Vav Proteins in Neutrophils Are Required for FcγR-Mediated Signaling to Rac GTPases and Nicotinamide Adenine Dinucleotide Phosphate Oxidase Component p40(phox)[1]

Ahmad Utomo,[2]* Xavier Cullere,[2]* Michael Glogauer,[†] Wojciech Swat,[‡] and Tanya N. Mayadas[3]*

Phagocytes generate reactive oxygen species, the regulation of which is important in eliminating ingested microbes while limiting tissue damage. Clustering of FcγRs results in the activation of Vav proteins, Rho/Rac guanine nucleotide exchange factors, and results in robust superoxide generation through the NADPH oxidase. In this study, studies in neutrophils isolated from mice deficient in Vav or Rac isoforms demonstrate a critical role for Vav3 in Rac2-dependent activation of the NADPH oxidase following FcγR clustering. However, studies in cytokine-primed cells revealed a strict requirement for Vav1 and Vav3 and Rac1 and Rac2 in the FcγR-mediated oxidative burst. In comparison, Vav was not essential for PMA or G protein-coupled receptor-mediated superoxide generation. The FcγR-mediated oxidative burst defect in Vav-deficient cells was linked to aberrant Rac activation as well as Rac- and actin-polymerization-independent, but PI3K-dependent, phosphorylation of the NADPH oxidase component p40(phox). In macrophages, Vav regulation of Rac GTPases was required specifically in FcγR-mediated activation of the oxidative burst, but not in phagocytosis. Thus, Vav proteins specifically couple FcγR signaling to NADPH oxidase function through a Rac-dependent as well as an unexpected Rac-independent signal that is proximal to NADPH oxidase activation and does not require actin polymerization. *The Journal of Immunology,* **2006, 177: 6388–6397.**

Innate immune responses require phagocytic leukocytes to destroy invading pathogens through the phagocytosis of microbes and the subsequent release of reactive oxygen radicals, digestive enzymes, and antimicrobial peptides. FcγR on neutrophils and macrophages are required for recognition of microbes opsonized with IgG resulting in immune complex (IC)[4] formation (1), and studies in knockout mice demonstrate a critical role for FcγRs in IC-triggered neutrophil-mediated tissue damage (2–4) and macrophage-mediated clearance of IgG-coated cells in autoimmune models (5). Thus, the tight regulation of FcγRs is essential for mounting an effective host immune response while avoiding tissue damage and autoimmune-related pathologies.

FcγR engagement leads to diverse cellular responses, including phagocytosis, reactive oxygen species (ROS) generation, degranulation, cytokine production, gene regulation, and apoptosis (5). Similar to TCR and BCR, signaling through FcγRs is initiated by the phosphorylation of ITAM motif, which triggers the assembly of protein complexes containing Syk- and Src-family tyrosine kinases (6). The precise sequence of events thereafter, although less clear, likely involves tyrosine phosphorylation of downstream targets of these kinases (6), including the Rho GTPase guanine exchange factor (GEF) Vav, PI3K, phospholipase Cγ (PLCγ), and scaffold and adaptor proteins (e.g., SLP-76, Cbl, and Gab2). Studies in knockout mice suggest a role for Syk, Src, PLCγ, SLP-76, and Gab2 in FcγR signaling in phagocytes (7). Vav1, Vav2, and Vav3 are expressed in hemopoietic cells and signal the activation of Rho GTPases, Rho, Rac, and Cdc42, as well as RhoA and RhoG in vitro. Vav proteins also may serve GEF-independent, adaptor-like functions, as they contain zinc finger and Src homology domains involved in protein–protein interactions in addition to the their GEF/Dbl homology and pleckstrin homology domains typical of members of the Dbl family of GEF (8). The Vav proteins play a central role in immunoreceptor (BCR and TCR) signaling leading to T cell development and activation (8). but little is known about their role in nonadaptive immune mechanisms. Recent work suggests that, in neutrophils, they are required for CD11b/CD18 integrin-mediated adhesive functions (9). Furthermore, fMLP-mediated NADPH oxidase generation in neutrophils was shown to be partially dependent on Vav1 (10).

Phagocytosis of IgG-opsonized microbes results in the generation of a vigorous oxidative burst by the multicomponent NADPH oxidase. NADPH oxidase-mediated superoxide generation also is triggered by the phagocytosis of zymosan or complement opsonized targets or the stimulation of G protein-coupled receptors (GPCR). A deficiency in components of the NADPH oxidase in humans (chronic granulomatous disease patients) (11) or genetically engineered mice (12, 13) is associated with life-threatening infections, which highlights the importance of this multisubunit

*Center of Excellence in Vascular Biology, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115; [†]Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada; and [‡]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110

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[2] A.U. and X.C. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Tanya N. Mayadas, Department of Pathology, Center of Excellence in Vascular Biology, Brigham and Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, New Research Building 07520, Boston, MA 02115. E-mail address: tmayadas@rics.bwh.harvard.edu

[4] Abbreviations used in this paper: IC, immune complex; ROS, reactive oxygen species; PLCγ, phospholipase Cγ; GEF, guanine exchange factor; WT, wild type; EGFP, enhanced GFP; LA, latrunculin A; PAK, p21-activated kinase; Wrt, wortmannin; BM, bone marrow; IgGSRBC, IgG opsonized sheep RBCs; BMDM, BM-derived macrophage; NBT, nitroblue tetrazolium; CD, cytochalasin D; GPCR, G protein-coupled receptor; p-p40, phospho-p40(phox); p, phospho; RLU, relative luminometer unit.

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enzyme complex in the regulation of innate immunity. The activation of the oxidase requires a molecular interaction between membrane-bound heterodimeric flavocytochrome b (558) (gp91/gp22) and cytosolic proteins p40(phox), p47(phox), and p67(phox) and Rac GTPases. PKC-dependent phosphorylation of phox47 and phox67 in response to activating stimuli is associated with their translocation to the membrane, steps required for the assembly of a functional NADPH oxidase complex (14). The phosphorylation of the newest member of the phox family, p40(phox), which primarily associates with p67(phox), has been implicated in the regulation of the NADPH oxidase and is required for FcγR-dependent ROS production (15). Moreover, the active oxidase requires the translocation of activated Rac GTPases. Rac1 or Rac2 GTPases promote oxidase activity in cell-free systems while, in intact neutrophils, Rac2 has been reported to be the sole isoform responsible for ROS generation (16, 17). Although not essential for the translocation of phox components, the active GTP-bound Rac functions by localizing to the membrane and serving as an adaptor molecule that aids p67(phox) binding to cytochrome b allowing the electron transfer from NADPH to molecular oxygen (18). The priming of cells with cytokines leads to significantly enhanced superoxide generation in response to IgG, complement, or GPCR agonists (19). Although the underlying mechanism of this is unclear, neutrophil priming may help restrict the NADPH oxidase activity to sites of inflammation, representing an important physiological mechanism to localize the inflammatory response.

In this study, we evaluated the role of Vav and Rac proteins in FcγR functions in neutrophils and macrophages. This was accomplished by analyzing mice deficient in Vav1 and/or Vav3 (9, 20, 21), the predominant Vav proteins in neutrophils and macrophages (9), mice with a deficiency in Rac2 (22), mice with a conditional knockout of Rac1 in cells of the granulocyte/monocyte lineage (17) and animals lacking both Rac isoforms (23). Our data demonstrate a role for Vav and Rac proteins in neutrophil FcγR-mediated spreading, phagocytosis, and oxidative burst, suggesting a broad role for these proteins in FcγR functions in this cell type. In addition, our studies reveal a strict requirement for specific Vav and Rac isoforms in FcγR-mediated ROS generation that is dictated by the priming status of the cell, while these proteins were not essential for a PMA- or fMLP-stimulated oxidative burst. In macrophages, Vav and Rac proteins played a role in FcγR-mediated phagocytosis-induced oxidative burst, but unlike neutrophils, were not required for the phagocytosis itself. This indicates a more restricted role for these proteins than predicted from previous studies (24–26) and provides an example of exquisite specificity in signaling two temporally and spatially linked functions. We find that the defect in the oxidative burst in macrophages and neutrophils was associated with impaired Vav GEF activity toward Rac. Furthermore, we provide evidence that Vav mediates Rac/actin-independent, PI3K-dependent activation of p40(phox) in neutrophils, thus demonstrating a new signaling pathway for the Vav proteins in these cells.

