The small GTPase Rap1b negatively regulates neutrophil chemotaxis and transcellular diapedesis by inhibiting Akt activation

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Neutrophils are the first line of cellular defense in response to infections and inflammatory injuries. However, neutrophil activation and accumulation into tissues trigger tissue damage due to release of a plethora of toxic oxidants and proteases, a cause of acute lung injury (ALI). Despite its clinical importance, the molecular regulation of neutrophil migration is poorly understood. The small GTPase Rap1b is generally viewed as a positive regulator of immune cell functions by controlling bidirectional integrin signaling. However, we found that Rap1b-deficient mice exhibited enhanced neutrophil recruitment to inflamed lungs and enhanced susceptibility to endotoxin shock. Unexpectedly, Rap1b deficiency promoted the transcellular route of diapedesis through endothelial cell. Increased transcellular migration of Rap1b-deficient neutrophils in vitro was selectively mediated by enhanced PI3K–Akt activation and invadopodia–like protrusions. Akt inhibition in vivo suppressed excessive Rap1b-deficient neutrophil migration and associated endotoxin shock. The inhibitory action of Rap1b on PI3K signaling may be mediated by activation of phosphatase SHP–1. Thus, this study reveals an unexpected role for Rap1b as a key suppressor of neutrophil migration and lung inflammation.

Abbreviations used: ALI, acute lung injury; BAL, bronchoalveolar lavage; DRM, detergent-resistant membrane; fMLP, formyl-methionyl-leucyl-phenylalanine; GAP, GTPase-activating protein; GEFs, guanine nucleotide exchange factors; ICAM, intercellular adhesion molecule 1; ITIM, immunoreceptor tyrosine-based inhibitory motif; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; MMP, metalloproteinase; PECAM, platelet endothelial cell adhesion molecule; Rap1, Ras homology region 2 domain-containing phosphatase-1; TEM, transendothelial migration; WCL, whole-cell lysate; WCL, whole-cell lysate.
adhesion of the neutrophils to the endothelium (Schenkel et al., 2004; Ley et al., 2007; Phillipson and Kubes, 2011). After arrest, neutrophils undergo actin-dependent polarization and lateral migration or crawling on endothelial cells in search for permissive sites, before final diapedesis out of blood vessels (Phillipson et al., 2006; Muller, 2011). Diapedesis or transendothelial migration (TEM) is the least explored step and can occur by two distinct routes: either through junctions between endothelial cells (the paracellular route) or directly through individual endothelial cells (the transcellular route; Feng et al., 1998; Schenkel et al., 2004; Phillipson et al., 2006; Carman et al., 2007; Carman and Springer, 2008; Sage and Carman, 2009; Shulman et al., 2009; Muller, 2011). Once at the site of inflammation, neutrophils release reactive oxygen species, pro-inflammatory cytokines, and various proteases that all contribute to tissue injury when the neutrophilic response remains uncontrolled. The signaling pathways that limit neutrophil responses are poorly understood.

Ras proximity 1 (Rap1) belongs to the Ras superfamily of GTPases that cycle between GTP-bound active and GDP-bound inactive forms through GEFs and GAPs (M’Rabet et al., 1998; Caron, 2003). The mammalian genome encodes two Rap1 genes, Rap1a and Rap1b, which are highly homologous and evolutionarily conserved. Mouse genetic studies have shown that Rap isoforms have both redundant and specific functions (Caron, 2003; Chrzanowska-Wodnicka et al., 2005; Li et al., 2007; Wittchen et al., 2011). Rap1 is historically known to control functional activation of integrins through inside-out signaling (Katagiri and Kinashi, 2012), and to promote cell adhesion, platelet aggregation, and phagocytosis in macrophages (Caron et al., 2000; Katagiri et al., 2003; Chrzanowska-Wodnicka et al., 2005; Boettner and Van Aelst, 2009; Katagiri and Kinashi, 2012). Rap1 also controls actin polarization, as well as migration and homing into tissue, in lymphocytes (Shimonaka et al., 2003). Furthermore, Rap1 regulates angiogenesis (Lakshmikanthan et al., 2011) via cross talk between VEGFR2 with integrin αvβ3. Rap1b is the predominant Rap1 isoform expressed in neutrophils, suggesting a preponderant role for Rap1b in these cells. Interestingly, CalDAG-GEFI, which is a Rap1-GEF, appears to control neutrophil chemotaxis independently on integrin functions (Carbo et al., 2010). However, the role of Rap1b in neutrophil functions has not been studied.

Here, we reveal that Rap1b deficiency conferred enhanced susceptibility to endotoxin shock. In addition, Rap1b-deficient neutrophils manifested enhanced emigration to inflamed lungs. This response was associated with increased transcellular diapedesis in vitro. Interestingly, Rap1b loss induced elevated PI3K-γ-p-Akt activity that promoted neutrophil invasiveness in vitro. Pharmacological inhibition of Akt activity completely reverted enhanced neutrophil migration and susceptibility to endotoxin shock. Our study characterizes a previously unknown role for Rap1b as a physiological suppressor of neutrophil emigration. This may represent an unappreciated regulatory pathway of neutrophil-related aberrant inflammatory responses.

RESULTS
A critical event of neutrophil migration is the adoption of a polarized shape. During neutrophil polarization, the plasma membrane reorganizes into detergent-resistant membrane (DRM) domains at the uropod. Recently, we showed that the small Rho GTPase Cdc42 regulates neutrophil polarity by controlling the composition of DRMs (Kumar et al., 2012). Proteomics analysis of WT and Cdc42−/− neutrophil DRM fractions revealed that the GTPase Rap1b was present in the DRM of WT but not Cdc42−/− neutrophils (not depicted), which was confirmed by immunoblotting (Fig. 1 A). Consistent with DRM association, Rap1b was enriched at the uropod (unpublished data), suggesting the participation of Rap1b signaling in neutrophil migration. Yet, the roles for Rap1b in neutrophil functions and inflammation remain largely unknown.

