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The Hemopoietic Rho/Rac Guanine Nucleotide Exchange Factor Vav1 Regulates *N*-Formyl-Methionyl-Leucyl-Phenylalanine-Activated Neutrophil Functions[1]

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Vav1 is a hemopoietic-specific Rho/Rac guanine nucleotide exchange factor that plays a prominent role in responses to multisubunit immune recognition receptors in lymphoid cells, but its contribution to regulation of neutrophil functions is unknown. Activated Rho family GTPases are critical participants in neutrophil signaling cascades initiated by binding of FMLP and other chemoattractants to their cognate G protein-coupled receptors. Therefore, we investigated whether Vav1 regulates chemoattractant-induced responses in neutrophils. We found that superoxide production elicited by FMLP in *Vav1*$^{-/-}$ murine neutrophils isolated from either bone marrow or from peritoneal exudates was substantially reduced compared with that of wild type. Filamentous actin generation in FMLP-stimulated *Vav1*$^{-/-}$ neutrophils was also markedly reduced, whereas it was normal in response to IL-8 or leukotriene B_4. FMLP induced tyrosine phosphorylation of Vav1, whereas IL-8 or leukotriene B_4 did not, correlating with the requirement for Vav1 in chemoattractant-stimulated filamentous actin generation. Neutrophil motility in vitro and neutrophil mobilization into peripheral blood in vivo elicited by FMLP were both decreased in *Vav1*$^{-/-}$ mice. Hence, this study defines a new role for Vav1 in regulating granulocytic leukocytes as well as linking Vav1 to specific cellular responses downstream of a seven transmembrane domain receptor. *The Journal of Immunology,* **2003, 171: 4425–4430.**

Activation of neutrophil effector functions by chemoattractants acting through G protein-coupled seven transmembrane domain receptors is an essential feature of the host response to infection and inflammation. Rho/Rac family GTPases play a central role in regulating responses downstream of these receptors (1–3). For example, Rac GTPases participate in chemoattractant-induced superoxide production by the NADPH oxidase and changes in the neutrophil cytoskeleton leading to lamellipodia formation and directed chemotaxis (4–6), whereas detachment during cellular migration requires RhoA (7).

Rho/Rac family GTPases alternate between inactive GDP-bound and active GTP-bound states to act as molecular switches that propagate receptor-induced signals. This cycle is controlled by guanine nucleotide exchange factors (GEFs)[3] that catalyze a DBL homology (DH) domain-mediated GDP-GTP exchange reaction and the opposing effect of GTPase-activating proteins. A large number of Rho family GEFs have been identified, including at least eight that activate Rac in vitro, and an emerging concept is that different GEFs are not functionally redundant but act in the context of selected signaling pathways linking receptors to specific cellular responses (8). A current challenge is to define these functional links.

Multiple signaling cascades are activated upon neutrophil chemoattractant binding to their cognate receptors, but the specific pathways leading to Rho/Rac GTPase activation and to downstream functions are incompletely understood (1, 2). At present, only one GEF that acts on Rho family proteins upon ligation of chemoattractant receptors has been firmly identified, a novel Rac activator termed P-Rex1 (9). P-Rex1 is directly activated by $G\beta\gamma$ subunits and by phosphatidylinositol 3,4,5-P_3 generated by phosphoinositide 3-kinases (PI3Ks), both in an independent and synergistic fashion, and antisense inhibition of P-Rex1 expression in a granulocyte-differentiated myeloid cell line reduced superoxide formation elicited by the chemoattractant C5a by 40–50% (9). However, Rac activation in either human or mouse neutrophils stimulated by the chemoattractant FMLP, a bacterial peptide, is inhibited by tyrosine kinase inhibitors, including those specific for the Src family (6, 10, 11). These findings suggested that other Rac GEFs regulated either directly or indirectly by tyrosine phosphorylation might act in a sequential or parallel pathway to P-Rex1 to activate Rac in response to chemoattractants.

The Vav family of GEFs are unique among Rho-GEFs in that their exchange activity is regulated by tyrosine phosphorylation (12, 13). There are three Vav isoforms in mammalian cells. Vav2 and Vav3 are broadly expressed, whereas Vav1 is expressed predominantly in hemopoietic cells. Vav GEFs are large multidomain proteins that contain a tandem DH and pleckstrin homology (PH) domain characteristic of Rho-GEFs. The DH and PH domains are flanked by a calponin homology domain and an acidic region at the N terminus, and a zinc finger motif, a short proline-rich region, and an Src homology (SH) SH3-SH2-SH3 module at the C terminus. These flanking domains are important for recruitment of Vav GEFs to specific signaling complexes and may also regulate some

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[3] Abbreviations used in this paper: GEF, guanine nucleotide exchange factor; DH, DBL homology; PH, pleckstrin homology; SH, Src homology; PI3K, phosphoinositide 3-kinase; BM, bone marrow; F-actin, filamentous actin; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]pyrimidine; LTB_4, leukotriene B_4; PEC, peritoneal exudate cell.

