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Vav GEFs are required for β_2 integrin-dependent functions of neutrophils

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Integrin regulation of neutrophils is essential for appropriate adhesion and transmigration into tissues. Vav proteins are Rho family guanine nucleotide exchange factors that become tyrosine phosphorylated in response to adhesion. Using Vav1/Vav3-deficient neutrophils (Vav1/3^{ko}), we show that Vav proteins are required for multiple β_2 integrin-dependent functions, including sustained adhesion, spreading, and complement-mediated phagocytosis. These defects are not attributable to a lack of initial β_2 activation as Vav1/3^{ko} neutrophils undergo chemoattractant-induced arrest on intercellular adhesion molecule-1 under flow. Accordingly,

in vivo, Vav1/3^{ko} leukocytes arrest on venular endothelium yet are unable to sustain adherence. Thus, Vav proteins are specifically required for stable adhesion. β_2 -induced activation of Cdc42, Rac1, and RhoA is defective in Vav1/3^{ko} neutrophils, and phosphorylation of Pyk2, paxillin, and Akt is also significantly reduced. In contrast, Vav proteins are largely dispensable for G protein-coupled receptor-induced signaling events and chemotaxis. Thus, Vav proteins play an essential role coupling β_2 to Rho GTPases and regulating multiple integrin-induced events important in leukocyte adhesion and phagocytosis.

Introduction

Neutrophil recruitment from the blood is a central event in the innate immune responses to invading pathogens and tissue damage. Regulation of cellular adhesive activity by the leukocyte β_2 integrins is an essential element of neutrophil recruitment. Circulating neutrophils maintain their integrins in a low affinity, nonadhesive state and, in response to local inflammatory stimuli, are rapidly activated to bind ligand, a process known as "inside-out" activation. Once arrested on venular endothelium, neutrophils must subsequently strengthen their adhesion to resist detachment by disruptive shear flow, while extravasating through the endothelial barrier for migration into target tissues and clearance of invading microbes by phagocytosis. Studies in mice have indicated that β_2 integrins are required for both neutrophil recruitment (Lu et al., 1997) and cytotoxic functions such as complement-mediated phagocytosis (Coxon et al., 1996). The importance of integrin-mediated adhesion for proper leukocyte function is emphasized in leukocyte adhesion defi-

ciency patients, who suffer from severe bacterial infections due to decreased β_2 integrin expression and/or function (Bunting et al., 2002). Thus, integrins are critical mediators of neutrophil function and innate immunity; however, the molecular mechanisms by which integrins regulate adhesive events in neutrophils are largely undefined.

The Vav family of guanine nucleotide exchange factors (GEFs) activates Rho GTPases (Rho, RhoG, Rac, and Cdc42) by catalyzing the exchange of GDP for GTP. The three mammalian Vav proteins (Vav1, -2, -3) differ in their tissue distribution: Vav1 is expressed predominantly in hematopoietic cells, whereas Vav2 and Vav3 are more broadly expressed. Each Vav protein shares the same set of structural domains (calponin homology, Dbl homology, pleckstrin homology, cysteine-rich, and Src homology 2 and 3 [SH2, SH3] domains) and display overall 50–60% sequence homology (Bustelo, 2000). Studies of lymphocytes from mice lacking *vav1*, *vav2*, *vav3*, or combinations of these genes indicate

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Key words: integrins; cell adhesion; Rho GTPases; chemotaxis; phagocytosis

Abbreviations used in this paper: AAM, adhesion assay media; fMLP, *formyl*-Met-Leu-Phe; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; ICAM, intercellular adhesion molecule; IRR, immune response receptor; LTB₄, leukotriene B₄; MLC, myosin light chain; SFK, Src family kinase; TCR, T cell receptor.

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that whereas certain functions of Vav proteins are redundant, individual Vav proteins also serve specialized functions in lymphocytes (Bustelo, 2000; Fujikawa et al., 2003). It has not been established which targets of individual Vav proteins mediate the specific functions in lymphocytes nor is it known which specific Rho family GTPases serve as direct substrates for each Vav family member *in vivo*.

The function of Vav proteins has been best characterized downstream of immune response receptors (IRRs). Targeted disruption of Vav1 results in severe impairment of T lymphocyte function in response to antigen receptor stimulation (Bustelo, 2000) and impaired Fc ϵ R-induced degranulation and cytokine production in mast cells (Manetz et al., 2001). In particular, T cell receptor (TCR)-induced IL-2 production, proliferation, and calcium mobilization are impaired in *vav1*^{-/-} T lymphocytes (Bustelo, 2000). Interestingly, actin cytoskeletal rearrangements such as TCR capping and actin patch formation are defective in *vav1*^{-/-} lymphocytes (Fischer et al., 1998; Holsinger et al., 1998), suggesting Vav1 is specifically required to couple the TCR to the actin cytoskeleton. A double deficiency in Vav1/2 or Vav1/3 causes distinct phenotypic effects in lymphocytes, with Vav2 loss causing more severe effects in B cells and Vav3 loss further enhancing T cell defects (Doody et al., 2001; Tedford et al., 2001; Fujikawa et al., 2003). Deletion of all Vav family members prevents production of all mature B and T cells (Fujikawa et al., 2003). Therefore, Vav proteins serve critical functions downstream of IRR signaling in lymphocytes. However, the role of Vav proteins downstream of receptors other than IRRs has not been extensively examined.

The exchange activity of Vav proteins is regulated by phosphorylation of regulatory tyrosine residues in the amino terminus (Bustelo, 2000). In addition to IRRs, stimulation of receptor tyrosine kinases, cytokine receptors, and G protein-coupled receptors (GPCRs) induces tyrosine phosphorylation of Vav1, the best characterized family member (Bustelo, 2000). Thus, Vav proteins can couple to diverse signaling receptors and, due to their exchange activity and adaptor domains, are ideally suited to transduce signals to Rho GTPases and the actin cytoskeleton. Previous studies have implicated a kinase cascade involving the tyrosine kinases Src and Syk in $\alpha_{IIb}\beta_3$ integrin-induced Vav1 phosphorylation and cytoskeletal rearrangements (Miranti et al., 1998; Obergfell et al., 2002). Inducible Vav1 phosphorylation in response to β_1 and β_2 integrin ligation has also been reported in myeloid cells (Gotoh et al., 1997), T cells (Yron et al., 1999), and platelets (Cichowski et al., 1996). In addition, overexpression studies have implicated Vav proteins in integrin-dependent cell spreading (Marignani and Carpenter, 2001; del Pozo et al., 2003). Thus, Vav proteins can be activated by integrins in multiple hematopoietic cell types and may regulate intracellular signaling pathways important for proper integrin function.