Rap1b deficiency enhances neutrophil recruitment into inflamed lungs
We first examined the role of Rap1b in neutrophil recruitment in vivo using a well-established model of LPS-driven ALI. Because Rap1b is expressed in all tissues, including endothelial cells, we used adoptive transplantation of BM cells from Rap1b−/− and control WT mice into lethally irradiated C57BL/6 WT mice to allow selective Rap1b deletion in blood cells (Filippi et al., 2007). 5 wk after hematopoietic reconstitution, Rap1b proteins were absent in neutrophils isolated from Rap1b−/− reconstituted mice (Fig. 1 B). In these cells, expression of total Rap1 protein was low, suggesting that Rap1a expression does not compensate for Rap1b loss (Fig. 1 B), as previously reported (Awasthi et al., 2010; Carbo et al., 2010). The total white blood count, including neutrophils, was normal in WT and Rap1b−/− reconstituted animals (unpublished data). Unexpectedly, bronchoalveolar lavage (BAL) of Rap1b−/− reconstituted mice that were inoculated with LPS contained more neutrophils than similarly challenged control mice (Fig. 1 C). Higher rate of hemorrhage, as indicated by increased red cell count (Fig. 1 C), and increased protein content (Fig. 1 D) were also noted in BAL from Rap1b−/− mice, whereas macrophages were comparable between the genotypes (Fig. 1 C). Lung histology showed enhanced neutrophil infiltration into interstitial tissues and increased neutrophil numbers in the alveoli of Rap1b−/− reconstituted mice, compared with WT mice (Fig. 1 E). An adoptive transfer of WT and Rap1b−/− neutrophils labeled with 2 different dyes confirmed the neutrophil cell-intrinsic nature of Rap1b function, as more Rap1b−/− neutrophils emigrated into inflamed lung alveoli than WT neutrophils (Fig. 1 F). Finally, Rap1b−/− reconstituted mice were more susceptible to higher dose of intraperitoneal LPS challenge than WT controls (Fig. 1 G). Hence, Rap1b is, surprisingly, a negative regulator of neutrophil emigration into lungs.

Rap1b deficiency increases neutrophil transmigration
To further investigate the role of Rap1b in neutrophil migration, chemotaxis toward fMLP was examined in Zigmond chamber using time lapse microscopy. In contrast to enhanced...
because, overall, the cells migrated toward the chemotactic gradient. To further understand the role of Rap1b in neutrophil migration, we used Boyden chamber assays in response to neutrophil chemokines, fMLP and MIP2. Random migration (chemokinesis) was assessed in a uniform fMLP concentration in both wells of the chamber, whereas directed migration (chemotaxis) was assessed in a gradient in which fMLP was placed only in the lower chamber. Interestingly, Rap1b\(^{-/-}\) neutrophil migration to fMLP or MIP2 in both chemotaxis and chemokinesis was similar to that of WT neutrophil emigration to lungs in vivo, time lapse analysis revealed that Rap1b\(^{-/-}\) neutrophils exhibited a modest decreased ability to efficiently migrate up the chemotactic gradient (Fig. 2A and Video 1). Rap1b\(^{-/-}\) cells extended transient multiple pseudopodia and made frequent turns leading to a 25% reduction in straightness relative to WT cells (Fig. 2A and Video 2), as previously reported (Carbo et al., 2010). However, the speed of migration was 15% increased whereas chemoattractant sensing of Rap1b\(^{-/-}\) was similar to that of WT because, overall, the cells migrated toward the chemotactic gradient. To further understand the role of Rap1b in neutrophil migration, we used Boyden chamber assays in response to neutrophil chemokines, fMLP and MIP2. Random migration (chemokinesis) was assessed in a uniform fMLP concentration in both wells of the chamber, whereas directed migration (chemotaxis) was assessed in a gradient in which fMLP was placed only in the lower chamber. Interestingly, Rap1b\(^{-/-}\) neutrophil migration to fMLP or MIP2 in both chemotaxis and chemokinesis...
Figure 2. **Rap1b−/−** neutrophils exhibit enhanced chemokinesis and chemotaxis. (A) Analysis of neutrophil migration using time lapse video microscopy in a gradient of fMLP in a Zigmond chamber. Cell trajectory analysis; the schema represents the migration trajectory of cells moving up fMLP gradient for 20 min. Speeds (Sp = µm/min) of migration are indicated at the bottom. Mean ± SD; n = 60 cells. Scatter plot of straightness of migration from 30 individual cells, representative of 3 independent experiments. Bar graph represents the percentage of cells exhibiting frequent changes in direction. Data are from 80 cells (mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001; 3 independent experiments). (B and C) Chemokinesis (uniform chemokine concentration) and chemotaxis (chemokine gradient) analysis of neutrophils using a Boyden chamber with 1 µM fMLP, 100 nM MIP2, or differential concentration of chemokines. The histogram represents the number of migrated neutrophils per field using a 40× objective (mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001; representative of 3 independent experiments). (D) Neutrophil migration using Transwell-coated with fibrinogen (Fg) or endothelial cells (HUVECs and bEND.3) grown on Transwell filters in uniform concentration or in a gradient of 10 µM fMLP. Histogram represents the total number of migrated neutrophils recovered from the bottom well (mean ± SD; **, P < 0.01; ***, P < 0.001; using unpaired Student’s t test; 3 independent experiments). (E) Adhesion analysis of WT and Rap1b−/− neutrophils that were stimulated with fMLP and plated on diverse integrin ligands. Expression of integrins on WT and Rap1b−/− neutrophils with or without fMLP stimulation, as quantified using flow cytometry (mean ± SD; n = 3 independent experiments). (F) Images and quantification of spreading from minimum 50 WT and Rap1b−/− neutrophils that were plated on CD11b-coated plates without fMLP. Bar, 10 µm. Mean ± SD; n = 3 independent experiments **, P < 0.01. (G) Superoxide generation analysis in suspension by using flow cytometry and...
assays was higher than WT cells (Fig. 2, B and C). Increased chemotaxis of Rap1b−/− neutrophils was also observed with a range of chemokine concentrations (Fig. 2, B and C). Migration was examined in Transwells coated with fibrinogen (Fg), a αMβ2 integrin ligand, to investigate transmigration or Transwell coated with murine (bEND.3) or human (HUVEC) endothelial cells to monitor the role of Rap1b in transendothelial migration (Gerard et al., 2009). Enhanced migratory responses of Rap1b−/− neutrophils to fMLP were seen in both assays (Fig. 2 D), and we noted higher neutrophil TEM across bEND.3 cells than HUVECs. Together, although straightness of migration may be modestly decreased, Rap1b−/− neutrophils exhibited enhanced transendothelial migration. This phenotype was specific to Rap1b isoform. Rap1a−/− neutrophils transmigrated similarly to WT cells, in Transwell assay and in vivo into lungs after neutrophil adoptive transfer (unpublished data), suggesting that Rap1a is dispensable for neutrophil migration.

Neutrophil migration across the endothelial barrier depends on chemokine release and on interaction with the endothelium through adhesion molecules, including integrins. At the site of inflammation, chemokine and integrin stimulation triggers various neutrophil responses, including spreading, oxidative burst, and degranulation, which are known as adhesion–dependent responses. Because Rap1 proteins were initially identified to regulate cell adhesion by controlling chemokine-induced inside-out integrin signaling (Caron et al., 2000; Katagiri et al., 2003), we then examined static adhesion to diverse extracellular matrix (ECM)–coated surfaces in response to the neutrophil chemokine fMLP. After fMLP stimulation, Rap1b−/− neutrophil adhesion on any tested surface was comparable to WT (Fig. 2 E). Consistently, regulated expression of different integrin subunits, including αL (LFA-1/CD11a), αM (Mac-1/CD11b), β1 (CD29), and β2 (CD18), in Rap1b−/− neutrophils were comparable to WT neutrophils (Fig. 2 E). Hence, in neutrophils, Rap1b appears not to be critical for integrin-mediated adhesion. However, we noted a significant reduction in Rap1b−/− neutrophil spreading onto CD11b-coated plates relative to WT (Fig. 2 F). fMLP-induced intracellular superoxide production was comparable between the genotypes (Fig. 2 G). However, it was lower in fibrinogen-mediated adherent Rap1b−/− than in WT neutrophils after fMLP stimulation, but not with PMA that mediates integrin-independent superoxide production (Fig. 2 H). Hence, Rap1b seems dispensable for static adhesion, but critical for adhesion-dependent neutrophil responses.

