Immunity 24, 305–316, March 2006 ©2006 Elsevier Inc. DOI 10.1016/j.immuni.2006.02.005

Requirements for Vav Guanine Nucleotide Exchange Factors and Rho GTPases in FcγR- and Complement-Mediated Phagocytosis

Amy B. Hall,[1,4] M. Angelica Martinez Gakidis,[1,4] Michael Glogauer,[2] Julie L. Wilsbacher,[1] Sizhen Gao,[1] Wojciech Swat,[3] and Joan S. Brugge[1,*]

[1] Department of Cell Biology
Harvard Medical School
Boston, Massachusetts 02115

[2] Faculty of Dentistry
University of Toronto
Toronto, Ontario M5S 1A8
Canada

[3] Washington University School of Medicine
St. Louis, Missouri 63110

Summary

Vav guanine nucleotide exchange factors (GEFs) have been implicated in cell adhesion by integrin and immune response receptors through the regulation of Rho GTPases. Here, we examine the role of Vav and Rho GTPases in phagocytosis by using primary murine macrophages. The genetic deletion of Rac1 and Rac2 prevents phagocytosis mediated by integrin and Fcγ receptors (FcγR), whereas the genetic deletion of Vav1 and Vav3 only prevents integrin-mediated phagocytosis through the complement receptor $\alpha_M\beta_2$. In addition, a Rac1/2 or Vav1/3 deficiency blocks Arp2/3 recruitment and actin polymerization at the complement-induced phagosome, indicating that these proteins regulate early steps in phagocytosis. Moreover, constitutively active Rac is able to rescue actin polymerization and complement-mediated phagocytosis in Vav-deficient macrophages. These studies indicate that Rac is critical for complement- and FcγR-mediated phagocytosis. In contrast, Vav is specifically required for complement-mediated phagocytosis, suggesting that Rac is regulated by GEFs other than Vav downstream of the FcγR.

Introduction

Phagocytosis, the process by which cells recognize and engulf large particles, usually greater than 0.5 μm, is a critical component of innate immunity, as it plays an important role in both host defense mechanisms and tissue repair. Phagocytosis is initiated by the binding of opsonized particles to cell surface receptors, which induce the formation of phagocytic cups at the cell membrane. Subsequently, the particles are engulfed, and the plasma membrane fuses to seal off a mature phagosome. Studies of phagocytosis have provided a useful model by which to study signaling receptor-induced events because the recruitment and concentration of proteins is restricted to the phagocytic cup and thus facilitates analysis of the spatiotemporal aspects of recruitment and activation of proteins engaged by ligand-receptor interactions (Cougoule et al., 2004).

Macrophages are professional phagocytes that are among the first lines of defense against invading pathogens. Macrophages express a diverse array of phagocytic receptors, including the integrin $\alpha_M\beta_2$ (also known as the complement receptor 3 or CR3) and the Fcγ receptor (FcγR), two of the best-characterized phagocytic receptors. The complement receptor binds to particles opsonized with complement C3bi, and FcγRs bind to immunoglobulin (IgG)-opsonized particles. One of the most striking features of both complement- and FcγR-mediated phagocytosis is the dynamic and rapid organization of the actin cytoskeleton to form the phagocytic cup upon receptor activation by opsonized particle binding (Chimini and Chavrier, 2000). The Arp2/3 complex, an actin nucleator, localizes to FcγR- and complement-mediated phagosomes and is required for both types of phagocytosis (May et al., 2000).

Despite the localized initiation of actin polymerization, previous studies have suggested that the mechanism of ingestion is distinct for these two types of phagocytosis. During FcγR-mediated phagocytosis, actin-rich projections known as pseudopods extend from the cell surface and surround the opsonized particle, whereas complement-opsonized particles passively "sink" into the cell (Kaplan, 1977). Several Rho family GTPases—Rac, Rho, and Cdc42—are recruited to the phagosome and have been implicated in different aspects of either FcγR- or complement-mediated phagocytosis (Caron and Hall, 1998). Dominant inhibitory variants of Rac and Cdc42 have been shown to inhibit FcγR-mediated phagocytosis in immortalized macrophage and mast cell lines, or in Cos cells reconstituted with phagocytic receptors (Caron and Hall, 1998; Cox et al., 1997; Massol et al., 1998). The role of Rho in phagocytosis is unclear, as the specific inhibition of Rho with C3 transferase was shown to block complement-, but not FcγR-mediated, phagocytosis in one study (Caron and Hall, 1998), but prevented FcγR-mediated phagocytosis in another (Hackam et al., 1997). Therefore, the role of Rho GTPases in phagocytosis remains to be clarified. The observed differences in the types of actin cytoskeletal rearrangements associated with complement- and FcγR-mediated phagocytosis may be due to engagement of distinct Rho GTPases or the activation of the Rho GTPases by specific guanine nucleotide exchange factors. However, the mechanisms linking the phagocytic receptors to activation of the Rho GTPases have not been elucidated.

Multiple Rho GTPases (Rac, Rho, Rho G, and Cdc42) are directly activated by the Vav family of guanine nucleotide exchange factors (GEFs), Vav1, Vav2, and Vav3, through the exchange of GDP for GTP (Bustelo, 2000). However, it is not known which specific Rho GTPase acts as a direct target of each Vav family member in vivo. Vav1 is found predominantly in hematopoietic cells, whereas Vav2 and Vav3 are more broadly expressed. Despite the differences in their tissue distribution, Vav family members share 50%–60% sequence

*Correspondence: joan_brugge@hms.harvard.edu
[4] These authors contributed equally to this work.

homology and similar structural domains (calponin homology [CH], Dbl homology [DH], pleckstrin homology [PH], cysteine-rich [CR], Src homology 2 and 3 [SH2 and SH3] domains) (Bustelo, 2000). These multidomain proteins are activated by a wide variety of cell surface receptors, including immune receptors, integrins, G protein-coupled receptors (GPCRs), and growth factor receptors, through the phosphorylation of regulatory tyrosines in the amino terminus. Thus, Vav proteins are potential targets ideally suited to link cell surface receptors to the intracellular Rho GTPases and the actin cytoskeleton during phagocytosis.

Several studies using Vav knockout mice have provided important insights into the function of Vav. In *vav1*$^{-/-}$ (Vav1^{ko}) lymphocytes, there is a decrease in T cell receptor (TCR)-induced IL-2 production, proliferation, and calcium mobilization (Bustelo, 2000). In addition, Vav1^{ko} lymphocytes have defects in TCR capping and actin patch formation, suggesting that Vav1 plays a role in coupling the TCR to the actin cytoskeleton (Fischer et al., 1998; Holsinger et al., 1998). In lymphocytes, the additional deletion of Vav2 results in further B cell defects, and the additional loss of Vav3 leads to more T cell defects (Doody et al., 2001; Fujikawa et al., 2003; Tedford et al., 2001). Furthermore, the deletion of all three Vav family members results in the loss of all mature T and B cells (Fujikawa et al., 2003). Recently, our laboratory has reported defects in integrin-mediated adhesion and Rho GTPase activation downstream of β_2 integrins in *vav1*$^{-/-}$*vav3*$^{-/-}$ (Vav1/3^{ko}) neutrophils (Gakidis et al., 2004). Thus, analysis of hematopoietic cells lacking *vav1*, *vav2*, or *vav3*, or the combination of these genes, has shown that certain functions of Vav family members are redundant and others play specific roles in distinct hematopoietic cell types.

Vav has been implicated in FcγR-mediated phagocytosis based on evidence that it is recruited to FcγR− phagosomes, and that expression of dominant inhibitory Vav1 mutants block FcγR-mediated phagocytosis in the J774 macrophage cell line (Patel et al., 2002). In the present study, we have investigated the role of Vav and Rho GTPases in phagocytosis by using primary murine macrophages. We demonstrate that Rac and Rho are required for FcγR- and complement-mediated phagocytosis. Specifically, Rac is required for Arp2/3 recruitment and actin polymerization, whereas Rho acts at a step distinct from actin polymerization. In addition, Vav is required for Arp2/3 recruitment and actin polymerization only during complement-mediated phagocytosis and is dispensable for FcγR-mediated phagocytosis. Furthermore, a constitutively active Rac mutant, but not a constitutively active Cdc42 mutant, is able to rescue actin polymerization and complement-mediated phagocytosis in the Vav1/3^{ko} macrophages. Moreover, ultrastructural studies reveal similar pseudopod extensions during both FcγR- and complement-mediated phagocytosis. Collectively, our findings indicate that Vav and Rac coordinate to regulate actin polymerization during complement-mediated phagocytosis and suggest that another GEF can activate Rac downstream of FcγR. Thus, our analysis of primary macrophages has revealed distinct requirements for both Vav and the Rho GTPase family members during FcγR- and complement-mediated phagocytosis.

