JCI The Journal of Clinical Investigation

Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-PLCγ2 signaling axis in mice

Daniel B. Graham, … , Craig M. Coopersmith, Wojciech Swat

J Clin Invest. 2007;117(11):3445-3452. https://doi.org/10.1172/JCI32729.

Research Article **Immunology**

Oxidative burst, a critical antimicrobial mechanism of neutrophils, involves the rapid generation and release of reactive oxygen intermediates (ROIs) by the NADPH oxidase complex. Genetic mutations in an NADPH oxidase subunit, gp91 (also referred to as NOX2), are associated with chronic granulomatous disease (CGD), which is characterized by recurrent and life-threatening microbial infections. To combat such infections, ROIs are produced by neutrophils after stimulation by integrin-dependent adhesion to the ECM in conjunction with stimulation from inflammatory mediators, or microbial components containing pathogen-associated molecular patterns. In this report, we provide genetic evidence that both the Vav family of Rho GTPase guanine nucleotide exchange factors (GEFs) and phospholipase C–γ2 (PLC-γ2) are critical mediators of adhesion-dependent ROI production by neutrophils in mice. We also demonstrated that Vav was critically required for neutrophil-dependent host defense against systemic infection by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, 2 common pathogens associated with fatal cases of hospital-acquired pneumonia. We identified a molecular pathway in which Vav GEFs linked integrin-mediated signaling with PLC-γ2 activation, release of intracellular Ca^{2+} cations, and generation of diacylglycerol to control assembly of the NADPH oxidase complex and ROI production by neutrophils. Taken together, our data indicate that integrin-dependent signals generated during neutrophil adhesion contribute to the activation of NADPH oxidase by a variety of distinct effector pathways, all of which require Vav.

Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-PLCγ2 signaling axis in mice

Daniel B. Graham,[1] Charles M. Robertson,[2] Jhoanne Bautista,[1] Francesca Mascarenhas,[1] M. Julia Diacovo,[1] Vivianne Montgrain,[1] Siu Kit Lam,[1] Viviana Cremasco,[3] W. Michael Dunne,[1] Roberta Faccio,[3] Craig M. Coopersmith,[2,4] and Wojciech Swat[1]

[1]Department of Pathology and Immunology, [2]Department of Surgery, [3]Department of Orthopedic Surgery and Department of Cell Biology and Physiology, and [4]Department of Anesthesiology, Washington University School of Medicine, St. Louis, Missouri, USA.

Oxidative burst, a critical antimicrobial mechanism of neutrophils, involves the rapid generation and release of reactive oxygen intermediates (ROIs) by the NADPH oxidase complex. Genetic mutations in an NADPH oxidase subunit, gp91 (also referred to as NOX2), are associated with chronic granulomatous disease (CGD), which is characterized by recurrent and life-threatening microbial infections. To combat such infections, ROIs are produced by neutrophils after stimulation by integrin-dependent adhesion to the ECM in conjunction with stimulation from inflammatory mediators, or microbial components containing pathogen-associated molecular patterns. In this report, we provide genetic evidence that both the Vav family of Rho GTPase guanine nucleotide exchange factors (GEFs) and phospholipase C–γ2 (PLC-γ2) are critical mediators of adhesion-dependent ROI production by neutrophils in mice. We also demonstrated that Vav was critically required for neutrophil-dependent host defense against systemic infection by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, 2 common pathogens associated with fatal cases of hospital-acquired pneumonia. We identified a molecular pathway in which Vav GEFs linked integrin-mediated signaling with PLC-γ2 activation, release of intracellular Ca^{2+} cations, and generation of diacylglycerol to control assembly of the NADPH oxidase complex and ROI production by neutrophils. Taken together, our data indicate that integrin-dependent signals generated during neutrophil adhesion contribute to the activation of NADPH oxidase by a variety of distinct effector pathways, all of which require Vav.

Introduction

Neutrophil oxidative burst, a critical antimicrobial mechanism, involves the catalytic conversion of dimolecular oxygen into superoxide anion by the NADPH oxidase complex. Superoxide and secondarily produced reactive oxygen intermediates (ROIs) and halide derivatives possess potent antimicrobial properties and serve to combat a diverse array of pathogens (1). In fact, human patients with genetic deficiencies in NADPH oxidase components develop chronic granulomatous disease (CGD), which is characterized by recurrent life-threatening infections with both Gram-negative and Gram-positive bacteria as well as fungi (2). The genetic basis of CGD has been extensively characterized, and mutations in many of the NADPH oxidase subunits have been reported in patients (2). In the most common form of CGD, which is X-linked, patients bear mutations in the membrane-bound gp91 (also known as NOX2) protein, which pairs with p22 to form cytochrome b558, the catalytic core of the NADPH oxidase complex (3). However, the cytochrome b558 complex is not catalytically active until it associates with the GTPase Rac and the NADPH oxidase regulatory subunits including $p67^{phox}$, $p47^{phox}$, and $p40^{phox}$. Activation of Rac can be performed by several different Rho guanine nucleotide exchange factors (GEFs) during neutrophil activation. In addition, the phox subunits become phosphorylated during neutrophil activation and translocate to the membrane, where they associate with gp91 and p22 catalytic subunits (4, 5). Reduced expression or loss-of-function mutations in gp91, p22, $p47^{phox}$, and $p67^{phox}$ have been causatively linked to CGD (3). In addition, a patient bearing a mutation in Rac2 was reported to exhibit severe neutrophil deficiencies including defective oxidative burst (6, 7). As a consequence of reduced or absent oxidative burst, CGD patients frequently develop lymphadenitis, skin abscesses, and pneumonia and contract infections with a variety of microbes including *Staphylococcus*, *Salmonella*, *Aspergillus*, and *Candida* species (8).

In the context of microbial infection, neutrophils become activated and undergo oxidative burst after encountering proinflammatory signals such as those from cytokines, chemokines, opsonized particles, or pathogen-associated molecular patterns. However, optimal neutrophil activation also requires signals generated by integrins as the cell undergoes adhesion to the extracellular matrix (9, 10). Thus, oxidative burst induced by proinflammatory signals is amplified by orders of magnitude in adherent neutrophils (9, 11, 12). Consistent with this observation, mice with genetic deletions of molecules involved in integrin signaling exhibit profound defects in adhesion-dependent oxidative burst (13–21). Notably, integrin signaling in neutrophils was recently shown to proceed via a canonical immunoreceptor tyrosine-based activation motif–mediated (ITAM-mediated) pathway in which Src family kinases phosphorylate ITAMs in DAP12 and FcRγ chains, thus creating docking sites for Syk family kinases (13), which phosphorylate numerous

Nonstandard abbreviations used: BAL, bronchoalveolar lavage; BAPTA, 1,2-bis(aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; CGD, chronic granulomatous disease; DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; ITAM, immunoreceptor tyrosine-based activation motif; PLC, phospholipase C–γ; pRGD, polyRGD; ROI, reactive oxygen intermediate.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **117**:3445–3452 (2007). doi:10.1172/JCI32729.