**Rap1b disruption enhances transendothelial migration through transcellular diapedesis**

During the extravasation cascade, initial neutrophil adhesion onto the endothelium is followed by lateral crawling to find the nearest endothelial cell junction and permissive sites for transmigration (Ley et al., 2007). To examine in more detail the role of Rap1b during transendothelial migration, we used a 3D transmigration model using either HUVECs (unpublished data; Schenkel et al., 2004; Muller, 2011) or murine endothelial cells (Gerard et al., 2009) that recapitulate these critical steps. Neutrophils were seeded onto nonactivated or LPS-activated bEND.3 cells that were grown on collagen gels (Fig. 3 A). Under these conditions, LPS stimulates expression of ICAM, a β2 integrin ligand, on endothelial cells (Fig. 3 A), triggering neutrophil adhesion and transmigration (Sikorski et al., 1993; Yang et al., 2005). Rap1b−/− neutrophil adhesion to activated endothelial cells was comparable to that of WT (Fig. 3 B). In contrast, significantly less Rap1b−/− than WT neutrophils (64.5 ± 2.3 vs. 83.0 ± 2.1) were found at the endothelial cell junctions (Fig. 3 C). Remarkably, transendothelial migration of Rap1b−/− neutrophils was persistently increased compared with WT neutrophils (Fig. 3 D). The integrity of endothelial junctions was examined using PECAM staining and dextran permeability assay (unpublished data). Similar enhanced transendothelial migration was observed when Rap1b−/− neutrophils were plated on HUVECs (unpublished data). These results confirmed enhanced transmigration of Rap1b−/− neutrophils.

Neutrophil diapedesis can occur by two distinct routes, either between two endothelial cells, i.e., a paracellular route, or directly through them, i.e., a transcellular route (Carman et al., 2007; Sage and Carman, 2009; Muller, 2011). Enhanced transcellular migration could account for increased transmigration of Rap1b−/− neutrophils while the cells remained away from the endothelial junction. To examine the nature of diapedesis, transmigrating neutrophils onto HUVEC (unpublished data) or mouse endothelial cells were stained for VE-Cadherin to visualize endothelial cell junctions and for Gr-1 to identify neutrophils. ICAM-1 staining allows identification of the so-called transmigration cup, which is comprised of endothelial ICAM-1–enriched projections surrounding transmigrating leukocytes that occur during diapedesis (Carman et al., 2003; Carman and Springer, 2004). A representative example of each migratory route, identified using VE-Cadherin, ICAM-1, and Gr-1 staining, is depicted in Fig. 4 A (Carman et al., 2003; Carman and Springer, 2004). Only 5–10% of WT neutrophils used the transcellular route of diapedesis (Fig. 4 A). Interestingly, up to 30–35% Rap1b−/− neutrophils transmigrated via the transcellular route (Fig. 4 A). More transmigratory cups were observed during Rap1b−/− transmigration than in WT counterparts (Fig. 4 B). Overall, the total number of cells that transmigrated was higher in Rap1b−/− than WT neutrophils (Fig. 3 B and Fig. 4 A). Similar results were obtained across HUVECs (unpublished data). Therefore, Rap1b−/− neutrophils were found away from the endothelial junctions and exhibited increased transmigration and transcellular diapedesis in vitro.
Proteolytic activity was identified by the appearance of black holes in fluorochrome-conjugated gelatin matrices (Artym et al., 2006). fMLP-stimulated Rap1b−/− neutrophils created more holes that were larger in size than those made by WT cells (Fig. 6 A). Likewise, using MMP zymography, we observed higher MMPs release from Rap1b−/− neutrophils than WT neutrophils, in a time-dependent manner (Fig. 6 B). Transmission electron microscopy confirmed increased podosome-like structures in Rap1b−/− neutrophils that penetrated deeper into endothelial surfaces than WT cells (Fig. 6 C). This was supported by observation of longer and more protrusive microvilli on surface of Rap1b−/− neutrophils than WT controls (Fig. 6 D). These results suggest that Rap1b inhibits invasive F-actin protrusions to limit transcellular diapedesis in vitro.

Loss of Rap1b leads to enhanced Akt and PtdIns (3,4,5) P3 signaling
F-actin protrusions are highly dependent on PI3K signaling generating inositol lipid phosphatidylinositol 3,4,5-trisphosphate (PtdIns (3,4,5)P3 or PIP3; Cicchetti et al., 2002; Stephens et al., 2002). Importantly, cellular invasion is strongly dependent on Akt signaling, a key downstream target of PI3K–PIP3 cascade (Stephens et al., 2002; Chin and Toker, 2009). To examine the role of PI3K signaling in our model, we first examined the localization of fMLP-elicited PIP3 production of cells plated on fibrinogen. As expected, PIP3 was localized at the leading edge of fMLP-stimulated and polarized WT neutrophils (Fig. 7 A). Conversely, PIP3 signals were higher in Rap1b−/− neutrophils than WT controls (Fig. 7 B). These results suggest that Rap1b inhibits invasive F-actin protrusions to limit transcellular diapedesis in vitro.
pathways, which are reportedly important for neutrophil migration (Liu et al., 2012), remained unchanged in Rap1b−/− neutrophils (Fig. 7 D). Screening of different signaling pathways important for matrix degradation/invasion using pharmacological inhibitors further suggested a critical role of PI3K-Akt pathway.