Results

Vav Is Required for Complement-Mediated Phagocytosis

To determine which Vav proteins may be involved in phagocytosis, we first examined the expression of all three family members in primary bone marrow-derived macrophages (BMMs) (Figure 1A). All three Vav family members are detected in BMMs. Based on the relative affinities of the Vav antibodies determined by using recombinant HA-tagged Vav proteins (Gakidis et al., 2004), these results indicate that Vav1 and Vav3 account for the majority of total Vav protein, suggesting that macrophages may be more dependent on these two family members. To address whether Vav is phosphorylated in response to phagocytic receptor activation in primary macrophages, we examined tyrosine phosphorylation of Vav, which generally correlates with activation (Crespo et al., 1997). Upon FcγR and $\alpha_M\beta_2$ ligation, all three Vav proteins are inducibly tyrosine phosphorylated (Figures 1C and 1D), indicating that Vav is activated downstream of these phagocytic receptors. In addition, we found that Vav1 is recruited to FcγR- and $\alpha_M\beta_2$-induced phagosomes (data not shown).

To determine if Vav plays a direct role in FcγR- and complement-mediated phagocytosis, wild-type (WT) and Vav-deficient BMMs were incubated with IgG- or C3bi-opsonized sheep red blood cells (RBCs) for 30 min at 37°C (Figure 1E). While no defects were observed in FcγR- or complement-mediated phagocytosis of BMMs lacking a single Vav family member (Figure S1; see the Supplemental Data available with this article online), Vav1/3^{ko} BMMs exhibit severe defects in complement-mediated phagocytosis, but no defect in FcγR-mediated phagocytosis (Figures 1E and 1F). The lack of a defect in the single Vav knockout macrophages suggests that Vav1 and Vav3 may have redundant functions in complement-mediated phagocytosis. Quantification of complement-mediated phagocytosis revealed that only 12% of Vav1/3^{ko} BMMs, compared to 90% of WT BMMs, contained at least one RBC/macrophage (Figure 1F). The complement-mediated phagocytosis defect in the Vav1/3^{ko} BMMs was comparable to that seen in $\alpha_M^{-/-}$ BMMs, which lack the complement receptor $\alpha_M\beta_2$ (data not shown). However, the complement-mediated phagocytosis defect in Vav1/3^{ko} BMMs was not due to defective particle binding since a similar percentage of WT and Vav1/3^{ko} BMMs contained bound RBCs and the amount of bound RBCs/cell was comparable (Figures 1G and 2A). In addition, FACS analysis revealed that the level of α_M on the surface of Vav1/3^{ko} BMMs was similar to WT BMMs (data not shown). Therefore, these results indicate that Vav is required for complement-mediated phagocytosis at a step downstream of opsonized particle binding.

The lack of a defect in FcγR-mediated phagocytosis in Vav1/3^{ko} BMMs was surprising because Vav is tyrosine phosphorylated downstream of FcγR ligation (Figure 1) and is recruited to FcγR-mediated phagosomes, and the dominant inhibitory Vav variants were previously shown to block FcγR-mediated phagocytosis in J774 cells (Patel et al., 2002). To address whether the residual Vav2 in Vav1/3^{ko} BMMs is sufficient to mediate FcγR phagocytosis, we examined FcγR-mediated

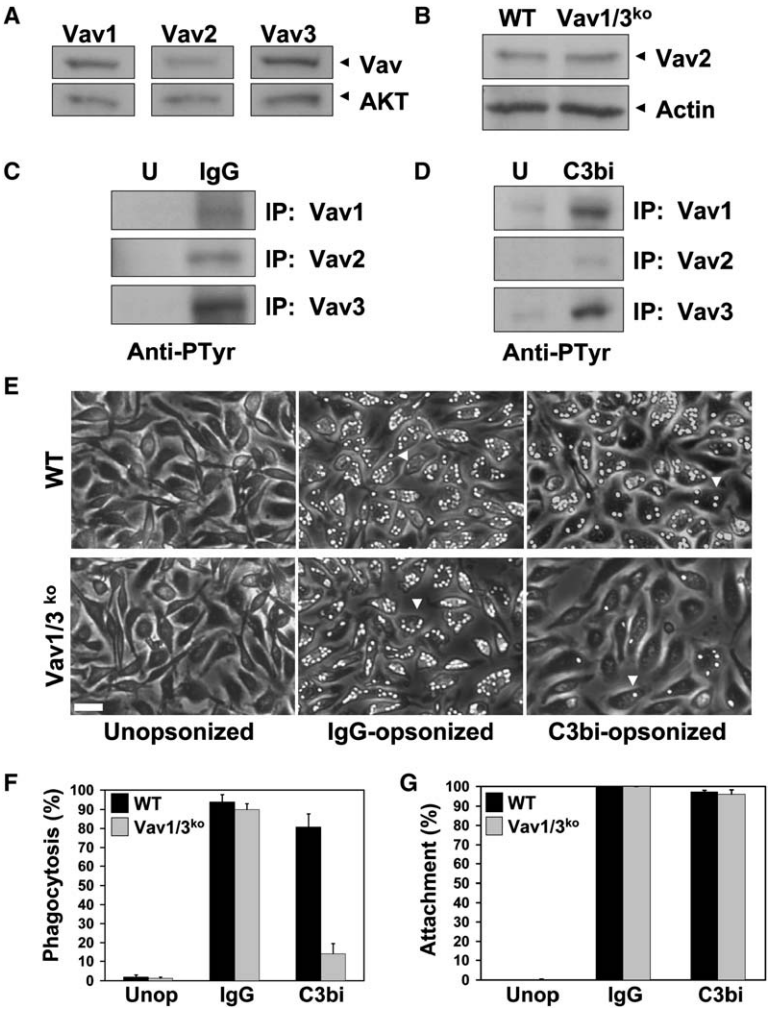

Figure 1. Vav Is Required for Complement-Mediated Phagocytosis in BMMs

(A) Wild-type (WT) BMM lysate was immunoblotted with non-crossreactive Vav1, Vav2, or Vav3 antibodies. The same blot was reprobed with anti-AKT as a loading control.

(B) WT and Vav1/3^{ko} BMM lysates were blotted with anti-Vav2 antibody. The same blot was reprobed with anti-actin as a loading control.

(C and D) WT BMMs were unstimulated (U) or stimulated with either IgG or C3bi for 5 min. Vav proteins were immunoprecipitated and immunoblotted with the indicated antibodies.

(C) The exposure time to detect Vav2 and Vav3 phosphorylation in response to IgG was longer than that for Vav1.

(E) Wild-type (WT) and Vav1/3^{ko} BMMs were incubated with unopsonized, IgG-opsonized, or C3bi-opsonized RBCs for 30 min. Phagocytosed RBCs appear as highly refractile circular structures (arrowheads). The scale bar represents 20 μm.

(F and G) Phagocytosis (%) and attachment (%) quantitation; the results represent the average of at least three independent experiments; >200 cells were counted per experiment ± standard deviations ($p \leq 0.001$).

phagocytosis in *vav1*$^{-/-}$*vav2*$^{-/-}$*vav3*$^{-/-}$ triple knockout (Vav1/2/3^{ko}) BMMs. As expected, there was a similar defect in complement-mediated phagocytosis as in Vav1/3^{ko} BMMs; however, FcγR-mediated phagocytosis in Vav1/2/3^{ko} BMMs was not impaired (Figure S1). Collectively, these results indicate that Vav can couple to both phagocytic receptors, as indicated by its inducible tyrosine phosphorylation and recruitment to FcγR- and complement-mediated phagosomes; however, Vav is specifically required for complement-mediated phagocytosis, and not FcγR-mediated phagocytosis.

Vav1/3^{ko} BMMs Fail to Recruit Arp2/3 and F-Actin to the Nascent Phagosome during Complement-Mediated Phagocytosis

Because the actin cytoskeleton plays a pivotal role in phagocytosis, we examined whether Vav is required for F-actin recruitment to the phagosome. To capture the early events of F-actin recruitment to phagosomes, opsonized RBCs were centrifuged onto WT and Vav1/3^{ko} BMMs and incubated for 3 min prior to fixation and staining with phalloidin. WT and Vav1/3^{ko} BMMs recruit F-actin to FcγR-induced phagosomes (Figure 2A, left panels; Figure 2B). However, Vav1/3^{ko} BMMs are unable to organize F-actin at the nascent phagosome during complement-mediated phagocytosis (Figure 2A, right panels). Only 3% of Vav1/3^{ko} BMMs, compared to 75% of WT, are able to recruit F-actin (Figure 2B). The decrease in F-actin at the complement-mediated phagosome is not due to the inability of opsonized RBCs to bind to the macrophages (Figure 2A, right panels; black arrows). Therefore, these data indicate that Vav provides a crucial link between the complement receptor $\alpha_M\beta_2$ and the actin cytoskeleton during complement-mediated phagocytosis.

The Arp2/3 complex nucleates actin and is recruited early in phagocytic cup formation during complement-mediated phagocytosis (May et al., 2000). To determine if Vav plays a role in actin nucleation during complement-mediated phagocytosis, we examined the recruitment of p34-Arc, a subunit of the Arp2/3 complex, to visualize Arp2/3 recruitment. As shown in Figures 2C and 2D, upon C3bi-opsonized RBC binding to Vav1/3^{ko} BMMs, p34-Arc is not recruited to the nascent phagosome. These results suggest that Vav is required for Arp2/3 recruitment and actin nucleation during complement-mediated phagocytosis.