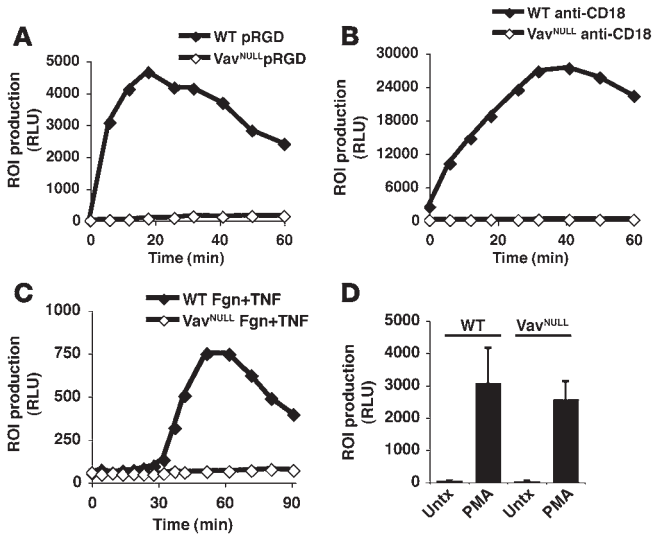

Figure 1
Adherent neutrophils require Vav to undergo oxidative burst in response to integrin ligands. ROI production in bone marrow–derived neutrophils was measured using lucigenin chemiluminescence. (**A** and **B**) WT and Vav^{NULL} neutrophils were stimulated in tubes coated with pRGD (**A**) or anti-CD18 (**B**). (**C**) Alternatively, neutrophils were stimulated in fibrinogen-coated tubes (100 μg/ml) with soluble TNF-α (50 ng/ml) (Fgn+TNF). (**D**) As a positive control, cells were stimulated with PMA (50 ng/ml) for 10–20 minutes or left untreated (Untx), and samples were performed in triplicate. Data shown are representative of at least 5 independent experiments. RLU, relative light units.

downstream targets leading to the assembly of a signaling complex composed of SLP-76, Vav, and phospholipase C–γ (PLC-γ) (22). However, the precise pathways that couple integrin signaling with NADPH oxidase assembly and activation remain to be elucidated. A particular issue is the involvement of the Vav family GEFs (Vav1, Vav2, and Vav3) and the PLC-γ family enzymes (PLC-γ1 and PLC-γ2) in neutrophil oxidative burst, which to date has been only partially characterized.

The importance of Vav proteins in Rac activation in neutrophils has been established (23); however, specific receptor-mediated signal transduction pathways can use distinct GEFs to activate Rac during oxidative burst. While Vav1 was originally implicated in regulating oxidative burst in neutrophils activated by formyl-methionyl-leucyl-phenylalanine (fMLP; refs. 24, 25), recent studies indicate that the Rac GEF P-Rex1 is required for fMLP-induced oxidative burst (26–28). Additionally, a recent report suggested the intriguing possibility that Vav1 could coordinate Rac and p67^{phox} activation during NADPH oxidase assembly in reconstituted CHO cells (25), although Vav1-deficient neutrophils and macrophages show no obvious defects in ROI production in response to a variety of stimuli (28). Furthermore, Vav has also been implicated in ROI production downstream of TLR4 and Fcγ receptors (24, 28, 29). However, it remains to be determined whether Vav proteins are generally required for adhesion-dependent augmentation of oxidative burst in neutrophils. In this regard, the expression of 3 functionally redundant Vav genes, Vav1, Vav2, and Vav3, has complicated the analysis of their requirement in neutrophils. Given that the function of individual Vav proteins is clearly redundant, a stringent analysis of the requirement for Vav in the regulation of oxidative burst necessitates the use of neutrophils that lack all Vav family proteins.

In addition to regulating Rac activation, Vav proteins can also play a critical role in the regulation of PLCγ-1 and PLCγ-2, for example during T and B cell activation downstream of ITAM-associated antigen receptors (30–32). In this context, the catalytic activity of PLC-γ enzymes mediates conversion of PIP_2 into IP_3 and diacylglycerol (DAG). Consequently, the generation of IP_3 leads to intracellular Ca^{2+} flux through opening of ER channels, which along with DAG production leads to the activation of PKCs and MAPKs. Such a Vav-dependent mechanism of PLC-γ regulation with subsequent release of intracellular Ca^{2+} cations and generation of DAG could conceivably be critical for oxidative burst in neutrophils in response to integrin- or adhesion-dependent stimuli, yet to our knowledge, the requirement for PLC-γ enzymes in the control of NADPH oxidase complex activation and ROI production has not been tested by gene knockout approaches.

Herein, we used genetic and biochemical approaches to demonstrate that the Vav GEFs and PLCγ-2 were essential for neutrophil oxidative burst induction by a variety of adhesion-dependent stimuli. Moreover, we provide in vivo evidence that Vav was required for clearance of *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections. Mechanistically, we identified Vav proteins and PLCγ-2 as critical components of a signal transduction pathway that links integrin-mediated adhesion to NADPH oxidase activation. In this context, we show that Vav regulation of neutrophil oxidative burst is mediated, at least in part, by Vav-dependent induction of PLC-γ2 activity.

Results

Vav is critically required for oxidative burst in response to adhesion-dependent stimuli in neutrophils. Full activation of microbicidal effector functions in neutrophils requires adhesion mediated by integrin binding to the extracellular matrix, which profoundly augments oxidative burst. Consistent with this view, strains of mice with genetic lesions disrupting integrin signaling exhibit severe defects in neutrophil function and oxidative burst (13, 14, 16, 18). In this context, our previous observations indicated that Vav proteins are not required for integrin affinity modulation (inside-out signaling) in response to inflammatory stimuli such as fMLP and LTB4, but are involved in control of integrin outside-in signaling that mediates firm adhesion (23). However, it remains unknown whether Vav proteins are required for adhesion-dependent oxidative burst. To test this hypothesis, we examined oxidative burst of WT neutrophils and neutrophils congenitally lacking Vav1, Vav2, and Vav3 GEFs (or the entire Vav family, referred to herein as Vav^{NULL}; ref. 30) upon adhesion to integrin ligands. Strikingly, Vav^{NULL} neutrophils failed to generate any detectable ROIs in response to various adhesion-dependent stimuli (Figure 1). In contrast to the response in WT neutrophils, Vav^{NULL} neutrophils did not generate ROIs after adhesion to the integrin ligand fibrinogen in the presence of TNF-α, a potent inducer of integrin activation by inside-out signaling (Figure 1A). Similarly, Vav^{NULL} neutrophils did not undergo oxidative burst in the presence of the strong integrin ligand polyRGD (pRGD), a synthetic peptide composed of multiple repeating Arg-Gly-Asp residues found in the integrin ligand fibronectin (Figure 1B). In addition, anti-CD18 antibody–mediated crosslinking of the integrin β2 chains induced oxidative burst in WT but not Vav^{NULL} neutrophils (Figure 1C). Importantly, WT and Vav^{NULL} neutrophils