Materials and Methods

Mice

Vav1$^{-/-}$ (20), Vav3$^{-/-}$, and Vav 1,3$^{-/-}$ mice (9, 21) and their wild-type (WT) counterparts were bred and maintained on a mixed C57BL/6 $\times$ 129Sv strain. Conditional Rac1$^{-/-}$ (17), Rac2$^{-/-}$ (22), and Rac1,2$^{-/-}$ (23) mice and their WT cohorts were on a C57BL/6 $\times$ 129Sv strain. These mice were bred and maintained in virus- and Ab-free facilities. Fcγ chain$^{-/-}$ mice (FcγR$^{-/-}$) on a C57BL/6 strain (27) and C57BL/6 WT controls were purchased from Taconic Biotechnology. Male and female mice (8–20 wk old) were used for experiments. All the experiments were approved by the Harvard Medical School Animal Care and Use Committee.

Reagents and Abs

GFP fusion of WT Vav1 and Vav1 with a point mutation in the Dbl homology domain (L278Q = VavΔGEF) were constructed on the MSCV backbone. The enhanced GFP (EGFP) retroviral vector was provided by Dr. D. Williams (Cincinnati Children's Hospital Medical Center and University of Cincinnati College of Medicine, Cincinnati, OH). Phalloidin rhodamine, C5a, fMLP, luminol, polyvinylpyrrolidone, cytochalasin D, and latrunculin A (LA) were obtained from Sigma-Aldrich; mouse GM-CSF and mouse TNF-α from R&D Systems; p-NBT from Calbiochem, and sheep RBC from ICN. p21-activated serine/threonine protein kinase/PAK-binding domain (PAK-PBD) agarose beads were from Cytoskeleton. Chemical inhibitors against PI3K were obtained as follows: LY294002 and LY303511 (EMD Bioscience) and wortmannin (Wrt) (Sigma-Aldrich).

The following Abs were purchased: mouse mAb against phosphotyrosine (4G10) (Upstate Biotechnology); rabbit anti-pAkt S473, rabbit anti-p38 MAPK, and rabbit anti-phospho-p40(phox) (p-p40) (Cell Signaling Technology); mouse anti-Rac (Chemicon International); rat anti-FcγR CD16/32 (eBioscience); goat anti-rat F(ab$'$)$_2$ (Jackson ImmunoResearch Laboratories); rabbit anti-BSA (Sigma-Aldrich); rabbit anti-sheep RBC and rabbit anti-OVA (both from Cappel); and rat anti-Gr-1 (BD Pharmingen). Rabbit anti-phospho-PAK was a gift from Dr. M. E. Greenberg (Children's Hospital, Harvard Medical School, Boston, MA).

Neutrophil isolation

For in vitro studies, mouse neutrophils were isolated from bone marrow (BM) as described (28, 29) with the following modifications: BM cells were layered on a 62.5% Percoll column, centrifuged at 1600 rpm (GS-6KR; Beckman Coulter) for 30 min at room temperature, and neutrophils in the pellet were resuspended in calcium- and magnesium-free PBS. The samples were subjected to hypotonic lysis of RBC with cold water. The neutrophil population was >90% pure as assessed by Wright-Giemsa stain of cytospun samples (30).

Neutrophil phagocytosis

The procedure was as described (31) and modified as follows. 20% sheep RBC (ICN) in PBS (v/v) were opsonized with rabbit anti-sheep IgG resuspended in PBS. The opsonized RBC were coincubated with neutrophils in the presence of fMLP (10 μM), C5a (5 μg/ml), GM-CSF (100 ng/ml), or PMA (1 μg/ml), and pelleted to promote binding. The cell mixture was resuspended in PBS plus calcium and magnesium and incubated at 37°C for 1 h. RBC were then lysed in H_2O, followed by the addition of cold PBS. The cells were visualized under a microscope to assess the total number of internalized IgG-opsonized sheep RBC (IgGSRBC) in 100 neutrophils. No internalized IgGSRBC were detected in unstimulated neutrophils.

Neutrophil IC adhesion assay

IC-coated plates were prepared as described (32) with the following modification: 0.5% polyvinylpyrrolidone (v/v) was added for 20 min at room temperature and washed rigorously with PBS. Neutrophils were allowed to bind at 37°C for 30 min. The plates were gently washed, fixed with 2.5% glutaraldehyde, and stained with rhodamine phalloidin.

PMA, fMLP, and FcγR cross-linking-mediated oxidative burst in neutrophils and macrophages

Chemiluminesence-based assays to monitor real-time ROS generation were performed using a six-channel bioluminat LB-953 tube luminometer (Berthold). Neutrophils or BM macrophages were incubated with 10 μg/ml rat anti-FcγRII/III on ice for 30 min. Cells (1×10^6) were washed, resuspended in 900 μl of PBS plus calcium and magnesium, and transferred to a tube with 30 μM luminol. The luminol solution was supplemented with 4 U/ml HRP when evaluating the oxidative burst in macrophages. FcγR cross-linking was initiated by injecting 100 μl of goat anti-rat F(ab$'$)$_2$ (100 μg/ml) at 37°C, and ROS generation was monitored for at least 4 min. In some experiments, GM-CSF (10 ng/ml), LA (1 μM), and cytochalasin D (CD; 5 μM) were included in the reaction tube. To determine PMA or fMLP-mediated oxidative burst, either PMA (1 μg/ml) or fMLP (10 μM) was injected directly into tubes containing cells, and ROS production was monitored in Ca^{2+}/Mg^{2+}-free buffer. For PI3K inhibition studies, neutrophils were preincubated for 25 min at 37°C without or with indicated doses of LY294002, 10 μM LY303511 (an inactive analog of LY294002), 20 nM Wrt, or DMSO (vehicle control). For Western blot analysis, cells were prepared and treated as described for ROS generation, and samples were lysed at indicated times (s) after the addition of goat anti-rat F(ab$'$)$_2$. The 0-s sample did not receive goat anti-rat F(ab$'$)$_2$.

Rac GTP pulldown assay

A total of either 10–20 × 10^6 macrophages (serum-starved overnight) or freshly isolated 2.5–5 × 10^6 neutrophils was incubated with 10 μg/ml rat anti-FcγRII/III on ice for 30 min. The cells were washed once and resuspended in DPBS $Ca^{2+}Mg^{2+}$. The cells were equilibrated in a 37°C water bath for 5 min, and goat anti-rat $F(ab')_2$ was added (final concentration 50 μg/ml). Cell lysis and Rac GTP pulldown were performed as described by the manufacturer's protocol (Cytoskeleton) with modifications as follows. Briefly, after indicated times, cells were lysed with an equal volume of 2× lysis buffer (100 mM Tris, 20 mM $MgCl_2$, 0.6 M NaCl, 4% IGEPAL plus protease inhibitor mixture) and supplemented with 1× lysis buffer up to a final volume of 700 μl. After a 10-min incubation on ice, extracts were spun for 10 min at 13,000 × *g*, and an aliquot of the supernatant was saved to analyze total Rac protein levels. The remaining supernatant was incubated with 20 μg of PAK-PBD beads and incubated for 30 min at 4°C with gentle shaking. The beads were washed three times with 1× lysis buffer and boiled in 50 μl of 2× Laemmli buffer.

Western blot analysis

Whole-cell lysate was prepared from cells by adding an equal volume of 2× SDS sample buffer containing 1 mM diisopropylfluorophosphate. The lysates were boiled, subjected to SDS-PAGE, and gels were transferred to nitrocellulose membranes. After blocking membranes with either 5% milk or 5% BSA for phosphotyrosine detection, membranes were incubated with primary Ab, followed by HRP-conjugated secondary Ab. Immunoreactive proteins were visualized by ECL (Pierce).

Mouse macrophage culture

BM-derived macrophages (BMDM) were cultured. Vav1,3$^{-/-}$ BMDM were retrovirally reconstituted with human Vav1-GFP fusion, the Vav1 mutant L278QΔGEF-GFP (VavΔGEF) fusion, or EGFP alone as described (33). The MIEG-3 expressing EGFP and MSCV-hVav1 and MSCV-hVav1ΔGEF retroviral vectors were transfected into PHOE cell lines and the supernatants were used to infect Vav1,3$^{-/-}$ macrophage progenitors. The retroviral infection efficiency was ~30%. To determine phagocytosis-induced oxidative burst, only green fluorescence cells were scored for NBT-formazan deposition and a minimum of 20 macrophages were counted from two to three random fields per experiment.