The Exchange Activity of Vav Is Required for Complement-Mediated Phagocytosis

The DH domain of Vav harbors the exchange activity required for the activation of the Rho GTPases

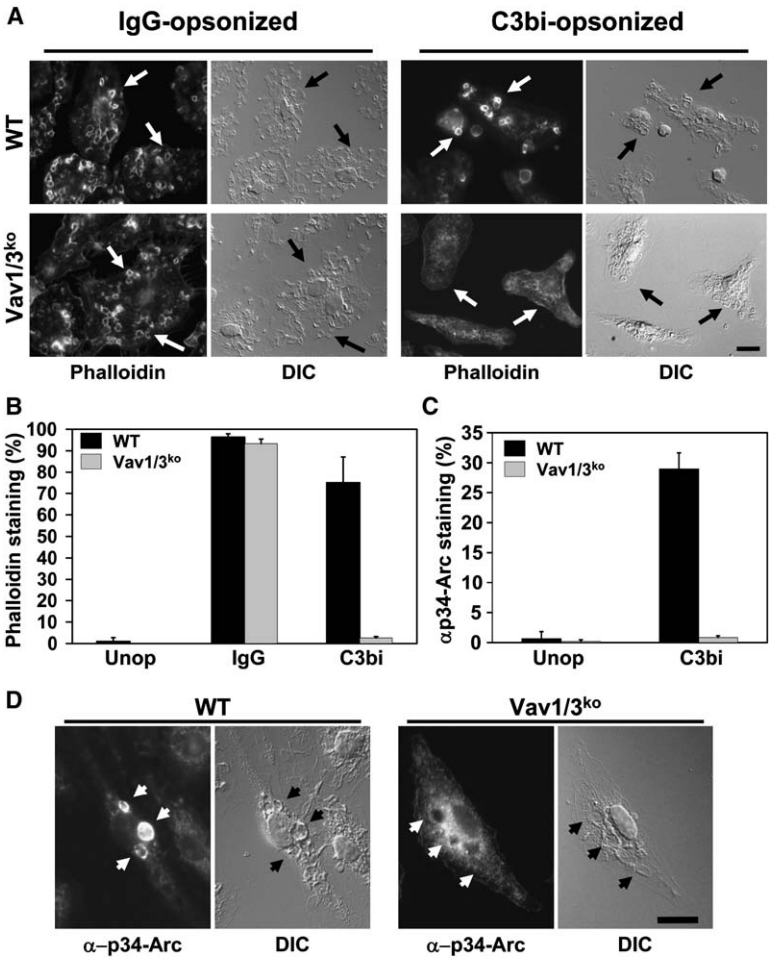

Figure 2. Vav1/3^{ko} BMMs Fail to Recruit F-Actin and Arp2/3 to the Nascent Phagosome during Complement-Mediated Phagocytosis

(A–D) Wild-type (WT) and Vav1/3^{ko} BMMs were incubated with either unopsonized, IgG-opsonized, or C3bi-opsonized RBCs for 3 min, fixed, and stained with Alexa 568-phalloidin or anti-p34-Arc antibody. (A) Differential interference contrast (DIC) images show the binding of the opsonized RBCs to the BMMs (black arrows). Alexa-568-phalloidin staining of WT and Vav1/3^{ko} BMMs incubated with either IgG- (left) or C3bi-opsonized (right) RBCs. White arrows indicate F-actin recruitment to the nascent phagosome. The scale bar represents 10 μm. (B and C) Quantitation of phalloidin and αp34-Arc staining (%); the results represent the average of at least three independent experiments, and >200 cells were counted per experiment ± standard deviations ($p \leq 0.001$). (D) Anti-p34-Arc antibody staining (white arrows) of WT and Vav1/3^{ko} BMMs incubated with C3bi-opsonized RBCs. Differential interference contrast (DIC) images show the binding of the opsonized RBCs to the BMMs (black arrows). The scale bar represents 10 μm.

(Bustelo, 2000). To determine if the exchange activity of Vav is required for complement-mediated phagocytosis, WT and Vav1/3^{ko} BMM precursors were infected with retroviral vectors encoding either GFP, Vav1-GFP, or Vav1L213Q-GFP. The latter Vav variant is a previously characterized catalytically inactive mutant (Zugaza et al., 2002) containing a point mutation in the DH domain. As shown in Figure 3A, Vav1-GFP rescued the complement-mediated phagocytosis defect in Vav1/3^{ko} BMMs, suggesting that this defect is due to a loss of Vav, and not to a defect in BMM differentiation. In contrast, Vav1L213Q-GFP failed to rescue Vav1/3^{ko} BMM complement-mediated phagocytosis (WT 70%; Vav1/3^{ko} 7%), indicating that the exchange activity of Vav, which mediates Rho GTPase activation, is required for complement-mediated phagocytosis (Figure 3A). The expression levels of Vav1-GFP and Vav1L213Q-GFP are low in comparison to endogenous Vav (data not shown), and Vav1L213Q-GFP is not acting as a dominant-negative in WT cells since the WT expressing cells do not exhibit a decrease in phagocytosis. In addition, Vav1-GFP, but not Vav1L213Q-GFP, rescued F-actin recruitment to the phagosome (Figure 3B). The inability of Vav1L213Q-GFP to rescue actin polymerization and complement-mediated phagocytosis was not due to lower expression levels compared to Vav1-GFP (Figures 3B and 3C). These results indicate that the exchange activity of Vav is required for F-actin recruitment to the phagosome and complement-mediated phagocytosis.

Rho Is Required for Both FcγR- and Complement-Mediated Phagocytosis, but Not Actin Polymerization

The inability of Vav1L213Q-GFP to rescue the complement-mediated phagocytosis defect in Vav1/3^{ko} BMMs suggests that the exchange activity of Vav, which mediates Rho GTPase activation, is required for F-actin recruitment and complement-mediated phagocytosis. To determine if the Rho GTPases are involved in FcγR- and complement-mediated phagocytosis in BMMs, we treated WT BMMs with the pan Rho GTPase (Rac, Rho, Cdc42) inhibitor *Clostridium difficile* toxin B. As expected, based on previous studies in immortalized cell lines (Caron and Hall, 1998; Massol et al., 1998), toxin B inhibited F-actin recruitment during both FcγR- and complement-mediated phagocytosis (data not shown). To further examine the role of specific Rho GTPases involved in each pathway, we used several strategies to selectively inhibit different Rho GTPases. To examine the role of Rho, we treated WT BMMs with increasing concentrations of TAT-C3 transferase, a cell-permeable inhibitor that inactivates Rho by ADP ribosylation (Sekine et al., 1989) (Figure 4). At 10 μg/ml TAT-C3, a significant reduction in the number of phagocytosed RBCs

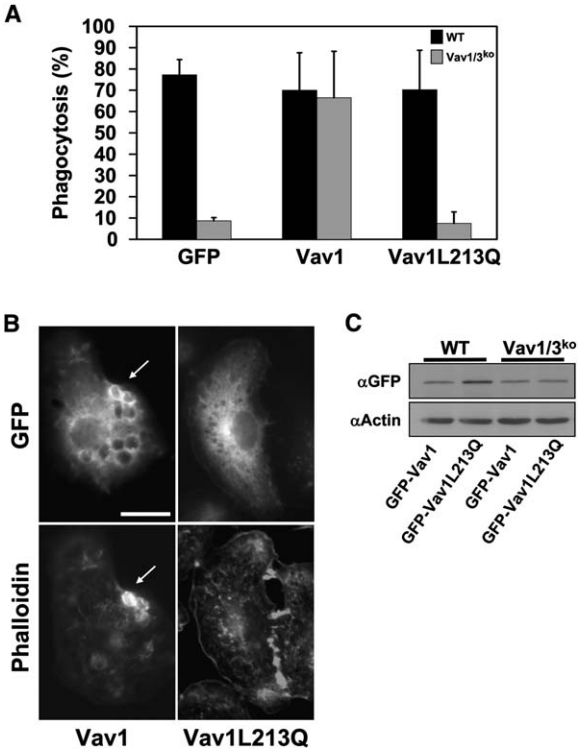

Figure 3. The Vav DH Domain Mutant Is Unable to Rescue Complement-Mediated Phagocytosis in Vav1/3^{ko} BMMs

(A–C) Wild-type (WT) and Vav1/3^{ko} BMMs were retrovirally infected with either pMX-uGFP, pMX-Vav1-uGFP, or pMX-Vav1L213Q-uGFP. (A) Infected WT and Vav1/3^{ko} BMMs were incubated with C3bi-opsonized RBCs for 30 min. The results represent the average of at least three independent experiments, and >100 cells were counted per experiment ± standard deviations ($p \leq 0.001$). (B) Infected Vav1/3^{ko} BMMs were incubated with C3bi-opsonized RBCs for 3 min, fixed, and stained with Alexa-568-phalloidin. Arrows indicate GFP (top left panel) and F-actin (bottom left panel) recruitment to the nascent phagosome. The scale bar represents 10 μm. (C) Lysates from infected WT and Vav1/3^{ko} BMMs were immunoblotted with anti-GFP antibody. The same blot was reprobed with anti-actin as a loading control.

