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Loss of SLP-76 Expression within Myeloid Cells Confers Resistance to Neutrophil-Mediated Tissue Damage while Maintaining Effective Bacterial Killing[1]

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The Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) is an adaptor molecule critical for immunoreceptor and integrin signaling in multiple hemopoietic lineages. We showed previously that SLP-76 is required for neutrophil function in vitro, including integrin-induced adhesion and production of reactive oxygen intermediates, and to a lesser extent, FcγR-induced calcium flux and reactive oxygen intermediate production. It has been difficult to determine whether SLP-76 regulates neutrophil responses in vivo, because *Slp-76*$^{-/-}$ mice exhibit marked defects in thymocyte and vascular development, as well as platelet and mast cell function. To circumvent these issues, we generated mice with targeted loss of SLP-76 expression within myeloid cells. Neutrophils obtained from these animals failed to respond to integrin activation in vitro, similar to *Slp-76*$^{-/-}$ cells. Despite these abnormalities, SLP-76-deficient neutrophils migrated normally in vivo in response to *Staphylococcus aureus* infection and efficiently cleared micro-organisms. Interestingly, SLP-76-deficient neutrophils did not induce a robust inflammatory response in the localized Shwartzman reaction. Collectively, these data suggest that disruption of integrin signaling via loss of SLP-76 expression differentially impairs neutrophil functions in vivo, with preservation of migration and killing of *S. aureus* but reduction in LPS-induced tissue damage and vascular injury. *The Journal of Immunology,* **2007, 178: 4606–4614.**

Neutrophils play a critical role in the host defense against bacterial pathogens. Comprising up to 80% of circulating leukocytes in humans, these cells migrate quickly to sites of infection, where they contain and eliminate invading micro-organisms through phagocytosis, production of reactive oxygen intermediates (ROI),[3] and release of granule contents. An effective immune response requires that neutrophils are plentiful, readily responsive, and potent. However, such highly reactive cells can be detrimental to host tissues, if their functions are not tightly controlled. This destructive potential is exemplified in humans by conditions characterized by neutrophil-mediated tissue pathology, including endotoxic shock (1), inflammatory arthritis (2), inflammatory bowel disease (3), and immune complex (IC)-mediated disorders (4). Animal models have further highlighted the importance of neutrophils in the pathogenesis of these diseases (2, 4–7).

Given the adverse consequences of neutrophil hyper- or hyporesponsiveness, it is not surprising that these cells use complementary regulatory mechanisms to maintain a balance between quiescence and activation. Integrins and FcγR for Ig are two receptor families that play critical roles in the regulation of neutrophil function. Integrins mediate cell-cell and cell-matrix attachment by binding to a variety of adhesion molecules. Circulating leukocytes constitutively express high levels of β_2 integrins and low levels of β_1 and β_3 integrins (8). β_1 and β_2 integrin expression is up-regulated upon neutrophil activation and emigration from the vasculature (9). β_2 integrins play a dominant role in neutrophil recruitment to sites of tissue injury and infection by promoting firm adhesion to molecules expressed on the endothelium, such as ICAM-1 and -2 and fibrinogen, and initiation of transendothelial migration (10–12). Subsequently, collagen-binding β_1 integrins facilitate neutrophil movement through the vessel basement membrane and interstitial tissues (8). Neutrophils express several members of the FcγR family, including receptors with stimulatory (FcγRI, FcγRIII, FcγRIV) or inhibitory (FcγRII) capability (13). These receptors bind Ab-opsonized bacteria and ICs and facilitate their internalization. Engagement of integrins and FcγR leads to activation of the Src and Syk family tyrosine kinases, which initiate intracellular signaling, including mobilization of calcium stores, activation of phospholipase Cγ (PLCγ) and PI3K, and activation of the Ras-MAPK pathway (14). It is less well understood how these proximal signals lead to more distal activation events in neutrophils.

The Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) is an adaptor molecule expressed throughout hemopoietic lineages. SLP-76 is comprised of several protein interaction domains that allow SLP-76 to act as a molecular scaffold to support the assembly of multiprotein signaling complexes

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[3] Abbreviations used in this paper: ROI, reactive oxygen intermediate; IC, immune complex; PLCγ, phospholipase Cγ; SLP-76, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa; GPVI, glycoprotein VI; PSA, passive systemic anaphylaxis; LSR, localized Shwartzman reaction; WT, wild type; HSA, human serum albumin; PMN, polymorphonuclear neutrophil; BMMC, bone marrow-derived mast cell; BMM, bone marrow-derived macrophage.

downstream of tyrosine kinase-associated immunoreceptors in T cells, mast cells, and platelets (15). Studies in cell lines and animals have highlighted the importance of SLP-76 for cell function. For example, SLP-76-deficient Jurkat T cells exhibit severe defects in TCR signaling, which in the *Slp-76*$^{-/-}$ mouse is reflected by a lack of peripheral T cells due to a complete block in thymocyte development (16, 17). Similarly, *Slp-76*$^{-/-}$ mast cells and platelets exhibit defects in FcεR and glycoprotein VI (GPVI) receptor signaling, respectively, which result in resistance to IgE-induced passive systemic anaphylaxis (PSA) and defective platelet aggregation (18–20). The role of SLP-76 in integrin signaling has been less well characterized; however, several studies indicate that SLP-76 is important for $\alpha_{IIb}\beta_3$-mediated platelet spreading (19, 21, 22).

Previously, we observed that SLP-76 is required in neutrophils for integrin-dependent ROI production and spreading, as well as for optimal FcγR-induced ROI production and calcium flux (23). These in vitro defects suggested that SLP-76 might also be important for neutrophil function in vivo. However, it has not been feasible to test this question directly, given the hemopoietic and vascular phenotypes of *Slp-76*$^{-/-}$ mice, which lead to perinatal lethality and circulatory abnormalities in surviving animals (24). In the current investigation, we used cre-lox technology to generate animals with myeloid lineage-specific loss of SLP-76 expression. To determine whether SLP-76 contributes to neutrophil activation during the physiological response to bacterial invasion or pathological inflammation, we examined two animal models of disease: bacterial killing within a s.c. abscess and the localized Shwartzman reaction (LSR). Prior studies have shown that these processes rely on functional neutrophils for the resolution or initiation/propagation of pathology, respectively (6, 25, 26).

We find that SLP-76-deficient neutrophils migrate normally into inflamed or bacterially infected tissues and kill *Staphylococcus aureus* in a manner comparable to wild-type (WT) cells. In contrast, mice lacking SLP-76 expression within myeloid cells are more resistant than WT mice to neutrophil-mediated vascular damage in the LSR. These data suggest that the selective loss of SLP-76 expression in myeloid cells differentially impacts upon neutrophil functions in vivo. Specifically, pathways of activation appear sufficiently redundant to allow for normal migration and killing of *S. aureus*. In contrast, pathological tissue inflammation is diminished, most likely due to reduced integrin-induced neutrophil functions (27).

Materials and Methods

Antibodies

Sheep antiserum directed against murine SLP-76 was generated as previously described (16). Anti-actin Abs were obtained from Santa Cruz Biotechnology; anti-Gr-1, Mac-1, FcεR, CD18, CD62P, and CD49b Abs were obtained from BD Pharmingen; and anti-human albumin Abs were obtained from Sigma-Aldrich. All other Abs were obtained from Cell Signaling Technology.