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pathways in a GEF-independent manner (12, 13). Tyrosine phosphorylation relieves the autoinhibition of the DH domain by the acidic region to activate GEF activity (14). In overexpression and in vitro studies, Rac1, Rac2, and RhoG are preferred targets of Vav1 GEF activity, whereas Vav2 and Vav3 are most active on RhoA, RhoB, and RhoG (13, 15). Vav proteins are additionally modulated by the PH domain via its binding to phospholipids and also possibly protein-protein interactions (12).

The biologic functions of Vav proteins have best been studied in lymphoid cells. Vav proteins are recruited to activate immune recognition receptors on lymphocytes, which is mediated by direct interaction of the Vav SH2 domain with receptor tyrosine kinases and with coreceptors or adaptor proteins (12, 13). These associations are important for activation of GEF activity (12, 13). Although all three Vav isoforms are expressed in hemopoietic cells, studies in gene-targeted mice indicate that these are not functionally redundant. Vav1 plays an important role in signaling though the T cell Ag receptor, which is essential for normal T cell development and function of mature T cells and NK cells, whereas Ag receptor signaling in B cells is dependent on both Vav1 and Vav2 (13). Vav1 also has been shown to function downstream of FcϵRs to mediate degranulation in mast cells and FcγR-mediated phagocytosis in macrophages (16, 17).

The role of Vav1 proteins in regulating neutrophil functions is not well characterized. Vav1 is activated upon ligation of neutrophil β_2 integrin receptors (18). Vav1 has also been proposed as a candidate GEF for Rac in neutrophils based on the sensitivity of FMLP-elicited Rac activation to tyrosine kinase inhibitors (6, 10, 11). Furthermore, microglial macrophages overexpressing the Vav-activating protein Nef, an HIV-encoded adaptor protein, have increased levels of activated Rac and a markedly enhanced production of superoxide in response to FMLP and other agonists (19). In COS7 cells engineered to respond to FMLP by transgenic expression of the formyl peptide receptor and PI3Kγ, FMLP-induced reorganization of the cytoskeleton was blocked by expression of inactive variants of Vav1 (20). In addition, we recently reported that expression of constitutively active derivatives of Vav1, Vav2, or Tiam1 in a COS7 line expressing transgenic phagocyte NADPH oxidase subunits activated robust NADPH oxidase activity, with Vav1 being the most effective relative to the level of activated Rac (21). Therefore, to obtain direct evidence for a role of Vav1 in regulating chemoattractant-induced NADPH oxidase activation and cytoskeletal reorganization in neutrophils, we examined whether chemoattractants were capable of inducing phosphorylation of Vav1, and whether these chemoattractant-induced responses in Vav1-deficient murine neutrophils were impaired.

Materials and Methods

Abs, reagents, and buffers

A rabbit polyclonal Ab against Vav1 (SC-132) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAbs against Vav1 (05-219) and phosphotyrosine (4G10; 05-321) were purchased from Upstate Biotechnology (Lake Placid, NY). R-PE-conjugated Ab for the formyl peptide receptor (556016) was from BD PharMingen (San Diego, CA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. PBS (pH 7.2), distilled water, glycerol, HBSS, and HEPES (125 mM; pH 7.5) were purchased from Life Technologies (Grand Island, NY). Other buffers used in this study include PBS-BG (PBS with 0.1% bovine albumin (BSA) and 1% glucose; pH 7.25–7.4), PBSG (PBS with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 7.5 mM glucose), and immunoprecipitation lysis buffer (10 mM Tri-Cl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 0.1% BSA, 50 mM NaF, 2 mM Na_3VO_4, 20 μg/ml chymostatin, 2 mM PMSF, 10 μM leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride). IgG immune complexes were prepared by mixing equal volumes of 625 μg/ml BSA with 6 mg/ml rabbit polyclonal Ab against BSA (ICN Pharmaceuticals, Aurora, OH) for overnight at 4°C with gentle mixing. BSA-Ab aggregates were sonicated for 45–60 s at 20% output, centrifuged for 5 min at 1000 rpm, and then washed and resuspended in PBS.

Animals

Vav1$^{-/-}$ mice (22) and *Rac2*$^{-/-}$ mice (4) were previously generated by targeted gene disruption and backcrossed into the C57BL/6J strain for >10 generations. C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for wild-type controls. Mice were housed in microisolator cages under specific pathogen-free conditions, fed autoclaved food and acidified water ad libitum, and used in experiments at 8–12 wk of age. All studies using mice were approved by the Indiana University Animal Care and Use Committee.