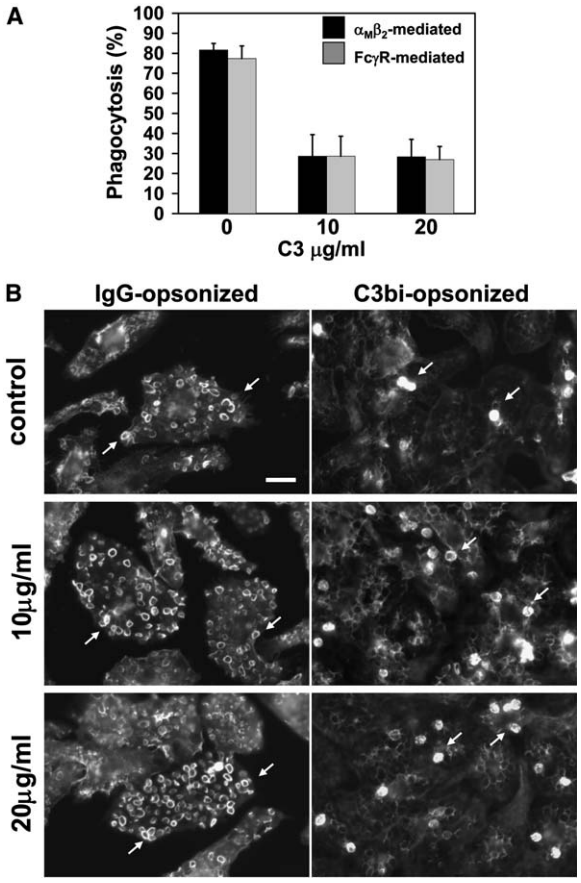

Figure 4. Rho Is Required for Both FcγR- and Complement-Mediated Phagocytosis, but Not Actin Polymerization

(A and B) Wild-type (WT) BMMs were treated with TAT-C3 for 4 hr and subsequently incubated with IgG-opsonized or C3bi-opsonized RBCs. (A) The results represent the average of at least three independent experiments, and >200 cells were counted per experiment ± standard deviations ($p \leq 0.001$). (B) After a 3 min incubation, the BMMs were fixed and stained with Alexa-568-phalloidin to visualize F-actin. The arrows indicate F-actin recruitment to the phagosome. The scale bar represents 10 μm.

during both FcγR- and complement-mediated phagocytosis was observed in WT BMMs (Figure 4A). Similarly, at lower concentrations (0.1 μg/ml and 1 μg/ml) of TAT-C3, the titration curves were identical for FcγR- and complement-mediated phagocytosis (Figure S2). In addition, TAT-C3 treatment resulted in a dose-dependent inhibition of Rho and a decrease in the phosphorylation of ERM, a downstream effector protein of Rho-kinase (Figure S3). Furthermore, BMMs treated with a control TAT recombinant protein, TAT-Cre, showed no decrease in phagocytosis (data not shown), indicating that the effects of TAT-C3 are specific to the inhibition of Rho. Interestingly, TAT-C3 did not cause a decrease in F-actin recruitment to nascent phagosomes (Figure 4B), suggesting that Rho is required for FcγR- and complement-mediated phagocytosis at a step distinct from actin polymerization. Since Rho inhibition does not phenocopy Vav1/3^{ko} BMMs, these results indicate that a defect in Rho activation most likely does not account for the reduction in actin polymerization observed in Vav1/3^{ko} BMMs during complement-mediated phagocytosis.

Rac1/2^{ko} BMMs Exhibit Defects in FcγR- and Complement-Mediated Phagocytosis

Dominant inhibitory Rac has been shown to inhibit FcγR- but not complement-mediated phagocytosis in macrophage and reconstituted phagocytic receptor cell lines (Caron and Hall, 1998). To further examine the role of Rac in phagocytosis, we examined FcγR- and complement-mediated phagocytosis in *rac1*$^{-/-}$ *rac2*$^{-/-}$ (Rac1/2^{ko}) BMMs. Because Rac1 null mice are embryonic lethal, the Rac1/2^{ko} mice were generated by mating Rac2 null mice with mice carrying a conditional allele of Rac1 and expressing Cre recombinase downstream of a specific myeloid promoter, Lysozyme M (Sun et al., 2004). Therefore, only cells of the granulocyte/monocyte lineage are Rac1 deficient. Incubation with opsonized RBCs revealed that both FcγR- and complement-mediated phagocytosis were severely impaired in Rac1/2^{ko} BMMs (Figures 5A and 5B). Quantitation of the defect indicated that, compared to WT BMMs, Rac1/2^{ko} BMMs showed an 85% and 82% decrease in FcγR- and complement-mediated

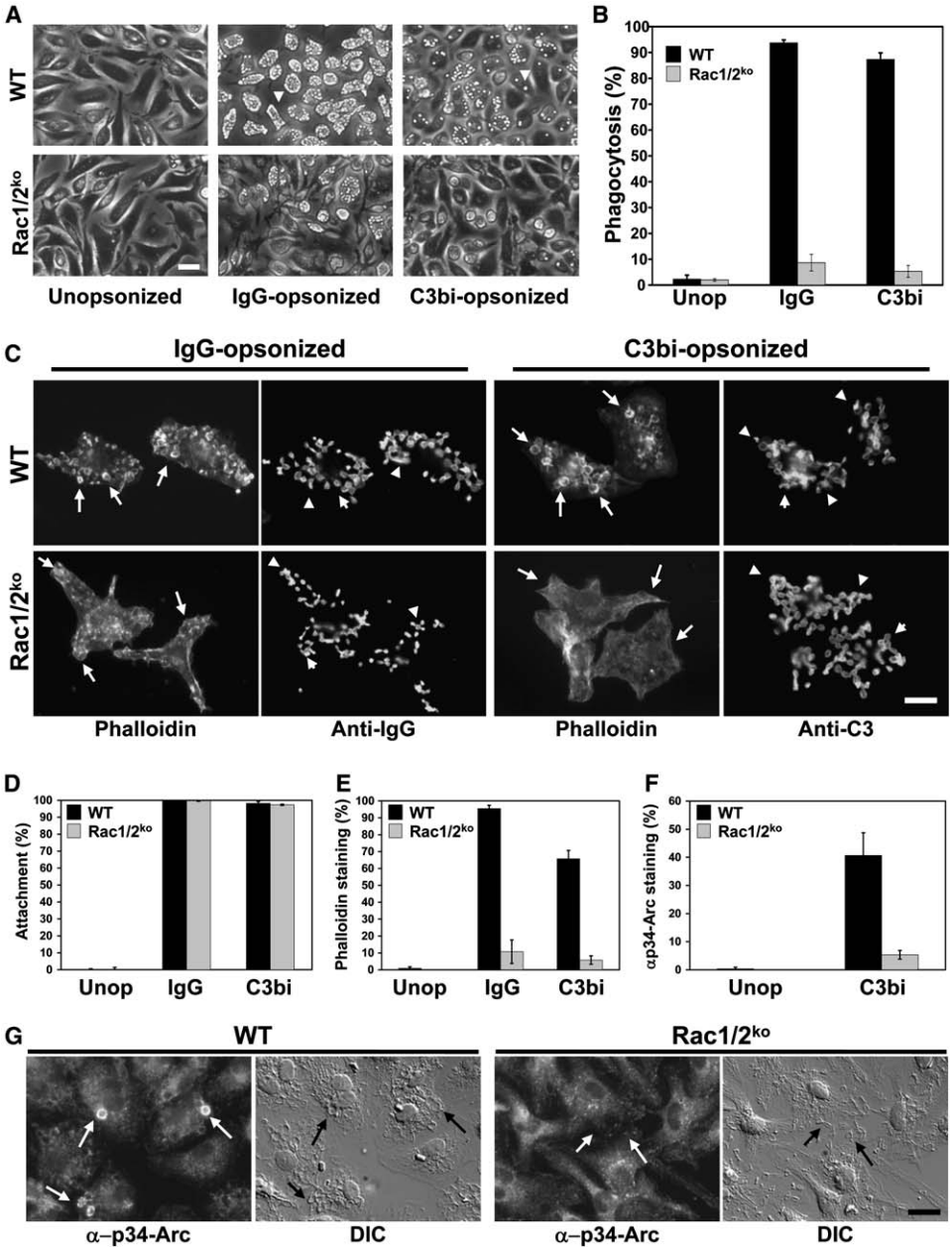

Figure 5. Rac1/2^{ko} BMMs Exhibit Defects in FcγR- and Complement-Mediated Phagocytosis

(A) Wild-type (WT) and Rac1/2^{ko} BMMs were incubated with unopsonized, IgG-opsonized, or C3bi-opsonized RBCs for 30 min. Ingested RBCs appear as highly refractile circular structures (arrowheads). The scale bar represents 20 μm.

(B) Phagocytosis (%) quantitation; the results represent the average of at least three independent experiments; >200 cells were counted per experiment ± standard deviations ($p \leq 0.001$).