Mice

Mice harboring an allele of *Slp-76* containing 3 loxP sites and a neomycin resistance cassette (*Slp-76*^{3lox}) have been described previously (28). *Slp-76*$^{3lox/+}$ mice were bred with protamine-1-Cre transgenic mice (29) to obtain progeny with two loxP sites flanking exon 3 of *Slp-76*, but lacking the neomycin cassette (*Slp-76*$^{2lox/+}$). In this study, *Slp-76*^{2lox} is termed *Slp-76*^{F}. *Slp-76*$^{F/F}$ mice were crossed with animals in which the Cre recombinase was inserted into the lysozyme M (*LysM*) genomic locus to generate progeny of genotype *Slp-76*$^{F/F}$ *LysM*^{Cre} (30). *Slp-76*$^{F/F}$ *LysM*$^{Cre/Cre}$ mice were mated with *Slp-76*$^{+/-}$ mice, which produced animals of genotypes *Slp-76*$^{F/-}$*LysM*$^{Cre/+}$ and *Slp-76*$^{F/+}$*LysM*$^{Cre/+}$, hereafter denoted *Slp-76*$^{F/-}$*Cre*$^{+}$ or *Slp-76*$^{F/+}$*Cre*$^{+}$. *W*^{sh}/*W*^{sh} and *W/W*^{v} mice were obtained from The Jackson Laboratory. Animal work was done in compliance with the Animal Care and Use Committee at the University of Pennsylvania.

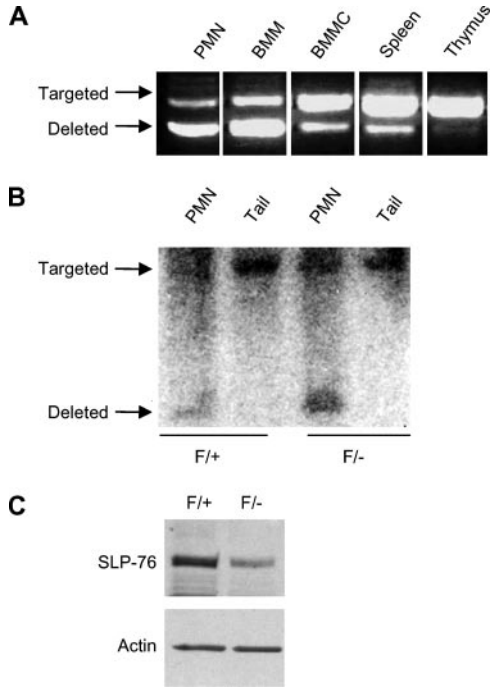

FIGURE 1. *Slp-76* excision is restricted to myeloid lineages in *Slp-76*$^{F/+}$*Cre*$^{+}$ and *Slp-76*$^{F/-}$*Cre*$^{+}$ mice. *A*, *Slp-76* gene excision was assessed by PCR using DNA isolated from neutrophils (PMN), BMM, BMMC, splenocytes, and thymocytes from *Slp-76*$^{F/-}$*Cre*$^{+}$ mice. These samples were run on the same gel; irrelevant lanes have been removed for clarity of presentation. *B*, Southern blot analysis using DNA isolated from neutrophils and tail snips from *Slp-76*$^{F/+}$*Cre*$^{+}$ (F/+) and *Slp-76*$^{F/-}$*Cre*$^{+}$(F/−) mice demonstrates deletion only within neutrophils. *C*, Neutrophils from the same mice shown in *B* were evaluated for SLP-76 and actin protein expression by Western blot. The data shown are representative of three separate experiments.

Isolation of bone marrow neutrophils

Bone marrow neutrophils were isolated as described previously (23). Cell preparations contained >90% neutrophils, as determined by expression of Gr-1 by flow cytometry.

Western blotting

Neutrophil integrin activation was induced on poly-RGD-coated dishes, and cells were lysed as described previously (23). Western blotting was performed using standard protocols.

Neutrophil adherent respiratory burst

Ninety-six-well Immulon 4 HBX plates (Thermo Labsystems) were coated with 150 μg/ml fibrinogen (Sigma-Aldrich) in PBS overnight at 4°C. One hundred microliters of prewarmed neutrophils suspended at 4×10^6 cells/ml in HBSS with 10 mM HEPES, 100 mM ferricytochrome C (Sigma-Aldrich), and 1 mM Ca^{2+} were added to wells containing 1.5 mM Mg^{2+} and 50 ng/ml murine TNF-α (PeproTech) or 100 nM PMA (Sigma-Aldrich). Respiratory burst was calculated through measurement of changes in ferricytochrome C absorbance as described previously (31).

Neutrophil degranulation

For integrin stimulation, 96-well Immulon 4 HBX flat-bottom plates were coated with 150 μg/ml fibrinogen in PBS or 50 μg/ml Chrono-par collagen (Chrono-Log) in PBS overnight at 4°C. For IC stimulation, wells were coated with human serum albumin (HSA; 20 μg/ml; Sigma-Aldrich) in PBS overnight at 4°C. The following day, plates were blocked with PBS containing 2% FCS for 30 min at room temperature and incubated with a 1/200 dilution of rabbit anti-HSA Ab in PBS containing 2% FCS for 1 h at room temperature. As a negative control, wells were blocked and coated with anti-HSA Ab alone (control-IC). Wells were washed three times with PBS before assay. One hundred microliters of prewarmed polymorphonuclear neutrophils (PMN) suspended at 3×10^6 cells/ml in HBSS with