Isolation of bone marrow and peritoneal exudate neutrophils (PEC)

Neutrophils were purified from the bone marrow (BM) and peritoneal cavity by density gradient centrifugation as described (5) except that PBS was used instead of HBSS. The final preparation was kept on ice in PBS (without Ca^{2+}, Mg^{2+}) until further processing. To elicit PEC, mice were injected with 1 ml of 3% thioglycolate, and peritoneal cells were harvested after 4 and 18 h as described (5).

NADPH oxidase activity

PMA-elicited superoxide production was measured in a quantitative kinetic assay based on the superoxide dismutase-inhibitable reduction of cytochrome *c* (5). Superoxide production upon FMLP stimulation was measured by an isoluminol chemiluminescence assay (6).

Flow cytometry

The content of filamentous actin (F-actin) in chemoattractant-stimulated neutrophils was measured by flow cytometry using FITC-phalloidin (4, 6). The relative amount of cell surface FMLP receptor expression in unstimulated BM neutrophils was evaluated using a mAb for the murine formyl peptide receptor (4).

Chemotaxis and neutrophil mobilization

A microchemotaxis device was used to assess chemotaxis in vitro (4, 6). Neutrophil mobilization to peripheral blood in vivo was performed similarly to the method described previously (23). Briefly, mice were injected s.c. with either 200 μl of 20 μM FMLP, 250 ng/ml IL-8 (35 nM), or 200 μl of sterile saline. Peripheral blood counts and differentials were determined from tail vein collections obtained 90 min after injection.

Measurement of Vav1 phosphorylation and Rac activation

Vav1 activation was assessed using a standard method based on phosphotyrosine staining of immunoprecipitates isolated from whole-cell lysates using an anti-Vav1 Ab (Santa Cruz Biotechnology). Briefly, 2×10^7 BM neutrophils in a total volume of 0.5 ml of PBSG incubated at 37°C for 2 min before incubation with 5 μl of DMSO, 10 μM FMLP, 250 ng/ml IL-8 (35 nM), or 100 nM leukotriene B_4 (LTB_4) for the indicated times. In some experiments, cells were pretreated with a final concentration of 10 and 100 μM genistein, 1 and 10 μM 4-amino-5-(4-methylphenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]pyrimidine (PP1), 2 and 20 μM LY294002, or 100 nM wortmannin at 37°C for 20 min before stimulation. For analysis of Vav1 phosphorylation after FcγR stimulation, neutrophils were incubated with IgG immune complexes for 1 h at 4°C with gentle shaking, and then for 5 min at 37°C. Following agonist stimulation, 800 μl of ice-cold PBS was added, and cells were pelleted, lysed with immunoprecipitation lysis buffer, and then clarified. Supernatant was incubated with 1 μg of Vav1 Ab overnight, and precipitated with protein A-conjugated Sepharose beads (Amersham, Arlington Heights, IL). No Vav1 was detected in the cell pellet following lysis of either resting or stimulated cells, and there was no evidence of Vav1 lability during the immunoprecipitation procedure (C. Kim and M. C. Dinauer, unpublished observations). Washed immunoprecipitates were electrophoresed on 10% SDS-PAGE. Immunoblots were probed using an anti-phosphotyrosine Ab or Vav1 Ab.

An affinity precipitation assay for Rac activation was performed as previously described (6). The p21-binding domain of p21-activated kinase 3 was expressed as a GST fusion protein using a vector obtained from R. Cerione (Cornell University, Ithaca, NY).

Statistical analysis

Statistical analysis was performed using Instat (GraphPad, San Diego, CA) or Excel (Microsoft, Redmond, WA) software. Data are expressed as mean ± SD unless otherwise indicated. The two-tailed Student's t test or Mann-Whitney test was used to determine the difference between groups. A value of $p < 0.05$ was considered significant.

Results

Vav1 is tyrosine phosphorylated in response to FMLP

Activation of Vav1 requires its tyrosine phosphorylation, which is mediated upon ligation of a variety of membrane tyrosine kinase receptors or receptors with linked tyrosine kinases of the Syk/Zap70, Jak, and Src families, including receptors for growth factors, cytokines, integrins, Igs, and Ag presentation (12). In platelets, Vav1 phosphorylation is also stimulated by the thrombin receptor, a seven transmembrane domain receptor coupled to trimeric G proteins (24). We examined whether Vav1 can be activated in murine neutrophils by FMLP and other chemoattractants, which also signal through G protein-coupled receptors. As shown in Fig. 1, the level of Vav1 phosphorylation was increased by 1.5- to 2-fold compared with vehicle-treated controls in neutrophils stimulated with FMLP. Increased Vav1 phosphorylation in FMLP-stimulated neutrophils was detected as early as 10 s of FMLP stimulation and peaked by 60–120 s (Fig. 1*B*). This increase was similar to that seen in neutrophils stimulated with IgG immune complexes (not shown). However, stimulation with two other neutrophil chemoattractants, IL-8 or LTB_4, did not induce Vav1 phosphorylation over the same time frame (Fig. 1*A* and data not shown).