(C) WT and Rac1/2^{ko} BMMs were incubated with either unopsonized, IgG-opsonized, or C3bi-opsonized RBCs for 3 min, fixed, and stained with Alexa-568-phalloidin to visualize F-actin recruitment to the phagosome (arrows) and with anti-IgG or anti-C3 to visualize opsonized RBC binding (arrowheads). The scale bar represents 10 μm.

(D–F) Attachment (%), phalloidin and αp34-Arc staining (%) quantitation; the results represent the average of at least three independent experiments, and >200 cells were counted per experiment ± standard deviations ($p \leq 0.001$).

(G) Anti-p34-Arc antibody staining of WT and Rac1/2^{ko} BMMs incubated with C3bi-opsonized RBCs (white arrows). Differential interference contrast (DIC) images show the binding of the C3bi-opsonized RBCs to BMMs (black arrows). The scale bar represents 10 μm.

phagocytosis, respectively. The phagocytosis impairment was not due to an inability to bind to opsonized RBCs, as observed by anti-IgG and anti-C3 staining of bound opsonized RBCs (Figures 5C and 5D). To determine if the defect was at the level of actin polymerization, Rac1/2^{ko} BMMs were incubated with opsonized RBCs for 3 min and subsequently stained with phalloidin to visualize F-actin. As seen in Figure 5C, Rac1/2^{ko} BMMs are unable to recruit F-actin to the nascent phagosome in either FcγR- or complement-mediated

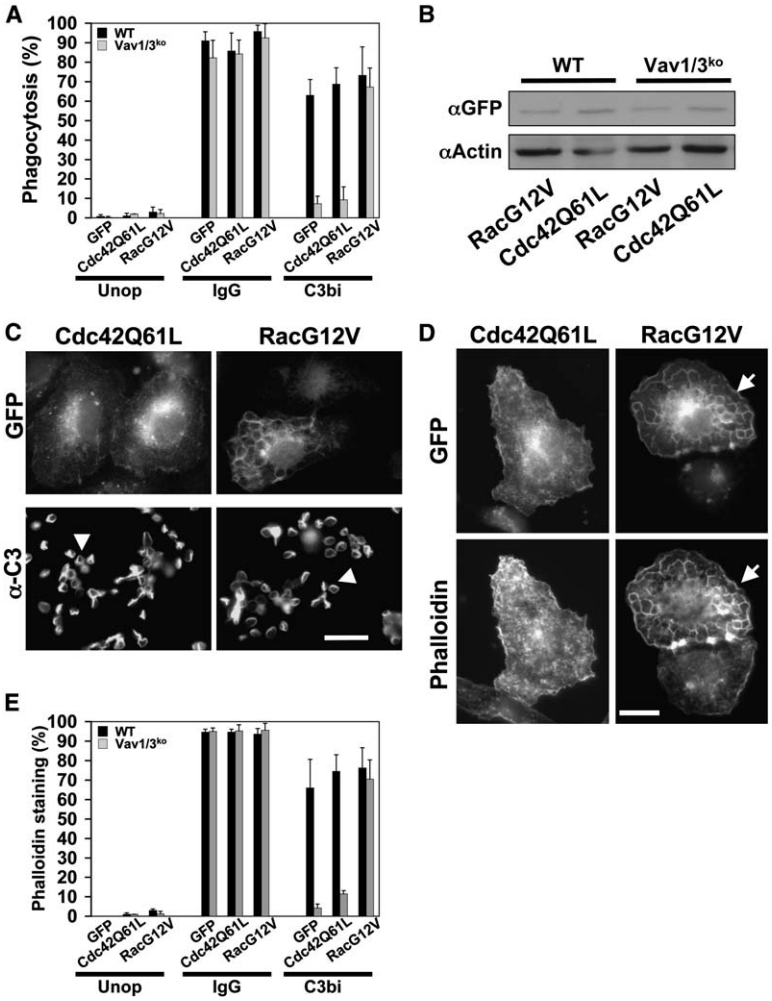

Figure 6. RacG12V Rescues the Complement-Mediated Phagocytosis Defect in Vav1/3^{ko} BMMs

(A–E) Wild-type (WT) and Vav1/3^{ko} BMMs were retrovirally infected with pMX-GFP, pMX-GFP-Cdc42Q61L, or pMX-GFP-RacG12V. (A) Infected BMMs were incubated with unopsonized, IgG-opsonized, or C3bi-opsonized RBCs for 30 min. Phagocytosis (%) quantitation; the results represent the average of at least three independent experiments, and >200 cells were counted per experiment ± standard deviations ($p \leq 0.001$). (B) Lysates from infected WT and Vav1/3^{ko} BMMs were immunoblotted with anti-GFP antibody. The same blot was reprobed with anti-actin as a loading control. (C) Infected Vav1/3^{ko} BMMs were incubated with C3bi-opsonized RBCs for 3 min., fixed, and stained with anti-C3 antibody to visualize opsonized RBC binding (arrowheads). (D) Infected Vav1/3^{ko} BMMs were incubated with C3bi-opsonized RBCs for 3 min, fixed, and stained with Alexa-568-phalloidin to visualize F-actin recruitment to the phagosome (arrows). (E) Phalloidin staining (%) quantitation; the results represent the average of at least three independent experiments; >200 cells were counted per experiment ± standard deviations ($p \leq 0.001$). The scale bar represents 10 μm.

phagocytosis. Only 6% of Rac1/2^{ko} BMMs were able to polymerize F-actin during complement-mediated phagocytosis, which is similar to the 3% seen in Vav1/3^{ko} BMMs (Figures 5E and 2B). Additionally, only 5% of Rac1/2^{ko} BMMs, as compared to 41% of WT, were able to recruit the Arp2/3 complex to the phagosome during complement-mediated phagocytosis, examined by anti-p34-Arc immunofluorescence (Figures 5F and 5G). These results indicate that Rac is required for actin polymerization downstream of opsonized RBC binding in both FcγR- and complement-mediated phagocytosis.

Activated RacG12V Rescues the Complement-Mediated Phagocytosis Defect in Vav1/3^{ko} BMMs

The defect in complement-mediated phagocytosis and actin polymerization of Rac1/2^{ko} BMMs is phenotypically similar to the defect in the Vav1/3^{ko} BMMs. These results, in addition to a requirement for the exchange activity of Vav, raised the possibility that Rac may act downstream of Vav in regulating complement-mediated phagocytosis. To address this possibility, we examined whether constitutively activated Rac would be able to rescue the complement-mediated phagocytosis defect in Vav1/3^{ko} BMMs. WT and Vav1/3^{ko} BMMs were infected with retroviral vectors encoding GFP-tagged, constitutively active forms of Rac (RacG12V) or Cdc42 (Cdc42Q61L). Interestingly, only GFP-RacG12V was able to rescue the complement-mediated phagocytosis defect in Vav1/3^{ko} BMMs, suggesting that Vav couples to Rac, not Cdc42, during phagocytosis (Figure 6A). The inability of activated Cdc42 to rescue complement-mediated phagocytosis in Vav1/3^{ko} was not due to a decrease in the expression of the protein or the inability of GFP-Cdc42Q61L-infected Vav1/3^{ko} BMMs to bind C3bi-opsonized RBCs (Figures 6B and 6C) or to its activity state in BMMs (Figure S4). In addition, RacG12V, but not Cdc42Q61L, was able to rescue the F-actin polymerization defect in Vav1/3^{ko} BMMs (Figures 6D and 6E). Quantitation revealed that 71% of Vav1/3^{ko} BMMs infected with GFP-RacG12V contain at least one F-actin-containing phagosome, as compared to 12% of GFP-Cdc42Q61L-expressing Vav1/3^{ko} BMMs (Figure 6E). Furthermore, the expression of RacG12V did not significantly increase FcγR- or complement-mediated phagocytosis in WT cells or nonspecifically increase the phagocytosis of unopsonized RBCs, suggesting that activated Rac alone cannot induce phagocytosis. These results suggest that Vav plays a critical role in coupling Rac activation downstream of