In this paper, we have examined the role of Vav proteins in β_2 integrin- and GPCR-dependent biological functions of neutrophils using mice deficient in both Vav1 and Vav3. We show that Vav proteins are required for multiple β_2 integrin-induced functions, including sustained adhesion, spreading, and complement-mediated phagocytosis, but are not essential for initial agonist-induced inside-out activation. β_2 inte-

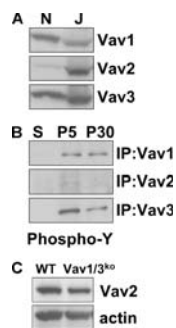


Figure 1. Expression and β_2 integrin-induced activation of Vav proteins in primary mouse neutrophils. (A) Neutrophils lysates (N) were analyzed for Vav expression using noncross-reactive Vav1, Vav2, or Vav3 antibodies. Jurkat cells (J) were included as a positive control. (B) Neutrophils were stimulated in suspension with TNF α (S) followed by plating onto C3bi-coated dishes for 5 and 30 min (P5, P30). Vav proteins were immunoprecipitated and blotted with anti-phosphotyrosine antibody. The exposure time for detection of Vav2 phosphorylation was significantly longer than that for Vav1 and Vav3.

(C) Vav2 levels in WT and Vav1/3^{ko} neutrophils were examined using anti-Vav2 antibody. Actin levels show equal loading.

grin-induced activation of Cdc42, Rac1, and RhoA is defective in the absence of Vav1 and Vav3. In addition, Pyk2 and paxillin phosphorylation, Akt activation, and phosphorylation of the Rho family GTPase effectors PAK and myosin light chain (MLC) are impaired in the absence of Vav proteins. The requirement for Vav is specific for integrin-dependent events, as GPCR-induced signaling pathways and chemotaxis are largely unaffected in Vav-deficient neutrophils. Thus, our work has defined a novel role for Vav proteins as critical regulators of integrin function in neutrophils.

Results

Activation of Vav proteins in response to adhesion in primary neutrophils

Vav GEFs can be activated by tyrosine phosphorylation via Syk and Src family kinases (SFKs) and previous work from our laboratory has implicated a signaling cascade involving these kinases in integrin-induced phosphorylation of Vav1 in $\alpha_{IIb}\beta_3$ -reconstituted CHO cells (Miranti et al., 1998; Moores et al., 2000). To determine whether integrin engagement induces Vav phosphorylation in primary phagocytes, we first examined which Vav proteins are expressed in neutrophils. Using noncross-reactive antibodies against specific family members, all three Vav proteins were detected in lysates of primary mouse neutrophils, with Vav1 and Vav3 in greatest abundance (Fig. 1 A). Quantitation based on the relative affinities of the Vav-specific antisera indicated that the relative abundance of the isoforms is Vav3 > Vav1 by approximately fourfold and that Vav1 > Vav2 by ~30-fold (unpublished data). To examine whether Vav proteins are activated in response to integrin engagement in leukocytes, we examined their tyrosine phosphorylation status after attachment of neutrophils to the physiological $\alpha_M\beta_2$ integrin ligand, complement fragment C3bi. Adhesion onto C3bi-coated surfaces is dependent on $\alpha_M\beta_2$, as α_M ^{-/-} neutrophils are unable to adhere to this ligand (Coxon et al., 1996; unpublished data). Because integrin receptors on resting neutrophils are in a low affinity state and require signals from proinflammatory stimuli to induce activation and adhesion to their ligands, cells were treated with TNF α to induce integrin receptor adhesive activity. TNF α stimulation in suspension did not result in phosphorylation of Vav proteins (Fig. 1 B). However, upon adhesion to C3bi, all three Vav family members became inducibly tyrosine phosphorylated

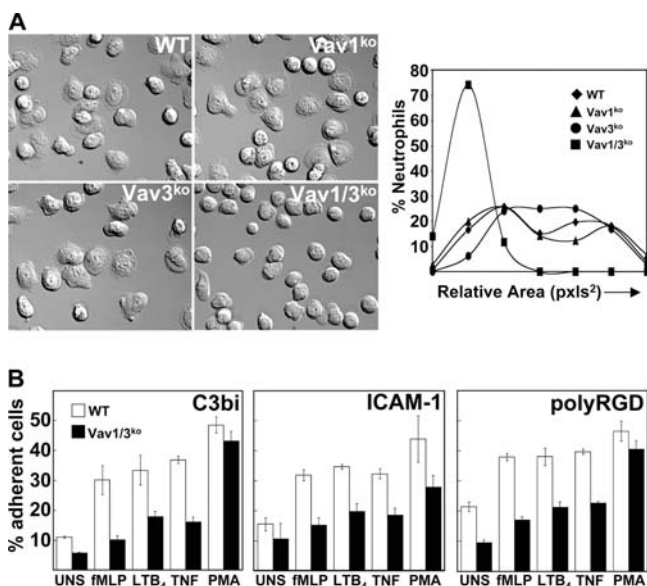


Figure 2. Vav1/3^{ko} neutrophils exhibit defective spreading and reduced strength of adhesion. (A) WT, Vav1^{ko}, Vav3^{ko}, and Vav1/3^{ko} neutrophils were plated on C3bi in the presence of TNFα for 30 min and imaged by DIC microscopy; cell spreading area of >100 cells was quantified. (B) WT or Vav1/3^{ko} neutrophils were plated on the indicated ligands with or without stimulation with fMLP, LTB₄, TNFα, or PMA for 30 min. The percentage of adherent cells after two static washes was quantified. The experiment was done in triplicate and values are mean ± SEM.

(Fig. 1 B), indicating that all three Vav proteins are activated in response to $\alpha_M\beta_2$ integrin ligation in neutrophils.

Vav proteins are required for spreading and sustained adhesion

To examine the role of Vav proteins in integrin-dependent neutrophil functions, *vav1*^{-/-} (Vav1^{ko}), *vav3*^{-/-} (Vav3^{ko}) and *vav1*^{-/-}*vav3*^{-/-} (Vav1/3^{ko}) neutrophils were analyzed for their ability to adhere and spread on two β_2 integrin ligands, C3bi and intercellular adhesion molecule (ICAM)-1 ($\alpha_M\beta_2$ and $\alpha_L\beta_2$ ligand), in response to multiple stimuli. In contrast to reported findings for *vav1*^{-/-} T lymphocytes (Krawczyk et al., 2002; Ardouin et al., 2003), neutrophils deficient in either Vav1 alone or Vav3 alone exhibited no defects in spreading (Fig. 2 A and not depicted). However, Vav1/3^{ko} neutrophils were severely defective in spreading on C3bi (Fig. 2 A) and ICAM-1 (not depicted) in response to *formyl*-Met-Leu-Phe (fMLP) and leukotriene B₄ (LTB₄; not depicted), and TNFα (Fig. 2 A). Neutrophil spreading on the synthetic integrin ligand polyRGD was also impaired (not depicted). The defect in spreading was not merely due to a delay in cell spreading because Vav1/3^{ko} neutrophils did not spread even after prolonged incubation in the presence of agonist (unpublished data). This spreading defect was associated with a significant reduction in the strength of adhesion, as fewer Vav1/3^{ko} neutrophils remained adherent on the ligand-coated surfaces after repeated washes (Fig. 2 B). The reduced adhesion was not attributable to decreased integrin expression nor impaired integrin receptor mobilization to the cell surface as we did not detect any differences in

the level of α_M or β_2 integrins in resting neutrophils and saw a similar increase after agonist stimulation based on FACS[®] analysis (unpublished data). Furthermore, neutrophils deficient in individual Vav proteins did not exhibit reduced adhesive strength over a range of ICAM-1-coating concentrations (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200404166/DC1>). Thus, Vav proteins may be functionally redundant in neutrophils and are required for sustained adhesion and spreading in vitro.