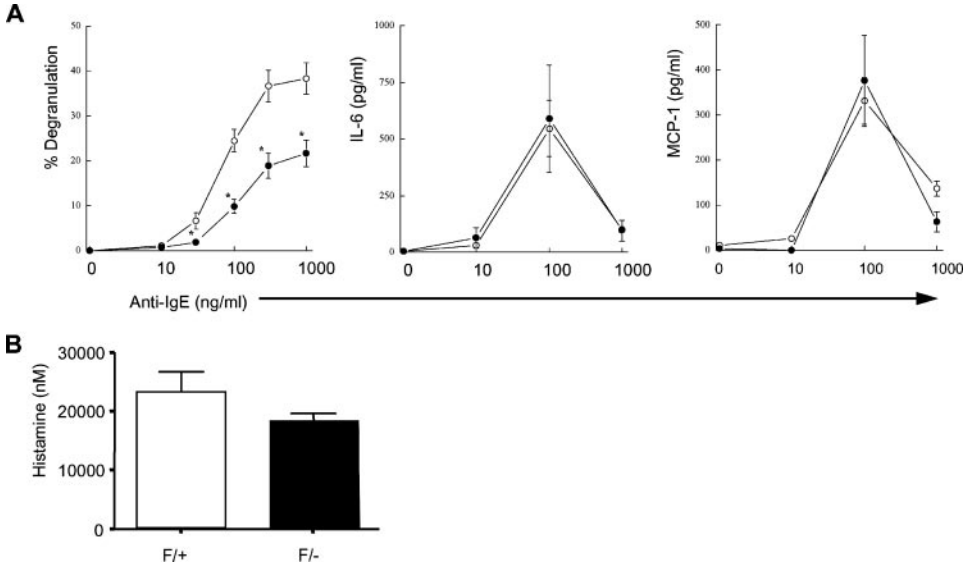

FIGURE 2. *Slp-76*$^{F/-}$*Cre*$^{+}$ mice demonstrate selective defects in mast cell function. BMMC from *Slp-76*$^{F/+}$*Cre*$^{+}$ (○) and *Slp-76*$^{F/-}$*Cre*$^{+}$ (●) mice were sensitized with IgE (1 μg/ml) for 4 h followed by stimulation with the indicated concentrations of anti-IgE Ab. One hour later, β-hexosaminidase activity in the culture supernatants was determined as a measure of degranulation (*A*, *left panel*). ∗, Significance of $p < 0.05$ compared with *Slp-76*$^{F/+}$*Cre*$^{+}$ controls. Twenty-four hours later, IL-6 (*A*, *middle panel*) and MCP-1 (*A*, *right panel*) release was determined by ELISA. Results are expressed as mean ± SEM using mast cells from four mice/genotype. One representative experiment of four independent experiments is shown. *B*, *Slp-76*$^{F/+}$*Cre*$^{+}$ (F/+; $n = 8$) and *Slp-76*$^{F/-}$*Cre*$^{+}$ (F/−; $n = 9$) mice were injected i.v. with 5 μg of anti-DNP IgE. Twenty-four hours later, mice were challenged with 100 μg of HSA-DNP i.v. and bled after 90 s. Plasma histamine concentrations were determined by immunoassay. Results are expressed as mean plasma histamine concentration ± SEM.

10 mM HEPES and 1 mM $CaCl_2$ were added to wells containing a final concentration of 1.5 mM $MgCl_2$ and 50 ng/ml murine TNF-α or 1 μM PMA. After incubation at 37°C for 30 min in a humidified chamber, cells were pelleted in ice-cold 96-well polypropylene plates and supernatant was collected to measure lactoferrin by ELISA (32).

Generation of a s.c. abscess

An air pouch was created by injecting 5 ml of air s.c. on the dorsum of an anesthetized mouse (day 0). Pouches were reinflated with 2.5 ml of air on day 3. On day 5, 5×10^7 *S. aureus* (clinical isolate) in the early log phase of growth were washed, resuspended in PBS, and injected into the pouch. At varying time points after injection, mice were euthanized, pouches lavaged with 5 ml of PBS, and kidneys excised. Lavage fluid and kidney homogenate were lysed in cold PBS with 0.1% Triton X-100, and serial dilutions were plated on brain-heart infusion agar plates to determine bacterial CFU. Immune cells within the fluid were counted, and receptor expression was evaluated using flow cytometry.

Induction of the LSR

To generate the LSR, 10 μg of LPS (*Escherichia coli* E157:B8; Sigma-Aldrich) in 20 μl of PBS with 2% FCS were injected intradermally into the left ear using a 25-μl Hamilton syringe (Hamilton). Twenty microliters of PBS/2% FCS were injected into the right ear as a negative control. Twenty hours later, animals were injected i.p. with LPS (150 μg in 200 μl of PBS). Five hours after the systemic injection, mice were sacrificed and the ears removed, measured for thickness, weighed, and fixed in 4% paraformaldehyde for histology. Samples were stained with H&E or toluidine blue to identify mast cells. In separate experiments, mice received 200 μl of 1% Evan's blue (Sigma-Aldrich) in PBS via retro-orbital injection along with the i.p. LPS injection. Five hours later, ears were removed, and the Evan's blue dye was extracted by incubation of ears in 1 ml of formamide for 48 h at 55°C. OD of the ear extract was measured at 610 nm to quantitate Evan's blue dye accumulation.

Measurement of mast cell FcεR-induced degranulation and cytokine production

Murine bone marrow-derived mast cells (BMMC) were generated as described previously (33) and studied after being in culture for 6–8 wk. Mast cells were incubated with anti-DNP IgE (Sigma-Aldrich) for 2–4 h in Tyrode's buffer (130 mM NaCl, 10 mM HEPES, 1 mM $MgCl_2$, 5 mM KCl, 1.4 mM $CaCl_2$, 5.6 mM glucose, and 1 mg/ml BSA (pH 7.4)). Unbound IgE was removed by washing in cold PBS, and cells were stimulated with anti-IgE for 1 and 24 h to measure degranulation and IL-6/MCP-1 release, respectively. To quantitate degranulation, β-hexosaminidase release into cell-free supernatants was measured by a colorimetric assay using the substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide as described previously (33). The percentage of specific release was determined using the following formula: percentage of specific release = (sample release − spontaneous release)/(total release − spontaneous release) × 100, where spontaneous release and total release were the amount of β-hexosaminidase released from unstimulated cells and cell lysates (1% Triton X-100), respectively. Mast cell secretion of IL-6 and MCP-1 were measured in cell-free supernatants using commercial ELISA kits (BD Biosciences).

Passive systemic anaphylaxis

Mice were injected i.v. with 5 μg of anti-DNP IgE in 200 μl of PBS. After 24 h, mice were i.v. injected with 100 μg of DNP-coupled HSA (DNP-HSA; Sigma-Aldrich) in 200 μl of PBS. Ninety seconds later, the mice were bled and plasma histamine concentration was determined using a commercial competitive immunoassay (Beckman Coulter).

Isolation and stimulation of mouse platelets

Whole blood was drawn into syringes containing heparin followed by a 2-fold dilution in platelet Tyrode's buffer (137 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 0.1% BSA, 0.1% glucose, and 5 mM HEPES (pH 7.4)). Platelet-rich preparations were prepared by centrifugation at 960 rpm for 8 min. For aggregation studies, platelets were resuspended at a concentration of 2×10^8/ml in Tyrode's buffer. Aggregation tests were conducted in a Born aggregometer (ChronoLog) following stimulation with 3–10 μg/ml collagen and 0.4–1 nM of the GPVI agonist convulxin (Alexis Biochemicals). For degranulation studies, 1×10^6 platelets were stimulated with 0.2 nM or 1 nM convulxin or 0.5 mM AYPGKF peptide (AYP; University of Pennsylvania Protein Core facility) in the presence of anti-CD62P and anti-CD49b Abs for 20 min at 37°C. P selectin surface expression was quantified by flow cytometry.

Statistical analyses

Studies of mast cell function, including in vitro degranulation and in vivo histamine release, were analyzed using the ANOVA (Excel software). Neutrophil functional studies, including in vitro degranulation and ROI production, and in vivo inflammatory responses were analyzed using a Student's unpaired *t* test, according to the GraphPad Prism 4 software package (GraphPad).