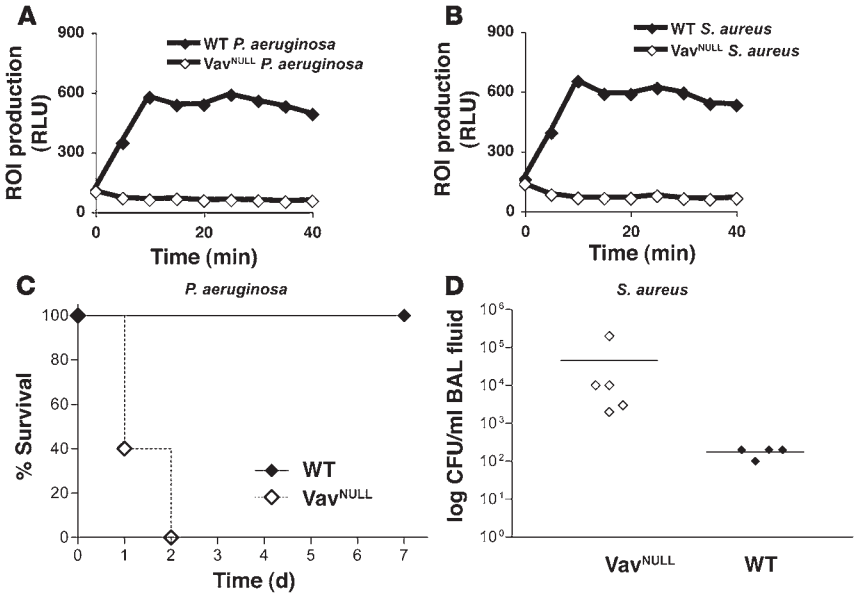

Figure 2
Vav^{NULL} mice exhibit severely compromised host defense against bacterial pneumonia. (**A** and **B**) WT and Vav^{NULL} bone marrow–derived neutrophils were stimulated with *P. aeruginosa* (**A**) or *S. aureus* (**B**) and monitored for oxidative burst by lucigenin chemiluminescence. (**C**) WT and Vav^{NULL} mice were infected with *P. aeruginosa* by intratracheal instillation, and mouse viability was monitored over 7 days. $n = 5$ per group. $P < 0.002$. (**D**) After intratracheal infection with *S. aureus*, WT and Vav^{NULL} mice were sacrificed at 20 hours, and BAL was collected for quantitation of bacterial load in the lungs. $n = 5$ per group. $P < 0.016$.

expressed similar levels of β2 integrin, a primary integrin expressed in neutrophils (data not shown). Notably, the requirement for Vav in adhesion-dependent oxidative burst mirrored the findings obtained with neutrophils lacking DAP12 and FcRγ chains, in which the integrin receptors are thought to be functionally uncoupled from downstream signaling pathways (ref. 13 and data not shown). Taken together, these results establish that Vav GEFs are critically required for induction of oxidative burst in response to adhesion-dependent stimuli in murine neutrophils. However, Vav^{NULL} neutrophils still produced ROIs in response to stimulation with PMA (Figure 1D). We interpret these data as indicating that Vav^{NULL} neutrophils express all essential components of the core NADPH oxidase complex, while the requirement for Vav in their activation can be bypassed by phorbol esters (presumably as a result of their direct effects on downstream effector pathways), which is consistent with previously published reports that PMA bypasses integrin-proximal signaling events required for NADPH oxidase activation (16, 18).

Vav is essential for neutrophil oxidative burst in response to S. aureus and P. aeruginosa and is required for their clearance in models of pneumonia-induced sepsis. The importance of neutrophil ROI production in host defense is highlighted in human CGD patients, who frequently develop microbial infections with *Staphylococcus* (8), and in mice lacking NADPH oxidase activity, which inefficiently clear *Staphylococcus* and *Pseudomonas* infections (33, 34). To determine whether Vav is required for oxidative burst in neutrophils in response to bacteria, we used WT and Vav^{NULL} neutrophils stimulated with heat-killed *S. aureus* and *P. aeruginosa* and measured neutrophil ROI production. In striking contrast to WT neutrophils, which produced a robust ROI response to this stimulation, Vav^{NULL} neutrophils completely failed to generate detectable levels of ROIs (Figure 2, A and B). Given that host defense against *P. aeruginosa* and *S. aureus* requires neutrophil oxidative burst, as evidenced by gp91-deficient and p40^{phox}-deficient mice, which inefficiently clear *S. aureus* infections (33, 35), and p47^{phox}-deficient mice, which inefficiently clear *P. aeruginosa* (34), we examined whether Vav proteins are required for clearance of these pathogens in 2 different murine models of pneumonia-induced sepsis. Notably, both of these pneumonia models are dependent on neutrophil function, because depletion of neutrophils leads to increased mortality after infection with *S. aureus* or *P. aeruginosa* (ref. 36 and our unpublished observations). To examine antimicrobial functions in vivo, we first subjected WT and Vav^{NULL} mice to intratracheal injection of *P. aeruginosa* and monitored them for survival over 7 days. Strikingly, while WT mice showed 100% survival at 7 days, Vav^{NULL} mice showed 100% mortality within the first 48 hours after infection (Figure 2C), indicating that deficiency in Vav renders sensitivity to *P. aeruginosa* infection. We next used a nonlethal model of pneumonia to examine the efficiency of bacterial clearance from the lungs of WT and Vav^{NULL} mice after infection with *S. aureus*. To this end, WT and Vav^{NULL} mice were subjected to intratracheal injection of *S. aureus* and killed at 20 hours after infection to assess bacterial counts in bronchoalveolar lavage (BAL) cultures. Strikingly, Vav^{NULL} mice showed approximately 100-fold increase over WT mice in bacterial counts assayed by BAL culture from infected lungs (Figure 2D). Histological examination demonstrated similar amounts of cellular infiltrate in the airways of *S. aureus*–infected WT and Vav^{NULL} mice (data not shown), indicating that an inflammatory response was initiated in Vav^{NULL} mice. Consistent with this observation, uninfected Vav^{NULL} mice were not neutropenic (data not shown), indicating that defective antibacterial responses in Vav^{NULL} mice are not due to reduced numbers of neutrophils but to defective neutrophil function. Importantly, although Vav^{NULL} mice eventually recovered and overcame infection with *S. aureus*, clearance of the bacteria from the lungs was inefficient. Taken together, these results demonstrated that the Vav GEFs were critical components of host defense mechanisms responsible for clearing systemic infections with common bacterial pathogens. Thus, while it is possible that Vav may also regulate other antibacterial mechanisms in vivo, our results clearly show that neutrophil oxidative burst, a requisite component of host defense against *S. aureus* and *P. aeruginosa* (33–35), critically depends on Vav function.