Figure 4. Rap1b loss promotes transendothelial migration via transcellular route. (A, top) Representative immunofluorescence images showing paracellular and transcellular migration of neutrophils, identified using VE-cadherin, ICAM-1, and Gr-1 staining. Bar, 20 µm. (bottom) Percentage of WT and Rap1b−/− neutrophils showing diapedesis via paracellular and transcellular route and relative percent neutrophils using paracellular and transcellular route (mean ± SD). Data are cumulative from 4 independent experiments with more than 750 cells showing transendothelial migration. (B, top) Representative images of transmigratory cups, percentage of WT and Rap1b−/− neutrophils encircled with ICAM-rich transmigratory cups. Mean ± SD. n = 3 independent experiments. **, P < 0.01; ***, P < 0.001; NS, not significant using unpaired Student’s t test. Bar, 10 µm.

enriched all around the cells (Fig. 7 A). Consistently, Akt phosphorylation was more substantially increased in Rap1b−/− neutrophils than in WT cells in response to fMLP and integrin activation (Fig. 7 B) or fMLP alone (Fig. 7 C), as shown in other cells (Lou et al., 2002). On the other hand, MAPK signaling pathways, which are reportedly important for neutrophil migration (Liu et al., 2012), remained unchanged in Rap1b−/− neutrophils (Fig. 7 D). Screening of different signaling pathways important for matrix degradation/invasion using pharmacological inhibitors further suggested a critical role of PI3K-Akt
Pharmacological inhibition of Akt activation rescued Rap1b−/− neutrophil phenotype

To examine the contribution of Akt signaling to Rap1b-null phenotype, we used a small molecule Akt inhibitor, MK-2206 (Hirai et al., 2010). MK-2206 treatment successfully inhibited Akt phosphorylation in Rap1b−/− and WT cells (Fig. 7E). Remarkably, MK-2206 treatment completely inhibited neutrophil infiltration. This suggests that Akt activation is a critical factor in neutrophil infiltration and that inhibition of Akt activity can prevent this process. These findings provide new insights into the mechanisms underlying neutrophil infiltration and suggest potential targets for therapeutic intervention.
Rap1b inhibits Akt activation via CD11b outside-in signaling

The neutrophil crawling and TEM steps are highly αMβ2-integrin–dependent (Phillipson et al., 2006). Because Rap1b controls some adhesion-dependent cellular functions, including superoxide production (Fig. 2), we investigated whether Rap1b migratory behavior was CD11b–dependent. Because CD11b associates with the DRMs at the cell uropod during migration (Kumar et al., 2012), and Rap1b can localize at the cell uropod and associate with DRMs, we examined the protein composition of DRMs of Rap1b−/− and CD11b−/− neutrophils. CD11b was present in the DRM fraction of Rap1b−/− neutrophils. However, Rap1b was completely absent in CD11b−/− DRMs (Fig. 9 A). Rap1b protein levels in whole-cell lysates (WCLs) of CD11b−/− PMN were comparable to WT cells (Fig. 9 A). We thus hypothesized that Rap1b may act downstream of CD11b to limit CD11b-induced Akt signals and associated matrix degradation. In support of this notion, we found Akt phosphorylation to be highly CD11b dependent. Akt phosphorylation was reversed elevated TEM across mouse endothelial cells of Rap1b−/− neutrophils to WT levels (Fig. 8 A). It reduced ECM degradation of Rap1b−/− neutrophils, and inhibited their multiple protrusions (Fig. 8, B and C). Furthermore, almost all Rap1b−/− cells treated with MK-2206 were found at the endothelial cell junctions (Fig. 8 D). However, MK-2206 had little effect on WT neutrophil transmigration. We also tested the effect of Src kinase inhibitor PP2, which inhibits neutrophil crawling and transmigration processes (Carman et al., 2007; Gerard et al., 2009). Interestingly, PP2, like MK-2206, completely rescued Rap1b−/− phenotypes (Fig. 8 A), but it also inhibited elevated p-Akt (Fig. 7 E). Remarkably, MK2206 pretreatment completely reversed LPS-induced Rap1b−/− neutrophil emigration into lung alveoli in vivo to WT levels (Fig. 8 E) and rescued survival of Rap1b−/− mice challenged with lethal dose of LPS, without any effect on WT (Fig. 8 F). Thus, Akt signaling is a major pathway mediating Rap1b loss phenotype.

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Rap1b regulates Akt activity via the phosphatase SHP-1

Because Rap1b is a GTPase, its effect on Akt activity is likely to be indirect and involve a phosphatase. The nontyrosine receptor SRC-homology region 2 domain-containing phosphatase-1 (SHP-1, also called PTPN6) is an important negative regulator of PI3K-Akt and Src signaling that associates with neutrophil DRMs (Zhang et al., 2005). It is activated by phosphorylation on tyrosine (Y536) residue (Tsui et al., 2006). fMLP-induced total tyrosine phosphorylation of SHP-1 was lower in Rap1b−/− neutrophils than in WT cells (Fig. 10 A), as was its association to DRMs in response to fMLP and fibrinogen (Fig. 10 B). Further, SHP-1 redistributed preferentially to the uropod in WT neutrophils stimulated with fMLP and on fibrinogen, but not in Rap1b−/− neutrophils, which exhibited homogenous SHP-1 distribution (Fig. 10 C). SHP-2 distribution was unchanged in Rap1b−/− neutrophils (unpublished data). Importantly, the SHP-1/2 inhibitor NSC-87877 (Nyström et al., 2009) substantially increased matrix degradation (Fig. 10 D) and Akt phosphorylation (Fig. 10 E) in WT cells, supporting the involvement of SHP-1 in neutrophil invasive behavior.

Adoptive transfer of neutrophils further supported impaired CD11b−/− neutrophil emigration into lungs (Fig. 9 G). Together, these results suggest that Rap1b acts downstream of CD11b to limit CD11b-induced Akt activation.

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Neutrophil depletion protects from LPS-induced toxicity (Hewett et al., 1992; Abraham et al., 2000). In general, tissue toxicity results from the uncontrolled accumulation of neutrophils into tissues and their subsequent activation. Neutrophils possess numerous granules that contain a large number of cytotoxic molecules, including numerous proteases, cytokines, elastase, myeloperoxidase and metalloproteinases. Neutrophil activation in tissue triggers neutrophil degranulation and release of these cytotoxic molecules into the extracellular space, as well as respiratory burst via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which is a cause of tissue damage. In addition, proinflammatory cytokines, such as IL-1β, TGF-β, and TNF-α, are locally released, hence further intensifying inflammation (Zemans et al., 2009). The increased death of LPS-challenged Rap1b−/−/−-reconstituted mice could thus be caused by multiple factors. We found increased Rap1b−/−/− neutrophil emigration into tissues. The passage of large numbers of activated neutrophils can result in damage to the epithelium, in part due to microscopic wounds in the epithelium (Nusrat et al., 1997). Intriguingly, Rap1b−/−/− neutrophils used (at least in vitro) an uncommon route of migration, i.e., transcellular diapedesis, during which neutrophils directly migrate through individual endothelial cells (Feng et al., 1998; Carman et al., 2007; Carman and Springer, 2008; Sage and Carman, 2009), although it is currently not known whether migrating transcellularly impacts the intensity of tissue damage and inflammation in vivo. However, there may be a discrepancy between the magnitude of the Rap1b−/−/− animal death and the increased Rap1b−/−/− mouse death which supports this possibility.

Therefore, Rap1b limits Akt signaling, likely via SHP-1. Together, these findings reveal Rap1b as a novel suppressor of neutrophil migration by limiting Akt signaling.