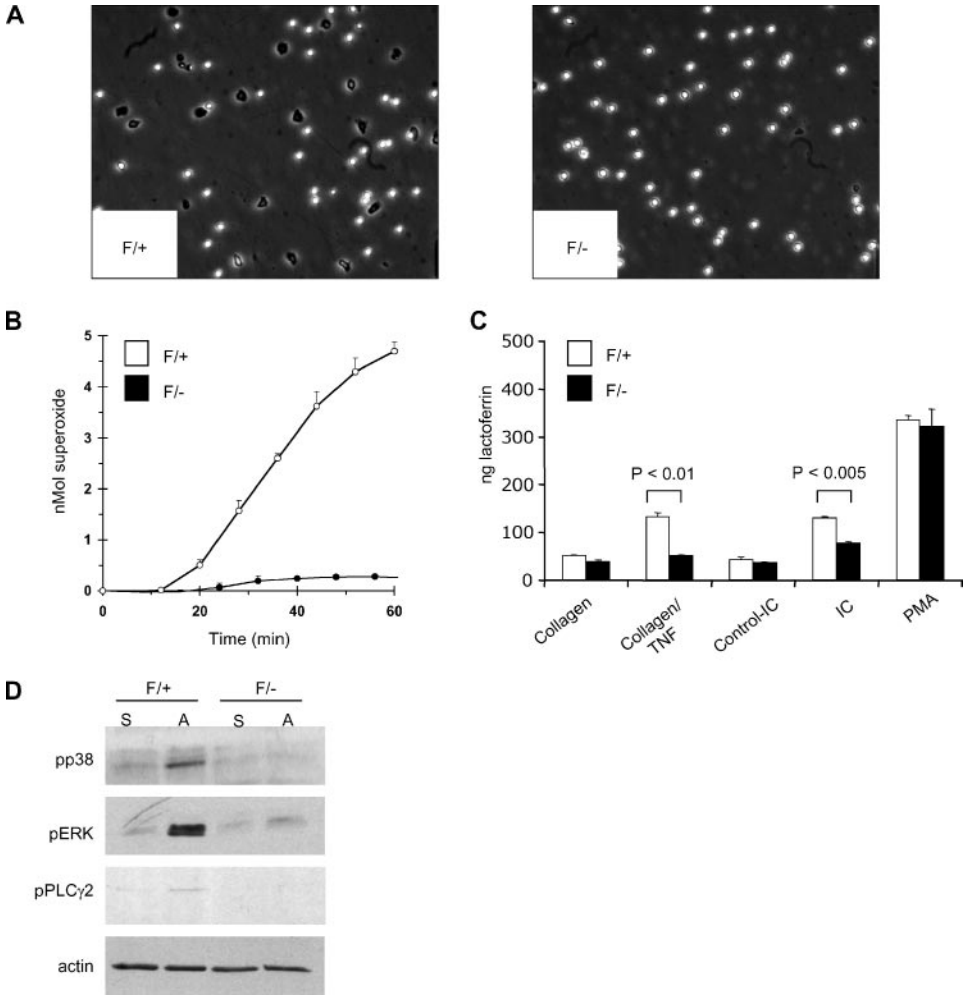

FIGURE 3. *Slp-76*$^{F/-}$*Cre*$^{+}$ neutrophils display defects in integrin-dependent activation. *A*, *Slp-76*$^{F/+}$*Cre*$^{+}$ (F/+) and *Slp-76*$^{F/-Cre+}$ (F/−) neutrophils were plated onto slides coated with poly-RGD and viewed by phase contrast microscopy after 30 min at 37°C. Spread cells (dark, flattened) were distinguished from nonspread cells (round, bright) based on morphology. *B*, ROI production by purified neutrophils was measured spectrophotometrically based on fibrinogen-induced ferricytochrome C oxidation. Results are presented as the average nanomole of superoxide produced/million neutrophils ± SD; $n = 2$ mice/genotype. *C*, Adhesion-dependent degranulation was assessed by plating neutrophils in wells coated with collagen in the presence or absence of TNF-α (labeled as Collagen and Collagen/TNF, respectively), or in wells coated with anti-HSA Abs or HSA/anti-HSA ICs (labeled Control-IC and IC, respectively). Cells were stimulated with PMA as a positive control. Lactoferrin release into the supernatants was measured by ELISA. Results are presented as the average nanogram per milliliter of lactoferrin ± SD; $n = 2$ mice/genotype. *D*, *Slp-76*$^{F/+}$*Cre*$^{+}$ and *Slp-76*$^{F/-}$*Cre*$^{+}$ neutrophils were plated onto poly-RGD-coated plates (adherent; A) or left in suspension (S). At 15 min, cells were lysed, and phosphorylation of the signaling molecules p38, ERK1/2, and PLCγ was evaluated by Western blotting of whole cell lysates. The data presented are representative of three separate experiments.

Results

Generation of mice with myeloid-specific reduction of SLP-76 expression

To investigate the role of SLP-76 during in vivo neutrophil function, we generated mice in which exon 3 of *Slp-76* is flanked by loxP sites (*Slp-76*^{F}) (28). *Slp-76*$^{F/F}$ mice were crossed with animals expressing the Cre recombinase downstream of the endogenous *LysM* promoter, which has produced deletion of floxed alleles at frequencies of 83–98% in macrophages and up to 100% in granulocytes (30, 34). *Slp-76*$^{-/-}$ mice exhibit significant perinatal mortality due to failed vascular-lymphatic separation (24). In contrast, *Slp-76*$^{F/-}$*Cre*$^{+}$ and *Slp-76*$^{F/F}$ *Cre*$^{+}$ mice were born at Mendelian ratios and displayed normal vascular morphology (data not shown).

PCR and Southern blot analysis of genomic DNA isolated from purified bone marrow neutrophils from *Slp-76*$^{F/-}$*Cre*$^{+}$ mice demonstrated 80–90% excision of *Slp-76* (Fig. 1, *A* and *B*). Western blotting of neutrophil lysates confirmed that gene excision was accompanied by a variable but comparable loss of SLP-76 protein expression (Fig. 1*C*). Protein quantification revealed that *Slp-76*$^{F/-}$*Cre*$^{+}$ neutrophils expressed ~80% less SLP-76 than *Slp-76*$^{+/+}$ cells. Specificity of excision was examined by PCR of genomic DNA isolated from *Slp-76*$^{F/-}$*Cre*$^{+}$ bone marrow-derived macrophages (BMM), BMMC, splenocytes, and thymocytes. As anticipated, BMM demonstrated significant deletion of SLP-76, whereas excision in thymus and spleen was minimal but detectable (Fig. 1*A*), likely reflecting the presence of resident tissue macrophages and/or neutrophils. Excision within mast cells has not been reported in *LysM*^{Cre} mice (30, 34–37). Interestingly, expression of *LysM*^{Cre} resulted in ~20% excision within mast cells (Fig. 1*A*), which did not lead to noticeable changes in protein expression by Western blotting (data not shown). *Slp-76*$^{F/-}$*Cre*$^{+}$ animals demonstrated no defects in T cell development or function, or in the number and surface phenotype of neutrophils (data not shown).

Slp-76$^{-/-}$ platelets do not degranulate or aggregate following treatment with convulxin or collagen, which activate cells via the

ITAM-bearing GPVI receptor, indicating that SLP-76 is required to support signaling downstream of this receptor (22). Loss or perturbation of platelet responses could complicate interpretation of in vivo models of neutrophil activation, such as the LSR. Therefore, we also examined whether platelets from *Slp-76*$^{F/+}$*Cre*$^{+}$ and *Slp-76*$^{F/-}$*Cre*$^{+}$ mice were capable of dense granule secretion and aggregation after stimulation with convulxin or collagen. Activation of platelets using these agonists resulted in similar granule release and aggregation from both genotypes of mice, whereas *SLP-76*$^{-/-}$ cells, which were used as a negative control, completely failed to function (data not shown). Thus, we conclude that the *LysM*-Cre transgene is not likely to be expressed in platelets or to perturb their function in response to ITAM receptor or integrin engagement.