Proinflammatory stimuli transduce “inside-out” signals that modulate the affinity and avidity of integrins toward their ligands by inducing a high affinity conformational state as well as lateral integrin mobilization/clustering. Once engaged, integrins transduce “outside-in” signals that mediate coupling to the actin cytoskeleton and the generation of an adhesive force. Visual observation of neutrophils after stimulation revealed that Vav1/3^{ko} neutrophils are indeed able to undergo an initial adhesion to the ligand-coated surfaces, suggesting that the integrin receptors are competent to bind ligand. In addition, unstimulated wild-type (WT) neutrophils displayed a low level of adhesion on polyRGD (Fig. 2 B), most likely due to the direct cross-linking of integrins by the high valency of this ligand, which allows adhesion in the absence of additional stimuli (Mocsai et al., 2002). However, adhesion of unstimulated Vav1/3^{ko} neutrophils to polyRGD was significantly reduced (Fig. 2 B), suggesting that Vav is required for signals downstream of direct integrin cross-linking. Furthermore, although treatment of Vav1/3^{ko} cells with phorbol ester (PMA), which can bypass upstream signaling events and induce inside-out integrin activation, could rescue the spreading defect of Vav1/3^{ko} neutrophils, this effect was significantly delayed relative to PMA-induced spreading of WT neutrophils—maximum spreading of Vav1/3^{ko} neutrophils occurred after ~30 min of stimulation versus 5 min in WT neutrophils (unpublished data). Therefore, this rescue may be a secondary effect of PMA stimulation and not integrin activation per se.

To investigate inside-out activation, we examined the distribution of β_2 integrins in suspended neutrophils stimulated with fMLP. First, $\alpha_M\beta_2$ was constitutively clustered in unstimulated WT neutrophils and was not notably altered by fMLP stimulation. In contrast, $\alpha_L\beta_2$ was uniformly distributed in unstimulated WT neutrophils; however, we were able to detect clustering in response to fMLP in only a very small percentage of cells (unpublished data). Therefore, chemoattractant-induced, ligand-independent β_2 integrin clustering does not seem to significantly contribute to neutrophil adhesion under the conditions examined in this work.

Neutrophils rolling on selectins in vitro and in vivo rapidly arrest in response to proinflammatory stimuli by binding to ICAMs such as ICAM-1 via inside-out activation of β_2 integrins. To address whether the phenotype of Vav1/3^{ko} neutrophils was attributable to reduced initial adhesion due to defective inside-out β_2 integrin activation, we compared the ability of WT and Vav1/3^{ko} neutrophils to arrest on coimmobilized P-selectin/ICAM-1 in response to LTB₄ under low shear (1 dyne/cm²) in vitro. LTB₄-mediated neutrophil arrest on ICAM-1 requires β_2 integrin function, as β_2 ^{-/-} neutrophils did not arrest in response to this stimulus (unpublished data). Vav1/3^{ko} neutrophils rapidly arrested on

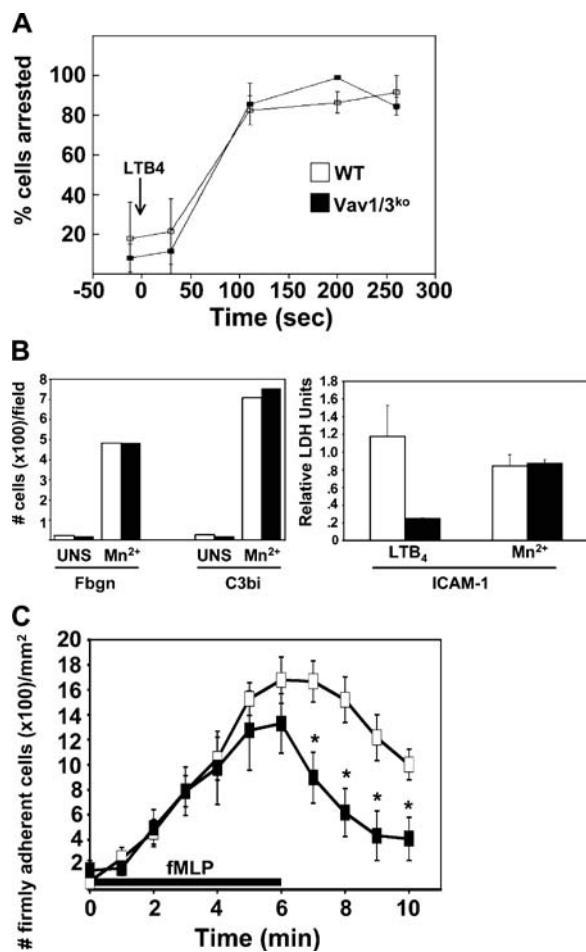


Figure 3. Vav1/3^{ko} neutrophils arrest in vitro and in vivo. (A) WT and Vav1/3^{ko} neutrophils were infused across immobilized P-selectin/ICAM-1 at 1.0 dyne/cm², followed by infusion of 100 nM LTB₄ (arrow). The percentage of arrested cells, defined as stationary for 6 s, shortly before and after exposure to LTB₄ was determined. The results were averaged from two experiments and bars indicate the values from both. (B) Adhesion of WT and Vav1/3^{ko} neutrophils to fibrinogen (Fbgn), C3bi, and ICAM-1 was induced with 1 mM Mn²⁺ treatment and quantified by counting the number of cells/field (Fbgn, C3bi) or by LDH assay (ICAM-1). (C) Intravital microscopy was performed on the cremaster muscle of WT or Vav1/3^{ko} mice in response to fMLP superfusion. The bar indicates the time of fMLP superfusion. The number of firmly adherent leukocytes, defined as stationary for 30 s, was determined and normalized by dividing by the cross-sectional area for each vessel. Values are mean ± SEM from 11 wild-type and six Vav1/3^{ko} venules from 3–5 mice/genotype. * indicates P < 0.05 (unpaired two-tailed *t* test).

ICAM-1 in response to LTB₄ indistinguishably from WT neutrophils, suggesting that initial inside-out integrin activation was not defective (Fig. 3 A). However, visual inspection of the video data indicated that the arrested Vav1/3^{ko} neutrophils frequently lifted from the surface if the flow rate was increased (unpublished data). These results suggest that Vav proteins are not essential for initial inside-out integrin activation induced by agonists but are required for sustained adhesion of neutrophils in vitro. To rule out any intrinsic defects in β_2 integrin ligand binding activity, neutrophil adhesion was induced by manganese, which binds to the extracellular metal ion-dependent adhesion site on integrins

and induces the active, high affinity conformation in the absence of additional stimuli. Comparable levels of WT and Vav1/3^{ko} neutrophils adhered to immobilized fibrinogen, C3bi and ICAM-1 in response to manganese treatment (Fig. 3 B), indicating that Vav deficiency does not affect the intrinsic ability of β_2 integrins to bind ligand.