The neutrophil formyl peptide receptor is coupled to trimeric GTP-binding proteins and transduces signals via multiple pathways that include PI3Ks and the Src-related tyrosine kinases Fgr, Hck, and Lyn (2, 25–28). To assess pathways that might regulate Vav1 activation, we examined the effects of pharmacologic inhibitors. The Src family kinase inhibitor PP1, which has been shown to inhibit at least Lyn and Hck activation in FMLP-stimulated human neutrophils (27), had the greatest effect on FMLP-induced Vav1 phosphorylation, which was substantially reduced even at the lowest concentration of PP1 tested (1 μM) (Fig. 1*C*). Incubation of neutrophils with PP1 also inhibited the basal level of Vav1 phosphorylation detected in our neutrophil preparations (not shown). Vav1 phosphorylation in FMLP-stimulated neutrophils was also attenuated in a dose-dependent manner by the tyrosine kinase inhibitor genistein (Fig. 1*C*). The PI3K inhibitors LY294002 (Fig. 1*C*) or wortmannin (not shown) produced only a slight decrease in Vav1 phosphorylation.

Vav1-deficient neutrophils have functional defects in FMLP-induced responses in vitro and in vivo

To examine whether Vav1 regulates functional responses of neutrophils to FMLP, we next examined NADPH oxidase activity in *Vav1*$^{-/-}$ neutrophils. As shown in Fig. 2*A*, *Vav1*$^{-/-}$ neutrophils isolated from BM had a ≈3-fold decrease in FMLP-elicited superoxide production compared with wild-type cells. NADPH oxidase activity was similarly reduced in FMLP-stimulated PEC (Fig. 2*A*), indicating that a role for Vav1 in regulating oxidase activity is independent of the activation state of the cell. Cell surface expression of the FMLP receptor was equivalent in resting wild-type and *Vav1*$^{-/-}$ neutrophils (not shown), and thus a reduction in FMLP receptor numbers did not account for the defect in FMLP-induced NADPH oxidase activity. In our hands, neither LTB_4 nor IL-8 elicited superoxide production even in wild-type murine neutrophils. The peak rate of superoxide release occurred at ≈30 s after the onset of FMLP stimulation in both wild-type and *Vav1*$^{-/-}$ neutrophils, but its amplitude was markedly reduced in Vav1-deficient cells (Fig. 2*B*). In contrast, *Rac2*$^{-/-}$ neutrophils, which have an 8- to 10-fold decrease in overall superoxide production with FMLP stimulation compared with wild-type cells (5, 6), exhibited both a prolonged lag in achieving maximal enzyme rates as well as an overall decrease in activity (Fig. 2*B*). These results suggest that Vav1 synergizes with or amplifies pathways acting through Rho/Rac GTPases and is required for full activation of FMLP-induced superoxide production, which is also consistent with the potent priming effect of Nef-activated Vav1 on FMLP-stimulated superoxide production in microglial macrophages (19).

We also examined NADPH oxidase activity elicited by PMA stimulation of *Vav1*$^{-/-}$ neutrophils, a potent pharmacologic activator of superoxide production acting via protein kinase C. Both BM and exudate *Vav1*$^{-/-}$ neutrophils exhibited a 25–35% decrease in PMA-induced NADPH oxidase activity compared with wild-type cells (Fig. 2*C*), suggesting that Vav1 is also required for full responsiveness to this stimulus. As for FMLP-elicited NADPH oxidase activity, Rac2-deficient neutrophils have a more severe defect in response to PMA, with ≈75 and 50% reduction in superoxide activity in bone marrow and exudate cells, respectively (4–6).

We next investigated the role of Vav1 in chemoattractant-induced actin polymerization and migration in vitro. The basal level of F-actin in *Vav1*$^{-/-}$ neutrophils was similar to wild-type cells (Fig. 3). However, the rapid generation of F-actin in response to FMLP was markedly reduced in *Vav1*$^{-/-}$ BM neutrophils, whereas it was unaffected in response to IL-8 or LTB_4 (Fig. 3).