Vav is required for membrane translocation of phox subunits. Given the strict requirement for Vav proteins in ROI production in response to adhesion-dependent stimuli, we examined whether Vav controls NADPH oxidase assembly and translocation of the

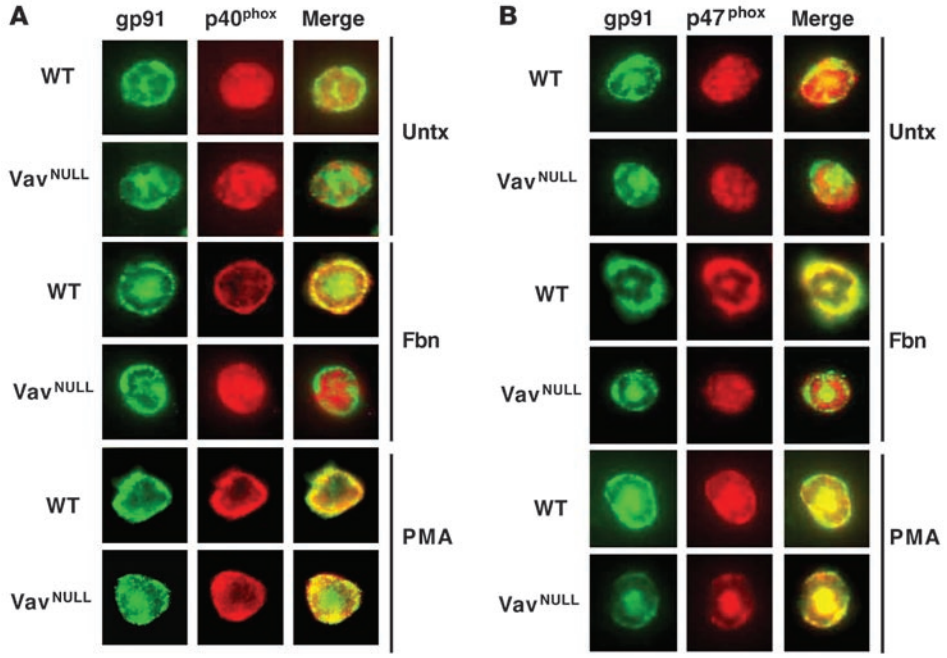

Figure 3
Vav is required for NADPH oxidase assembly during adhesion. (**A**) Bone marrow–derived neutrophils from WT and Vav^{NULL} mice were distributed onto untreated glass coverslips, coverslips coated with fibronectin (Fbn, 1 μg/ml), or uncoated coverslips with soluble PMA (50 ng/ml). After 30 minutes, cells were fixed, permeabilized, and stained with anti-gp91 (green) and anti-p40^{phox} (red). (**B**) Neutrophils were treated as in **A** and stained with anti-gp91 (green) and anti-p47 (red). Data shown are representative of at least 100 cells examined per condition.

cytoplasmic NADPH oxidase regulatory subunits to the plasma membrane in adherent neutrophils. To this end, we analyzed translocation of p40^{phox} and p47^{phox} to the membrane in neutrophils undergoing adhesion to coverslips coated with fibronectin. In contrast to WT neutrophils, which showed rapid translocation of p40^{phox} to the membrane and colocalization with gp91 under these conditions, Vav^{NULL} neutrophils failed to exhibit such changes in p40^{phox} localization (Figure 3A). Furthermore, p47^{phox} translocation and colocalization with gp91 was observed in WT neutrophils adhering to fibronectin, but was not detectable in Vav^{NULL} neutrophils (Figure 3B). However, both WT and Vav^{NULL} neutrophils showed rapid translocation of p40^{phox} and p47^{phox} in response to PMA treatment (Figure 3, A and B), consistent with normal production of ROIs in PMA-treated Vav^{NULL} neutrophils. Taken together, these data show that Vav proteins were required for the translocation of phox subunits during NADPH oxidase activation. Given that p40^{phox} and p47^{phox} translocation occurs independently of Rac activation (37), these results indicate a broader role for Vav proteins in regulating neutrophil oxidative burst beyond functioning as a Rac GEF. Specifically, translocation of the p40^{phox} and p47^{phox} subunits is thought to involve their phosphorylation by PKC isoforms and tethering to the membrane through interactions between their PX domains and phosphoinositides (38, 39), all of which could be mediated by PLC-γ enzymes.

Vav is required for activation of PLC-γ2 in neutrophils. Given previously published studies implicating Vav proteins as essential upstream regulators of PLC-γ1 and PLC-γ2 in T and B cells stimulated via their ITAM-containing antigen receptor complexes (30–32) and a recent discovery that integrin-dependent ROI production also involves ITAM-based signaling (13), we hypothesized that Vav may also control PLC-γ activation downstream of integrin receptors upon neutrophil adhesion. To test this hypothesis, we first examined tyrosine phosphorylation of PLC-γ2, a major PLC-γ isoform expressed in neutrophils, and observed robust phosphorylation of PLC-γ2 in WT neutrophils upon adhesion to pRGD (Figure 4A). Strikingly, however, phosphorylation of PLC-γ2 was drastically diminished in Vav^{NULL} neutrophils under identical conditions. Consistent with these findings, phosphorylation of ERK was severely diminished in Vav^{NULL} neutrophils undergoing adhesion to pRGD compared with WT neutrophils (Figure 4B). Therefore, these results indicate that Vav is required for adhesion-dependent activation of PLC-γ2 and its downstream effectors. However, it is not known at present whether PLC-γ2, or its activity, is required for the activation of NADPH oxidase and generation of oxidative burst in neutrophils.

The PLC-γ2 effectors Ca^{2+} and DAG regulate ROI production and oxidative burst in neutrophils. To determine whether the catalytic activity of PLC-γ2 and generation of DAG and/or IP_3 and Ca^{2+} flux are required for adhesion-dependent oxidative burst in neutrophils, we first used pharmacological inhibitors to block intracellular Ca^{2+} flux with the selective Ca^{2+} chelator 1,2-bis(aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA).