DISCUSSION

Rap1b is an evolutionarily conserved protein of the Ras-like GTPase superfamily (Caron, 2003; Boettner and Van Aelst, 2009; Raaijmakers and Bos, 2009; Katagiri and Kinashi, 2012). The general view has been that Rap1 positively regulates cell adhesion, cell polarity, cell junction formation, and secretion (Caron et al., 2000; Chrzanowska-Wodnicka et al., 2005; Lakshmikanthan et al., 2011; Wittchen et al., 2011; Katagiri and Kinashi, 2012). Rap1 loss often causes cellular migratory and homing defects, as seen in epithelial cells, endothelial cells, and lymphocytes (Caron et al., 2000; Katagiri et al., 2003; Shimonaka et al., 2003; Wittchen et al., 2011). Here, we unexpectedly found that Rap1b is a key suppressor of neutrophil migration and inflammation. Mice reconstituted with Rap1b−/− hematopoietic cells were more susceptible to LPS-induced endotoxin shock than WT. Rap1b−/− neutrophils exhibited increased emigration into lungs, and increased transmigration in vitro, due to enhanced Akt signaling. Further, this Rap1b pathway appears to selectively limit the formation of invasive protrusions to restrain what may be abnormal transcellular migration in vitro. Our work uncovers a novel functional role for Rap1b in immune cells.

Neutrophils are major effector cells of systemic inflammation induced by endotoxin challenge, illustrated by the fact that neutrophil depletion protects from LPS-induced toxicity (Hewett et al., 1992; Abraham et al., 2000). In general, tissue toxicity results from the uncontrolled accumulation of neutrophils into tissues and their subsequent activation. Neutrophils possess numerous granules that contain a large number of cytotoxic molecules, including numerous proteases, cytokines, elastase, myeloperoxidase and metalloproteinases. Neutrophil activation in tissue triggers neutrophil degranulation and release of these cytotoxic molecules into the extracellular space, as well as respiratory burst via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which is a cause of tissue damage. In addition, proinflammatory cytokines, such as IL-1β, TGF-β, and TNF-α, are locally released, hence further intensifying inflammation (Zemans et al., 2009). The increased death of LPS-challenged Rap1b−/−/−-reconstituted mice could thus be caused by multiple factors. We found increased Rap1b−/− neutrophil emigration into tissues. The passage of large numbers of activated neutrophils can result in damage to the epithelium, in part due to microscopic wounds in the epithelium (Nusrat et al., 1997). Intriguingly, Rap1b−/− neutrophils used (at least in vitro) an uncommon route of migration, i.e., transcellular diapedesis, during which neutrophils directly migrate through individual endothelial cells (Feng et al., 1998; Carman et al., 2007; Carman and Springer, 2008; Sage and Carman, 2009), although it is currently not known whether migrating transcellularly impacts the intensity of tissue damage and inflammation in vivo. However, there may be a discrepancy between the magnitude of the Rap1b−/−/− animal death and the increased...
neutrophil tissue infiltration, thus suggesting the involvement of additional mechanisms. Increased release of metalloproteinases of Rap1b−/− neutrophils may directly contribute to the high mortality of Rap1b−/− mice due to their tissue matrix degradation activity (Plitas et al., 2003). Because metalloproteinases are released by degranulation, it is possible that Rap1b−/− neutrophils additionally release numerous cytotoxic agents, including elastase, defensins, leukotriene B4. Other Rap1b−/− hematopoietic cells, such as macrophages, could also secrete inflammatory mediators, e.g., TNF, IL-1β, and IL-6. These molecules play key roles in the pathogenesis of organ damage (Zemans et al., 2009). For instance, in animal models, elastase administration causes lung injury and elastase inhibition has a protective effect on lung injury (Delacourt et al., 2002; Tremblay et al., 2002). The mechanism by which elastase causes tissue injury could be caused by basement membrane degradation, increased epithelial permeability, or epithelial ulcerations. Defensins are another major component of neutrophil granules and have been shown to cause endothelial injury (Okrent et al., 1990). TNF is a key component of the pathogenesis of inflammation by increasing endothelial cell permeability (Hamacher et al., 2002; Petrache et al., 2003). Overall these

Figure 9. Rap1b negatively regulates PI3K/Akt signaling via CD11b outside-in signaling. (A) Immunoblot analysis of CD11b and Rap1b in DRM fractions or WCL of Rap1b−/− or CD11b−/− neutrophils with respective WT controls. (B) Immunoblot analysis of p-Akt in WT and CD11b−/− neutrophils stimulated on uncoated plates. (C) p-Akt immunoblots analysis of WT and Rap1b−/− neutrophils that were stimulated with fMLP and plated on fibrinogen-coated slides or on slides coated with CD11b Ab. (D) Analysis of cells with more than one protrusion using F-actin staining of WT and Rap1b−/− neutrophils that were stimulated with fMLP and plated on fibrinogen-coated slides or on slides coated with anti-CD11b. (E) Immunoblot analysis of p-Akt from WT and Rap1b−/− neutrophils incubated with or without a monoclonal blocking anti-CD11b antibody after stimulation with fMLP and on fibrinogen-coated plates. Blots are representative of 3 independent experiments and densitometric analyses are cumulative of 3 experiments (A–C and E). Mean ± SD; n = 3 independent experiments (A–C and E). *, P < 0.05; **, P < 0.01; ***, P < 0.001; NS, not significant using unpaired Student’s t test. (F) Matrix degradation analysis in CD11b−/− neutrophils or WT and Rap1b−/− neutrophils treated with blocking anti-CD11b antibody. Mean ± SD, n = 3 independent experiments (D and F). *, P < 0.05; **, P < 0.01; NS, not significant using unpaired Student’s t test. (G) Relative recruitment of CSFE and SNARF-1 labeled and adoptively transferred WT and CD11b−/− neutrophils in BAL of LPS challenged mice. Mean ± SD; n = 3 independent experiments. ***, P < 0.001 (unpaired Student’s t test).
insults enhance tissue degradation and permeability that, in the case of the lungs, can lead to lethal edema. Hence, the possible secretion of such factors by Rap1b−/− hematopoietic cells would increase LPS-driven tissue toxicity and animal death. Finally, platelet–neutrophil interaction via CD11b is key to vascular injury (Hidalgo et al., 2009). As Rap1b is a crucial regulator of platelet functions (Lakshmikanthan et al., 2011), defective functions and/or interaction of Rap1b−/− platelets with neutrophils may also contribute to the increased mortality of Rap1b−/− mice. These possibilities will be interesting to investigate in the future.