Macrophage phagocytosis and phagocytosis-induced oxidative burst assay

IgGSRBC in serum-free medium were mixed with adherent macrophages at 4°C for 10 min to synchronize binding. The unbound IgGSRBC were then washed with cold PBS followed by the addition of prewarmed medium, and cells were incubated for an additional 1 h at 37°C. The noninternalized IgGSRBC were lysed with cold water for 10 s, aspirated, and replaced with PBS. The number of internalized IgGSRBC in 100–300 macrophages per sample was counted. For analysis of the oxidative burst, phagocytosis was performed in cell culture medium (10% FCS in DMEM) supplemented with 1 mg/ml NBT. Noninternalized IgGSRBC were lysed with water for 10 s, and tonicity was restored with PBS. Macrophages with blue NBT deposition surrounding internalized RBC were scored as positive.

Results

Vav-deficient neutrophils are defective in FcγR-mediated neutrophil spreading and phagocytosis

BM WT neutrophils adhered and spread on immobilized BSA-anti-BSA IgG-coated coverslips in a process sometimes referred to as "frustrated phagocytosis." This process was FcγR dependent, as neutrophils deficient in the Fcγ chain (FcγR$^{-/-}$), and therefore lacking activating FcγRs, failed to bind immobilized ICs (data not shown). Neutrophils deficient in Vav1 or Vav3 adhered normally to IC-coated plates, while neutrophils lacking both Vav1 and 3 failed to stably adhere and spread (Fig. 1*A*). Flow cytometric analysis showed that Vav1,3$^{-/-}$ and WT neutrophils expressed equal levels of FcγRs and displayed similar binding to soluble ICs in suspension (data not shown). Thus, the observed defects in Vav1,3$^{-/-}$ cells are attributable to events downstream of FcγR binding to ICs.

Next, FcγR-mediated phagocytosis was examined in murine neutrophils. Human neutrophil phagocytosis of IgG-opsonized targets is enhanced by GM-CSF and TNF (31). We found that GM-CSF, C5a, or fMLP stimulation/priming of murine neutrophils was required for phagocytosis of IgGSRBC (Fig. 1*B*), and phagocytosis under these conditions was FcγR dependent, as shown by the inability of FcγR-deficient neutrophils to perform this function (data not shown). IgGSRBC phagocytosis was normal in Vav1$^{-/-}$ cells and was significantly reduced in Vav3$^{-/-}$ neutrophils. Phagocytosis in Vav1,3$^{-/-}$ neutrophils was barely detectable (Fig. 1*B*) despite comparable levels of IgGSRBC binding (data not shown). Additionally, the requirement for Rac proteins, downstream effectors of Vav GEF activity, was evaluated. Rac2$^{-/-}$ neutrophils had impaired phagocytosis of IgGSRBC, while phagocytosis in Rac1$^{-/-}$ neutrophils was largely intact (Fig. 1*C*). Thus, neutrophil IgG-mediated phagocytosis primarily requires Vav3 and Rac2.

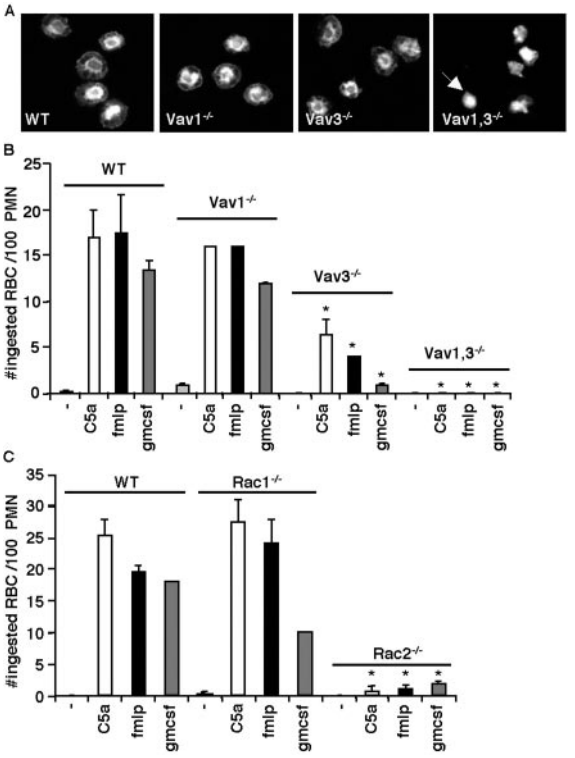
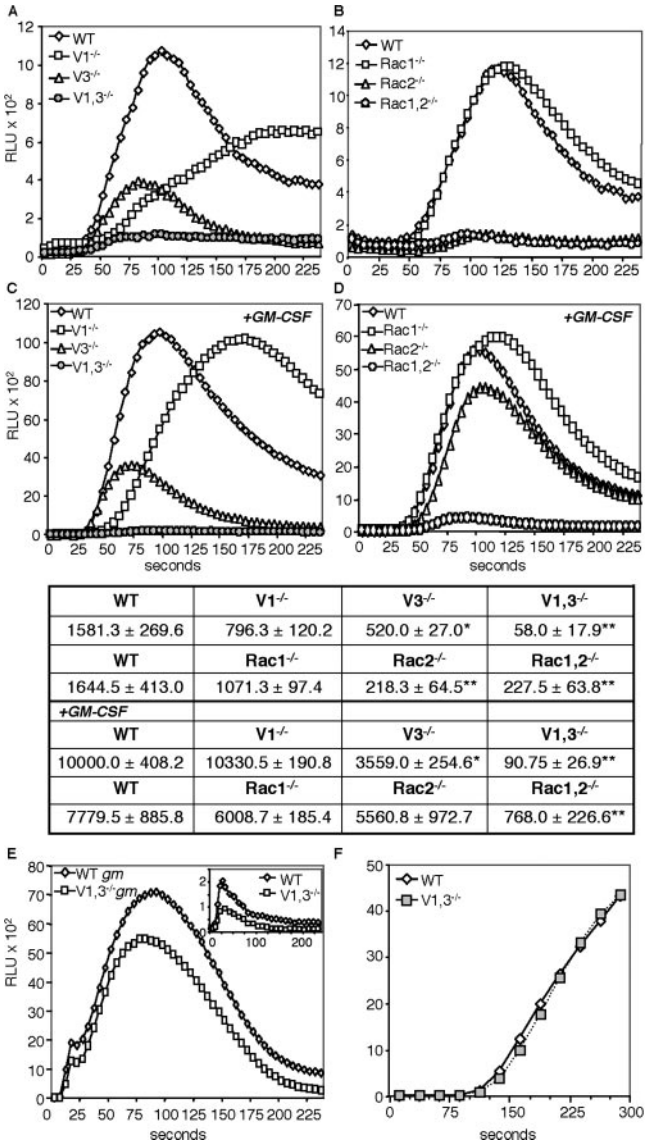


FIGURE 1. Immune complex-mediated spreading and phagocytosis. *A*, Analysis of WT, Vav1$^{-/-}$, Vav3$^{-/-}$ and Vav1,3$^{-/-}$ neutrophils spreading on IC-coated plates and stained with rhodamine-phalloidin. *B* and *C*, Phagocytosis of IgGSRBC by neutrophils deficient in Vav (*B*) or Rac isoforms (*C*) and their WT counterparts after priming of cells with C5a, fMLP or GM-CSF (gmcsf). The results are the average of four experiments ± SEM; ∗, $p < 0.05$, compared with WT.