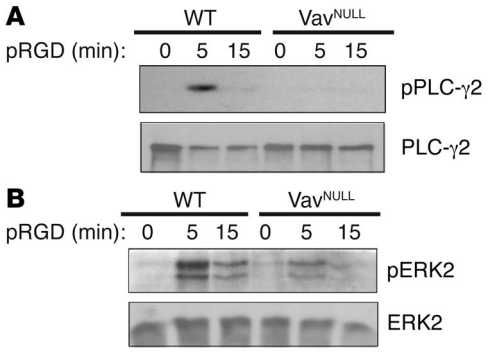

Figure 4
Vav regulates PLC-γ2 signaling during neutrophil adhesion. (**A** and **B**) WT and Vav^{NULL} neutrophils were stimulated on pRGD-coated tissue culture plates and lysed at the indicated time points. Lysates were resolved by SDS-PAGE and analyzed by Western blot for activated phosphorylated PLC-γ2 (pPLC-γ2) followed by total PLC-γ2 (**A**) and phosphorylated ERK (pERK) followed by total ERK2 (**B**). Data shown are representative of at least 3 independent experiments.

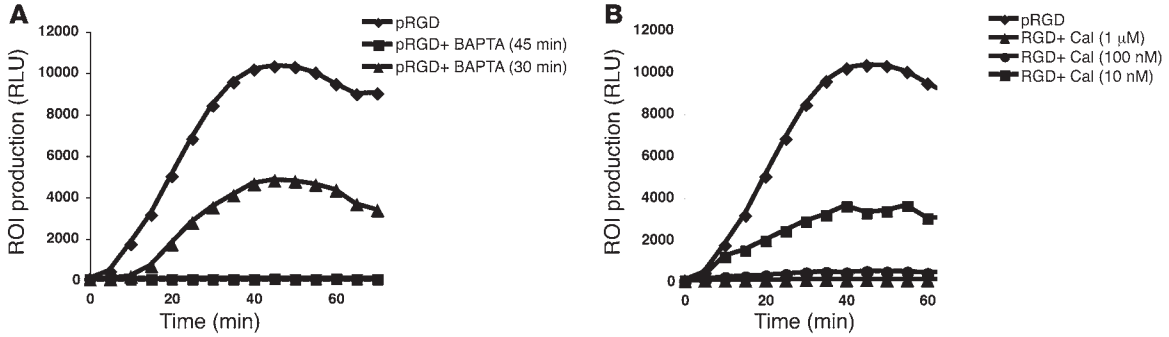

Figure 5
PLC-γ2 effector pathways through calcium and DAG are required for adhesion-dependent oxidative burst. (**A**) Neutrophils were loaded for the indicated time periods in a 10-μM solution of the calcium chelator BAPTA and stimulated in luminometer tubes coated with pRGD (1 μg/ml). (**B**) Neutrophils were stimulated with the indicated concentrations of the PKC inhibitor calphostin C (Cal) and stimulated in luminometer tubes coated with pRGD (1 μg/ml). ROI production was measured using lucigenin chemiluminescence as in Figure 1. Data shown are representative of at least 3 independent experiments.

These experiments showed that pretreatment of WT neutrophils with BAPTA completely blocked their ability to generate ROIs in response to adhesion to pRGD (Figure 5A). These data indicate that the mobilization of Ca^{2+} cations is required for ROI production in neutrophils. To address the requirement for DAG-mediated activation of PKCs during neutrophil oxidative burst, we used calphostin C, an antagonist of the DAG-binding site on PKCs. Strikingly, calphostin C treatment showed a dose-dependent inhibition of ROI production in WT neutrophils upon activation with pRGD (Figure 5B). Taken together, these data indicate that the PLC-γ2 signaling effectors Ca^{2+} and DAG mediate adhesion-dependent oxidative burst in neutrophils.

PLC-γ2 is essential for ROI production and oxidative burst in neutrophils. Having established that Ca^{2+} and DAG are required in the control of ROI production in adherent neutrophils, we sought to determine whether PLC-γ2 itself is essential in this process. To this end, we used neutrophils from mice congenitally lacking PLC-γ2 expression (40). Importantly, PLC-γ2 did not appear to be required for neutrophil development, because PLC-γ2$^{-/-}$ mice had normal numbers of neutrophils in peripheral blood and bone marrow and showed morphology indistinguishable from that of WT neutrophils (data not shown). Strikingly, however, PLC-γ2$^{-/-}$ neutrophils completely failed to generate any detectable levels of ROIs after stimulation with various adhesion-dependent stimuli, including fibrinogen and TNF-α, pRGD, and anti-CD18 antibodies (Figure 6). Thus, PLC-γ2$^{-/-}$ neutrophils exhibited defects in oxidative burst similar to those of Vav^{NULL} (Figure 1) or DAP12/FcRγ-deficient neutrophils (ref. 13 and data not shown). These data indicate that adhesion-dependent stimuli transduced by ITAM-associated receptors in neutrophils induce NADPH oxidase and activate ROI production via a signaling pathway involving Vav GEFs and PLC-γ2. In agreement with this model, PLC-γ2 was not required for oxidative burst induced in response to PMA, because PLC-γ2$^{-/-}$ neutrophils efficiently produced ROIs in response to PMA stimulation (Figure 6). Together, these data established the requirement for PLC-γ2 in adhesion-dependent oxidative burst in neutrophils and identified Vav GEFs as key regulators of PLC-γ2 and NADPH oxidase.

Discussion

Among the most common bacterial infections observed in patients with CGD are those involving *Staphylococcus* species; similarly, NADPH oxidase–deficient mice inefficiently clear *S. aureus* and *P. aeruginosa* infections (2, 8, 33, 34). Moreover, in human patients, *S. aureus* and *P. aeruginosa* account for 33%–62% of all intensive care unit pneumonias and are associated with significant morbidity and mortality (41–43). In this context, we observed a striking

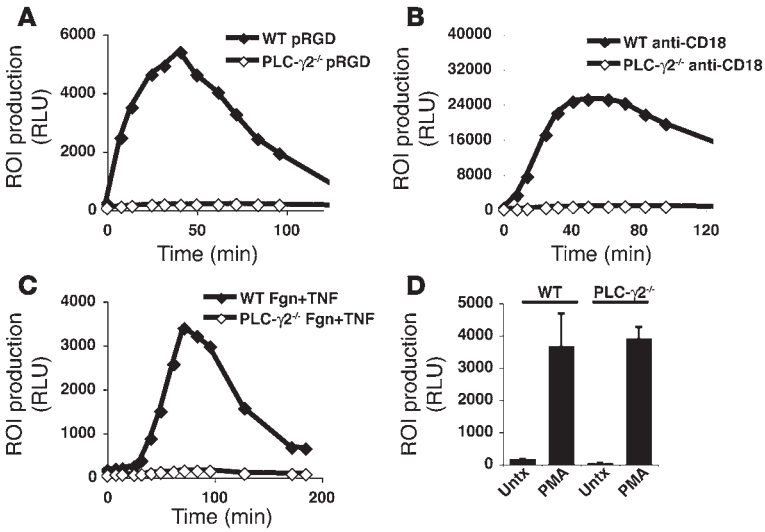

Figure 6
Adherent neutrophils require PLC-γ2 to undergo oxidative burst in response to integrin ligands. ROI production in bone marrow–derived neutrophils was measured using lucigenin chemiluminescence. (**A** and **B**) WT and PLC-γ2$^{-/-}$ neutrophils were stimulated in tubes coated with pRGD (**A**) or anti-CD18 (**B**). (**C**) Alternatively, neutrophils were stimulated in fibrinogen-coated tubes (100 μg/ml) with soluble TNF-α (50 ng/ml). (**D**) As a positive control, cells were stimulated with PMA (50 ng/ml) for 10–20 minutes or were left untreated, and samples were performed in triplicate. Data shown are representative of 3 independent experiments.