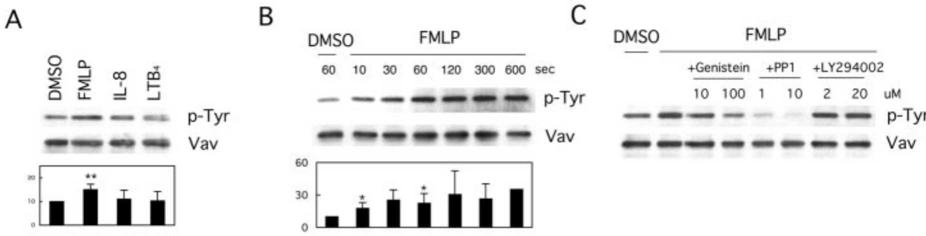

FIGURE 1. Tyrosine phosphorylation of Vav in chemoattractant-stimulated neutrophils. Lysates from vehicle- or chemoattractant-treated wild-type BM neutrophils were immunoprecipitated with a polyclonal Vav1 Ab and analyzed by immunoblotting with a p-Tyr Ab (*upper panels*) or a monoclonal anti-Vav1 Ab (*middle panels*). Immunoblots are representative of three to four independent experiments. Densitometry of p-Tyr signal normalized to Vav expression as averaged from all experiments in each group is shown in *lower panels*, except 1*C*. *A*, Neutrophils were treated with 10 μM FMLP, 250 ng/ml IL-8, or 100 nM LTB_4 for 60 s at 37°C ($n = 4$). *B*, Neutrophils were incubated with either DMSO for 60 s or 10 μM FMLP for 10–600 s at 37°C ($n = 4$, except for 600 s time point where $n = 1$). *C*, Neutrophils were incubated with 10 and 100 μM genistein, 1 and 10 μM PP1, or 2 and 20 μM LY294002 for 20 min before stimulation with 10 μM FMLP for 60 s at 37°C ($n = 4$). $*$, $p < 0.05$; $**$, $p < 0.02$, DMSO control vs FMLP stimulation (paired t test).