Vav is required for the FcγR-dependent oxidative burst but is nonessential for fMLP and PMA-induced superoxide generation

Ab-mediated cross-linking of FcγRs resulted in significant ROS generation in WT neutrophils (Fig. 2*A*) that was absent in Fcγ chain-deficient neutrophils (data not shown). This method of ROS generation was chosen over phagocytosis-induced ROS to evaluate the role of Vav and Rac proteins in superoxide production, as it results in a more robust oxidative burst and would bypass the observed phagocytic defect in neutrophils lacking these proteins. Vav1$^{-/-}$ neutrophils consistently had a delay in the kinetics of ROS production that was followed by a more sustained burst that reached WT levels (Fig. 2*A*). Vav3$^{-/-}$ cells exhibited a major reduction in the magnitude of the oxidative burst at all time points,



WT	V1$^{-/-}$	V3$^{-/-}$	V1,3$^{-/-}$
1581.3 ± 269.6	796.3 ± 120.2	520.0 ± 27.0*	58.0 ± 17.9**
WT	**Rac1$^{-/-}$**	**Rac2$^{-/-}$**	**Rac1,2$^{-/-}$**
1644.5 ± 413.0	1071.3 ± 97.4	218.3 ± 64.5**	227.5 ± 63.8**
+GM-CSF			
WT	**V1$^{-/-}$**	**V3$^{-/-}$**	**V1,3$^{-/-}$**
10000.0 ± 408.2	10330.5 ± 190.8	3559.0 ± 254.6*	90.75 ± 26.9**
WT	**Rac1$^{-/-}$**	**Rac2$^{-/-}$**	**Rac1,2$^{-/-}$**
7779.5 ± 885.8	6008.7 ± 185.4	5560.8 ± 972.7	768.0 ± 226.6**

FIGURE 2. Neutrophil FcγR cross-linking induced ROS generation. Neutrophils were preincubated with rat anti-FcγRII/III and stimulated with F(ab′)$_2$ of goat anti-rat IgG. Real-time generation of ROS was monitored using a luminol-based assay. Incubation with isotype control IgG and cross-linking F(ab′)$_2$ secondary Ab exhibited no ROS generation (data not shown). *A*, ROS profile of WT, Vav1 (V1$^{-/-}$), Vav3 (V3$^{-/-}$), and Vav1,3 (V1,3$^{-/-}$)-deficient neutrophils subjected to FcγR cross-linking. *B*, ROS release in WT, Rac1$^{-/-}$, Rac2$^{-/-}$, and Rac1,2$^{-/-}$ neutrophils after FcγR cross-linking. *C* and *D*, Neutrophils were primed with GM-CSF before FcγR cross-linking. ROS profile in GM-CSF primed WT, V1$^{-/-}$, V3$^{-/-}$, and V1,3$^{-/-}$ (*C*) and WT, Rac1$^{-/-}$, Rac2$^{-/-}$ and Rac1,2$^{-/-}$ (*D*) neutrophils is shown. A table of the average ± SEM of the RLU peak value for samples depicted in *A–D* is given. $n = 4$–7 independent experiments, *, $p < 0.05$; **, $p < 0.005$ compared with WT controls in the same group. *E*, ROS generation in Vav1,3$^{-/-}$ and WT neutrophils that were preincubated with GM-CSF (gm) or without GM-CSF (*inset*), followed by fMLP stimulation. *F*, ROS generation in Vav1,3$^{-/-}$ (V1,3$^{-/-}$) and WT neutrophils following PMA stimulation alone. One representative of more than four independent experiments is shown for each panel.

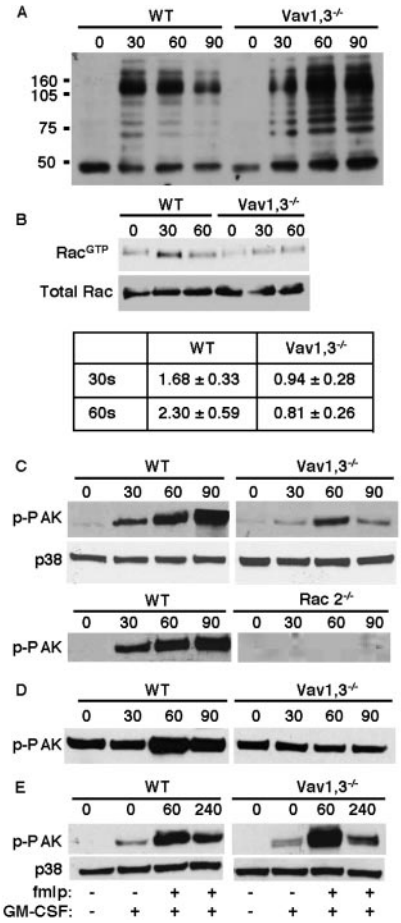
while Vav1,3$^{-/-}$ cells completely failed to produce ROS (Fig. 2*A*). Rac1$^{-/-}$ neutrophils produced normal levels of ROS, while Rac2$^{-/-}$ or Rac1,2$^{-/-}$ cells failed to generate an oxidative burst following FcγR cross-linking (Fig. 2*B*). Thus, Vav3 and Rac2 are required for FcγR-dependent NADPH oxidase activation in neutrophils.

During inflammation, neutrophils encounter cytokines such as TNF or GM-CSF. These alone do not trigger ROS generation but significantly enhance GPCR, FcγR, or integrin-driven ROS production and are thus referred to as priming agents. FcγR cross-linking after GM-CSF priming of Vav3$^{-/-}$ neutrophils resulted in an enhanced oxidative burst relative to Vav3$^{-/-}$ unprimed cells, but the overall ROS generation remained significantly diminished, compared with primed WT neutrophils. Primed Vav1,3$^{-/-}$ neutrophils remained unable to generate any detectable FcγR-induced ROS under these conditions (Fig. 2*C*). Importantly, priming of Rac2$^{-/-}$ neutrophils overcame the defect observed in unprimed cells after FcγR cross-linking. Indeed, only a deficiency in both Rac1 and Rac2 abrogated the primed FcγR-dependent oxidative burst (Fig. 2*D*). The use of TNF as the priming agent yielded similar results (data not shown). Thus, in the context of a primed FcγR-dependent oxidative burst, Vav1 and Rac1 could compensate for the Vav3 and Rac2 deficiency, respectively.

Stimulation of neutrophils with the GPCR agonist fMLP results in NADPH oxidase activation that is dependent on Rac2 GTPases (34) and Vav 1 (10). In primary murine neutrophils, a deficiency in Vav1,3 resulted in a partial reduction of fMLP-mediated ROS production (Fig. 2*E*, *inset*). However, following GM-CSF priming, Vav proteins were no longer required for this process (Fig. 2*E*). Furthermore, the PMA-stimulated superoxide generation, which also was previously shown to be Rac2 dependent (34), was not impaired in Vav1,3$^{-/-}$ cells (Fig. 2*F*). Thus, unlike the critical role for Vav in FcγR-mediated NADPH oxidase activation, Vav is nonessential for fMLP- or PMA-mediated ROS generation.

Vav is required for activation of Rac GTPase

To examine signaling pathways in neutrophils that are Vav and/or Rac dependent, neutrophils deficient in Vav1,3 or Rac 2 were subjected to biochemical analysis after cross-linking FcγRs. Analysis of total tyrosine phosphorylation in WT and Vav1,3$^{-/-}$ cells showed a similar increase following FcγR clustering (Fig. 3*A*), indicating that early tyrosine kinase activation events after FcγR engagement are unaffected by Vav deficiency. Affinity isolation of the GTP-bound form of Rac revealed that FcγR-mediated Rac activation was significantly reduced in Vav1,3$^{-/-}$ neutrophils (Fig. 3*B*). The Ab used for Rac detection recognizes both Rac 1 and Rac2 as confirmed by analysis of lysates from Rac1$^{-/-}$, Rac2$^{-/-}$, and Rac 1,2$^{-/-}$ cells (data not shown), suggesting that FcγR signaling to both Rac isoforms is Vav dependent. Furthermore, the serine-threonine phosphorylation of an immediate Rac-effector, PAK, was considerably decreased in Vav1,3$^{-/-}$ neutrophils. FcγR-induced PAK activation was nearly undetectable in Rac2-deficient neutrophils, demonstrating that PAK phosphorylation is a bona fide readout of Rac activity (Fig. 3*C*). The requirement for Vav and Rac proteins in PAK phosphorylation (p-PAK) was recapitulated under priming conditions. GM-CSF treatment alone resulted in a significant increase in phosphorylation of PAK. Following FcγR engagement, p-PAK was further increased in WT cells, but not in Vav1,3$^{-/-}$ cells (Fig. 3*D*), and p-PAK remained undetectable in Rac 1,2$^{-/-}$ neutrophils (data not shown). In contrast with the defects in PAK phosphorylation, the activation of p38 MAPK after FcγR cross-linking was similar in WT and Vav1,3$^{-/-}$ neutrophils (data not shown). Following fMLP stimulation of GM-CSF-primed cells, PAK phosphorylation was intact in Vav-deficient cells (Fig. 3*E*). Thus, Vav proteins are specifically required for FcγR-induced Rac activation, a finding that is consistent with the critical role for Vav in the primed FcγR but not fMLP-mediated ROS production.



