Immunity
Article

Integrin-Induced PIP5K1C Kinase Polarization Regulates Neutrophil Polarization, Directionality, and In Vivo Infiltration

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DOI 10.1016/j.immuni.2010.08.015

SUMMARY

Neutrophils are important in innate immunity and acute inflammatory responses. However, the regulation of their recruitment to sites of inflammation has not been well characterized. Here, we investigated the kinase PIP5K1C and showed that PIP5K1C deficiency impaired neutrophil recruitment because of an adhesion defect. PIP5K1C regulated the adhesion through facilitating RhoA GTPase and integrin activation by chemoattractants. Integrins could induce polarization of an isoform of PIP5K1C, PIP5K1C-90, in neutrophils through intracellular vesicle transport independently of exogenous chemoattractant. PIP5K1C-90 polarization was required for polarized RhoA activation at uropods and provided an initial directional cue for neutrophil polarization on the endothelium. Importantly, the polarization was also required for circumventing the inhibition of lamellipodium formation by RhoA so that neutrophils could form leading edges required for transendothelial migration. Because integrins are not known to regulate neutrophil polarization, our study revealed a previously underappreciated role of integrin signaling in neutrophil regulation.

INTRODUCTION

Neutrophils are one of the key players in acute inflammatory responses. They play an important role in host defense and contribute to inflammation-related tissue injuries. During the inflammation, neutrophils extravasate across the endothelium that lines the blood vessel wall through a multistep process (Luo et al., 2007; Rose et al., 2007), including the rolling on and subsequent firm adhesion to endothelial cells. In mouse neutrophils, the β2 integrins are important in mediating the adhesion of neutrophils to endothelial cells. After neutrophils transmigrate through the endothelium, they migrate to the sites of injury and infection in response to chemoattractant gradients. Thus, understanding the mechanisms for neutrophil recruitment is of great physiological and pathophysiological significance.

Neutrophils are the most motile cells in higher organisms and can efficiently interpret and chemotax under a shallow chemoattractant gradient. Although it remains unclear how, these cells can translate a small chemoattractant gradient into intracellular biochemical polarization and align the biochemical polarity with the chemoattractant gradients (Franca-Koh et al., 2007; Janetopoulos and Firtel, 2008; Rericha and Parent, 2008; Wu, 2005). In neutrophils, the biochemical polarization includes the localization of molecules such as phosphatidylinositol 3,4,5-trisphosphate and the small GTPases Rac and Cdc42 in the front and the GTPase RhoA, phosphorylated myosin light chain (pMLC) (Weiner, 2002; Wu, 2005; Xu et al., 2003), the RhoA guanine nucleotide exchange factor PDZRhoGEF (Wong et al., 2007), phosphorylated ezrin-radixin-moesin (pERM) (Lacalle et al., 2007; Lokuta et al., 2007), and phosphatase and tensin homolog (PTEN) (Heit et al., 2008; Li et al., 2003, 2005; Wu et al., 2004) in the back. The biochemical polarization subsequently leads to cellular polarization into a leading edge (front) and uropod (back). The leading edge contains lamellipodia composed of unbundled F-actin, continuous formation of which provides a driving force for cell locomotion. The uropod contains actomyosin filaments. Contraction of these filaments can provide another locomotive force to push cells forward. Thus far, chemoattractant signaling is believed to be exclusively responsible for regulating the directionality of neutrophil migration.

Phosphatidylinositol 4,5-bisphosphate [PtdIns (4,5)P_2] represents about 1% of plasma membrane phospholipids and is important in various cellular functions (De Matteis and Godi, 2004; Di Paolo and De Camilli, 2006; Ling et al., 2006). In mammalian cells, PtdIns (4,5)P_2 is primarily synthesized by

sequential phosphorylation of PtdIns by two PtdIns kinases, PI4K and PIP5K1. To date, three mammalian PIP5K1 isoforms (A, B, and C) have been identified. PIP5K1C has two major splicing variants: a short 87 kDa protein (PIP5K1C-87) and a longer one with 28 additional amino acids at its C terminus (PIP5K1C-90). PIP5K1C-90 was shown to be localized in the uropods of chemotaxing neutrophils (Lokuta et al., 2007), whereas PIP5K1B was found in the uropods of polarized human neutrophil-like HL-60 cells (Lacalle et al., 2007). There are two reported *Pip5k1c*$^{-/-}$ mouse lines, both of which show early lethality (Di Paolo et al., 2004; Wang et al., 2007a).