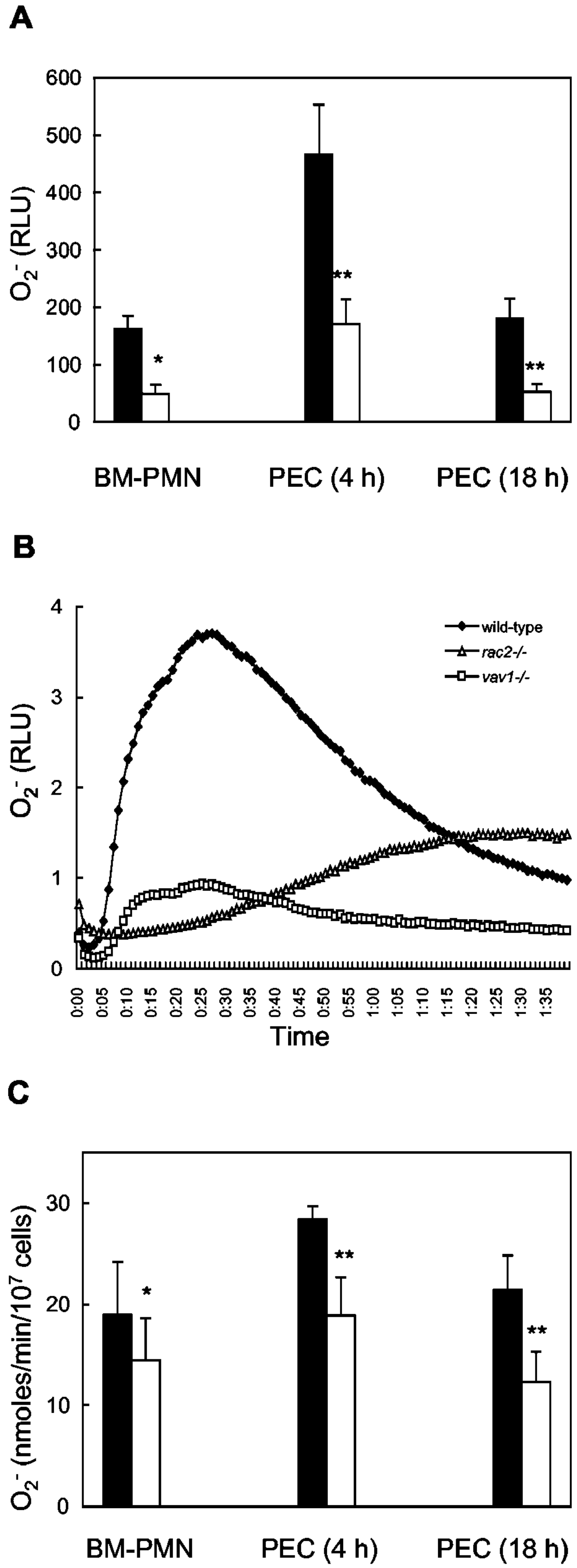

FIGURE 2. NADPH oxidase activity in FMLP- or PMA-stimulated neutrophils. Superoxide dismutase-inhibitable superoxide anion production was measured in BM polymorphonuclear neutrophils (BM-PMN) or PEC harvested 4 or 18 h after i.p. thioglycolate. ■, Wild type; □, *Vav1*$^{-/-}$. *A*, Isoluminol chemiluminescence was used to measure superoxide production in BM-PMN ($n = 6$) and PEC ($n = 4$–5) following stimulation with 10 μM FMLP. Data shows relative luminescence units (RLU) integrated over 100 s in wild-type or *Vav1*$^{-/-}$ neutrophils. *B*, Time course of superoxide production in BM-PMN following stimulation with 10 μM FMLP for wild-type, *Vav1*$^{-/-}$, or *Rac2*$^{-/-}$ neutrophils, as indicated. *C*, Superoxide production was measured by cytochrome *c* reduction assay in BM-PMN ($n = 8$) and PEC ($n = 4$–5) following stimulation with 200 ng/ml PMA. Data for *A* and *C* are expressed as mean ± SD. $*$, $p < 0.001$; $**$, $p < 0.005$, wild type vs *Vav1*$^{-/-}$ (unpaired *t* test).

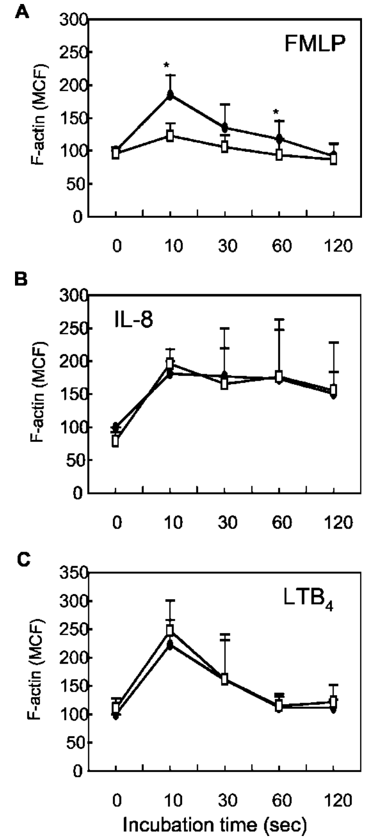

FIGURE 3. F-actin generation by chemoattractant-stimulated neutrophils. Freshly isolated BM neutrophils were warmed at 37°C in HBSS for 3 min before stimulation with 10 μM FMLP (*A*), 250 ng/ml IL-8 (*B*), or 100 nM LTB_4 (*C*). ●, Wild type; □, *Vav1*$^{-/-}$. Treatment with carrier (0.5% DMSO for FMLP, PBS for IL-8, and ethanol for LTB_4) did not stimulate F-actin generation. Wild-type and *Vav1*$^{-/-}$ cells were analyzed in pairs. Results are expressed as mean channel fluorescence (MCF), with the baseline wild-type fluorescence arbitrarily assigned a value of 100. Mean ± SD of two to four independent experiments per group. $*$, $p < 0.02$, wild type vs *Vav1*$^{-/-}$ (paired *t* test).

Vav1 has also been shown to regulate actin polymerization downstream of the TCR (12, 13). The selective requirement for Vav1 in FMLP-stimulated F-actin generation correlated with the finding that FMLP induced tyrosine phosphorylation of Vav1, whereas IL-8 or LTB_4 did not (Fig. 1). Transmigration of *Vav1*$^{-/-}$ BM neutrophils in the presence of buffer alone was reduced by ≈2-fold compared with wild-type cells (14 ± 11 vs 27 ± 10; $n = 8$; $p < 0.01$; *Vav1*$^{-/-}$ vs wild type). Migration elicited by 1 and 10 μM FMLP, but not 0.1 μM FMLP, was reduced by an additional 15–25% in *Vav1*$^{-/-}$ neutrophils (Fig. 4*A*). Hence, absence of Vav1 results in impaired FMLP-induced signals that regulate changes in the neutrophil actin cytoskeleton and cell motility, although *Vav1*$^{-/-}$ neutrophils exhibited only a small change in directed migration to FMLP.