	WT	Vav1,3$^{-/-}$
30s	1.68 ± 0.33	0.94 ± 0.28
60s	2.30 ± 0.59	0.81 ± 0.26

FIGURE 3. Identification of signaling molecules participating in FcγR signaling through Vav and Rac. Neutrophils in suspension were subjected to FcγR cross-linking for the indicated time (s). *A*, Evaluation of total cellular tyrosine phosphorylation (p-Tyr) in WT and Vav1,3$^{-/-}$ neutrophils. *B*, Analysis of Rac^{GTP} loading after FcγR cross-linking in WT or Vav1,3$^{-/-}$ neutrophils. Densitometric analysis of Western blots for Rac activation was performed. The average value of Rac^{GTP} as a percent of total Rac was determined. Next, the fold induction following FcγR cross-linking for 30 and 60 s, compared with WT cells (0 s), was determined and the average value ± SD from two independent experiments is reported. A third experiment showed a large induction of Rac^{GTP} loading (>10-fold) following cross-linking of WT cells with no induction in Vav1,3$^{-/-}$ cells. Although this experiment supports the conclusion of our study, it was not included in the analysis because the increase in Rac^{GTP} in WT cells following FcγR cross-linking was atypical. *C* and *D*, p-PAK and p38 (as loading control) were analyzed in Vav1,3$^{-/-}$ or Rac2$^{-/-}$ neutrophils and their corresponding WT counterparts after FcγR cross-linking in the absence (*C*) and presence (*D*) of GM-CSF. *E*, WT and Vav 1,3$^{-/-}$ neutrophils were stimulated without (−) or with (+) GM-CSF before fMLP treatment for the indicated times (s), and cell lysates were analyzed for p-PAK and p38. Each panel is one representative of three individual experiments.

A requirement for Vav in PI3K activation and PI3K-dependent phosphorylation of p40(phox)

It is well accepted that PI3K activation is required for the oxidative burst following binding of either opsonized particles or chemotactic factors such as fMLP (35–37). In this study, we show that a pharmacological inhibitor of PI3K, LY294002, dose-dependently inhibited the FcγR-induced oxidative burst in both resting (Fig. 4*A*) and GM-CSF-primed (data not shown) WT neutrophils, and that this was associated with a dose-dependent reduction of Akt phosphorylation, a well-known PI3K-dependent event (Fig. 4*B*).

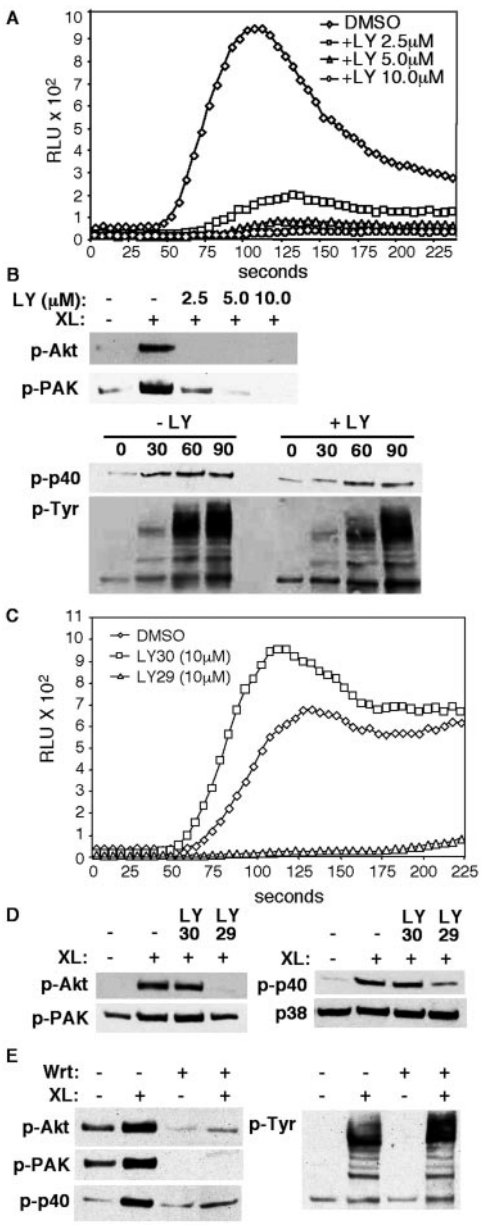


FIGURE 4. Analysis of PI3K-dependent NADPH oxidase activation. *A*, The effect of PI3K inhibition on ROS production. Real-time generation of ROS was evaluated following FcγR cross-linking in neutrophils pretreated with vehicle (DMSO) or the indicated concentrations of the PI3K inhibitor LY294002. One of three representative experiments is shown. *B*, Biochemical analysis. *Top panels*, Neutrophils, minus and plus the indicated concentrations of LY294002, were subjected to FcγR cross-linking and p-Akt or p-PAK were evaluated. *Bottom panels*, Neutrophils were treated with 2.5 μM LY and subjected to FcγR cross-linking (XL) for the indicated times in seconds followed by analysis of p-p40 and total tyrosine (p-Tyr) phosphorylation. *C*, The effect of LY294002 or its inactive analog LY303511 on real-time generation of ROS was evaluated following FcγR cross-linking. Only LY294002 treatment resulted in a significant blockade of ROS generation compared with vehicle (DMSO) control. One representative of three experiments is shown. *D*, Neutrophils treated with DMSO (−), 10 μM LY294002 or LY303511 were subjected to 90 s FcγR cross-linking (XL) as indicated, and p-Akt, p-PAK (*left panels*), p-p40, and p38 (loading control) (*right panels*) were analyzed. One representative of two experiments is shown. *E*, Neutrophils were treated without or with 20 nM Wrt and subjected to cross-linking (XL) followed by analysis of p-Akt, p-PAK, p-p40, and total tyrosine phosphorylation (p-Tyr). One representative of three independent experiments is shown.

Importantly, the NADPH oxidase component p40(phox) (Thr 154) and PAK were rapidly phosphorylated following FcγR cross-linking, and these required PI3K activity while overall tyrosine phosphorylation was unaffected by LY294002 (Fig. 4*B*). The relative specificity of LY294002 effects was evaluated by repeating these studies with its inactive analog LY303511. Only LY294002 treatment resulted in a reduction in FcγR-induced oxidative burst (Fig. 4*C*) and phosphorylation of Akt, PAK, and p40(phox) (Fig. 4*D*). Similarly, another unrelated PI3K inhibitor, Wrt, inhibited the FcγR-cross-linking (XL)-induced oxidative burst in WT neutrophils (relative light unit (RLU) peak value (average ± SEM): XL, 901.7 ± 125.9; XL + Wrt, 44.0 ± 18.3*, $n = 3$, *, $p < 0.005$) as well as the phosphorylation of PAK and p40(phox) without effects on overall tyrosine phosphorylation (Fig. 4*E*). These data indicate that PI3K activation is upstream of FcγR-induced Rac activation and p40(phox) phosphorylation.