In this report, we studied PIP5K1C-deficient mouse neutrophils and found that PIP5K1C deficiency did not impair neutrophil chemotactic activities in vitro, but it compromised neutrophil infiltration in vivo. In our investigation, we discovered that integrins could induce PIP5K1C-90 polarization independently of chemoattractants. This integrin-induced PIP5K1C-90 polarization works together with chemoattractant signaling in regulating neutrophil polarization and directionality in vitro and infiltration in vivo.

RESULTS

PIP5K1C Deficiency Impairs Neutrophil Recruitment In Vivo

To investigate the role of PIP5K1C in neutrophil regulation, we generated neutrophils lacking PIP5K1C by reconstituting lethally irradiated mice with fetal liver cells from a *Pip5k1c*$^{-/-}$ line (Di Paolo et al., 2004). Neither PIP5K1C mRNA nor protein could be detected in neutrophils prepared from the transplanted mice (Figures S1A and S1B available online).

The regulation of PtdIns(4,5)P_2 by PIP5K1C in mouse neutrophils was examined by visualizing the localization of the phospholipase C δ-pleckstrin homology (PLC δ-PH) domain, which binds PtdIns(4,5)P_2 in a specific manner (Lemmon et al., 1995). Most of wild-type neutrophils undergoing chemotaxis exhibited uropod polarized PLC δ-PH GFP distribution (Figure S1C upper panels and Movie S1). However, in *Pip5k1c*$^{-/-}$ neutrophils, there was a lack of increased localization of PLC δ-PH-GFP in the uropods (Figure S1C lower panels and Movie S2), suggesting that PIP5K1C may be responsible for the increased amount of PtdIns(4,5)P_2 at the uropods.

Next, we investigated the effect of PIP5K1C deficiency on neutrophil recruitment in vivo by using a peritonitis model. Purified wild-type neutrophils and *Pip5k1c*$^{-/-}$ neutrophils were labeled with different cell tracing dyes or vice versa (Jia et al., 2007) and mixed at a 1:1 ratio. The mixed cells were injected into tail veins of wild-type mice, in which acute peritoneal inflammation was induced by intraperitoneal injection of thioglycolate. Significantly lower numbers of *Pip5k1c*$^{-/-}$ than wild-type transplanted neutrophils were recruited into the peritonea (Figures 1A and 1B). We next extended our observations to a gout model by examining the effects of PIP5K1C deficiency in neutrophil recruitment into preformed air pouches after injection of monosodium urate (MSU) crystals (Chen et al., 2006). There were significantly lower numbers of neutrophils (CD11b$^+$Ly6G$^+$) in the lavages from *Pip5k1c*$^{-/-}$ cell transplanted mice than those from the wild-type cell transplanted mice (Figure 1C). Thus, PIP5K1C deficiency impairs the in vivo neutrophil infiltration in both of the models.

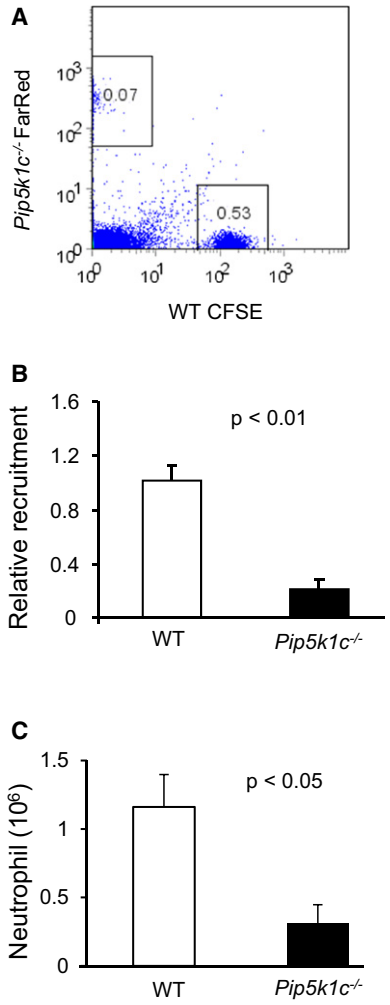

Figure 1. Effect of PIP5K1C Deficiency on Neutrophil Infiltration In Vivo

(A and B) The peritonitis model. Mice were injected with labeled wild-type (WT) and *Pip5k1c*$^{-/-}$ neutrophils. The number of the WT cells (taken as 1) was compared with that of the mutant cells in the inflamed peritonea. A representative flow cytometric chart is shown in (A). Summary shown in (B) (n = 3, Student's t test).