increase in mortality in Vav^{NULL} mice compared with WT mice after infection with *P. aeruginosa*. In addition, Vav^{NULL} mice failed to efficiently clear *S. aureus*, as evidenced by a 100-fold increase in CFUs derived from BAL in Vav^{NULL} mice compared with WT mice. Although many mechanisms are responsible for bacterial clearance in vivo, one critical effector pathway required for *S. aureus* and *P. aeruginosa* clearance is neutrophil oxidative burst (33–35). For example, mice lacking the gp91 subunit of NADPH oxidase exhibit an increased susceptibility to infection by *S. aureus* (33). In addition, p40^{phox}-deficient mice fail to clear *S. aureus* infections (35), while p47^{phox}-deficient mice show increased bacterial load in a model of *P. aeruginosa*–induced pneumonia (34).

Neutrophil adhesion is fundamentally important for oxidative burst induction by various proinflammatory agents associated with bacterial infections (9). In this context, we identified integrin signaling defects in Vav^{NULL} neutrophils that were accompanied by an absence of ROI production in these cells in response to various adhesion-dependent stimuli. Notably, a recent report identified an ITAM signaling pathway involving DAP12 and FcRγ adaptors triggered by integrin adhesion, which is critical for ROI production in neutrophils (13). Thus, while additional inflammatory signals mediated by cytokines, opsonins, and pathogen-associated molecular patterns costimulate or synergize with integrin signals (9), our results presented here indicate that Vav is a critical mediator of such adhesion-dependent signals and plays a key role in the neutrophil activation program.

In addition, we identified what we believe to be a novel signaling pathway controlled by Vav that regulates PLC-γ2 activation in the context of NADPH oxidase activation. We demonstrated that PLC-γ2 and its effector pathways mediated by intracellular Ca^{2+} flux and DAG production were required for adhesion-dependent oxidative burst in neutrophils. Both Ca^{2+} and DAG activate PKC isoforms that are capable of phosphorylating the cytosolic regulatory subunits of the NADPH oxidase complex (5). In this context, the adaptor molecule SLP-76 has been shown to interact with both Vav and PLC-γ (44), and SLP-76 was also implicated in oxidative burst in neutrophils (17, 18). Moreover, consistent with the notion that Vav is involved in PLCγ-2 activation during neutrophil adhesion, Vav proteins have previously been shown to regulate PLC-γ1 and PLC-γ2 downstream of ITAM-associated antigen receptors in T and B lymphocytes (30–32).

While our previous work indicated that Vav is required for integrin-mediated Rac activation in neutrophils (23), Vav and its GEF activity are also required for LPS- and FcRγ-mediated oxidative burst in macrophages and neutrophils (28, 29). In this context, a recent report suggested that Vav1 coordinates Rac and p67^{phox} activation during NADPH oxidase assembly (25); however, Vav1-deficient neutrophils and macrophages show no obvious defects in ROI production (ref. 28 and data not shown). Therefore, while the significance of the findings linking Vav1 to p67^{phox} and Rac still remains to be established, the function of individual Vav proteins is clearly redundant in the regulation of phagocyte oxidative burst.

Nevertheless, taken together with other published reports, our data indicate that integrin-dependent ITAM signals generated during neutrophil adhesion contribute to the activation of NADPH oxidase by a variety of distinct effector pathways, all of which require Vav. For example, Vav contributes to the integrin-dependent activation of Rac GTPases, which are directly involved in activation of the NADPH oxidase complex (6, 7, 45–51). Another Vav-dependent pathway leads to the activation of PLC-γ2, and intracellular Ca^{2+} flux and DAG production by PLC-γ2 are critical for NADPH oxidase activation. In this context, we provide what we believe to be the first direct demonstration of the essential role of PLC-γ2 in this process, because PLC-γ2-deficient neutrophils showed profound defects in ROI production and oxidative burst. Thus, by controlling the activation of Rac and PLC-γ2, Vav serves as a key regulator of adhesion-dependent signaling pathways that converge to activate NADPH oxidase during neutrophil oxidative burst.

Methods

Mice. Vav^{NULL} mice genetically deficient in all 3 Vav proteins (Vav1$^{-/-}$, Vav2$^{-/-}$, and Vav3$^{-/-}$) have been previously described (30), as have PLC-γ2$^{-/-}$ mice (40). Strain-matched B6 mice were used as controls for knockout animals. All protocols involving mice were approved by the Institutional Animal Care and Use Committee at Washington University School of Medicine and were carried out in accordance with institutional guidelines and regulations.

Neutrophil purification. Bone marrow was harvested from femurs and tibias prior to red blood cell lysis in hypotonic saline solution. Cells were then resuspended in HBSS (without Ca^{2+} and Mg^{2+}) and overlaid on a discontinuous Percoll gradient composed of 70% and 60% Percoll fractions in HBSS. Neutrophils were recovered from between the 60% and 70% fractions, then washed and resuspended in HBSS (with 1.3 mM $CaCl_2$ and 1 mM $MgSO_4$). Neutrophil purity was typically greater than 90% as determined by FACS and morphological analysis.

Oxidative burst. Neutrophils were washed and resuspended in HBSS (with Ca^{2+} and Mg^{2+}) at a concentration of 2.5×10^5 cells/ml. Lucigenin (Molecular Probes) was added to the cells at a final concentration of 150 μM. Cells were immediately distributed into luminometer tubes and stimulated with the indicated stimuli. TNF-α was obtained from Peprotech and used at 50 ng/ml. Where indicated, sheep fibrinogen (Sigma-Aldrich) was used at a concentration of 100 μg/ml in PBS to coat luminometer tubes for 2 hours at 37°C. Alternatively, pRGD peptide (Sigma-Aldrich) was used at a concentration of 1 μg/ml, or anti-CD18 (BD Biosciences — Pharmingen clone C71/16) was used at a concentration of 5 μg/ml in PBS to coat luminometer tubes for 2 hours at 37°C. The Ca^{2+} chelator BAPTA was obtained from Sigma-Aldrich. The PKC inhibitor calphostin C (Calbiochem) was activated under a 50-W fluorescent light for 15 minutes prior to use at the indicated concentrations. During stimulation, lucigenin chemiluminescence was measured in each sample for 10 seconds in an OptocompII luminometer (MGM Instruments Inc.). Samples were read over the indicated time course.