In vivo, circulating leukocytes survey the environment for proinflammatory stimuli by selectin-mediated rolling on the endothelium. At sites of tissue inflammation, rolling leukocytes are quickly converted to firmly adherent leukocytes through β_2 integrin-dependent attachment to ICAM-1, a major cell adhesion molecule on endothelial cells mediating chemoattractant-induced leukocyte arrest in vivo (Foy and Ley, 2000). To directly determine whether neutrophil adhesion was affected in vivo, leukocyte adhesion was induced in WT and Vav1/3^{ko} mice by superfusion of fMLP onto the exteriorized cremaster muscle and quantified in individual venules by intravital microscopy. Both WT and Vav1/3^{ko} neutrophils arrested in response to fMLP in vivo (Fig. 3 C). However, arrested neutrophils from Vav1/3^{ko} mice displayed a significant reduction in stable adhesion, as fewer cells remained attached over time. Thus, as observed in vitro, neutrophils from Vav1/3^{ko} mice arrest in response to fMLP but exhibit significantly reduced sustained adhesion in vivo.

Defective complement-mediated phagocytosis in Vav1/3^{ko} neutrophils

Neutrophils are professional phagocytes that efficiently bind and clear foreign particles. Serum opsonization leads to efficient fixation of complement C3bi on the surface of bacteria and renders them susceptible to complement-mediated phagocytosis via $\alpha_M\beta_2$. To examine whether Vav is required for this process, we compared the phagocytic activity of WT and Vav-deficient neutrophils using serum-opsonized fluorescent *E. coli*. Although there was no detectable defect in Vav1^{ko} or Vav3^{ko} neutrophils (not depicted), phagocytosis was severely impaired in Vav1/3^{ko} neutrophils (Fig. 4, A and B). PMA pretreatment was able to only partially rescue this defect in that Vav1/3^{ko} cells contained significantly fewer fluorescent bacteria per neutrophil (Fig. 4 A). The cells were imaged in the presence of trypan blue to quench any extracellular, unphagocytosed bacteria, as demonstrated by the absence of signal in cells incubated with unopsonized *E. coli* (Fig. 4 A). To determine whether the impaired phagocytic activity of Vav1/3^{ko} neutrophils was due to reduced particle binding, neutrophils were incubated with serum-opsonized fluorescent *E. coli* in the presence of the actin polymerization inhibitor, Latrunculin B, which blocks particle ingestion (Fig. 4 A) but not binding. A similar percentage of WT and Vav1/3^{ko} neutrophils contained bound *E. coli* (76% WT vs. 81% Vav1/3^{ko}) and the amount of bound *E. coli* per cell was also comparable (Fig. 4 C). Thus, the defect in complement-mediated phagocytosis of Vav1/3^{ko} neutrophils is not attributable to decreased particle binding.

Multiple β_2 integrin-dependent signaling pathways are affected in Vav1/3^{ko} neutrophils

Vav family proteins have multiple structural domains in addition to the catalytic Dbl homology domain that mediates

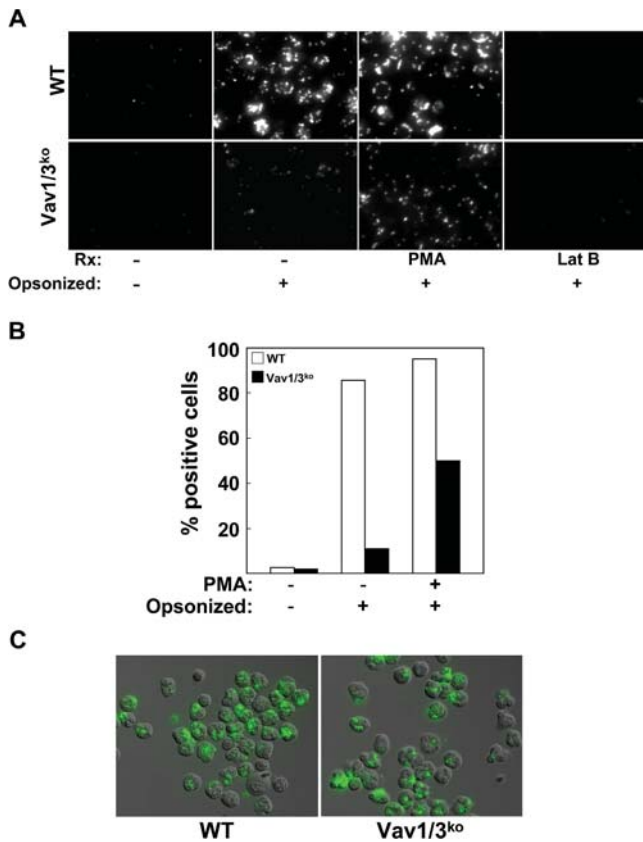


Figure 4. Defective complement-mediated phagocytosis in Vav1/3^{ko} neutrophils. (A) Phagocytic activity of WT and Vav1/3^{ko} toward unopsonized or serum-opsonized FITC-*E. coli*. Trypan blue was used to quench extracellular *E. coli*. Where indicated, cells were treated with PMA or Latrunculin. (B) Quantification of phagocytosis. Neutrophils containing at least one *E. coli* were scored by fluorescence microscopy. The experiment was repeated three times, and values shown are from a representative experiment in which >200 cells were scored. (C) Binding activity of WT and Vav1/3^{ko} neutrophils to serum-opsonized FITC-*E. coli* in the presence of Latrunculin.

GEF activity. To examine Vav-dependent signaling pathways downstream of integrin ligation, WT and Vav1/3^{ko} neutrophils were primed in suspension with TNF α and adhered to C3bi. Adhesion-induced tyrosine phosphorylation was significantly reduced in lysates from Vav1/3^{ko} neutrophils (Fig. 5 A). Because two of the tyrosine phosphorylated bands displayed the same mobility as the focal adhesion-associated proteins Pyk2 and paxillin, we directly examined the phosphorylation status of these proteins using phospho-specific antibodies. In accordance with the phosphotyrosine profile, the phosphorylation of both Pyk2 and paxillin was significantly reduced in Vav1/3^{ko} neutrophils (Fig. 5 B), but not in Vav1^{ko} or Vav3^{ko} neutrophils (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200404166/DC1>). Integrin ligation results in the accumulation of 3-phosphorylated phosphoinositides, catalyzed by the enzyme PI 3-kinase (King et al., 1997). Activation of the S/T kinase Akt requires 3-phosphorylated phosphoinositides and can therefore be used as a read out for PI 3-kinase activity. Adhesion-induced activation of PI 3-kinase/Akt was also decreased in Vav1/3^{ko} neutrophils, but not singly deficient