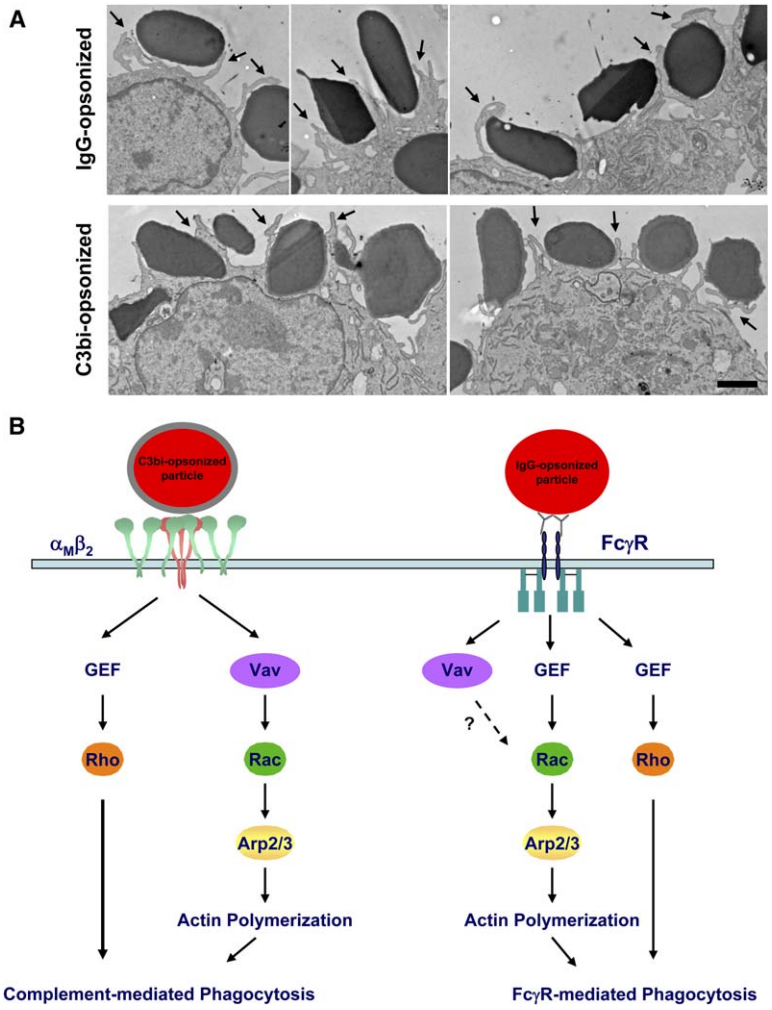

Figure 7. Transmission Electron Microscopy Reveals Similar Pseudopod Extensions in FcγR- and Complement-Mediated Phagocytosis and a Model of Vav Action in FcγR- and Complement-Mediated Phagocytosis

(A) Wild-type (WT) BMMs were incubated with IgG-opsonized (top panels) or C3bi-opsonized (bottom panels) RBCs for 3 min, fixed, and analyzed by TEM. Long, thin pseudopods extend around the RBC (arrows). The results shown are representative of two independent experiments, each containing triplicate samples that gave similar results. Magnification, 2900×. The scale bar represents 2 μm.

(B) Rac and Rho are required for complement- and FcγR-mediated phagocytosis. Rac is required for Arp2/3 recruitment and actin polymerization, whereas Rho acts at a step distinct from that of actin polymerization. While IgG- and C3bi-opsonized particle binding results in Vav phosphorylation and recruitment to FcγR- and complement-mediated phagosomes, respectively, Vav is only required for complement-induced Rac activation and is dispensable for FcγR-mediated phagocytosis. We propose that Rac is activated by a distinct GEF downstream of FcγR in Vav1/3^{ko} BMMs. However, it remains to be determined whether Vav can also activate Rac downstream of FcγR in WT primary BMMs.

complement receptor ligation to regulate actin polymerization during complement-mediated phagocytosis.

Transmission Electron Microscopy Reveals Similar Pseudopod Extensions in FcγR- and Complement-Mediated Phagocytosis

A previous study in mouse peritoneal macrophages indicated that the mechanism of ingestion during FcγR- and complement-mediated phagocytosis is different (Kaplan, 1977). During FcγR-mediated phagocytosis, actin-rich pseudopods extend around IgG-opsonized particles, whereas during complement-mediated phagocytosis, C3bi-opsonized particles passively "sink" into the cells. To determine if there is a similar distinction between FcγR- and complement-mediated phagocytosis in WT bone marrow-derived macrophages, we examined both phagocytic processes by transmission electron microscopy (TEM). As shown in Figure 7A, both FcγR- and complement-induced phagosomes contain similar pseudopods that extend along the surface of the opsonized RBCs. At the ultrastructural level, the spectrum of morphological features of the processes was comparable in terms of its diameter, the proximity of the pseudopod extension to the RBC, and the pseudopod length. Thus, we were unable to distinguish early phagocytic steps in the two types of phagocytosis based on this analysis.

Discussion

In this report, we have defined the role of Vav and several Rho GTPases in FcγR- and complement-mediated phagocytosis in primary bone marrow-derived macrophages. These studies identified novel, to our knowledge, functions of Vav and Rac in coupling the integrin receptor $\alpha_M\beta_2$ to the actin cytoskeleton during complement-mediated phagocytosis. Vav is tyrosine phosphorylated in response to both FcγR and $\alpha_M\beta_2$ ligation and is recruited to FcγR- and complement-induced phagosomes; however, Vav is only required for complement-mediated phagocytosis and is dispensable for FcγR-mediated phagocytosis. Furthermore, Rac and Rho are required for both FcγR- and complement-mediated phagocytosis; however, they regulate distinct events associated with this process. Specifically, Rac1/2^{ko} BMMs, like Vav1/3^{ko} BMMs, exhibited defects in Arp2/3 recruitment and F-actin polymerization at complement-mediated phagosomes, whereas specific inhibition of Rho did not inhibit actin polymerization. Moreover, constitutively active Rac rescues the complement-mediated

phagocytosis defect in Vav1/3^{ko} BMMs. Electron microscopy reveals similar pseudopod extensions surrounding opsonized particles during FcγR- and complement-mediated phagocytosis. Collectively, these data suggest that Rac and Rho are required for both FcγR- and complement-mediated phagocytosis, and that Rac and Vav regulate actin polymerization downstream of the complement receptor $\alpha_M\beta_2$.

Defects in complement-mediated phagocytosis were not observed in BMMs lacking a single Vav family member (Figure S1), but defects were observed with the combined deficiency of Vav1 and Vav3 (Figure 1), suggesting that Vav1 and Vav3 have redundant functions in BMMs. In addition, our laboratory has shown that Vav1 and Vav3 play redundant functions in regulating adhesion downstream of β2 integrins in neutrophils (Gakidis et al., 2004). Therefore, based on these studies and the absence of a compensatory increase in Vav2 expression in Vav1/3^{ko} macrophages (Figure 1B), we conclude that Vav2 does not play a critical role downstream of β2 integrins in leukocytes.

Our results establish a critical role for Vav in regulating the actin cytoskeletal rearrangements that are required for complement-mediated phagocytosis. Specifically, Vav1 and Vav3 are required to recruit Arp2/3 and polymerize F-actin at the nascent phagosome based on the inhibition of these processes in Vav1/3^{ko} BMMs (Figure 2). In addition, the exchange activity of Vav, which mediates Rho GTPase activation, is required for actin polymerization during complement-mediated phagocytosis (Figure 3).

Although, Vav family members have been shown to activate the Rho family of GTPases (Bustelo, 2000), the specificity of Vav's exchange activity in vivo has not been resolved. Interestingly, in T cells, Vav is necessary for TCR-dependent activation of Rac and actin patch formation (Fischer et al., 1998; Holsinger et al., 1998). In this report, several lines of evidence suggest that Vav is the guanine nucleotide exchange factor for Rac downstream of the complement receptor $\alpha_M\beta_2$ in BMMs. The phenotypes of Rac1/2^{ko} and Vav1/3^{ko} BMMs are similar with respect to defects in complement-mediated phagocytosis, Arp2/3 recruitment, and F-actin polymerization at the complement-induced phagosome (Figures 1, 2, and 5). In addition, our results show that a constitutively active mutant of Rac, but not a constitutively active mutant of Cdc42, rescues actin polymerization and complement-mediated phagocytosis in Vav1/3^{ko} BMMs (Figure 6). These data suggest that both Vav and Rac regulate the actin cytoskeletal rearrangements necessary for complement-mediated phagocytosis. Furthermore, the inability of activated Cdc42 to rescue the defect in Vav1/3^{ko} BMMs suggests that the hierarchical Rho GTPase activation cascade (Cdc42-Rac) found in other cell types (Nobes and Hall, 1995), such as fibroblasts, is not functional in primary BMMs downstream of the complement receptor $\alpha_M\beta_2$. Previous studies have suggested that this hierarchy is also not observed downstream of the FcγR (Massol et al., 1998; Patel et al., 2002). In addition to the defect in F-actin recruitment during complement-mediated phagocytosis, Rac1/2^{ko} BMMs exhibit actin polymerization defects in FcγR-mediated phagocytosis (Figure 5), indicating that Rac is required for both types of phagocytosis in BMMs. However, Vav1/3^{ko} and Vav1/2/3^{ko} macrophages only exhibit defects in complement-mediated phagocytosis, suggesting that Vav is not a critical regulator of FcγR-mediated phagocytosis. Therefore, another guanine nucleotide exchange factor must be activating Rac downstream of the FcγR in the absence of Vav1, Vav2, and Vav3. In addition to Vav, several other Rac GEFs have been identified in leukocytes such as Tiam1, PRex1, PIX, and DOCK2 (Bokoch, 2005). Thus, guanine nucleotide exchange factors may be regulating signaling specificity downstream of the phagocytic receptors. Further studies will be needed to identify the GEF necessary for FcγR-mediated phagocytosis.

In primary BMMs, the specific inhibition of Rho with C3 transferase blocked both FcγR- and complement-mediated phagocytosis, but not actin polymerization, during either of these processes (Figure 4). These results in primary BMMs indicate that Rho is required for both FcγR- and complement-mediated phagocytosis at a step distinct from actin polymerization. Since Rho inhibition does not affect actin polymerization, and the loss of Vav1/3 inhibits actin polymerization during complement-mediated phagocytosis, the defect in actin polymerization in the Vav1/3^{ko} BMMs cannot be explained by a defect in Rho activation. These data indicate that Rho is not the substrate of Vav involved in actin polymerization at the phagosome.