The potential role of Vav and Rac in Akt and p40(phox) phosphorylation (p-p40) was examined in Vav $1,3^{-/-}$, $Rac2^{-/-}$, and $Rac1,2^{-/-}$ neutrophils following FcγR cross-linking. Phosphorylation of both proteins was significantly diminished in $Vav1,3^{-/-}$ cells (Fig. 5*A*), while $Rac2^{-/-}$ cells exhibited normal levels of p-Akt and p-p-p40 following FcγR cross-linking (Fig. 5*A*). Under GM-CSF priming conditions, p-Akt production in Vav $1,3^{-/-}$ cells reached near WT levels following FcγR engagement (Fig. 5*B*). However, $Vav1,3^{-/-}$ cells continued to exhibit a failure to phosphorylate p40(phox) (Fig. 5*B*). Thus, the inability of $Vav1,3^{-/-}$ cells, either resting or primed, to produce ROS following FcγR cross-linking directly correlated with the poor phosphorylation of p40(phox). As observed in unprimed $Rac2^{-/-}$ neutrophils (Fig. 5*A*), primed $Rac1,2^{-/-}$ cells subjected to FcγR cross-linking showed no impairments in either p-Akt or p-p40 formation (Fig. 5*B*). These data demonstrate a role for Vav1,3 in signaling the phosphorylation of proteins that is not dependent on Rac proteins. This indicates that Vav proteins serve functions in neutrophils in addition to their GEF activity toward Rac. In contrast with the significant effects of Vav deficiency on FcγR-mediated signaling, Vav 1,3 played no role in the phosphorylation of Akt or p40(phox) following fMLP stimulation of GM-CSF primed neutrophils (Fig. 5*C*). This is consistent with the normal ROS generation (Fig. 2*E*) and PAK phosphorylation (Fig. 3*E*) observed in Vav $1,3^{-/-}$ cells under priming conditions. Together, our findings suggest a substantial role for Vav proteins in FcγR, but not GPCR-mediated signal transduction.

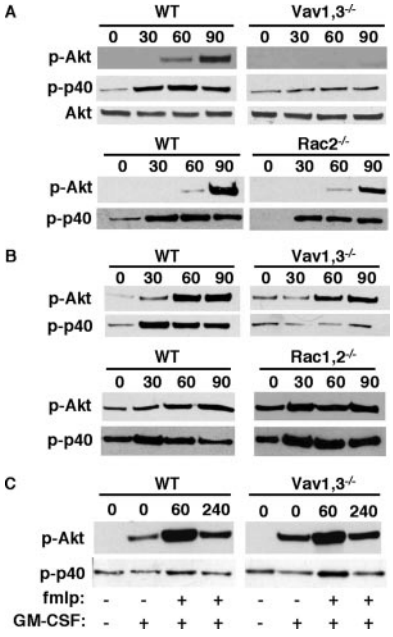


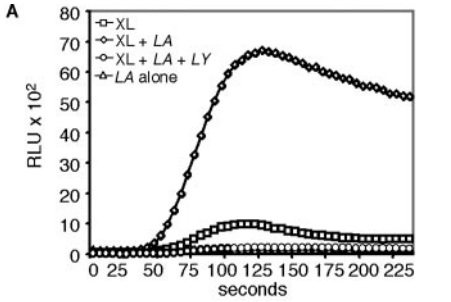
FIGURE 5. The effect of Vav or Rac deficiency on phosphorylation of Akt and p40(phox). *A*, Analysis of p-Akt, p-p-p40, and Akt (loading control) was undertaken in $Vav1,3^{-/-}$ and $Rac2^{-/-}$ neutrophils and their WT cohorts following FcγR cross-linking for the indicated times (s). *B*, WT, $Vav1,3^{-/-}$ and $Rac1,2^{-/-}$ neutrophils were prestimulated with GM-CSF followed by FcγR cross-linking for the indicated times in seconds and analyzed for p-PAK, p-Akt, and p-p40. *C*, WT and Vav $1,3^{-/-}$ neutrophils were stimulated with (+) or without (−) GM-CSF before fMLP treatment for the indicated times (s), and cell lysates were analyzed for p-Akt and p-p-p40. Each panel in this figure is one representative of three individual experiments.

PI3K-dependent Rac activation and p40(phox) phosphorylation following actin depolymerization requires Vav

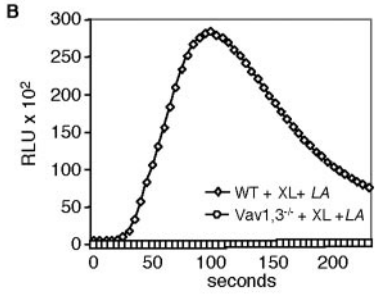
Previous reports link activation of Vav and Rac proteins to actin polymerization and Vav in lymphocytes couple Ag-receptor signaling to the actin cytoskeleton (38). Thus, the dependence of Vav-mediated ROS generation on the actin cytoskeleton was evaluated. It is noteworthy that activation of the NADPH oxidase following fMLP stimulation occurs even in the presence of actin depolymerizing agents and indeed is enhanced following these treatments (39). Furthermore, a recent observation suggests that the oxidative burst that takes place in the phagosome coincides with actin depolymerization (40). In this study, we show that FcγR-dependent oxidative burst not only occurred in cells treated with actin depolymerization agents LA (Fig. 6*A*) or CD (data not shown) but also was significantly elevated, compared with cells subjected to FcγR cross-linking alone. Importantly, the ROS generation remained PI3K dependent as both LY294002 (Fig. 6*A*) and Wrt (data not shown) treatment inhibited the oxidative burst. However, neither LA (Fig. 6*B*) nor CD (data not shown) was able to restore normal ROS production in $Vav1,3^{-/-}$ neutrophils. Biochemical analysis showed an induction of p-Akt, p-PAK, and p-p40 following FcγR cross-linking and treatment with LA or CD in WT cells (Fig. 6*C*). In contrast, actin depolymerization did not rescue the poor phosphorylation of PAK or p40(phox) in $Vav1,3^{-/-}$ cells. However, it reconstituted p-Akt levels in Vav-deficient cells (Fig. 6*C*). Despite an increase in p-Akt levels, Vav $1,3^{-/-}$ cells continued to fail to phosphorylate PAK and p40(phox). Thus, PI3K is necessary, but not sufficient, for FcγR-mediated p40(phox) phosphorylation, while Vav protein signaling is essential for both Rac activation and p40(phox) phosphorylation.

Macrophage FcγR-dependent phagocytosis-induced oxidative burst is dependent on Vav and Rac activity

Phagocytosis of IgGSRBC and the associated oxidative burst were evaluated in adherent BM-derived macrophages. Phagocytosis was FcγR dependent, as macrophages isolated from FcγR-deficient mice failed to phagocytose IgGSRBC (data not shown). Macrophages deficient in Vav1, Vav3, or both Vav1 and 3 (Vav1,3), as well as Rac-deficient macrophages, were competent for phagocytosis of IgG-opsonized SRBC (Fig. 7*A*). In contrast, phagocytosis-induced oxygen radical generation, as assessed by NBT reduction, was significantly impaired in $Vav1,3^{-/-}$ macrophages, but not in macrophages lacking Vav1 or Vav3 alone (Fig. 7*B*). In contrast, the PMA-induced oxidative burst in Vav $1,3^{-/-}$ macrophages was intact (Fig. 7*B*). Importantly, the phagocytosis-induced oxidative burst also was greatly reduced in $Rac1,2^{-/-}$ macrophages (Fig. 7*C*). Thus, Vav1,3, and Rac1,2 are dispensable for FcγR-dependent phagocytosis, but are essential for the associated phagocytosis-induced oxidative burst. Vav also played an essential role in FcγR-dependent oxidative burst following cross-linking of FcγRs



XL	XL + *LA*
1166.7 ± 441.0	8000.0 ± 816.5*
XL + *LA* + LY	***LA***
50.0 ± 8.1#	173.0



WT+XL+*LA*	Vav1,3$^{-/-}$+XL+*LA*
44,056.7 ± 4263.6	90.0 ± 3.3**

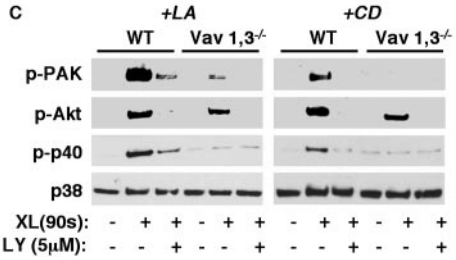


FIGURE 6. Analysis of actin disrupting agents on NADPH oxidase activation. *A*, The effect of 1 μM LA on FcγR-mediated ROS production in WT cells was evaluated in the presence or absence of the PI3K inhibitor LY294002 (5 μM). Minimal ROS generation was observed following LA treatment alone, while it significantly increased FcγR cross-linking (XL) induced superoxide production. The RLU peak value for each condition is given. *, $p < 0.05$, compared with XL alone; #, $p < 0.005$, compared with XL + *LA* samples. *B*, The ROS profile of WT and Vav1,3$^{-/-}$ neutrophils following FcγR cross-linking (XL) and treatment with LA. The RLU peak value (average ± SEM) for each condition is given, **, $p < 0.005$. *C*, Biochemical analysis of neutrophils, pretreated with either 1 μM LA or 5 μM CD with or without LY and subjected to FcγR cross-linking (XL) for the indicated times. p-PAK, p-Akt, p-p40, and p38 (loading control) were evaluated. *A–C*, Shown are one representative of three independent experiments except for LA alone (*A*), which is $n = 1$.