Akt signaling appears to mediate the effect of Rap1b deletion on neutrophil functions. The mechanism by which Rap1b limits Akt signaling is unprecedented. Rap1b is known to positively regulate ERK/MAPK and p38MAPK activities through the assembly of IQGAP-mediated signalosome (Awasthi et al., 2010). Rap1b can be activated downstream of PI3K and regulate Cdc42-mediated polarity (Schwamborn and Püschel, 2004). We found no clear evidence of adhesion defects in Rap1b−/− neutrophils. Instead, Rap1b negatively regulates PI3K-Akt signaling pathways in response to both GPCR and outside-in CD11b signaling. Rap1b can be recruited to the triton-insoluble plasma membrane domains by CD11b and, in turn, limits CD11b-induced PI3K-Akt signaling. Rap1b itself is not acting between CD11b and PI3K-Akt, as CD11b cross-linking can enhance Akt phosphorylation in Rap1b−/− neutrophils. Instead, Rap1b acts as a counterpart regulatory pathway to limit ligand-induced Akt activation. Because Rap1b−/− neutrophils are not in an activated state without ligand stimulation, Rap1b integrates GPCR and integrin signaling to limit excessive ligand-induced activation.

Rap1b, like Ras–related small GTPases, functions through activation of effector kinase proteins (Raaijmakers and Bos, 2009). The elevated PI3k-Akt signals in Rap1b−/− neutrophils likely results from failure to activate a negative regulator. We provide evidence that Rap1b may act through the non-receptor protein-tyrosine phosphatase SHP-1. SHP-1 is a negative regulator of innate immune cell functions by down-modulating signaling pathways, including Src and Akt signaling (Tsui et al., 2006). Neutrophils from mice with genetic deletion of shp-1 or with null mutations in Ptpn6, which encodes SHP-1, have increased actin polymerization, and chemotaxis (Zhang et al., 2005). Thus, impaired SHP-1 activity in Rap1b−/− neutrophils may maximize Akt signaling. SHP-1 is a negative regulator of innate immune cell functions by down-modulating signaling pathways, including Src and Akt signaling (Tsui et al., 2006). Neutrophils from mice with genetic deletion of shp-1 or with null mutations in Ptpn6, which encodes SHP-1, have increased actin polymerization, and chemotaxis (Zhang et al., 2005). It would be interesting to investigate possible cross talk between Rap1b and ITIMs bearing receptors in SHP-1 regulation and transcellular migration.

These findings raise an interesting question about what triggers a transcellular mode of migration over paracellular diapedesis. The local vascular environment seems important.
Carman and Springer suggested that cells take the path of least resistance. During cell locomotion, podosome-like protrusions would sense endothelial cells for area of least resistance at which cells can extend longer invadopodia-like structures triggering transcellular migration (Carman et al., 2007; Carman and Springer, 2008). As such, neutrophils use transcellular diapedesis to migrate through the blood–brain barrier, where endothelial junctions are particularly tight (von Wedel-Parlow et al., 2011). In addition, endothelial cells enriched in ICAM tend to promote the transcellular route (Yang et al., 2005). However, this cannot be the only mechanism, as transcellular migration can also occur on low-resistant endothelial cells. In this case, the level of neutrophil activation and subsequent activation of specific intracellular signals may be an important determinant factor. The fact that intradermal injection of fMLP stimulates transcellular migration in vivo (Feng et al., 1998), or that direct activation by fMLP of neutrophils plated on endothelial cells increases transcellular migration events in vitro (Mamdouh et al., 2009) support this idea that transcellular migration can be promoted when neutrophils are highly activated. Because the strength of stimuli-induced intracellular signals can trigger various cellular responses, it is possible that high neutrophil activation may promote invasive actin protrusions to form in tissue environment with loose endothelial junctions, and increase their ability to migrate transcellularly. Our findings suggest that neutrophil invasive protrusions and transcellular migration in vitro depends on high Akt signal intensity. Rap1b−/− neutrophils seem to become more invasive with increased actin polymerization, invadopodia and MMP release in vitro, in a manner dependent on high Akt signals. Inhibition of Akt signaling in these cells blocks transcellular but not paracellular migration in vitro. Hence, Akt signal robustness seems to trigger a neutrophil response that will specifically determine transcellular migration in vitro. Akt signal strength may promote MMP release and convert noninvasive lamellipodial protrusions into invasive podosome structures. Because transcellular migration requires the formation of a transcellular channel and membrane fusion of the endothelial lateral border recycling compartment (LBRC; Mamdouh et al., 2009), Akt-driven invasive protrusions may send signals to endothelial cells to recruit the LBRC and initiate the formation of the transcellular pore away from the junction. Our findings strongly suggest that Rap1b loss directly increased the ability of neutrophils to exploit the transcellular pathway by enhancing intracellular signaling intensity. Rap1b–Akt signaling pathway may operate as a switch signal that controls the occurrence of invasive protrusions to balance paracellular and transcellular migration, at least in vitro.

ALL is triggered by excessive neutrophil infiltration of the airway wall. Neutrophil emigration into the lung takes place via the small capillaries, in contrast to the systemic circulation where neutrophils enter tissue via postcapillary venules. The lumen of the pulmonary capillary is extremely narrow (2–15 µm) preventing rolling along the endothelium (Brown et al., 2006; Grommes and Soehnlein, 2011); thus, neutrophil emigration in the lungs is largely integrin dependent (Brown et al., 2006). Hence, Rap1b regulatory signaling in immune cells has far reaching importance for inflammatory processes. Such understanding may help in designing effective therapeutics for treating acute and chronic inflammatory diseases.

MATERIALS AND METHODS

Antibodies and reagents. Antibodies against CD/LFA-1 (2D7), o/α-Mac1/CD11b (M1/70), B2 (C17/16), and Rap1 were obtained from BD. Human anti-ICAM-1 (HAI8) and B1 (EBtensor1-1) were obtained from ebioscience. Phospho–Akt (Ser473), Akt, p-p38, p38, p-Erk, Erk, Rap1b, and anti–VE-cadherin (clone 55-7H1) were purchased from Cell Signaling Technology. Anti–SHP-1 antibody was obtained from Santa Cruz Biotechnology, Inc. Rhodamine-labeled phallolidin, IgG conjugated to Alexa Fluor 488, Alexa Fluor 594, Alexa 6Fluor 47, or Cy5 were obtained from Invitrogen. Anti–PIP3 antibody and PTEN inhibitor SF-1670 were from Echelon Biosciences. Antibody used for immunoblotting of CD11b was from Abcam, Src-inhibitor PP2, SHP-1/2 inhibitor NSC87877, and serine–protease inhibitor DFP were obtained from EMDBillipore. Akt-inhibitor MK2206 was obtained from Selleckchem. Rac inhibitor NSC23766 was a gift from Y. Zheng (Cincinnati Children’s Hospital Medical Center, Cincinnati, USA). Anti–β2-integrin antibody and PEG inhibitor Ly294002 were obtained from Sigma-Aldrich.

Mice strains. The Rap1b-null mice (Rap1b−/−) C57BL/6 background > 10 generations) were described previously (Chrzanowska-Wodnicka et al., 2005). CD11b-deficient animals (B6) were purchased from The Jackson Laboratory. All experimental procedures and animal protocol were approved by the Cincinnati Children’s Institutional Animal Care and Use Committee in accordance with AAALAC accreditation standards.