Pneumonia models. *P. aeruginosa* (ATCC strain 27853) was placed in trypticase soy broth with constant shaking overnight. The resulting culture was then centrifuged at 6,000 *g* and washed twice with 0.9% NaCl. The washed bacteria were resuspended in 0.9% NaCl to a density of 0.1 A_{600nm}. *S. aureus* strain 313 (methicillin-resistant, Panton-Valentine leukocidin–negative, multilocus sequence type 5) was prepared in a similar manner to a density of 0.5 A_{600nm}. Under isoflurane anesthesia, mice had a midline cervical incision and received an intratracheal injection of 40 μl of a solution containing either *P. aeruginosa* or *S. aureus*. The final density of *P. aeruginosa* inoculum was 1.5×10^8 CFU/ml, as determined by serial dilution and colony counts. The final density of *S. aureus* inoculum was 5×10^8 CFU/ml. After instillation of bacteria, animals were held vertically for 10 seconds to enhance bacterial delivery into the lung. Mice were either sacrificed at 20 hours for BAL and lung histology or followed for survival for 7 days.

Bacterial cultures and histology. NaCl (0.9%, 1 ml) was instilled into the trachea and then returned. The resultant BAL fluid was serially diluted and cultured overnight on blood agar plates at 37°C using standard microbio-

logical techniques. After BAL was performed, lungs were removed, fixed in formalin, and stained with hematoxylin and eosin. Slides were evaluated in a blinded fashion for severity and distribution of pneumonia on a scale of 0–4 (no abnormality to most severe pneumonia) and 0–3 (no abnormality to most widespread pneumonia), respectively.

Neutrophil imaging. Glass coverslips were left unmanipulated or coated with bovine fibronectin (Sigma-Aldrich) at 1 μg/ml in PBS for 2 hours at 37°C. Neutrophils were distributed onto coverslips, incubated at 37°C for 30 minutes, and fixed in 4% paraformaldehyde. Cells were then permeabilized in 0.1% Triton X-100 and stained with the indicated antibodies. Mouse anti-mouse gp91 (clone 53) was obtained from BD Biosciences — Pharmingen. Rabbit anti-mouse $p47^{phox}$ and $p40^{phox}$ were purchased from Upstate Biotech. Alexa 594–conjugated goat anti-rabbit IgG and Alexa 488–conjugated goat anti-mouse IgG were purchased from Molecular Probes. Cells were mounted using Vectashield medium (Vector Laboratories) and were visualized using a Nikon Eclipse E400 microscope equipped with a ×60/1.4 numerical aperture oil objective. Images were captured using a Magnafire camera (Optronics) and processed with Nikon Elements software and Adobe Photoshop CS software (Adobe Systems).

Biochemistry. Each well in 6-well plates was coated with pRGD (Sigma-Aldrich) at 1 μg/ml in PBS for 2 hours at 37°C. Cells were distributed onto pRGD-coated plates at a concentration of 2–10 × 10^6 cells/ml DMEM/well and incubated at 37°C for the indicated time course, after which each sample was processed. Media containing nonadherent cells was collected from the well, and cells were pelleted. The remaining adherent cells were lysed in the well in 75 μl RIPA buffer (PBS, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 10 mM EDTA, protease inhibitor cocktail [Boehringer], 10 mM NaF, and 1 mM Na_3VO_4), and this lysate was then added to the pelleted nonadherent cells. Clarified lysates were separated by PAGE and transferred to PVDF membranes (Millipore) for Western blotting. Rabbit anti–phosphorylated PLCγ2 (Tyr759) and mouse anti–phosphorylated p44/42 MAPK (ERK) T202/Y204 were obtained from Cell Signaling. Rabbit anti-PLCγ2 and rabbit anti-ERK2 were purchased from Santa Cruz Biotechnology Inc. HRP-conjugated secondary antibodies raised against mouse-IgG (Zymed) and rabbit-IgG (Amersham Biosciences) were used with enhanced chemiluminescence (Amersham Biosciences) to develop blots.

Statistics. Group survival differences were analyzed by log-rank analysis. BAL and quantitative histology scores were compared by Mann-Whitney test. Data analysis was performed using Prism 3.0 (GraphPad Software). A P value of less than 0.02 was considered significant.

Acknowledgments

This work was supported by NIH grants AI06107703 and AI06302402 (to W. Swat), T32 CA009547 (to D.B. Graham), and GM66202 and GM072808 (to C.M. Coopersmith).

Received for publication May 18, 2007, and accepted in revised form July 18, 2007.

Address correspondence to: Wojciech Swat, Washington University School of Medicine, Box 8118, 660 S. Euclid Ave., St. Louis, Missouri 63110, USA. Phone: (314) 747-8889; Fax: (314) 362-4096; E-mail: swat@wustl.edu.