(Fig. 7*D*) and Vav-regulated Rac GTPase activation following cross-linking was significantly reduced in Vav1,3-deficient, compared with WT, macrophages (Fig. 7*E*). This shows a requirement for Vav in FcγR-mediated Rac activation in macrophages. To directly evaluate the relative contribution of Vav's GEF activity to FcγR-induced ROS generation, Vav1,3$^{-/-}$ macrophages were retrovirally transduced with human Vav1 or Vav1 with a point mutation in the Dbl domain (Vav-ΔGEF), which abolishes Vav1's GEF activity (41). Expression of WT Vav1 restored the oxidative

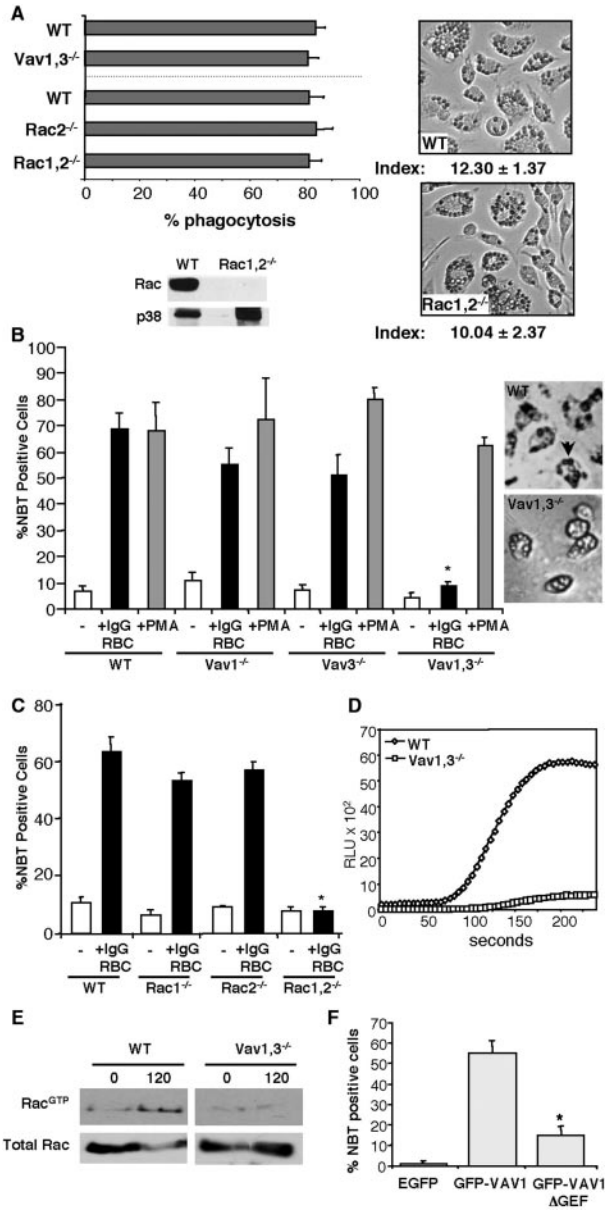


FIGURE 7. Macrophage FcγR-mediated phagocytosis and associated oxidative burst. *A*, Phagocytosis of IgGSRBC by Vav1,3$^{-/-}$, Rac2$^{-/-}$, and Rac1,2$^{-/-}$ macrophages and their WT cohorts is shown as the percentage of cells that ingest ≥2 IgGSRBC for 1 h at 37°C. Representative pictures of WT or Rac1,2$^{-/-}$ macrophages are shown, and the average number of SRBC ± SEM ingested per macrophage (index) is given. $n = 4$ independent experiments. Rac deficiency was confirmed in Rac1,2$^{-/-}$ macrophages by Western blot analysis. *B*, BMDM from WT, Vav1$^{-/-}$, Vav3$^{-/-}$, and Vav1,3$^{-/-}$ were incubated with IgGSRBC or PMA and oxygen radical generation was evaluated by counting the percentage of cells with NBT deposition ($n = 8$ experiments; *, $p < 0.001$, compared with WT). Representative pictures of one of eight experiments are shown. An arrow points to NBT deposition (black deposits) around internalized SRBC observed in WT but not Vav1,3$^{-/-}$ macrophages. *C*, BM-derived WT, Rac1$^{-/-}$, Rac2$^{-/-}$, or Rac1,2$^{-/-}$ macrophages were incubated with IgGSRBC and ROS generation was quantitated as in *B* ($n = 3$ experiments; $p < 0.05$, compared with WT). Data in *B* and *C* are average percent ± SEM. *D*, FcγR cross-linking in WT or Vav1,3$^{-/-}$ macrophages and ROS generation was measured in real-time as in Fig. 2, *A–D*. *E*, Rac GTP loading in WT or Vav1,3$^{-/-}$ macrophages after FcγR cross-linking (XL) was analyzed at the indicated times. *F*, Vav1,3$^{-/-}$ macrophages were retrovirally transduced with GFP alone, GFP-Vav1 or GFP-Vav1 with a mutation in its GEF domain (Vav1ΔGEF) and incubated with IgGSRBC. GFP-positive cells were scored for NBT reduction. $n = 6$ independent experiments with >600 GFP-positive cells scored per group. *, $p < 0.05$, compared with GFP-Vav1.

burst in Vav1,3$^{-/-}$ macrophages, while the Vav1-Dbl domain mutant only partially restored the oxidase activity (Fig. 6*F*). Thus, GEF activity of Vav toward Rac1,2 is primarily responsible for the oxidative burst, although GEF-independent functions of Vav may also contribute to this function.

Discussion

More than 40 GEF have been described for the Rho family of GTPases, but how they couple distinct receptor-mediated pathways to defined downstream functions in vivo remains poorly understood. In GPCR signaling, the GEF P-Rex has been implicated in NADPH oxidase activation in neutrophils (42). In this study, we show that Vav1,3 were essential for FcγR-dependent activation of the NADPH oxidase in neutrophils and macrophages. Indeed, in macrophages, Vav1,3 were dispensable for phagocytosis of IgG-opsonized targets but were critical for the associated oxidative burst, providing an example of exquisite specificity in signaling two temporally and spatially linked functions. In neutrophils, Vav3 contributed significantly to the NADPH oxidase activation, demonstrating differential functions for Vav family members in FcγR-mediated oxidative burst. This is unique, because past studies tend to emphasize that these proteins play overlapping functions in neutrophils (9).

Rac GTPases play important roles in several GPCR-mediated signaling pathways in neutrophils (22, 23) and in FcγR functions, Rac2 was implicated in the phagocytosis-induced burst in macrophages (26) and neutrophils (34). Unlike previous reports, in this study, we examined FcγR-dependent ROS production following FcγR cross-linking to rule out defects in oxidative burst as a consequence of inability to phagocytose. The profiles of ROS generation in neutrophils suggest that FcγRs may primarily useVav1 to initiate the oxidative burst, while Vav3 sustains it. This may reflect differential subcellular compartmentalization of different isoforms or the formation of distinct molecular complexes in neutrophils. Current data in the literature point to Rac2 as the major regulator of the oxidative burst in phagocytes (16, 18, 23). Yet, the requirement for Rac isoforms in ROS production following FcγR clustering was dependent on the presence of priming stimuli. Indeed, in cytokine-primed neutrophils, both Vav1 and Rac1 could compensate for the Vav3 or Rac2 deficiency, respectively. Cultured, adherent macrophages also may exist in a primed state, and therefore, like primed neutrophils, use either of the two Rac or Vav isoforms for the phagocytosis-induced oxidative burst.