LPS-induced lung inflammation. For BAL and histology, the mice were challenged with 1.25 mg/kg LPS from Escherichia coli O111:B4 by intratracheal instillation after ketamine and xylazine anesthesia. BALs and lung histology were performed as previously described (Filippi et al., 2007; Kumar et al., 2012). For survival curve analysis, 20 mg/kg LPS was injected i.p. in 300 µl saline solution. When indicated, mice were pretreated with MK2206 (15 mg/kg) or vehicle control DMSO one hour before LPS challenge. Mice were followed for 6 d, and then, survived mice were sacrificed.

Neutrophil migration in vitro. Neutrophil isolation was previously described (Szczur et al., 2009; Kumar et al., 2012). Chemokinesis or random migration was analyzed by using chemotactrant in both well of Boyden system and Transwell plates, whereas chemotaxis was recorded by adding chemotactrant in the lower well as described previously (Szczur et al., 2009). Time lapse video microscopy was performed in a Sigmund chamber (Neuro Probe) on a surface coated with fibrinogen in gradient of 10 µM fMLP as previously described (Szczur et al., 2009). Migration was recorded with an Axiovert 200 microscope (Carl Zeiss) at 10×/0.3 NA objective, equipped with ORCA-ER camera (Hamamatsu) and driven by ImageJ software (National Institutes of Health). Analysis of cell migration, speed (Sp), and straightness (Sr; distance from origin to total distance covered) of migration was performed in the motile population that had moved >20 µm using ImageJ software. Quantifications were performed on at least 30 cells from individual experiment/video and from 3 independent experiments.

Neutrophil adoptive transfer. Neutrophils isolated from WT or Rap1b−/− bone marrow were labeled with CFSE (green; 2.5 µM; Invitrogen) and chloromethyl SNARF-1 acetate (red; 5 µM; Invitrogen) or vice-versa, at 37°C for 10 min. The labeled cells were mixed at a 1:1 ratio and transfer into WT recipient though i.v. injection at the time of LPS challenge. The relative amounts of labeled neutrophils of the mixed population recovered after transfer in the blood and in BALF were analyzed by flow cytometer (FACS-Canto; BD; Kumar et al., 2012). Relative neutrophil recruitment was calculated as the ratio of Rap1b−/− relative to WT neutrophils.
Immunofluorescence. Neutrophils were stimulated with fMLP in HBSS containing 0.1% BSA, 1 mM Ca²⁺, 1 mM Mg²⁺, and on fibrinogen-coated slides or anti-CD11b-coated slides for 0–10 min at 37°C. The anti-CD11b-coated slides were used for antibody-mediated cross-linking experiments to enforce CD11b activation (Kumar et al., 2012). All samples were fixed with 2% PFA at room temperature, until specified. To examine the intracellular distribution of various proteins, the cells were permeabilized with 0.1% Triton X-100 and stained for the intracellular protein. For PI3P staining, cells were fixed with 4% PFA and permeabilized with 0.1% saponin for 10 min and stained with PIP3 and appropriate fluorescently labeled secondary in the presence of 0.05% saponin. Fluorescence images or Z series of fluorescence images were captured using a Leica DMi6000 fluorescence microscope at 63x/1.3 NA objective, with ORCA-ER C4742-95 camera (Hamamatsu) driven by Openlab software (Szzczur et al., 2009; Kumar et al., 2012). Z series were analyzed by deconvolution using Velocity (Improvement). Fluorescence intensity quantifications were performed in Openlab, Velocity, and ImageJ softwares (Szzczur et al., 2009) on cells that show increased spreading and positive F-actin polymerization in response to the stimulation. Quantifications shown in the study are represented as mean ± SD and are from at least 30 cells per experiment and representative of at least 3 independent experiments.

Crawling/transendothelial migration assay. Confluent monolayers of endothelial cells (HUVECs and hEnd3 murine brain endothelial cells) were grown on hydrated collagen gels in DMEM (American Type Culture Collection [ATCC]) plus 20% FBS, as in previous works (Muller, 2011; Schenklen et al., 2004). Endothelium monolayers were incubated with or without LPS (2 µg/ml) for 4–6 h in a CO₂ incubator for activation. Neutrophils were washed in HBSS plus 0.1% bovine serum albumin, were resuspended at a density of 2 × 10⁶ cells/ml in DMEM plus 0.5% FBS for crawling and transendothelial migration. Neutrophils were incubated on activated endothelial cells for 15–30 min, after incubation monolayers were washed twice with PBS for removal of nonadherent cells and the remaining adherent and transmigrated cells were fixed in place on the endothelial monolayer by overnight incubation in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. Total number of cells per high-power field was determined by counting cells both on the apical surface and in the collagen. For quantification of transendothelial migration, neutrophils that were below endothelial monolayers were counted and divided by the total number of cells per high-power field of bright-field microscope. For identification of neutrophil position on endothelial monolayer, endothelial cell junctions were stained with silver staining. Endothelial monolayers with neutrophils were washed once in PBS and twice in 5% glucose, and then stained with 0.5% silver nitrate/1% ammonium bromide/3% cobalt bromide before fixation in 5% phosphate buffered formalin. The cells were examined by microscope for quantification of neutrophil position on endothelial cells. Where indicated, neutrophils were pretreated with the Src-inhibitor PP2 (10 µM), Akt-inhibitor MK2206 (2 µM), or an equal dilution of vehicle (DMSO) for 30 min.

Quantification of transcellular and paracellular diapedesis. Endothelial hEnd3 and HUVEC monolayers were grown on 0.2% gelatin-coated 30-mm glass bottom discs in DMEM medium (ATCC) with 20% FBS and were not activated or activated for 4–6 h with LPS (2 µg/ml) before use. Endothelial cells were washed five times before addition of neutrophils with secondary fluorescence antibodies for 45 min in the presence of 0.05% saponin. After washing, stained monolayers were mounted with slow-fade containing DAPI to visualize nuclei.

Image acquisition and processing. Confocal imaging was performed with Nikon A1R. Confocal Laser Scanning Microscope (Nikon) using a 40x and 60x water objective. For serial z-stacks, the section thickness was between 0.2 and 0.4 µm. Image processing, including background subtraction, three-dimensional reconstruction, tiles view, and projection of Z-stacks were performed with NIH-elements image analysis software version 4.01. Images were then exported to Photoshop software for preparation of final images. For identification of neutrophils with transmigratory cups that were enriched with ICAM-1 microvilli, large images (4 × 4 fields) were captured with an open pinhole on Nikon A1R. Confocal Laser Scanning Microscope using 60X water objective with 15% overlap. Percentages of WT and Rap1b−/− neutrophils with transmigratory cups were calculated from 4 independent experiments.