(C) The gout model. The numbers of neutrophils in the lavages from the pouches are shown as means ± SEM (n = 4, Student's t test).

PIP5K1C Deficiency Does Not Impair Neutrophil Chemotaxis In Vitro

Previous studies with overexpression of PIP5K mutants suggest that PIP5K including PIP5K1C plays a positive role in regulating neutrophil chemotaxis (Lacalle et al., 2007; Lokuta et al., 2007). We found that *Pip5k1c*$^{-/-}$ cells showed no defects in their directionality and motility compared to wild-type cells under an fMLP gradient (Figures 2A and 2B), though the mutant cells appeared to be more elongated than the wild-type cells (data not shown). On the contrary, *Pip5k1c*$^{-/-}$ neutrophils appeared to follow the chemoattractant gradient more faithfully than the wild-type cells because they had significantly smaller average directional errors than the wild-type cells on fibrinogen-coated surface (Figure 2C). However, this directional error difference became less significant

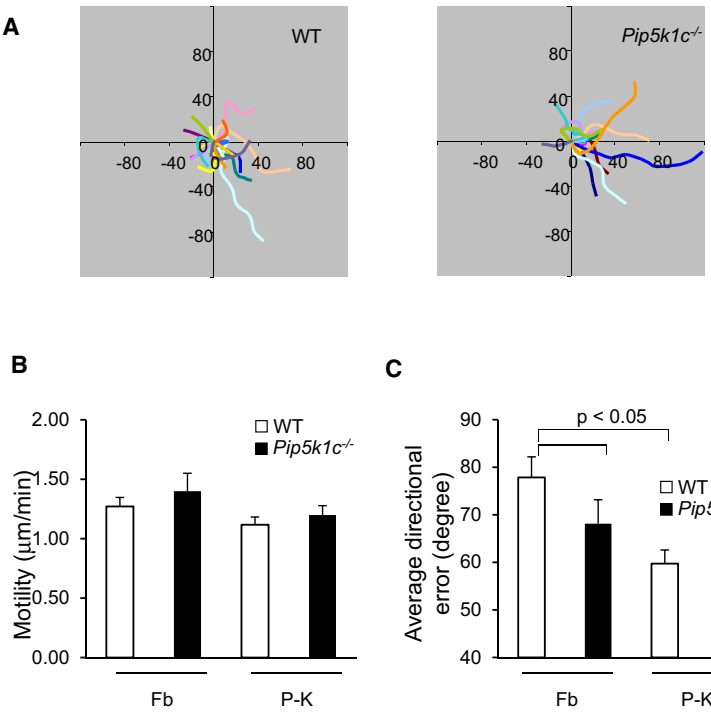

Figure 2. Effect of PIP5K1C Deficiency on Neutrophil Chemotaxis

Chemotaxis of wild-type or PIP5K1C-deficient neutrophils in response to an fMLP gradient was analyzed with a Dunn Chamber coated with fibrinogen (Fb) or polylysine (P-K).

(A) Cell migration traces of a representative experiment on a fibrinogen surface. Seven independent experiments with seven pairs of wild-type and mutant mice were performed.

(B and C) Motility (B), which reflects the speed of cell movement, and average gradient errors (C), which reflect how well cells follow the chemoattractant gradient, were calculated from the these migration traces as described in Experimental Procedures. Mean ± SEM; paired Student's t test, n = 7.

on the polylysine surface (Figure 2C). These results in Figure 2C can also be interpreted to suggest that there is a substantial difference in gradient errors or directionality between fibrinogen-coated and polylysine-coated surfaces for wild-type neutrophils, whereas the difference became insignificant for *Pip5k1c*$^{-/-}$ cells. Because there may be more integrin activation in cells on fibrinogen than on polylysine (Kim et al., 2006), these results imply that there might be a connection between integrins and PIP5K1C in regulating neutrophil directionality.

