are limiting elements of a biochemical pathway regulating BCR signaling and selection. Immunity 8: 497–508.