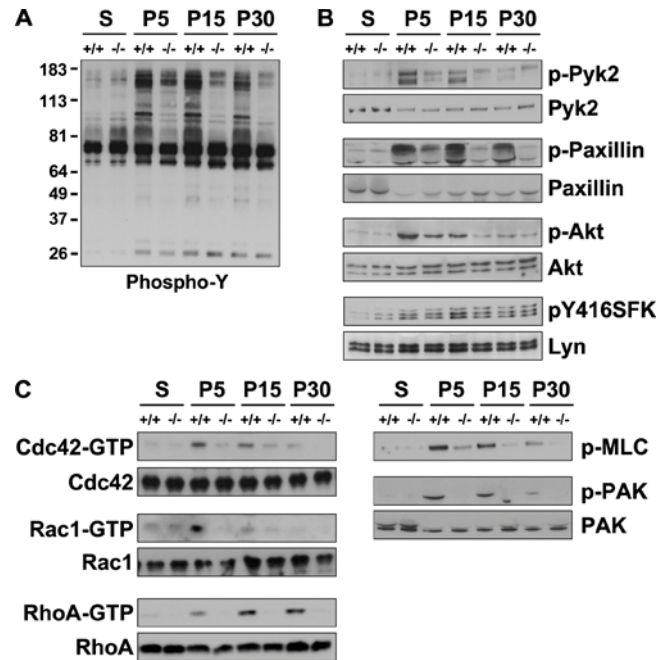


Figure 5. Multiple β_2 integrin-activated signaling pathways are affected in Vav1/3^{ko} neutrophils. WT (+/+) or Vav1/3^{ko} (-/-) neutrophils were primed with TNF α in suspension (S) and plated (P) on C3bi for the indicated time (min). Lysates were analyzed for total phosphotyrosine (A); pY881 Pyk2, pY118 Paxillin, pS473 Akt, and pY416 Src (B). (C) Adhesion-induced activation of the Rho GTPases Cdc42, Rac1, and RhoA was analyzed by GST-PBD and GST-RBD pull-down. Activation of the downstream effectors PAK α and MLC was analyzed using pS198/S203 and pS19 antibodies, respectively.

neutrophils (Fig. 5 B and Fig. S2). In contrast, β_2 integrin-induced activation of SFKs in Vav1/3^{ko} neutrophils was comparable to WT (Fig. 5 B), indicating that signaling pathways upstream and independent of Vav remain intact.

It is well documented that Rho family GTPases play an important role in integrin-mediated adhesion, spreading, and migration by regulating the formation and maturation of focal adhesions and of actin-based structures (Ridley, 1999). Vav GEFs can induce activation of multiple members of the Rho family when overexpressed in fibroblasts (Bustelo, 2000) and reduced Rac-GTP levels have been observed in *vav1*^{-/-} thymocytes after TCR stimulation, implicating endogenous Vav proteins as Rac GEFs in T cells (Reynolds et al., 2002). Therefore, we examined the activation status of Rho GTPases after integrin engagement by affinity isolating the GTP-bound form using GST-PBD (p21-binding domain of PAK) or GST-RBD (Rho binding domain of rhotekin). Adhesion onto C3bi induced the activation of Cdc42, Rac1, and RhoA in WT neutrophils. However, activation of all three GTPases was impaired in Vav1/3^{ko} neutrophils (Fig. 5 C). We were unable to detect adhesion-induced activation of Rac2 in WT neutrophils by this assay (unpublished data). Accordingly, activation of the Rac/Cdc42 effector, the S/T kinase PAK, was almost completely absent in Vav1/3^{ko} neutrophils (Fig. 5 C). Phosphorylation of MLC, which is mediated by the Rho effector ROCK, was also significantly reduced in the Vav1/3^{ko} neutrophils (Fig. 5 C). Unlike Vav1/3^{ko} neutrophils, Vav1^{ko} and

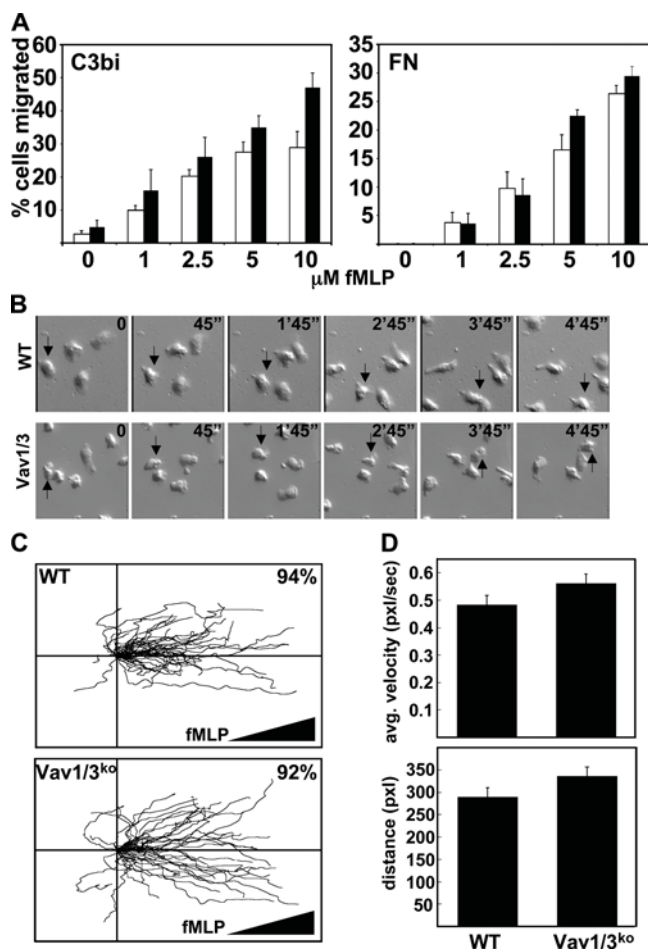


Figure 6. In vitro chemotaxis of Vav1/3^{ko} neutrophils. (A) Chemotaxis of WT (white bars) and Vav1/3^{ko} (black bars) in response to fMLP on C3bi- or fibronectin (FN)-coated transwell filters. Values are the mean percentage of cells migrated \pm SEM. (B) Video microscopy of neutrophils plated on C3bi-coated Zigmond chambers. fMLP increases in concentration from left to right. Arrows indicate the migration of a particular neutrophil. See online supplemental material to view videos. (C) The migration paths of cells from B were tracked and the percentage of cells that underwent a net positive displacement in the direction of the gradient is indicated. (D) The average velocity and distance migrated were quantified with Metamorph. Values are mean \pm SEM of 50 cell tracks.

Vav3^{ko} neutrophils did not exhibit reduced phosphorylation of PAK or MLC (Fig. S2), indicating that individual Vav proteins are functionally redundant in neutrophils. Thus, β_2 integrin-dependent activation of Rac1, Cdc42, and RhoA and downstream effectors of these GTPases is dependent on Vav1 and Vav3 in neutrophils.