Slp-76$^{-/-}$ mast cells also fail to degranulate or release cytokines following FcεR ligation in vitro, and SLP-76-deficient mice are resistant to IgE-mediated PSA (20). To determine whether *Slp-76* gene deletion within a subset of mast cells was associated with functional consequences, we examined in vitro and in vivo mast cell activation. When stimulated via the FcεR, BMMC from *Slp-76*$^{F/-}$*Cre*$^{+}$ animals exhibited a 30–50% reduction in degranulation, as measured by the release of β-hexosaminidase (Fig. 2*A*, *left panel*), but normal secretion of IL-6 (*middle panel*) and MCP-1 (CCL2; *right panel*). In vivo analyses of *Slp-76*$^{F/-}$*Cre*$^{+}$ mice revealed a nonsignificant reduction in histamine release during PSA (Fig. 2*B*). Therefore, *Slp-76* gene deletion within a subset of mast cells differentially influences FcεR-induced functions, with moderate reductions in degranulation but no effect on cytokine or histamine release.

Slp-76$^{F/-}$Cre^{+} *neutrophils phenocopy* Slp-76$^{-/-}$ *neutrophils in vitro*

Slp-76$^{-/-}$ neutrophils display defects in immunoreceptor- and integrin-dependent responses (23). To confirm that targeted deletion of SLP-76 within myeloid lineages resulted in similar cellular dysfunction, we evaluated the in vitro function of neutrophils from *Slp-76*$^{F/-}$*Cre*$^{+}$ mice. Unlike *Slp-76*$^{F/+}$*Cre*$^{+}$ cells, 30–50% of which spread rapidly when settled onto surfaces coated with integrin ligands, only rare SLP-76-deficient neutrophils adhered to or spread on slides coated with poly-RGD, an engineered polymer of the fibronectin-binding motif (Arg-Glu-Asp) that directly stimulates integrins (Fig. 3*A*). To further examine adhesion-dependent neutrophil responses, we plated cells onto integrin ligand-coated dishes in the presence of TNF-α, a proinflammatory cytokine that increases integrin affinity and avidity for ligand binding, and measured ROI production and degranulation. Fibrinogen-induced ROI production (Fig. 3*B*) and collagen-induced degranulation (measured by the release of lactoferrin; Fig. 3*C*) were reduced >90% in *Slp-76*$^{F/-}$*Cre*$^{+}$ cells, indicating that elimination of SLP-76 expression severely compromises β_2 and β_1 integrin functions, respectively. In further support of this notion, SLP-76-deficient cells showed marked reduction in poly-RGD-induced phosphorylation of the signaling mediators ERK1/2, PLCγ2, and p38 (Fig. 3*D*). In contrast, when *Slp-76*$^{F/-}$*Cre*$^{+}$ cells were stimulated via the FcγR using anti-HSA IC, degranulation was significantly diminished but not abolished (Fig. 3*C*), and calcium flux was minimally compromised (data not shown). These data are in agreement with prior results obtained using *Slp-76*$^{-/-}$ neutrophils (23) and indicate that in the absence of SLP-76, alternative mechanisms exist in neutrophils to propagate intracellular signaling events initiated by FcγR engagement.

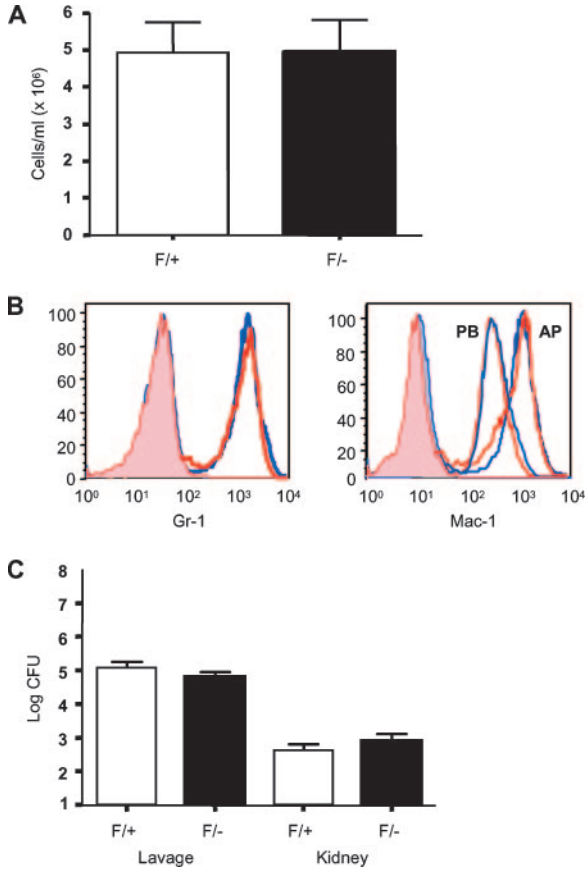

FIGURE 4. *Slp-76*$^{F/-}$*Cre*$^{+}$ mice maintain efficient killing of *S. aureus* in vivo. Ten million S. *aureus* organisms were injected into air pouches raised on the backs of *Slp-76*$^{F/+}$*Cre*$^{+}$ (F/+) and *Slp-76*$^{F/-}$*Cre*$^{+}$ (F/−) mice. Two days later, mice were sacrificed and pouches lavaged with PBS. *A*, Lavaged cells were harvested and enumerated. Cells from *Slp-76*$^{F/+}$*Cre*$^{+}$ (red) or *Slp-76*$^{F/-}$*Cre*$^{+}$ (blue) mice were stained using an Ab recognizing the neutrophil marker Gr-1 (open histograms) or with isotype controls (filled histograms) (*B*, *left panel*). Cells from air pouches (AP) were also stained with Abs recognizing the β_2 integrin Mac-1 (*B*, *right panel*). The level of Mac-1 staining was compared with that observed on resting peripheral blood (PB) neutrophils. To quantify bacterial killing, lavage fluid and kidney homogenates from *Slp-76*$^{F/+}$*Cre*$^{+}$ and *Slp-76*$^{F/-}$*Cre*$^{+}$ mice were plated and bacterial colonies enumerated after 12 h at 37°C (*C*). The results presented in *A* and *C* are the average ± SEM from five experiments. In each experiment, 6 mice of each genotype were examined for a total of 30 mice/genotype.

Slp-76$^{F/-}$Cre^{+} *mice maintain efficient killing of the bacterial strain* S. aureus *in vivo*

Because *Slp-76*$^{F/-}$*Cre*$^{+}$ mice lack the immune and vascular abnormalities that are typical of *Slp-76*$^{-/-}$ animals, we reasoned that these mice could be used to probe the physiological role of SLP-76 during integrin-induced neutrophil activation. We first examined whether SLP-76-deficient neutrophils could migrate into an infected site and kill invading organisms. To address this question, we injected *S. aureus* in early log phase growth into day-5 air pouches raised on the backs of *Slp-76*$^{F/+}$*Cre*$^{+}$ and *Slp-76*$^{F/-}$*Cre*$^{+}$ mice. At varying times after injection, mice were sacrificed and the pouches lavaged to evaluate the number and phenotype of elicited immune cells and the number of remaining bacteria. We found that the kinetics of leukocyte migration into infected air pouches was comparable in *Slp-76*$^{F/-}$*Cre*$^{+}$ mice and control littermates (Fig. 4*A* and data not shown) and that the majority of migrating cells were neutrophils, as demonstrated by expression of the neutrophil