Integrins Induce Polarized Localization of PIP5K1C-90 Independently of Chemoattractants

PIP5K1C-90, which is expressed five times more than PIP5K1C-87 in mouse neutrophils based on quantitative RT-PCR analysis (data not shown), was shown to polarize in neutrophils upon fMLP stimulation (Lokuta et al., 2007). In reproducing these findings, we observe that GFP-PIP5K1C-90 could polarize in mouse neutrophils in the absence of any exogenous chemoattractant (Figure 3A). Up to 80% of neutrophils expressing GFP-PIP5K1C-90 showed the polarized localization of PIP5K1C on the fibrinogen surface compared to less than 20% on the polylysine surface (Figure 3B). Figure S2A and Movie S3 show three-dimensional reconstruction of a neutrophil in which GFP-PIP5K1C-90 is polarized. Moreover, endogenous PIP5K1C-90 could polarize on fibrinogen (Figure S2B). It is important to note that, in the absence of a chemoattractant, fibrinogen did not overtly polarize the distribution of F-actin, which was primarily localized in the cortex (Figure 3A; Figures S2A and S2B). The differential effects of fibrinogen and polylysine prompted us to hypothesize that integrins may regulate PIP5K1C-90 polarization. We therefore tested ICAM-1, a ligand for the β2 integrins abundantly found on neutrophils. ICAM-1 could also induce GFP-PIP5K1C-90 polarization (Figure S2C). In addition, ICAM-induced polarization was reduced in neutrophils isolated from a mouse line in which β_2-intergrin is expressed at ~10% of the normal amount (Wilson et al., 1993). Moreover, a neutralizing β_2 integrin antibody or expression of an integrin dominant-negative mutant, in which the intracellular domain of the IL-2 receptor was replaced with the β_2 integrin intracellular domain (Smilenov et al., 1994), inhibited PIP5K1C-90 polarization (Figure 3B; Figure S2C). Thus, integrins, particularly β_2-integrin, are involved in polarized localization of PIP5K1C-90 in mouse neutrophils. This conclusion is further confirmed by the fact that neutralizing α_L or α_M integrin antibody inhibited ICAM1-induced PIP5K1C-90 polarization (Figure S2D). Thus, both $\alpha_L\beta_2$ (LFA-1) and $\alpha_M\beta_2$ (MAC-1) integrins participate in PIP5K1C-90 polarization.

To examine the kinetics of GFP-PIP5K1C-90 polarization, we acquired time-lapsed images of GFP-PIP5K1C-90-expressing neutrophils that flowed over fibrinogen-coated surfaces. Polarization appeared to start only when cells stopped or were close to stopping moving, presumably because of integrin engagement (Figure 3C; Movies S4 and S5). The polarization process was completed in 1–2 min. Of note, during this polarization process, the shape of these cells remained largely round rather than overtly elongated, as found in chemoattractant-stimulated cells. Intriguingly, GFP-PIP5K1C-90 always polarized in the alignment of the cell movement direction in all of the cells we examined. We also tested neutrophils expressing GFP-PIP5K1C-90 with a flow chamber coated with mouse endothelial cells. In all of the cells we observed (n = 35), GFP-PIP5K1C-90 was polarized in general alignment of the flow direction (Figure 3D). These results together suggest that the cell movement direction on the substrate prior to their arrest may provide a directional cue for GFP-PIP5K1C-90 polarization.

The C Terminus of PIP5K1C-90 Has an Important Role in Its Polarization

We tested the short splicing variant of PIP5K1C, PIP5K1C-87 (Figure S3A) and showed that it could not polarize (Figure 3B; Figure S3B), suggesting that the 28 extra amino acids found only in PIP5K1C-90 are required for integrin-induced polarization. These 28 amino acids are required for the binding to talin and adaptin-2 (AP2) proteins, and various point mutations in this