Vav proteins are not required for chemotaxis

One of the first steps of directional motility is the formation of a leading edge which is stabilized by integrin attachments to the substratum. Furthermore, the creation and turnover of these integrin-based focal adhesions is a tightly coordinated process during cell migration (Webb et al., 2002). Given the defects in spreading and firm adhesion in Vav1/3^{ko} neutrophils, we examined fMLP-dependent chemotaxis of these cells in vitro using transwell filters coated with inte-

grin ligands. Surprisingly, we observed no defects in chemotaxis of Vav1/3^{ko} neutrophils over a range of chemoattractant concentrations on either C3bi- or fibronectin-coated transwell filters (Fig. 6 A). To specifically examine whether Vav1/3^{ko} neutrophils exhibited altered cell polarization, speed, or distance of migration, we monitored chemotaxis by video microscopy using Zigmond chambers (Zigmond, 1988) coated with C3bi. Similar to the transwell assays, Vav1/3^{ko} neutrophils migrated efficiently in the direction of the gradient (Fig. 6 B; Videos 1 and 2, available at <http://www.jcb.org/cgi/content/full/jcb.200404166/DC1>). Furthermore, analysis of the migration tracks of these cells revealed that similar percentages of both WT and Vav1/3^{ko} neutrophils polarized and migrated up the chemotactic gradient of fMLP (Fig. 6 C, 94% vs. 92%). Notably, Vav1/3^{ko} neutrophils on average migrated slightly faster than WT cells and traversed a longer distance (Fig. 6 D). Similar results were obtained with LTB₄ (not depicted). Thus, despite significant β_2 integrin adhesion defects, Vav1/3^{ko} neutrophils are able to efficiently polarize and migrate in response to a chemotactic gradient.

Activation of multiple GPCR-dependent signaling pathways in Vav1/3^{ko} neutrophils

Because Vav1 can couple to diverse classes of signaling receptors, it is possible that Vav proteins may also be required for integrin-independent signaling pathways in neutrophils. Many neutrophil chemoattractants are GPCRs agonists and require Rac activity to mediate multiple biological effects (Roberts et al., 1999; Glogauer et al., 2003). Therefore, we examined whether GPCR-induced signaling pathways were affected in Vav1/3^{ko} neutrophils in response to fMLP and LTB₄. fMLP stimulation induced a robust activation of Rac1, Rac2, and Cdc42 in Vav1/3^{ko} neutrophils that was indistinguishable from WT (Fig. 7 A). Similarly, activation of PAK, Akt, and Erk1/2 occurred normally in Vav1/3^{ko} neutrophils stimulated with fMLP (Fig. 7 B) or LTB₄ (not depicted). We were unable to detect activation of RhoA in WT neutrophils in response to fMLP (not depicted). These data suggest that Vav proteins are dispensable for many adhesion-independent signaling events in mouse neutrophils.

Discussion

In this paper, we demonstrate that Vav GEFs are required for multiple β_2 integrin-dependent functions of neutrophils. Although all three Vav proteins are expressed in neutrophils, Vav1 and Vav3 account for \sim 99% of total Vav protein. Deficiency in a single family member does not lead to any detectable neutrophil dysfunction; however, a combined deficiency in Vav1 and Vav3 results in defective chemoattractant-induced sustained adhesion and integrin-mediated phagocytosis. Adhesion assays under flow and intravital microscopy of the cremaster muscle revealed that chemoattractant-induced arrest is unaffected in Vav1/3^{ko} neutrophils, indicating that the adhesion defect is likely not due to a loss in initial inside-out integrin activation. β_2 integrin-dependent activation of multiple Rho GTPases—Rac1, Cdc42, and RhoA—is impaired in the absence of

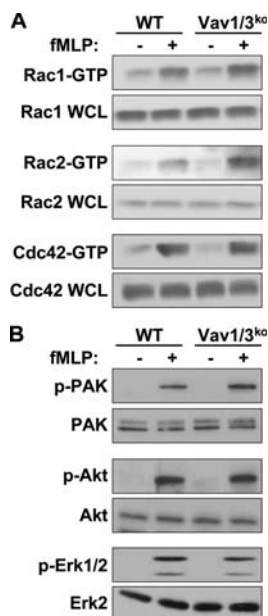


Figure 7. fMLP-activated signaling pathways are unaffected in Vav1/3^{ko} neutrophils. (A) WT and Vav1/3^{ko} neutrophils were stimulated with fMLP for 15s and activation of Rac1, Rac2, and Cdc42 was analyzed by GST-PBD pull-downs. (B) WT and Vav1/3^{ko} neutrophils were stimulated with fMLP for 5 min and analysis of PAK, Akt, and Erk1/2 was performed as described in Fig. 5.

Vav1 and Vav3. In addition, reduced activation of multiple integrin-induced intracellular signaling proteins is also evident. In contrast, fMLP- and LTB₄-induced activation of intracellular signaling pathways and chemotaxis are unaffected in Vav1/3^{ko} neutrophils. Thus, Vav GEFs are specifically required downstream of β_2 integrins in neutrophils.

Although distinct functions have been attributed to different Vav family members in certain cell types (e.g., T and B lymphocytes), our results clearly suggest that Vav1 and Vav3 play redundant roles in regulating neutrophil functions downstream of integrins and that the contribution of Vav2 appears to be minimal. Vav2 expression represents only a small fraction of total Vav protein, its expression does not increase in Vav1/3^{ko} neutrophils (Fig. 1 C), and neutrophils deficient in all three *vav* genes do not exhibit a more severe phenotype than those deficient in both *vav1* and *vav3*, based on adhesion, spreading, and chemotaxis (unpublished data).

Vav regulation of integrin-mediated adhesion

One critical aspect of leukocyte adhesion in response to inflammatory stimuli is the induction of integrin binding activity through inside-out signaling events that convert integrins from a low to a high affinity/avidity ligand-binding state. Our evidence that Vav1/3^{ko} neutrophils are able to undergo chemoattractant-induced arrest under flow in vitro and in circulating blood in vivo suggests that Vav is not required for initial events associated with inside-out integrin activation. However, given the complexity of this response (Ley, 2003), we cannot rule out that Vav contributes to full activation of integrins, thus affecting the strength and maintenance of adhesion.

Impaired integrin-mediated adhesion has also been reported in Vav1-deficient T lymphocytes (Krawczyk et al., 2002; Ardouin et al., 2003). This conclusion was based on defects in static adhesion and in $\alpha_1\beta_2$ clustering on the cell surface in response to TCR stimulation. Because Vav1 plays a critical role in transducing early TCR signals required for most TCR-induced biological activities, including actin polymerization and activation of many downstream effectors of in vitro (Bustelo, 2000), the specific effects of Vav deficiency on adhesion and integrin activation per se are difficult to distinguish from those required to activate early receptor-proximal events. In neutrophils, Vav is not required for fMLP or LTB₄-induced chemotaxis, initial adhesion, or activation of Rac, Cdc42, Erk, or Akt. Therefore, loss of Vav does not have broad effects on early receptor-induced events in chemoattractant-stimulated neutrophils as in TCR-stimulated T cells, and our data supports the possibility that Vav specifically regulates integrin-mediated adhesion events.