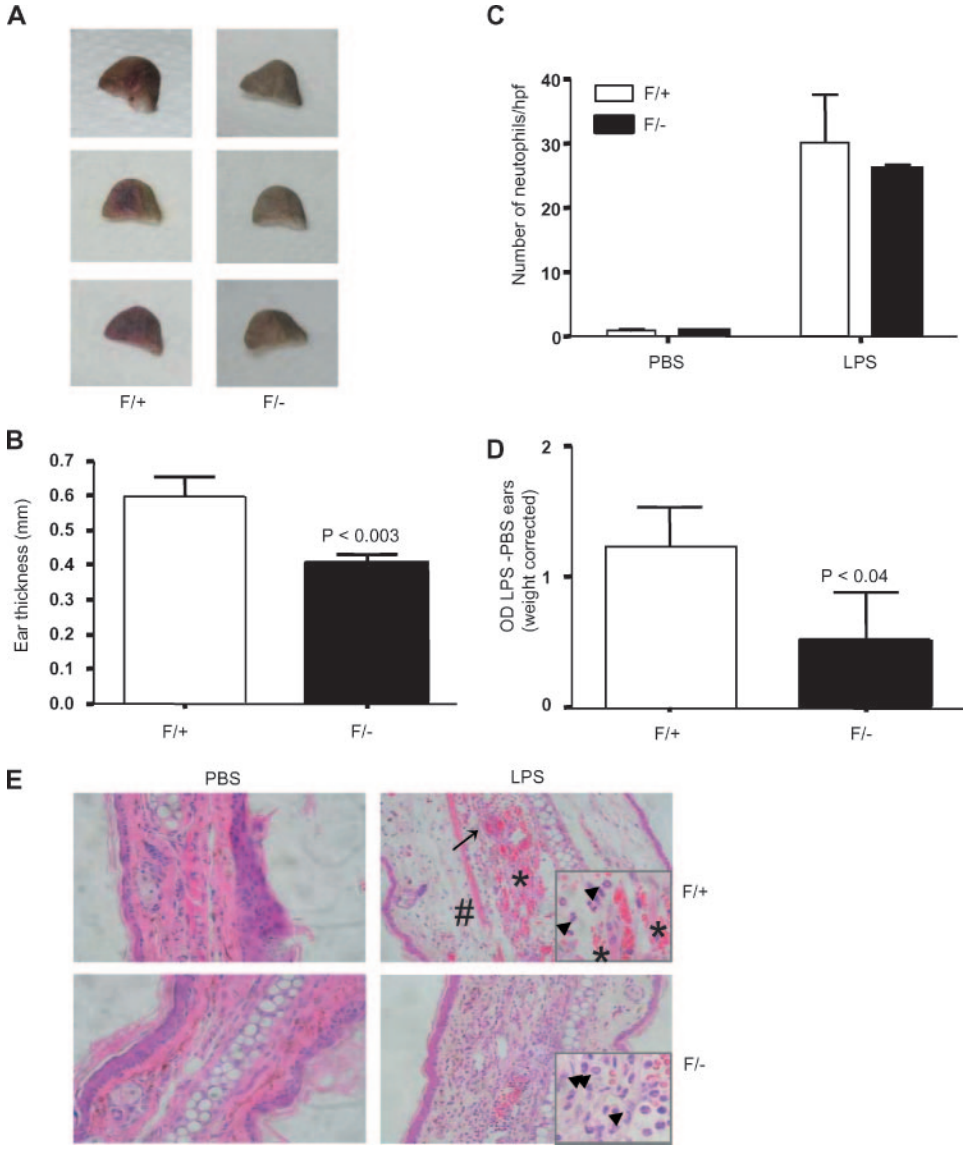

FIGURE 5. Mice lacking SLP-76 expression in neutrophils exhibit reduced inflammation in the LSR. The LSR was induced in *Slp-76*$^{F/+}$*Cre*$^{+}$ (F/+) and *Slp-76*$^{F/-}$*Cre*$^{+}$ (F/−) mice, as described in *Materials and Methods*. *Slp-76*$^{F/-}$*Cre*$^{+}$ mice demonstrated reduced redness (*A*; ears from three representative pairs of mice are shown) and swelling (*B*) in LPS-injected ears. Ear sections from SLP-76$^{F/+}$Cre^{+} (F/+) and SLP-76$^{F/-}$Cre^{+} (F/−) mice were stained with H&E and examined for the number of infiltrating PMN per 40× high-power field (*C*) and (*E*; arrowheads). Ears were also examined for the presence of thrombosis (*E*; arrow), edema (*E*; #), and for presence of hemorrhage (*E*; ∗). Histology was viewed using a Nikon Eclipse E400 microscope and images acquired with a Nikon Coolpix5000 digital camera (Nikon). Vascular permeability was determined by measuring the extravasation of Evan's blue dye into inflamed tissues. The OD of the ear extract was read at 610 nm and represented as OD of LPS ear/weight-OD PBS ear/weight (*D*). The data presented in *B–D* are the average ± SEM of four experiments. In each experiment, 5 mice of each genotype were examined for a total of 20 mice/genotype.

marker Gr-1 on >99% of cells (Fig. 4*B*, *left panel*). Air pouch-infiltrating neutrophils isolated from *Slp-76*$^{F/+}$*Cre*$^{+}$ and *Slp-76*$^{F/-}$*Cre*$^{+}$ mice expressed equivalent levels of the β_2 integrin Mac-1 (Fig. 4*B*, *right panel*, marked as "AP") that was increased ~1 log over resting peripheral blood neutrophils (Fig. 4*B*, *right panel*, "PB"). *Slp-76*$^{F/-}$*Cre*$^{+}$ and *Slp-76*$^{F/+}$*Cre*$^{+}$ mice demonstrated comparable bacterial clearance from infected air pouches, as well as control of bacterial dissemination to the kidneys (Fig. 4*C*). Together, these data suggest that SLP-76 is not required for neutrophil migration or killing of the bacterial strain *S. aureus* in vivo.

Loss of expression of SLP-76 minimizes neutrophil-mediated tissue damage in the LSR

During inflammatory reactions, toxic neutrophil products, such as ROI and granule contents, can inflict significant damage to host tissues. We hypothesized that in the setting of inflammation, the elimination of SLP-76 expression in neutrophils might perturb integrin signaling and thereby lessen neutrophil-mediated tissue damage. The LSR is a reverse-Arthus type reaction in which a local sensitizing injection of LPS is followed by a systemic LPS challenge to induce a neutrophil-dependent microangiopathy characterized by hemorrhage, thrombosis, and vascular damage at the site of the local LPS injection (38, 39). The LSR is dependent on neutrophil β_2 integrin-dependent adhesion, because neutrophil depletion or use of CD18-blocking Abs ameliorates the pathogenic response (26, 38, 40). Furthermore, deficient expression of gp49B1, a negative regulator of integrins, enhances tissue inflammation in the LSR due to basal integrin activation (26). Most recently, Hirahashi et al. (27) demonstrated that the CD18 integrin Mac-1 plays a dominant role in LSR pathogenesis via recognition of the complement component C3 deposited within affected vessel walls. Engagement of Mac-1 on neutrophils triggers a signaling cascade involving the Src family kinase Hck and the Syk tyrosine kinase, which leads to the release of neutrophil elastase and the generation of vasculopathy and thrombosis (27).