Systemic administration of chemoattractants produces an increase in the peripheral blood neutrophil concentration, commonly

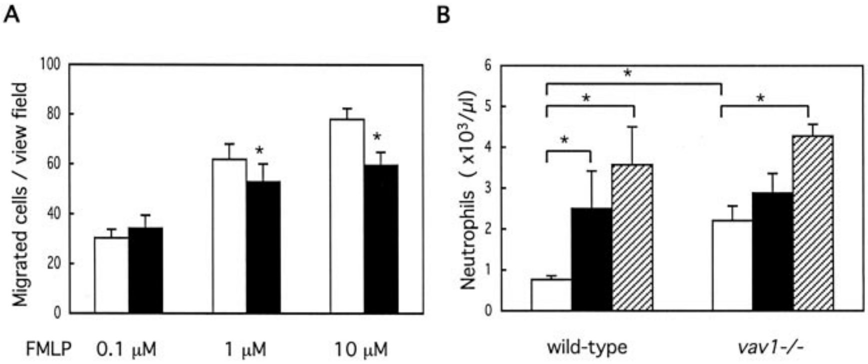

FIGURE 4. Neutrophil migration in response to FMLP. *A*, Migration of BM neutrophils was assayed in a modified Boyden chamber using FMLP (10^{-7}–10^{-5} μM). The numbers of cells migrating to the lower side of the filter in chambers containing buffer alone were also counted in samples assayed in parallel (27 ± 10 for wild-type neutrophils and 14 ± 11 for *Vav1*$^{-/-}$; $n = 8$; $p < 0.01$; wild type vs *Vav1*$^{-/-}$), and have been subtracted from the data shown for FMLP-treated groups. ■, Wild type; □, *Vav1*$^{-/-}$. Data are expressed as mean ± SEM. $*$, $p < 0.02$, wild type vs *Vav1*$^{-/-}$ (paired *t* test). *B*, Peripheral blood neutrophil concentrations in wild-type or *Vav1*$^{-/-}$ mice 90 min following s.c. injection of 200 μl of sterile saline (□), 20 μM FMLP (■), or 250 ng/ml IL-8 (hatched bars) (n = 5–9 mice in each group). Data are expressed as mean ± SEM. $*$, $p < 0.01$ (Mann-Whitney test).

referred to as mobilization, which reflects release of mature neutrophils from the BM storage pool (29, 30). Mice with targeted disruption of the high affinity *N*-formyl peptide receptor lack this response (23). The mechanisms underlying chemoattractant-induced neutrophil mobilization, which does not seem to require any known adhesion molecules, are not fully understood but have been speculated to involve chemoattractant-induced changes in the actin cytoskeleton (reviewed in Ref. 30). Similar to Rac2-null mice (4), we found a modest increase in baseline peripheral blood neutrophil counts in *Vav1*$^{-/-}$ mice (1890 ± 1180 neutrophils/μl; $n = 7$) compared with wild-type cohorts (706 ± 670; $n = 14$; $p < 0.01$; *Vav1*$^{-/-}$ vs wild type). Administration of sterile saline alone did not result in a change in peripheral blood neutrophil counts in either wild-type or *Vav1*$^{-/-}$ mice (Fig. 4*B*). Subcutaneous injection of FMLP induced a ~3-fold increase in the peripheral blood neutrophil concentration in wild-type mice after 90 min, whereas no significant increase was detected in *Vav1*$^{-/-}$ mice (Fig. 4*B*). In contrast, s.c. injection of IL-8 produced a substantial increase in peripheral blood neutrophil counts in both wild-type and *Vav1*$^{-/-}$ mice (Fig. 4*B*). This is consistent with our findings that IL-8 does not induce phosphorylation of Vav1 and that IL-8-induced F-actin generation is intact in Vav1-null mice. These data demonstrate that Vav1 is involved in FMLP-induced neutrophil mobilization in vivo, and indicate that the impairment in *Vav1*$^{-/-}$ mice is selective rather than reflecting a more general defect in this response. Also consistent with a selective defect in FMLP-induced neutrophil trafficking in vivo, the number of neutrophils in peritoneal exudate at 4 and 18 h following i.p. injection of thioglycolate, a nonspecific inflammatory stimulus, was similar in *Vav1*$^{-/-}$ and wild-type neutrophils (data not shown).

Regulation of cellular signaling by Vav1 is mediated in large part via activation of Rho family GTPases, and Rac and RhoG appear to be its preferred substrates (13, 15). The defects identified above in Vav1-null neutrophils resemble those of Rac2-null neutrophils, although the functional deficiencies in Rac2-null neutrophils are more severe (4–6). We previously showed that wild-type murine BM neutrophils contain similar amounts of Rac1 and Rac2, and that stimulation with FMLP induced an increase in both Rac1-GTP and Rac2-GTP as measured by an affinity precipitation assay, with the Rac2-GTP levels being ≈4-fold greater than Rac1-GTP (6). We did not observe a detectable difference in the FMLP-induced increase in either Rac1-GTP or Rac2-GTP in *Vav1*$^{-/-}$ neutrophils compared with wild-type neutrophils (not shown). However, FMLP is likely to activate other Rac GEFs such as P-Rex1 (9), and we cannot rule out the possibility that there is a small decrease in Rac activation in the absence of Vav1.