In neutrophils, Vav proteins played a broader role in FcγR-mediated functions. Spreading on ICs was abrogated only in neutrophils lacking both Vav 1 and 3, while Vav 3 deficiency alone led to a significant reduction in IgG-mediated phagocytosis. Thus spreading on ICs, a process sometimes referred to as frustrated phagocytosis, appears to have additional requirements for Vav-dependent signaling. Spreading of murine neutrophils on ICs requires signaling from the CD18 integrin CD11b/CD18 (32), while phagocytosis of densely IgG-coated RBC does not (X. Cullere and T. N. Mayadas, unpublished data). Hence, the requirement for both Vav1 and 3 in the spreading response may reflect its dual role in signaling through the FcγR and CD18 integrin (9) pathways. Additional experiments will be required to sort this out. In contrast with the results in neutrophils, we observed no role for Rac1 or Rac2 in macrophage FcγR-mediated phagocytosis, and a previous study suggested that Rac2 deficiency only partially inhibited this process (26). Previous studies in a macrophage cell line suggest that Vav proteins were required for IgG-phagocytosis (24, 25). However, a deficiency in Vav1 and Vav 3 did not affect phagocytosis of IgG-coated targets, and Vav1,2,3$^{-/-}$ macrophages also have been reported to have normal FcγR-mediated phagocytosis (43). It is possible that primary macrophages express additional Rho family GEF, which could signal to other Rho GTPases to initiate phagocytosis (44). It is well accepted that phagocytosis requires actin remodeling mediated by Rho GTPases (45), and all other studies of ITAM-mediated signaling, particularly in lymphocytes, would predict that Vav/Rac signaling is required for actin cytoskeletal changes. The fact that macrophages do not rely on Vav or Rac family members for such a well-defined, ITAM-based signaling pathway, is highly unexpected and warrants further study.

Our studies indicate that a primary function of Vav proteins in phagocytes is to specifically link FcγR to activation of Rac proteins: Vav- and Rac-deficient cells phenocopied each other in all functions examined. Vav deficiency was linked to aberrant Rac activation, and reconstitution studies suggest a requirement for Vav GEF activity in phagocytosis-induced oxidative burst. In contrast, Vav played a nonessential role in GPCR-mediated activation of the NADPH oxidase, a Rac2-dependent process.

Our analysis revealed a role for Vav 1,3 in PI3K activation in neutrophils, as also reported in other hemopoietic cells (46–49), and may partly explain the lack of NADPH oxidase activation. However, prestimulation of Vav1,3$^{-/-}$ neutrophils with priming agents such as GM-CSF (Fig. 2) or TNF (data not shown) as well as LA and CD did not rescue the defect in activation of the NADPH oxidase, Rac, and p40(phox), despite the up-regulation of PI3K activity. Therefore, activation of PI3K, necessary for NADPH oxidase activation (37, 50), is not sufficient to rescue the phenotype in Vav-deficient cells. Our experiments in neutrophils favor a model of a positive feedback loop between GTPase activation and PI3K, because PI3K inhibition during FcγR cross-linking results in diminished Rac activation. In T cells, PI3K inhibition also affects Vav1-dependent Rac activation after TCR engagement (51). More recently, Vav2 and Vav3 have been shown to be essential constituents of a feedback loop between PI3K and Rac1/Cdc42 during neurite outgrowth, which was dependent on organized actin filaments (52). In contrast, disruption of the actin cytoskeleton after FcγR cross-linking did not interfere with the activation of the PI3K-Vav feedback loop. This is consistent with our finding that actin depolymerizing agents did not inhibit, and indeed enhanced, the neutrophil oxidative burst after FcγR cross-linking. Possible mechanisms for this feedback loop would include the direct interaction of active GTPases and PI3K as described previously (53, 54). However, our data indicate that Rac 1,2 proteins are not required for this cycle. Further studies are needed to determine whether Cdc42 plays a role in this process. Alternatively, Vav proteins could contribute to PI3K activation by their direct interaction, as has been proposed to occur during the neutrophil-like maturation of myeloid cells (55).

Our study uncovered novel functions for Vav in regulating p40(phox) phosphorylation independently of Rac family members. The strict correlation between the requirements for Vav proteins in oxidative burst with the FcγR-mediated signaling to p40(phox) phosphorylation suggests that this Vav signaling pathway may be equally important to the Vav/Rac signaling axis in initiating ROS production (Fig. 8). The lipid product of PI3K, PI3-phosphate was shown to regulate the oxidase complex by binding to the PX domain of p40(phox) (56) and promoting phox phosphorylation through activation of PKC isoforms and/or Akt (14). Consistent with this, FcγR-mediated p40(phox), phosphorylation was PI3K dependent. A positive regulatory role for p40(phox) has been suggested following fMLP and PMA stimulation in heterologous cell systems, and in neutrophils, the onset and extent of p40(phox) phosphorylation strongly correlated with the level of fMLP and PMA-mediated ROS generation (15, 57, 58). In addition, a study

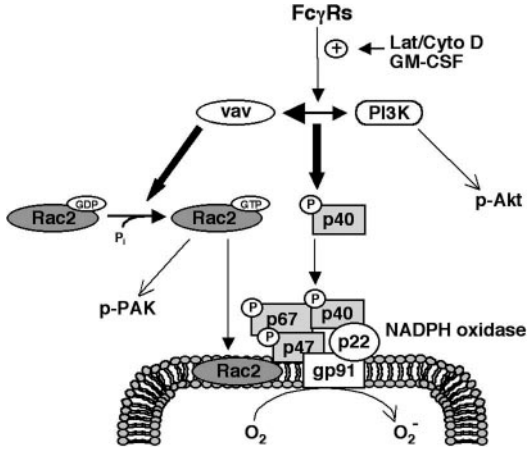


FIGURE 8. Model of Vav-dependent activation of the NADPH oxidase in neutrophils. FcγR engagement leads to Vav/Rac and PI3K activation and establishes a positive feedback loop. This is enhanced in the presence of priming agents and is independent of actin remodeling. Both Vav and PI3K are required for optimal Rac2 GTP loading and the Rac-independent phosphorylation of p40(phox). Activation of Rac and phosphorylation of p40(phox) and other phox subunits leads to their translocation to the membrane, and association with the membrane-bound cytochrome *b* (gp91/gp22), leading to the assembly of a functional NADPH oxidase.

exploiting p40(phox) expression in a heterologous cell line reported a critical role for this protein in FcγR-dependent NADPH oxidase activation (15). p40(phox) also has been shown to inhibit NADPH oxidase activation in cell-free systems and whole cell models, although in the former, the presence of PI3-phosphate p40(phox) enhanced superoxide production (15, 58). Thus, although the functional role of p40(phox) in NADPH oxidase activity is not well defined, its phosphorylation parallels superoxide generation in ours and other systems (57, 59) and thus may serve as a useful readout of NADPH oxidase activation. It is possible that Vav proteins serve an adapter-type function to facilitate the interaction of p40(phox) and the PKC kinases, which would maximize rapid formation of p40(phox) and assembly of the NADPH complex. This is an obvious area for future investigation.

In conclusion, Vav GEF play an essential role in regulating Rac activity required for FcγR-dependent responses. Vav regulation of Rac is indispensable for FcγR-mediated ROS production in both neutrophils and macrophages, while their requirement in phagocytosis is cell-context dependent. The uncoupling of pathways leading to phagocytosis vs oxidative burst in macrophages at the level of Vav/Rac suggests that signaling to these two functions may be differentiated. The observed activation of the NADPH oxidase in Vav-deficient neutrophils following stimulation with the GPCR agonist fMLP demonstrates a strict requirement for Vav proteins in FcγR- but not GPCR-mediated signaling. Vav directly affects two core constituents of the NADPH oxidase, Rac proteins and p40(phox), following FcγR clustering. Phosphorylation of p40(phox) specifically upon FcγR clustering may be reinforced by the proposed PI3K-Vav positive feedback loop as depicted in our model in Fig. 8. The demonstration that Vav plays a role in these processes that are not dependent on actin remodeling is unique. The dependence of neutrophil FcγR-mediated phagocytosis and oxidative burst primarily on Vav3 suggests the possibility of developing strategies that target Vav3 to selectively inhibit these FcγR functions in IC-induced inflammatory diseases in patients.

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Disclosures

The authors have no financial conflict of interest.

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