Because SLP-76 is known to function downstream of Src and Syk kinases, and SLP-76-deficient neutrophils exhibit defects in response to integrin engagement in vitro, we were interested in evaluating whether loss of SLP-76 in neutrophils would ameliorate in vivo integrin-dependent inflammatory responses. Therefore, the LSR was induced in *Slp-76*$^{F/-}$*Cre*$^{+}$ and *Slp-76*$^{F/+}$*Cre*$^{+}$ mice. Within 5 h, the LPS-injected ears of *Slp-76*$^{F/+}$*Cre*$^{+}$ mice exhibited redness and swelling (Fig. 5, *A* and *B*), findings that were significantly reduced in *Slp-76*$^{F/-}$*Cre*$^{+}$ animals. Blinded evaluation revealed no difference in neutrophil influx into the LPS-injected ears of *Slp-76*$^{F/+}$ or *Slp-76*$^{F/-}$ mice (Fig. 5*C*). To assess vascular damage, we

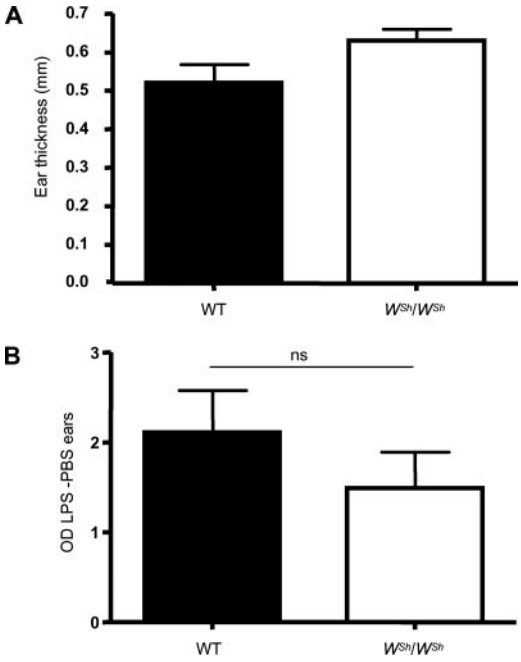

FIGURE 6. Mast cell depletion does not influence development of the LSR. The LSR was induced in W^{Sh}/W^{Sh} mast cell-deficient mice and littermate controls (WT), as described in *Materials and Methods*. Ear thickness (*A*) and vascular permeability (*B*) were measured as in Fig. 4. ns, Not significant. The data presented in *A* and *B* reflect the average ± SEM of four mice/genotype and are representative of three separate experiments.

measured the accumulation of Evan's blue dye into the injected ears of *Slp-76*$^{F/+}$*Cre*$^{+}$ and *Slp-76*$^{F/-}$*Cre*$^{+}$ mice. Evan's blue is normally restricted within the circulation, but it can extravasate under conditions of increased vascular permeability and/or damage. We observed that *Slp-76*$^{F/-}$*Cre*$^{+}$ mice exhibited significantly less Evan's blue accumulation within their ears, compared with their *Slp-76*$^{F/+}$*Cre*$^{+}$ counterparts (Fig. 5*D*). On histological examination, there was less extravasation of RBC and edema in *Slp-76*$^{F/-}$*Cre*$^{+}$ mice (Fig. 5*E*), further confirming that there is reduced vascular injury in the absence of SLP-76. Together, these data are consistent with those reported by Hirahashi et al. (27) and support the notion that the elimination of SLP-76 expression does not affect neutrophil migration in vivo. However, absence of SLP-76 lessens neutrophil-induced tissue and vascular damage in a model of LPS-induced acute inflammation, most likely by reducing integrin-induced release of granule constituents, such as elastase.

Tissue-resident mast cells are present in the ear and express TLR4, the surface receptor for LPS. Because BMMC from *Slp-76*$^{F/+}$*Cre*$^{+}$ mice exhibited mild defects in IgE-mediated degranulation in vitro (Fig. 2*A*), we assessed whether mast cells participate in induction of the LSR and whether defects in mast cell function could be one of the factors contributing to the reduced tissue inflammation observed in *Slp-76*$^{F/-}$*Cre*$^{+}$ mice. Therefore, we induced the LSR in WT animals and in W^{Sh}/W^{Sh} mice, which have markedly reduced numbers of mast cells. We found no significant difference in hemorrhage (data not shown), ear swelling (Fig. 6*A*), or vascular permeability (Fig. 6*B*) following induction of the LSR in either genotype of mice. Similar results were obtained using W/W^{v} mice, another mast cell-deficient mouse strain (data not shown). These data indicate that the resistance of *Slp-76*$^{F/-}$*Cre*$^{+}$ mice to LPS-induced tissue damage is due to deficits in neutrophil rather than mast cell function.

Discussion

Neutrophil integrins are adhesive molecules that also act as receptors transducing activating signals during adhesion-dependent inflammatory responses. β_2 integrins, such as Mac-1 ($\alpha_M\beta_2$), are abundantly expressed on neutrophils and interact with ligands, including ICAM-1, fibrinogen, the yeast product zymosan, and complement-coated bacteria. Integrin engagement results in cell adhesion, cytoskeletal reorganization, phagocytosis, respiratory burst, release of granule contents, and cytokine production (41). Although these processes are critical for host defense, they can impart to the activated neutrophil the potential to damage host tissues. Thus, it is imperative that integrin-mediated neutrophil functions remain tightly regulated.

We previously showed that SLP-76 is necessary for integrin-induced neutrophil activation in vitro. To study the physiological role of SLP-76 in neutrophils, we generated a mouse strain that lacks SLP-76 selectively within the myeloid compartment by driving Cre-mediated excision of the *Slp-76* gene via the *LysM* promoter. Despite residual SLP-76 expression in a minority of neutrophils, *Slp-76*$^{F/-}$*Cre*$^{+}$ cells recapitulated the in vitro functional defects previously observed using *Slp-76*$^{-/-}$ neutrophils. To further determine whether loss of SLP-76 expression would impact upon neutrophil activity in vivo, we examined bacterial killing in s.c. air pouches and the induction of LPS-induced tissue inflammation. Interestingly, *Slp-76*$^{F/-}$*Cre*$^{+}$ mice demonstrated significant resistance to neutrophil-mediated tissue damage in the LSR but showed no increase in susceptibility to infection. The results of this study suggest that myeloid lineage-specific loss of SLP-76 expression reduces neutrophil-induced tissue inflammation but preserves bactericidal activity against *S. aureus*.

One potential caveat in studying animals that express the Cre recombinase under control of the *LysM* promoter is that myeloid cells other than neutrophils may express LysM. Resolving this issue is particularly important for lineages such as mast cells and platelets, because these cells originate from a common myeloid progenitor along with neutrophils and macrophages. In this study, we illustrate by PCR and Western blotting that *Slp-76*$^{F/-}$*Cre*$^{+}$ mice exhibit gene deletion in 80–90% of neutrophils and macrophages, consistent with prior reports (30, 34). We also demonstrate *Slp-76* gene deletion within 10–20% of mast cells and provide evidence that partial gene deletion within mast cells leads to selective defects in FcεR function, with moderately impaired degranulation but normal cytokine production. Although these defects are not as striking as in *Slp-76*$^{-/-}$ mast cells, these findings indicate that the *LysM* promoter is sufficiently active in mast cells or mast cell precursors to result in mildly altered function. In contrast to mast cells, in vitro analyses of platelets revealed normal immunoreceptor-induced activation, indicating that minimal or no excision occurs within megakaryocytes.