1. Lambeth, J.D. 2004. NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol.* **4**:181–189.
2. Kume, A., and Dinauer, M.C. 2000. Gene therapy for chronic granulomatous disease. *J. Lab. Clin. Med.* **135**:122–128.
3. Assari, T. 2006. Chronic Granulomatous Disease; fundamental stages in our understanding of CGD. *Med. Immunol.* **5**:4.
4. Bokoch, G.M. 2005. Regulation of innate immunity by Rho GTPases. *Trends Cell Biol.* **15**:163–171.
5. Bokoch, G.M., and Knaus, U.G. 2003. NADPH oxidases: not just for leukocytes anymore! *Trends Biochem. Sci.* **28**:502–508.
6. Williams, D.A., et al. 2000. Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. *Blood.* **96**:1646–1654.
7. Ambruso, D.R., et al. 2000. Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. *Proc. Natl. Acad. Sci. U. S. A.* **97**:4654–4659.
8. Liese, J., et al. 2000. Long-term follow-up and outcome of 39 patients with chronic granulomatous disease. *J. Pediatr.* **137**:687–693.
9. Nathan, C. 2006. Neutrophils and immunity: challenges and opportunities. *Nat. Rev. Immunol.* **6**:173–182.
10. Abram, C.L., and Lowell, C.A. 2007. The expanding role for ITAM-based signaling pathways in immune cells. *Sci. STKE.* **2007**:re2.
11. Nathan, C., et al. 1989. Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J. Cell Biol.* **109**:1341–1349.
12. Nathan, C.F. 1989. Respiratory burst in adherent human neutrophils: triggering by colony-stimulating factors CSF-GM and CSF-G. *Blood.* **73**:301–306.
13. Mocsai, A., et al. 2006. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat. Immunol.* **7**:1326–1333.
14. Lowell, C.A., Fumagalli, L., and Berton, G. 1996. Deficiency of Src family kinases p59/61hck and p58c-fgr results in defective adhesion-dependent neutrophil functions. *J. Cell Biol.* **133**:895–910.
15. Mocsai, A., Ligeti, E., Lowell, C.A., and Berton, G. 1999. Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. *J. Immunol.* **162**:1120–1126.
16. Mocsai, A., Zhou, M., Meng, F., Tybulewicz, V.L., and Lowell, C.A. 2002. Syk is required for integrin signaling in neutrophils. *Immunity.* **16**:547–558.
17. Clemens, R.A., et al. 2007. Loss of SLP-76 expression within myeloid cells confers resistance to neutrophil-mediated tissue damage while maintaining effective bacterial killing. *J. Immunol.* **178**:4606–4614.
18. Newbrough, S.A., et al. 2003. SLP-76 regulates Fcgamma receptor and integrin signaling in neutrophils. *Immunity.* **19**:761–769.
19. Clemens, R.A., et al. 2004. PRAM-1 is required for optimal integrin-dependent neutrophil function. *Mol. Cell. Biol.* **24**:10923–10932.
20. Chen, H., et al. 2003. Role for plastin in host defense distinguishes integrin signaling from cell adhesion and spreading. *Immunity.* **19**:95–104.
21. Zhang, H., et al. 2006. Impaired integrin-dependent function in Wiskott-Aldrich syndrome protein-deficient murine and human neutrophils. *Immunity.* **25**:285–295.
22. Koretzky, G.A., Abtahian, F., and Silverman, M.A. 2006. SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat. Rev. Immunol.* **6**:67–78.
23. Gakidis, M.A., et al. 2004. Vav GEFs are required for beta2 integrin-dependent functions of neutrophils. *J. Cell Biol.* **166**:273–282.
24. Kim, C., Marchal, C.C., Penninger, J., and Dinauer, M.C. 2003. The hemopoietic Rho/Rac guanine nucleotide exchange factor Vav1 regulates N-formyl-methionyl-leucyl-phenylalanine-activated neutrophil functions. *J. Immunol.* **171**:4425–4430.
25. Ming, W., Li, S., Billadeau, D.D., Quilliam, L.A., and Dinauer, M.C. 2007. The Rac effector p67phox regulates phagocyte NADPH oxidase by stimulating Vav1 guanine nucleotide exchange activity. *Mol. Cell. Biol.* **27**:312–323.
26. Welch, H.C., et al. 2005. P-Rex1 regulates neutrophil function. *Curr. Biol.* **15**:1867–1873.
27. Dong, X., Mo, Z., Bokoch, G., Guo, C., Li, Z., and Wu, D. 2005. P-Rex1 is a primary Rac2 guanine nucleotide exchange factor in mouse neutrophils. *Curr. Biol.* **15**:1874–1879.
28. Miletic, A.V., et al. 2007. Vav proteins control MyD88-dependent oxidative burst. *Blood.* **109**:3360–3368.
29. Utomo, A., Cullere, X., Glogauer, M., Swat, W., and Mayadas, T.N. 2006. Vav proteins in neutrophils are required for FcgammaR-mediated signaling to Rac GTPases and nicotinamide adenine dinucleotide phosphate oxidase component p40(phox). *J. Immunol.* **177**:6388–6397.
30. Fujikawa, K., et al. 2003. Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells. *J. Exp. Med.* **198**:1595–1608.
31. Reynolds, L.F., et al. 2002. Vav1 transduces T cell receptor signals to the activation of phospholipase C-gamma1 via phosphoinositide 3-kinase-dependent and -independent pathways. *J. Exp. Med.* **195**:1103–1114.
32. Vigorito, E., Kovesdi, D., and Turner, M. 2006. Synergistic activation of PKD by the B cell antigen receptor and CD19 requires PI3K, Vav1 and PLC-gamma. *Cell. Signal.* **18**:1455–1460.
33. Pollock, J.D., et al. 1995. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat. Genet.* **9**:202–209.
34. Sadikot, R.T., et al. 2004. p47phox deficiency impairs NF-kappa B activation and host defense in Pseudomonas pneumonia. *J. Immunol.* **172**:1801–1808.
35. Ellson, C.D., et al. 2006. Neutrophils from p40phox–/– mice exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing. *J. Exp. Med.* **203**:1927–1937.

36. Kimura, K., et al. 1999. Factors affecting the course and severity of transnasally induced Staphylococcus aureus pneumonia in mice. *J. Med. Microbiol.* **48**:1005–1010.
37. Kim, C., and Dinauer, M.C. 2006. Impaired NADPH oxidase activity in Rac2-deficient murine neutrophils does not result from defective translocation of p47phox and p67phox and can be rescued by exogenous arachidonic acid. *J. Leukoc. Biol.* **79**:223–234.
38. Kanai, F., et al. 2001. The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat. Cell Biol.* **3**:675–678.
39. Sumimoto, H., Miyano, K., and Takeya, R. 2005. Molecular composition and regulation of the Nox family NAD(P)H oxidases. *Biochem. Biophys. Res. Commun.* **338**:677–686.
40. Wang, D., et al. 2000. Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. *Immunity.* **13**:25–35.
41. Eggimann, P., and Pittet, D. 2001. Infection control in the ICU. *Chest.* **120**:2059–2093.
42. National Nosocomial Infections Surveillance. 1999. National Nosocomial Infections Surveillance (NNIS) System report, data summary from January 1990–May 1999, issued June 1999. *Am. J. Infect. Control.* **27**:520–532.
43. American Thoracic Society, and Infectious Diseases Society of America. 2005. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. *Am. J. Respir. Crit. Care Med.* **171**:388–416.
44. Baldari, C.T., Koretzky, G., Telford, J.L., and Acuto, O. 2005. Antigen receptor signaling: the Tuscan chronicles. *Nat. Immunol.* **6**:3–5.
45. Knaus, U.G., Heyworth, P.G., Evans, T., Curnutte, J.T., and Bokoch, G.M. 1991. Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2. *Science.* **254**:1512–1515.
46. Abo, A., et al. 1991. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature.* **353**:668–670.
47. Roberts, A.W., et al. 1999. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity.* **10**:183–196.
48. Li, S., et al. 2002. Chemoattractant-stimulated Rac activation in wild-type and Rac2-deficient murine neutrophils: preferential activation of Rac2 and Rac2 gene dosage effect on neutrophil functions. *J. Immunol.* **169**:5043–5051.
49. Carstanjen, D., et al. 2005. Rac2 regulates neutrophil chemotaxis, superoxide production, and myeloid colony formation through multiple distinct effector pathways. *J. Immunol.* **174**:4613–4620.
50. Kim, C., and Dinauer, M.C. 2001. Rac2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. *J. Immunol.* **166**:1223–1232.
51. Yamauchi, A., et al. 2004. Rac2-deficient murine macrophages have selective defects in superoxide production and phagocytosis of opsonized particles. *J. Immunol.* **173**:5971–5979.