Vav requirement for integrin-dependent Rho GTPase activation

Rho GTPases are key mediators of actin cytoskeletal rearrangements associated with adhesion and spreading as they nucleate events involved in formation of focal adhesions, lamellipodia, and filopodia (Ridley, 1999). However, the mechanisms responsible for integrin-mediated Rho GTPase activation are poorly defined. Here, we show that β_2 integrin-dependent activation of Rac1, Cdc42, and RhoA is defective in Vav1/3^{ko} neutrophils, implicating Vav proteins as critical Rho family GEFs downstream of integrins. The loss of integrin-induced activation of RhoA, Rac1, and Cdc42 in Vav1/3^{ko} neutrophils could be a consequence of direct effects of Vav-mediated GTP exchange on multiple Rho family members or from indirect Vav regulation of multiple Rho GTPases, for example, due to a cascade of Rho GTPase activation or to generalized effects of Vav deficiency on adhesion and spreading. Vav family proteins have been reported to serve as GEFs for RhoA, RhoG, Rac, and Cdc42; however, the specificity of individual Vav proteins has been difficult to establish for many reasons: lack of consistent results from in vitro experiments in different laboratories, use of distinct domains of Vav for in vitro exchange assays, and complications of interpreting experiments in cultured cells in which distinct Vav proteins have been overexpressed (Bustelo, 2000). Thus, it is not feasible to distinguish, based on previous studies, whether the phenotypic effects observed here are due to direct or indirect effects of Vav on these GTPases. Studies from Olson and coworkers demonstrated that a mutant, constitutively activated variant of Vav1 can independently activate Rho, Rac, and Cdc42 in Swiss 3T3 cells (Olson et al., 1996); however, one cannot extrapolate these findings to the endogenous Rho GTPases activated by integrins in neutrophils. There are also precedents for a hierarchical cascade linking Rho GTPases (Ridley and Hall, 1992; Nobes and Hall, 1995; Price et al., 1998), yet coupling between these proteins has not been examined in neutrophils after attachment to integrin ligands. Thus, further studies will be required to establish the basis for loss of activation of RhoA, Rac1, and Cdc42 in Vav1/3^{ko} neutrophils.

Regardless of whether the loss of RhoA, Rac1, and Cdc42 activation is a direct or indirect result of Vav deficiency, impaired Rho GTPase activation may be the major contributing factor in the phenotypes exhibited in Vav1/3^{ko} neutrophils. As previously discussed, Vav1/3^{ko} neutrophils are able to make initial contacts with immobilized integrin ligands, yet these neutrophils are unable to spread. Although the precise downstream intracellular events that coordinate cell spreading are poorly understood, a role for Rac and Cdc42 has been well documented in hematopoietic and nonhematopoietic cells (Allen et al., 1997; Clark et al., 1998; D'Souza-Schorey et al., 1998). In addition, Rac1- and Rac2-deficient neutrophils exhibit defects in integrin-mediated cell spreading (Roberts et al., 1999; Glogauer et al., 2003). In contrast, Rho has been implicated in events required for integrin-mediated firm adhesion and aggregation in lymphocytes and platelets (Morii et al., 1992; Laudanna et al., 1996; Woodside et al., 1998), but not for cell spreading (Ren et al., 1999). Interestingly, Rho, but not Rac or Cdc42, has been shown to be required for complement-mediated phagocytosis in macrophages (Caron and Hall, 1998), suggesting that loss of Rho activation contributes to the defect in phagocytosis in Vav1/3^{ko} neutrophils. Thus, failure to activate RhoA, Rac1, and Cdc42 may differentially contribute to the loss of distinct integrin-mediated events in neutrophils.

Phenotypic similarities with Syk- and SFK-deficient neutrophils

In both platelets (Oberfell et al., 2002) and neutrophils (Mocsai et al., 2002), Syk is required for adhesion-induced Vav phosphorylation, implicating Syk as an upstream regulator of Vav. Not surprisingly, Syk- and Vav-deficient neutrophils display multiple phenotypic similarities, including impaired spreading on multiple integrin ligands and reduced Pyk2 activation (Mocsai et al., 2002). Moreover, despite impaired integrin function, SFK-, Syk-, and Vav-deficient neutrophils do not exhibit defects in chemotaxis. The requirement for Syk in neutrophils is limited to integrin-dependent functions, as GPCR-mediated responses are unaffected in *syk*^{-/-} (Mocsai et al., 2003). This evidence, coupled with the data in this paper, suggests that Vav proteins are critical downstream mediators of integrin-Syk signaling in neutrophils.

Vav1/3^{ko} neutrophil migration in vitro and in vivo

Neutrophils polarize in response to shallow chemotactic gradients by extending a leading edge lamellipodium that is stabilized via integrin-mediated adhesion to the substratum. The evidence that β_2 integrins are required for leukocyte migration in multiple mouse inflammatory model systems (Mizgerd et al., 1997; Walzog et al., 1999) demonstrates the importance of these receptors for optimal migration in vivo. Surprisingly, despite significant adhesion defects, Vav1/3^{ko} neutrophils efficiently polarize and migrate on C3bi-coated glass in response to a chemotactic gradient of either fMLP or LTB₄. In comparison to WT, Vav1/3^{ko} neutrophils on average migrate slightly faster, most likely due to their decreased adhesiveness. These data, coupled with the phenotypes of *syk*^{-/-} and *sfk*^{-/-} neutrophils, indicate that optimal integrin function is not required for the amoeboid movement of neutrophils in che-

motactic models in vitro. It is possible that the minimal adhesion that occurs in Vav1/3^{ko} neutrophils is sufficient to mediate this form of chemotaxis in vitro. However, in vivo leukocytes must survey the environment by rolling on venular endothelium and arrest at sites of tissue inflammation firmly enough to withstand the constant shear force from blood flow. Intravital microscopy of the cremaster muscle indicates that Vav1/3^{ko} leukocytes are not as stably attached as WT to the venular endothelium in response to fMLP. Despite this difference, we have observed similar levels of neutrophil extravasation to the peritoneum in WT and Vav1/3^{ko} mice treated with thioglycollate in preliminary experiments (unpublished data). Similar results have been reported for *syk*^{-/-} and *sfk*^{-/-} mice (Mocsai et al., 2002); however Rac1- and Rac2-deficient mice display defects in chemotaxis in vitro and inflammatory recruitment in vivo (Roberts et al., 1999; Li et al., 2002; Glogauer et al., 2003). Although it is difficult to resolve this discrepancy, these studies suggest that minimal integrin activity may suffice for both in vivo and in vitro neutrophil migration. Alternatively, thioglycollate, an extremely potent inflammatory stimulus, may bypass the need for Syk and Vav signaling in neutrophils, analogous to phorbol ester stimulation in vitro. Therefore, alternative in vivo models are required to resolve this discrepancy.

Vav proteins in GPCR signaling

Binding of GPCR agonists to their cognate receptors initiates signaling cascades leading to the activation of Rho GTPases, presumably via activation of a Rho exchange factor. Specifically, Rac2 activity is required for multiple GPCR-induced events in neutrophils, including chemotaxis to fMLP and LTB₄ (Roberts et al., 1999). The fact that Vav1/3^{ko} neutrophils undergo normal chemotaxis in response to these agonists suggests that Vav1 and Vav3 are not critical mediators of GPCR signaling. In addition, GPCR-induced calcium flux, integrin up-regulation, and initial inside-out integrin activation are unaffected in Vav1/3^{ko} neutrophils. fMLP- and LTB₄-induced activation of Rho GTPases, Akt, and Erk are also unaffected, and preliminary experiments with Vav triple knockout neutrophils suggest Vav proteins are completely dispensable for fMLP- and LTB₄-induced chemotaxis (unpublished data). Thus, Vav proteins are not critical mediators of many GPCR-induced signaling events in mouse neutrophils, raising the possibility that another GEF, potentially the G $\beta\gamma$ -dependent P-Rex (Welch et al., 2002), regulates Rac activation downstream of GPCRs.