Neutrophil-mediated bacterial killing requires multiple coordinately organized steps, including migration to infected sites, followed by recognition and engulfment of pathogens. Phagocytes identify bacteria by a variety of mechanisms, including binding to pathogen-associated molecular patterns via the TLR and lectin receptors or by binding to bacterial particles that are coated with complement or Ig via surface β_2 integrins and FcγRs, respectively. Engagement of these receptors activates a host of inflammatory responses that facilitate bacterial killing, such as activation of the NADPH oxidase leading to production of ROI and activation and/or release of granule constituents (14). To study bacterial clearance by SLP-76-deficient neutrophils in vivo, we used a model involving the injection of bacteria into a s.c. air pouch. This

model has been shown to mimic infection within a superficial abscess and allows simultaneous measure of neutrophil migration and bacterial killing in a self-contained region (25, 42). Neutrophil migration in this model appears to be integrin dependent, because a recent report demonstrates that bacterial killing within an air pouch requires the integrin signaling molecule L-plastin (25). Using this model, we demonstrate that *Slp-76*$^{F/-}$*Cre*$^{+}$ neutrophils migrate normally into infected sites and effectively contain and kill the pathogenic bacterium *S. aureus*. This finding was surprising, because ex vivo integrin function is significantly impaired in *Slp-76*$^{F/-}$*Cre*$^{+}$ neutrophils.

There are several potential explanations why the killing of *S. aureus* may be preserved in SLP-76-deficient neutrophils. First, excision in the Cre-lox system is not complete. Therefore, residual SLP-76 protein expression within a subset of cells may support sufficient signaling to allow bacterial killing. Second, neutrophils express additional receptors such as the TLR, which may offer alternative routes of activation by *S. aureus*. It is well established that TLR signals lead to potent activation of the NADPH oxidase complex, which is required for clearance of *S. aureus*. Therefore, it is possible that TLR-initiated signals, which occur independently of SLP-76, facilitate the production of ROI and elimination of *S. aureus* in mice that lack SLP-76 expression in neutrophils. Third, SLP-76-deficient neutrophils may be afforded residual signaling capabilities due to expression of the SLP-76 homolog, SLP-65/BLNK/BASH. Although SLP-65 was originally described in B cells (43), we have observed abundant expression of SLP-65 in neutrophils (data not shown). It will be interesting to determine whether neutrophils that lack expression of both SLP-76 and SLP-65 exhibit more pronounced functional defects.

The LSR is a classic example of how adhesive interactions between activated endothelial cells and neutrophils elicit damage to blood vessels, which is followed by platelet activation and thrombus formation (26, 38, 40). Injury is further augmented by recruitment of circulating neutrophils that adhere to the growing thrombus and damaged endothelium. Vascular occlusion and stasis lead to local tissue hypoxia, vascular degradation, and hemorrhage. Adherent neutrophils continue to produce ROI and release granule enzymes such as elastase that further propagate tissue damage.

Neutrophils from *Slp-76*$^{F/-}$*Cre*$^{+}$ mice migrate normally into LPS-treated ear tissue in the LSR, similar to their movement into bacterially infected air pouches. SLP-76-deficient neutrophils, however, induce less vascular damage, thrombosis, hemorrhage and induration, when compared with neutrophils that express SLP-76. The finding that SLP-76-deficient neutrophils migrate normally to the site of inflammation yet are unable to elicit tissue damage is intriguing. These results resemble data obtained using Syk-deficient neutrophils, which fail to produce ROI or to spread following integrin ligation in vitro but demonstrate normal migration in vivo (44). These data are also similar to what was observed using Mac-1-deficent mice, suggesting that the molecular mechanisms supporting integrin-mediated migration and inflammatory responses may differ (27). There are several possible explanations for these seemingly divergent findings. First, integrin-mediated locomotion might require the adhesive function of the receptor and not receptor-induced intracellular signaling. Thus, neutrophils deficient in signaling mediators might migrate normally but exhibit defects in effector function. Alternatively, it is known that neutrophils express two β_2 integrin receptors, LFA-1 (CD11a) and Mac-1 (CD11b). Although integrin-mediated inflammatory responses are attributed predominantly to Mac-1, some studies suggest that LFA-1 is preferentially used for migration (10, 45). Thus, the discrepancy in migratory vs inflammatory signals might also be due to differing signaling requirements for SLP-76 downstream of LFA-1 vs Mac-1.

Because neutrophil recruitment is normal in the *Slp-76*$^{F/-}$*Cre*$^{+}$ mice, the resistance of these animals to LPS-mediated pathology is likely due to the reduced ability of SLP-76-deficient neutrophils to degranulate in vivo. Indeed, a proinflammatory role for neutrophil granule enzymes is supported by reports showing inhibition of the LSR by protease inhibitors or use of elastase-deficient mice, and by induction of the LSR following injection of neutrophil lysosomal extracts (27, 39, 46). It should be noted that other myeloid cells, such as macrophages and mast cells, lack SLP-76 to varying degrees in *Slp-76*$^{F/-}$*Cre*$^{+}$ mice. Therefore, defects of these other immune cell lineages might also diminish tissue inflammation induced by the LSR in *Slp-76*$^{F/-}$*Cre*$^{+}$ animals. We have previously shown that SLP-76-deficient macrophages function normally in response to FcγR and complement receptor stimulation (47, 48), but we have not examined integrin function in detail. Thus, it remains possible that SLP-76-dependent macrophage functions might contribute to the LSR. There are several lines of evidence suggesting that mast cells do not contribute significantly to the LSR. First, our examination of W^{Sh}/W^{Sh} and W/W^{v} mast cell-deficient mice revealed that induction of the LSR was temporally and qualitatively comparable to WT controls (Fig. 6). Second, studies of SLP-76 have uncovered a role for this adaptor only in FcεR signaling pathways in mast cells. In the LSR, however, there is no source provided for FcεR stimulation. Furthermore, in a study of mice lacking the inhibitory receptor gp49B1, mast cells did not appear to contribute to the LSR, despite hyperactive FcεR responses in vitro (26). Together, these results suggest that reduced expression of SLP-76 within myeloid cells imparts protection against the LSR by specifically dampening neutrophil adhesion-dependent responses.

To our knowledge, this is the first report of mice that lack expression of an adaptor molecule selectively within the myeloid compartment. In this study, we show that loss of SLP-76 within neutrophils leads to marked perturbations in integrin function in vitro. Furthermore, mice lacking SLP-76 in neutrophils exhibit reduced tissue damage in the LSR in vivo. We conclude from these data that SLP-76 plays a physiological role in neutrophils by promoting integrin-dependent proinflammatory responses. In contrast, loss of SLP-76 in neutrophils does not appear to impede killing of the bacterial strain *S. aureus*, suggesting that the molecular pathways underlying this critical function must be sufficiently redundant to compensate for the absence of SLP-76. Future studies of SLP-76 signaling in neutrophils will improve our understanding of innate immune cell function and may facilitate development of therapeutic agents with which to modulate neutrophil activation to prevent tissue damage but preserve bacterial killing.

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Disclosures

The authors have no financial conflict of interest.

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