Our TEM analysis of FcγR- and complement-mediated phagocytosis in WT BMMs indicates that thin pseudopods extend around opsonized particles during both processes (Figure 7A). The similar nature of these structures is consistent with our finding that Rac is required for actin polymerization during both FcγR- and complement-mediated phagocytosis. Based on previous ultrastructural studies in peritoneal macrophages indicating a phenotypic difference between the ingestion of opsonized particles during FcγR- and complement-mediated phagocytosis (Kaplan, 1977), others have suggested that this difference was due to the activation of distinct Rho GTPases by Fcγ and complement receptors in macrophage cell lines and immortalized cell lines reconstituted with phagocytic receptors. In contrast, in our model of primary mouse bone marrow-derived macrophages, early phagocytic pseudopod extension is similar during FcγR- and complement-mediated phagocytosis; therefore, it is not surprising that Rac was found to be required for actin polymerization and engulfment during both types of phagocytosis. These studies raise the possibility that primary bone marrow-derived macrophages may use a different mechanism for complement-mediated phagocytosis than peritoneal macrophages and immortalized cell lines.

A previous study from our laboratory has shown that, upon integrin ligation, Src and Syk kinases phosphorylate Vav, relieving an autoinhibitory interaction (Aghazadeh et al., 2000) and resulting in Vav activation and actin cytoskeletal rearrangements (Miranti et al., 1998). In this study, we have shown that Vav is tyrosine phosphorylated in response to $\alpha_M\beta_2$ ligation (Figure 1D), and that it is required for actin cytoskeletal rearrangements during complement-mediated phagocytosis in BMMs (Figure 2). In Syk and SFK (Hck, Fgr, Lyn, Src)-deficient leukocytes Vav phosphorylation after adhesion is impaired (Mocsai et al., 2002). However, Syk- or SFK-deficient

macrophages are not deficient in complement-mediated phagocytosis (Fitzer-Attas et al., 2000; Kiefer et al., 1998). In addition, inhibitor studies have found that tyrosine kinases are not required for complement-mediated phagocytosis (Allen and Aderem, 1996). These results suggest a tyrosine kinase-independent mechanism of Vav activation downstream of the complement receptor $\alpha_M\beta_2$. In primary monocytes and macrophages, pertussis toxin, a $G\alpha_i$ inhibitor, prevents complement-mediated phagocytosis (Brown et al., 1987; Hazeki et al., 1994). Thus, one could envision a mechanism in which a heterotrimeric G protein mediates Vav activation downstream of $\alpha_M\beta_2$. We are currently investigating the alternative tyrosine kinase-independent mechanisms of Vav activation downstream of the complement receptor.

Our results implicating Vav in complement-mediated phagocytosis, but not FcγR-mediated phagocytosis, differ from those in a previous study indicating that Vav is recruited to FcγR-mediated phagosomes and regulates FcγR-mediated phagocytosis through the activation of Rac in an immortalized macrophage cell line and phagocytic receptor-reconstituted Cos cells (Patel et al., 2002). In our study, we also observe Vav recruitment to FcγR-induced phagosomes, but Vav is not required for FcγR-mediated phagocytosis. One explanation for this discrepancy could be that primary BMMs contain a Rac GEF that is redundant with Vav, whereas macrophage cell lines do not. If this were the case, the loss of Vav would not necessarily prevent FcγR-mediated Rac activation in primary BMMs. In addition, these results were based on experiments with dominant-negative mutants of Vav, such as the carboxy-terminal SH3-SH2-SH3 domains (adaptor region), in the J774 macrophage cell line, and phagocytic receptor-reconstituted Cos cells (Patel et al., 2002). The SH2 domain of Vav has been shown to bind to multiple tyrosine-phosphorylated proteins (Bustelo, 2000). Therefore, overexpression of only the adaptor region of Vav may lead to promiscuous binding or nonspecific sequestering of proteins that are involved in FcγR-mediated phagocytosis, resulting in a false inhibition of FcγR-mediated phagocytosis.

The report from Patel et al. (2002) also provided evidence that Vav is not required for complement-mediated phagocytosis in J774 cells. This discrepancy with our data may also be explained by differences between primary BMMs and immortalized cell lines. As described previously, several lines of evidence suggest that the well-characterized pathway of $\alpha_M\beta_2$ integrin activation of Vav involving Src and Syk kinases is not conserved in complement-mediated phagocytosis. Therefore, it is possible that this tyrosine kinase-independent mechanism of Vav activation is also not retained in macrophage cell lines or phagocytic receptor ($\alpha_M\beta_2$)-reconstituted Cos cells and that these cell lines may not accurately recapitulate the complexity of this signaling pathway in primary macrophages. Thus, Vav knockout mice have enabled us to look at the individual or combined contributions of the Vav isoforms in FcγR- and complement-mediated phagocytosis. Our results with Vav1/3^{ko} and Vav1/2/3^{ko} BMMs definitively indicate that Vav is required for complement-mediated phagocytosis and is not required for FcγR-phagocytosis.

Previous studies have shown that Rac is recruited to FcγR− phagosomes, and that dominant-negative mutants of Rac and Cdc42 block FcγR phagocytosis in J774 and RAW 264.7 macrophages or reconstituted phagocytic receptor Cos cells (Caron and Hall, 1998; Cox et al., 1997). However, dominant inhibitory N17Rac did not block complement-mediated phagocytosis in J774 cells (Caron and Hall, 1998). In agreement, our studies support a role for Rac in FcγR-mediated phagocytosis since Rac1/2^{ko} macrophages are defective in actin polymerization during this process. However, in contrast, our studies show that Rac1/2^{ko} BMMs also have defects in actin polymerization and Arp2/3 recruitment during complement-mediated phagocytosis, suggesting that Rac plays a role in both types of phagocytosis (Figures 5C, 5E, 5F, and 5G). In addition, the role of Rho in complement- and FcγR-mediated phagocytosis and actin recruitment is controversial. In J774 cells and phagocytic receptor-reconstituted Cos cells, Rho was found to be required for complement-, but not FcγR-mediated, phagocytosis (Caron and Hall, 1998). Furthermore, one study found Rho to be required for F-actin recruitment during FcγR-mediated phagocytosis (Hackam et al., 1997), whereas another found Rho to be required for F-actin recruitment during complement-mediated phagocytosis (May et al., 2000). Our studies in primary BMMs indicate that Rho inhibition by C3 transferase blocks both forms of phagocytosis independent of actin recruitment. It is possible that the observed differences may be due to the inherent signaling differences between primary BMMs and immortalized or phagocytic receptor-reconstituted cells. Significantly, this report reveals differences in the signaling networks and receptor complexes operative in primary macrophages and immortalized cell lines.

In conclusion, we have shown that Vav is required for complement-mediated phagocytosis, but not for FcγR-mediated phagocytosis (Figure 7B). Vav regulates Arp2/3 recruitment and actin polymerization during complement-mediated phagocytosis via the small GTPase Rac. Furthermore, Rac is required for actin cytoskeletal rearrangements during FcγR- and complement-mediated phagocytosis, whereas Rho is necessary for both types of phagocytosis at a step distinct from that of actin polymerization in primary BMMs. Rac and Rho play broader roles in both FcγR- and complement-mediated phagocytosis than previously revealed in immortalized cell lines. Thus, primary BMMs from knockout mice have allowed us to analyze the specific contributions of Vav and Rac in FcγR- and complement-mediated phagocytosis. Further studies are needed to identify the GEF required for Rac activation downstream of the FcγR.

Experimental Procedures

Bone Marrow-Derived Macrophage Culture

Rac1/2^{ko} mice were generated as described in Sun et al. (2004). Briefly, Rac2 null mice were crossed with mice carrying a conditional allele for Rac1 expressing Cre recombinase downstream of a specific myeloid promoter, Lysozyme M. Vav1/3^{ko} mice were generated as described in Gakidis et al. (2004). To generate the Vav1/2/3^{ko} mice, Vav1/3^{ko} mice were crossed with Vav2^{ko} mice (Doody et al., 2001) to generate Vav1$^{+/-}$Vav2$^{+/-}$Vav3$^{+/-}$ mice. These triple Vav

heterozygous mice were crossed to generate Vav1$^{+/-}$Vav2$^{-/-}$ Vav3$^{-/-}$ mice, and these mice were crossed to each other to generate Vav1/2/3^{ko} mice. Age- and gender-matched mice (10–12 weeks old) were used for experiments. All experimental procedures were performed in accordance with institutional guidelines. Briefly, bone marrow progenitor cells were isolated from femurs and tibias and were cultured in macrophage media: DMEM high glucose (Invitrogen) supplemented with 15% heat-inactivated FBS (GIBCO-BRL), 1 mM nonessential amino acids (Invitrogen), 55 μM 2-mercaptoethanol (Invitrogen), and 25 ng/ml CSF-1 (R&D Systems). A total of 4 days after the initial isolation, bone marrow-derived macrophages were lifted from polystyrene flasks with 5 mM EDTA/PBS. The macrophages were plated, and the phagocytosis experiments were performed the following day.