Transmission electron microscopy. Endothelial (HUVEC) monolayers grown on gelatin-coated ALCAR membranes, with or without LPS activation, were incubated with neutrophils for 10 min at 37°C. Cells were fixed with 2.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h, postfixed in 1.5% buffered OsO₄ for 1 h, dehydrated in a series of graded alcohols, and embedded in Epon 812. ALCAR membranes were then separated from the endothelial monolayer. Thin sections of 100 nm were cut perpendicular to the monolayer or long axis of the block (and to the plane of the monolayer) and collected on copper single-slot (1 × 2 mm) grids coated with Formvar and stained with uranyl acetate. Cells were visualized with a Philips CM-10 electron microscope at 80 kV (Shulman et al., 2009). Quantification of length of microvillus protrusions and invasive protrusions were performed in ImageJ software from at least 30 cells from 3 experiments with >120 protrusions.

F-actin quantitation by flow cytometry. Flow cytometry was used to measure the relative amount of filamentous actin per neutrophil, as reported previously (Roberts et al., 1999; Kumar et al., 2012). Neutrophils were stimulated with fMLP in HBSS containing 0.1% BSA, 1 mM Ca²⁺, and 1 mM Mg²⁺ at 37°C. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with rhodamine/phalloidin (1:40). The results are reported as arbitrary units of mean cellular fluorescence (AU). To normalize data between experiments, the mean cellular fluorescence of untreated wild-type neutrophils was arbitrarily assigned a value of 100.

ECM degradation assay. Matrix degradation was performed by using fluorescent gelatin (Oregon Green; Molecular Probes) as previously described (Artym et al., 2006). To assess the ability of cells to degrade matrix, 5 × 10⁴ WT and Rap1b−/− neutrophils were stimulated on coverslips coated with fluorescent gelatin matrix for 10 min at 37°C. Cells were then fixed with 4% paraformaldehyde for 15 min at room temperature and followed by permeabilization with 0.1% Triton X-100 in PBS. Cells were then washed with PBS, labeled with rhodamine/phalloidin for F-actin staining. Foci of degraded matrix were identified as dark areas that lack fluorescence and appear as holes in the bright fluorescent gelatin matrix. Number of holes per 20X field and degraded area were calculated from three to five independent experiments.

Zymography assay. Neutrophils (10⁶ cells/ml) were stimulated with fMLP (5 µM) in HBSS-0.1% BSA for indicated time points at 37°C. Supernatants were collected after centrifugation at 13,000 rpm for 5 min at 4°C. The supernatants (5 µl) were mixed with a nonreducing buffer (40% glycerol, 1 M Tris-HCl, pH 6.8, and 8% SDS) and separated on 7.5% acrylamide gels containing 0.1% Triton X-100 in buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM CaCl₂) for 5 min at 37°C. Supernatants were incubated overnight in digestion buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and

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5 mM CaCl2) for digestion of gelatin. The gels were stained with Coomassie blue 0.1% and destained. Densitometric analysis of clear band at 92 kD that corresponds to gelatinase was performed by using ImageJ software.

**Adhesion assay.** WT and Rap1b−/− neutrophils were resuspended in HBSS buffer containing 0.1% BSA. Cells were added to the coverslips coated with different ligands and stimulated for 5 min at 37°C. The assay was stopped by immersing the coverslip in 4% formaldehyde solution. The adherent cells were counted (the mean number of cells per field of view was calculated from five fields of view) using an Axiovert 200 (Carl Zeiss) with a 40× objective.

**Preparation of WCLs and DRM fractions, immunoprecipitation, and Western blotting.** To examine protein expression in various cellular domains, neutrophils were either maintained in suspension without stimulation or stimulated with fMLP in suspension or plated on fibrinogen-coated/anti-CD11b-coated plates for specific times, as indicated in the figures. WCL was performed using standard triton-based lysis buffer. Triton insoluble or DRM fractions were prepared as described before (Kumar et al., 2012). Cell lysates containing equal amounts of protein were separated by SDS-PAGE and probed for p-Akt, Akt, p-p38, p38, Erk, and Rap1b (all from Cell Signaling Technology, Boston, MA), actin (Sigma-Aldrich), CD11b (Abcam), and Rap1 (BD). For immunoprecipitation, rested or stimulated WT and Rap1b−/− neutrophils were lysed in 1% NP-40 lysis buffer and 500 µg protein was subjected to each immunoprecipitation with 2 µg anti-SHP-1 antibody, followed by protein A/G agarose beads. Blots were probed with anti-phospho-tyrosine 4G10 antibody, followed by anti-SHP-1 (Santa Cruz Biotechnology, Inc.) antibody.

**Measurement of calcium signaling.** WT and Rap1b−/− neutrophils were loaded with Fura-3 AM (1 µg/ml) for 30 min at 37°C in HBSS. Cells were analyzed by flow cytometry (FACScanto II) for time kinetics and activated by different concentration of fMLP or Ionomycin. Intracellular calcium release kinetics were analyzed using FlowJo software (Tree Star).

**Proteomics analysis of DRMs.** Proteomics analysis of WT and Cdc42−/− neutrophil DRM fractions was performed using LCMS/MS orbitrap according to standard protocol at Ohio State University, Ohio.

**Superoxide generation.** WT and Rap1b−/− neutrophils (1 × 106) in suspension were incubated with H2-DCFDA in accordance with the manufacturer’s instructions (Molecular Probes). Cells were then stimulated with 1–10 µM fMLP (Sigma-Aldrich) for 15 min before FACS analysis for mean fluorescence intensity. Fold change stimulation was calculated by normalization of fMLP-stimulated neutrophils fluorescence intensity to unstimulated control WT cells. For superoxide production under adherent conditions, neutrophils (2 × 105) were incubated with 10 µM of advanced luminol derivative L012 (8-amino-5-chloro-7-phenylpyrrolo [3,4-d] pyridazine-1,4(2H,3H) dione), 10 nM horseradish peroxidase (HRP; Sigma-Aldrich) in HBSS buffer for 10 min on a fibrinogen-coated 96-well plate (Greiner Bio One). Reactions were started by addition of 30 µl of stimuli fMLP (0.5–10 µM) or PMA (20 nM). Chemiluminescence was measured at every 5 s for 20 min using GloMax-Microplate Luminometer (Promega).

**Statistics.** All the experiments were performed at least three times. An unpaired Student’s t test (normally distributed) was performed as statistics using Prism 5 software (GraphPad) for comparison of experimental groups unless specified. For survival curve statistics analysis, Log-rank test was used. Data are mean ± SD. The p-value of *P < 0.05; **P < 0.01; and ***P < 0.001 were considered as significant.

**Online supplemental material.** Video 1 shows migration of Rap1b−/− neutrophils in gradient of fMLP and on fibrinogen–coated surface. Video 2 shows migration of WT neutrophil in gradient of fMLP and on fibrinogen–coated surface. In both videos, fMLP concentration increases from the left to the right. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131706/DC1.
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Video 1. Rap1b−/− neutrophil migration was examined in Boyden chamber in a gradient of fMLP. fMLP concentration increases from the left to the right.

Video 2. WT neutrophil migration was examined in Boyden chamber in a gradient of fMLP. fMLP concentration increases from the left to the right.