Our results have defined a novel requirement for Vav GEFs as critical mediators of β_2 integrin-dependent Rho GTPase activation in neutrophils, in addition to their well-documented role downstream of IRRs. The regulation of neutrophil adhesion and phagocytosis is an important aspect of innate immunity. Thus, Vav proteins may be attractive therapeutic targets for inflammatory diseases.

Materials and methods

Animals

Germline *vav1*^{-/-} mice (Turner et al., 1997) were provided by V. Tybulewicz (National Institute for Medical Research, London, UK). Vav3^{ko} (provided by F. Alt, Children's Hospital, Boston, MA) and Vav1/3^{ko} mice

are described elsewhere (Fujikawa et al., 2003). Vav1/3^{ko} mice were bred with 129Sv wild-type mice (Taconic) to generate Vav1/3^{+/-} and were crossed to generate all lines. 8–12 wk old age- and gender-matched mice were used for experiments. All mice were maintained under specific pathogen-free conditions.

Isolation of bone marrow neutrophils

Neutrophils were isolated by Percoll density centrifugation as described previously (Mocsai et al., 2003). For a detailed protocol see online supplemental material. Equal cell equivalents were analyzed for Vav expression with rabbit anti-Vav1 (C-14; Santa Cruz Biotechnology, Inc.), anti-Vav2 (2203), and anti-Vav3 (2206; Oberfell et al., 2002). Antiserum 2203 to Vav2 was produced by rabbit immunization (Covance) with a bacterially expressed GST-Vav2 fusion protein containing human Vav2 amino acids 573–878. It does not cross react with endogenous or recombinant Vav1 or Vav3 by Western blotting.

Neutrophil adhesion assays

All reagents were purchased from Sigma-Aldrich, unless otherwise indicated. For C3bi coating, 10 µg/ml mouse IgM (BD Biosciences) was absorbed onto 96-well plates (Immulon) overnight at 4°C, washed with PBS (Cellgro) and coated with 33–50% mouse serum. 10 µg/ml mICAM-1:Fc (R&D Systems) was bound to protein A-coated wells (Pierce Chemical Co.) according to the manufacturer's instructions. 20 µg/ml polyRGD was coated overnight at 4°C. Neutrophils in adhesion assay media (AAM) [HBSS⁺/20 mM Hepes, pH 7.4, 0.1% low endotoxin HSA] were stimulated with 10 µM fMLP, 100 nM LTB₄, 10 ng/ml mTNFα (R&D Systems), or 100 ng/ml PMA for 30 min at 37°C, washed 2× with AAM, and adherent neutrophils quantified by LDH content using the Cytotox Kit (Promega). For flow assays, the flow apparatus used has been described previously (Luscinskas et al., 1994) with the modifications described in online supplemental material. Cells were infused onto co-immobilized P-selectin/ICAM-1 followed by 100 nM LTB₄ and recorded by video microscopy. Arrested cells were defined as those that remained stationary for 6 s and the percentage was calculated by dividing the number of arrested cells by the number of interacting cells (rolling and adherent). Intravital experiments were performed in accordance with protocols approved by the University of Virginia Health Science Center institutional committee for animal use and as described previously (Ley et al., 1995). For a detailed protocol see online supplemental material.

Complement-mediated phagocytosis assays

FITC-*E. coli* (Molecular Probes) were opsonized with mouse serum for 30 min at 37°C. Neutrophils were treated with 100 ng/ml PMA or 500 nM Latrunculin B (Calbiochem) for 15 min and incubated with *E. coli* for 30 min. Extracellular fluorescence was quenched with 0.2% trypan blue and representative images were captured using a microscope (model TE300; Nikon) and 40× objective. For quantification, cells were fixed with 3.7% formalin and manually scored by fluorescence microscopy. Cells containing at least one bacterium were scored positive and >200 cells were counted. For binding activity, cells were treated with Latrunculin B and incubated with *E. coli* for 30 min at 37°C, washed with PBS, and fixed. Cells containing bound *E. coli* were scored by fluorescence microscopy. Representative images were captured with an ORCA1 CCD camera (Hamamatsu) and 60× objective and overlaid with Metamorph (Universal Imaging Corp.).

Chemotaxis assays

Transwell filters were coated with C3bi or with fibronectin. Assays were performed as described previously (Mocsai et al., 2002), except migrated cells were scored by hemocytometer. For detailed information, see online supplemental material. For video microscopy, cells were plated on C3bi-coated coverslips and mounted onto Zigmund slides (Neuroprobe). The gradient was established with 10 µM fMLP for 10 min. Images were captured at 15-s intervals for >10 min with a 20× DIC objective on a microscope (model TE300; Nikon) modified with a heated stage. Migration paths, velocity, and distance were calculated with Metamorph. 50 tracks/genotype were analyzed.

Biochemistry experiments

DFP-washed cells were primed with TNFα and plated on C3bi-coated dishes at 37°C. Unattached cells were removed by aspiration. Cells were lysed and lysates normalized by LDH content using the Cytotox kit and analyzed by immunoblotting against: phosphotyrosine (4G10; provided by T. Roberts, Dana Farber Cancer Institute, Boston, MA); pY881 Pyk2 and pY118 paxillin (Biosource International); pY416 Src, pS473 Akt, and pS19 MLC2 (Cell Signaling); PAKα (Santa Cruz); Pyk2 and paxillin (Transduction

Laboratories); and pS198/S203 PAKα (a gift from M. Greenberg, Children's Hospital, Boston, MA). For Vav IPs, equal amounts of lysate were incubated with Vav1 (C-14), Vav2 (2203), and Vav3 (2206) antibodies for 2–3 h. For GTPase pull-down assays, neutrophils were plated on C3bi and PBD assays performed as described previously (Benard and Bokoch, 2002). Rho assays were performed as described previously (Ren et al., 1999) except cells were lysed in PBD lysis buffer. Pull-downs were blotted with Rac1 and Cdc42 (Transduction Laboratories) or RhoA (Santa Cruz Biotechnology, Inc.) antibodies. Rac2 antibody was a gift from G. Bokoch (Scripps Research Institute, La Jolla, CA). For fMLP stimulation, suspended neutrophils were treated with 10 µM fMLP followed by immediate lysis with 2× lysis buffer.

Online supplemental material

The accompanying videos to the still clips in Fig. 6 show wild-type (Video 1) and Vav1/3^{ko} (Video 2) neutrophils migrating towards a gradient of fMLP in C3bi-coated Zigmund chambers. Analysis of adhesion of single Vav knockout neutrophils to varying concentrations of ICAM-1 is shown in Fig. S1. In addition, activation of multiple integrin signaling pathways in Vav single knockout neutrophils in response to adhesion to C3bi is shown in Fig. S2. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200404166/DC1>.

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