Discussion

Although Vav1 is well-known participant in tyrosine kinase-linked immunoreceptor signaling in leukocytes, particularly in T lymphocytes and NK cells, this study is the first to describe a functional role for the Vav1 in the neutrophil lineage and also to establish a function for Vav1 downstream of a seven transmembrane domain receptor. We found that FMLP stimulated tyrosine phosphorylation of Vav1, and analysis of Vav1-null mice provided genetic evidence linking this hemopoietic-specific GEF to regulation of NADPH oxidase activation in both BM and exudate neutrophils, changes in the neutrophil actin cytoskeleton, and neutrophil trafficking in vivo in response to FMLP. That the requirement for Vav1 was selective for FMLP, but not IL-8 or LTB_4, is consistent with other studies showing that functional responses stimulated by these chemoattractants are not identical (e.g., Refs. 31 and 32). These differential effects have been postulated to reflect qualitative differences in downstream signaling constituents (31, 32), and the current study suggests that the involvement of Vav1 in responses initiated by FMLP, but not IL-8 or LTB_4, may contribute to these differences.

FMLP-induced Vav1 phosphorylation was markedly sensitive to the Src family kinase inhibitor PP1, and G protein-coupled receptors are known to activate Src kinase family members in a variety of cells, including chemoattractant-stimulated neutrophils (25, 27). Src kinases have also been implicated in phosphorylation of Vav1 downstream of immunoreceptors in lymphocytes (13). In contrast, PI3K inhibitors had only a minimal effect on Vav1 phosphorylation, which has also been observed in other settings (13).

The regulatory and signaling properties of Vav1 have been most intensively studied in lymphocytes, where its role is complex. Some aspects of lymphoid Vav1 signaling appear to be independent of its GEF activity, including activation of PLC-γ1 and calcium mobilization, which leads to activation of the transcription factor NFAT (12, 13). Calcium mobilization also accompanies chemoattractant signaling in neutrophils, but calcium transients induced by FMLP were normal in *Vav1*$^{-/-}$ neutrophils (J. Travers and M. C. Dinauer, unpublished data). Most of the effects of Vav1 in lymphoid cells appear to be related to its activation of Rho/Rac GTPases. Vav1 is most active on Rac and RhoG GTPases in model systems, whereas Cdc42 appears not to be a preferred substrate (12, 13). Expression of either activated or dominant-negative forms of GTPases in lymphoid cells or NK cells suggest that the exchange activity toward Rac is crucial for many aspects of Vav1 function in these cells (13).

Additional work will be needed to establish precisely how Vav1 is functioning in FMLP-induced signaling in neutrophils. The defects in FMLP-elicited responses in *Vav1*$^{-/-}$ neutrophils paralleled those observed in Rac2-null neutrophils, although the defects in superoxide production and chemotaxis were not as severe as seen in the absence of Rac2. We were unable to demonstrate any differences in FMLP-stimulated Rac1-GTP or Rac2-GTP formation in *Vav1*$^{-/-}$ neutrophils compared with wild-type neutrophils. There are a number of possible reasons for this. First, FMLP is likely to stimulate other Rac GEFs in addition to Vav, such as P-Rex1 (9). Hence, absence of Vav may result in only a small decrease in overall Rac activation in FMLP-stimulated neutrophils that is difficult to detect; alternatively, there may be a compensatory increase in the activity of other GEFs that act on Rac. In addition, that Vav-deficient neutrophils have significant functional defects in FMLP-stimulated superoxide production and F-actin generation despite apparently normal levels of activated Rac is consistent with the concept that GEFs are not functionally redundant but act in the context of specific downstream signaling pathways (8). Second, Vav1 is also an exchange factor for RhoG, whose functions are not well understood at present, but has recently been implicated in a pathway paralleling Rac that regulates actin remodeling and gene expression in lymphocytes (33). An assay sufficiently sensitive to detect RhoG activation induced by physiologic signals has not yet been established (33). Finally, Vav1 regulates some lymphocyte functions independent of its GEF activity, as noted above. Although future studies will need to address the underlying mechanism(s) by which Vav acts in neutrophils, the phenotypic defects in Vav1-deficient neutrophils observed in the current study indicate that Vav1 synergizes with or amplifies pathways acting through Rho/Rac GTPases, and is essential for fully intact coupling of FMLP-induced signaling to downstream neutrophil functions.

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