FcγR- and Complement-Mediated Phagocytosis Assays

Sheep red blood cells (RBCs) (ICN) were washed and resuspended in veronal-buffered saline (Sigma). For FcγR-mediated phagocytosis, the RBCs were incubated with rabbit anti-sheep RBC IgG (1:5000, Cappel) for 1 hr at 37ºC. For complement-mediated phagocytosis, the RBCs were incubated with rabbit anti-sheep RBC IgM (1:1000, Accurate Scientific) for 1 hr at 37ºC, followed by an incubation with 10% C5-deficient serum (Sigma) for 20 min. The opsonized RBCs were washed and resuspended in DMEM. Prior to the addition of opsonized RBCs for complement-mediated phagocytosis, the BMMs were treated with 100 ng/ml PMA (CalBiochem) for 15 min to activate the complement receptor $\alpha_M\beta_2$. Phagocytosis was initiated by centrifuging the opsonized RBCs onto the BMMs for 2 min, at 1800 rpm, and incubating at 37ºC for the indicated times. Phagocytosis was terminated by lysing the nonphagocytosed RBCs with H_2O for 30 s. The BMMs were photographed with a Nikon TE300 microscope (20× phase objective), and phagocytosis (%) was quantified by counting the number of BMMs with at least one internalized RBC as a proportion of the total number of cells in 5 random fields (~50 cells/field).

Inhibitor Assays

TAT-C3 was purified as described in Coleman et al. (2001). Wild-type BMMs were incubated with TAT-C3 for 4 hr prior to the addition of opsonized RBCs for the phagocytosis assays.

Information on plasmids, biochemical and immunoblot analysis, immunofluorescence microscopy, transmission electron microscopy, and retroviral infection can be found in the Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data including four figures and Experimental Procedures are available at http://www.immunity.com/cgi/content/full/24/3/305/DC1/.

Acknowledgments

We would like to thank V. Tybulewicz for the Vav1^{ko} mice, M. Turner for the Vav2^{ko} mice, and T. Mayadas for the $\alpha_M{}^{-/-}$ mice; Elizabeth J. Benecchi and Maria Ericsson for expert assistance with TEM; C.J. Marshall for the TAT-C3 plasmid; Eugene Koh for pMX-uGFP; and Rebecca Hillenbrand and Eugene Yim for mouse husbandry and help with macrophage preparation. This work was supported by National Institutes of Health grant HL059561 (J.S.B.), a National Research Service Award fellowship (J.L.W.), and the Charles A. King Trust, Bank of America, N.A., Co-Trustee (Boston, MA) fellowship (A.B.H.).

Received: October 6, 2005
Revised: January 20, 2006
Accepted: February 1, 2006
Published: March 21, 2006

References

Aghazadeh, B., Lowry, W.E., Huang, X.Y., and Rosen, M.K. (2000). Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation. Cell *102*, 625–633.

Allen, L.A., and Aderem, A. (1996). Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. J. Exp. Med. *184*, 627–637.

Bokoch, G.M. (2005). Regulation of innate immunity by Rho GTPases. Trends Cell Biol. *15*, 163–171.

Brown, E.J., Newell, A.M., and Gresham, H.D. (1987). Molecular regulation of phagocyte function. Evidence for involvement of a guanosine triphosphate-binding protein in opsonin-mediated phagocytosis by monocytes. J. Immunol. *139*, 3777–3782.

Bustelo, X.R. (2000). Regulatory and signaling properties of the Vav family. Mol. Cell. Biol. *20*, 1461–1477.

Caron, E., and Hall, A. (1998). Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science *282*, 1717–1721.

Chimini, G., and Chavrier, P. (2000). Function of Rho family proteins in actin dynamics during phagocytosis and engulfment. Nat. Cell Biol. *2*, E191–E196.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A., and Olson, M.F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat. Cell Biol. *3*, 339–345.

Cougoule, C., Wiedemann, A., Lim, J., and Caron, E. (2004). Phagocytosis, an alternative model system for the study of cell adhesion. Semin. Cell Dev. Biol. *15*, 679–689.

Cox, D., Chang, P., Zhang, Q., Reddy, P.G., Bokoch, G.M., and Greenberg, S. (1997). Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. J. Exp. Med. *186*, 1487–1494.

Crespo, P., Schuebel, K.E., Ostrom, A.A., Gutkind, J.S., and Bustelo, X.R. (1997). Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. Nature *385*, 169–172.

Doody, G.M., Bell, S.E., Vigorito, E., Clayton, E., McAdam, S., Tooze, R., Fernandez, C., Lee, I.J., and Turner, M. (2001). Signal transduction through Vav-2 participates in humoral immune responses and B cell maturation. Nat. Immunol. *2*, 542–547.

Fischer, K.D., Kong, Y.Y., Nishina, H., Tedford, K., Marengere, L.E., Kozieradzki, I., Sasaki, T., Starr, M., Chan, G., Gardener, S., et al. (1998). Vav is a regulator of cytoskeletal reorganization mediated by the T-cell receptor. Curr. Biol. *8*, 554–562.

Fitzer-Attas, C.J., Lowry, M., Crowley, M.T., Finn, A.J., Meng, F., DeFranco, A.L., and Lowell, C.A. (2000). Fcγ receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. J. Exp. Med. *191*, 669–682.

Fujikawa, K., Miletic, A.V., Alt, F.W., Faccio, R., Brown, T., Hoog, J., Fredericks, J., Nishi, S., Mildiner, S., Moores, S.L., 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.

Gakidis, M.A., Cullere, X., Olson, T., Wilsbacher, J.L., Zhang, B., Moores, S.L., Ley, K., Swat, W., Mayadas, T., and Brugge, J.S. (2004). Vav GEFs are required for β2 integrin-dependent functions of neutrophils. J. Cell Biol. *166*, 273–282.

Hackam, D.J., Rotstein, O.D., Schreiber, A., Zhang, W., and Grinstein, S. (1997). Rho is required for the initiation of calcium signaling and phagocytosis by Fcγ receptors in macrophages. J. Exp. Med. *186*, 955–966.

Hazeki, K., Tamoto, K., Ui, M., and Mori, Y. (1994). Dual pertussis toxin-sensitive pathway of zymosan-induced activation in guinea pig macrophages. An anti-CR3 antibody-inhibitable stimulation of phagocytosis and -resistant stimulation of O2- production and arachidonate release. FEBS Lett. *342*, 29–32.

Holsinger, L.J., Graef, I.A., Swat, W., Chi, T., Bautista, D.M., Davidson, L., Lewis, R.S., Alt, F.W., and Crabtree, G.R. (1998). Defects in actin-cap formation in Vav-deficient mice implicate an actin requirement for lymphocyte signal transduction. Curr. Biol. *8*, 563–572.

Kaplan, G. (1977). Differences in the mode of phagocytosis with Fc and C3 receptors in macrophages. Scand. J. Immunol. *6*, 797–807.

Kiefer, F., Brumell, J., Al-Alawi, N., Latour, S., Cheng, A., Veillette, A., Grinstein, S., and Pawson, T. (1998). The Syk protein tyrosine kinase

is essential for Fcγ receptor signaling in macrophages and neutrophils. Mol. Cell. Biol. *18*, 4209–4220.

Massol, P., Montcourrier, P., Guillemot, J.C., and Chavrier, P. (1998). Fc receptor-mediated phagocytosis requires CDC42 and Rac1. EMBO J. *17*, 6219–6229.

May, R.C., Caron, E., Hall, A., and Machesky, L.M. (2000). Involvement of the Arp2/3 complex in phagocytosis mediated by FcγR or CR3. Nat. Cell Biol. *2*, 246–248.

Miranti, C.K., Leng, L., Maschberger, P., Brugge, J.S., and Shattil, S.J. (1998). Identification of a novel integrin signaling pathway involving the kinase Syk and the guanine nucleotide exchange factor Vav1. Curr. Biol. *8*, 1289–1299.

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.

Nobes, C.D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell *81*, 53–62.

Patel, J.C., Hall, A., and Caron, E. (2002). Vav regulates activation of Rac but not Cdc42 during FcγR-mediated phagocytosis. Mol. Biol. Cell *13*, 1215–1226.

Sekine, A., Fujiwara, M., and Narumiya, S. (1989). Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. J. Biol. Chem. *264*, 8602–8605.

Sun, C.X., Downey, G.P., Zhu, F., Koh, A.L., Thang, H., and Glogauer, M. (2004). Rac1 is the small GTPase responsible for regulating the neutrophil chemotaxis compass. Blood *104*, 3758–3765.

Tedford, K., Nitschke, L., Girkontaite, I., Charlesworth, A., Chan, G., Sakk, V., Barbacid, M., and Fischer, K.D. (2001). Compensation between Vav-1 and Vav-2 in B cell development and antigen receptor signaling. Nat. Immunol. *2*, 548–555.

Zugaza, J.L., Lopez-Lago, M.A., Caloca, M.J., Dosil, M., Movilla, N., and Bustelo, X.R. (2002). Structural determinants for the biological activity of Vav proteins. J. Biol. Chem. *277*, 45377–45392.