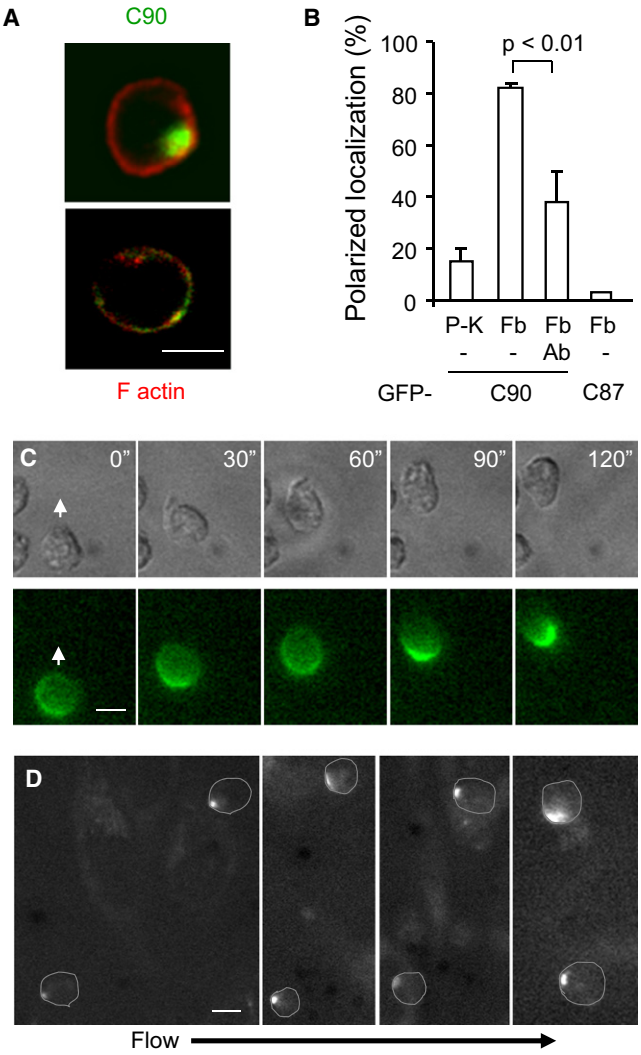

Figure 3. PIP5K1C-90 Polarization in Mouse Neutrophils

(A) Localization of GFP-PIP5K1C-90 (C90) and F-actin (Alex633-phalloidin staining) in mouse neutrophils placed on fibrinogen (top) or polylysine (bottom).

(B) Quantification of cells showing polarized distribution of GFP-PIP5K1C-90 and GFP-PIP5K1C-87 (C87) in the presence or absence of a neutralizing β2 integrin antibody (Ab) on different surfaces. Quantification was done in triplicate, and at least 50 cells were examined for each data point (mean ± SD, Student's t test).

(C) Time-lapsed phase (top) and fluorescence (bottom) images of a representative GFP-PIP5K1C-90-expressing neutrophil moving on a fibrinogen surface (n = 8). The arrow indicates the moving direction. Selected images from Movies S4 and S5 are shown.

(D) Directionality of GFP-PIP5K1C-90 polarization in neutrophils adhered to endothelial cells under a flow condition. Thirty-five GFP-PIP5K1C-90-expressing cells were examined. Eight randomly selected cells are shown.

Scale bars represent 8 μm.

sequence can disrupt these interactions (Di Paolo et al., 2002; Lee et al., 2005; Ling et al., 2002; Thieman et al., 2009). We confirmed that L652S, S650D, Y649F, and W647F mutations disrupted the interaction of PIP5K1C-90 with the AP2β subunit (Figure S3C), and these mutations except Y649F also disrupted the interaction with talin (Figure S3D). Because all of these mutations including Y649F impaired the polarization (Figure 4A), we suspected that the interaction of PIP5K1C-90 with AP2 might have an important role in PIP5K1C-90 polarization upon integrin engagement.

Involvement of Vesicle Transport in PIP5K1C-90 Polarization

AP2 is a key adaptor protein in the formation of intracellular transport vesicles, including those coated with clathrin. The localization of AP2 and clathrin relative to that of PIP5K1C-90 in neutrophils was examined. AP2 also showed polarized distribution in neutrophils placed on fibrinogen, but not polylysine, and there was colocalization of AP2 and PIP5K1C-90 (Figure 4B). Similar observations were also made with endogenous clathrin (Figure S3E) and clathrin-GFP (Figure S3F). There is also a partial colocalization of β_2-integrin and PIP5K1C-90 (Figure S3G). These results are consistent with the idea of an involvement of vesicle transport in PIP5K1C-90 polarization. Vesicle transport is regulated by various small GTPases including Arfs (ADP-ribosylation factor) and Rabs (D'Souza-Schorey and Chavrier, 2006; Myers and Casanova, 2008). We tested several dominant-negative (dn) mutants of small GTPases and found that dnArf6, but not dnArf4 (Figure 4C) or dnRab5 (data not shown), inhibited integrin-dependent PIP5K1C-90 polarization.

Actin filaments also play an important role in vesicle transport (Myers and Casanova, 2008). Treatment with Cytochalasin D, an actin polymerization inhibitor, resulted in reduction in GFP-PIP5K1C-90 polarization on fibrinogen (Figure 4D). Along the same line, expression of dnRac1, a small GTPase known to stimulate actin polymerization in neutrophils (Gu et al., 2003), also inhibited PIP5K1C-90 polarization on fibrinogen (Figure 4E). We also tested pertussis toxin, which inhibits the Gi class of heterotrimeric G proteins. It failed to inhibit PIP5K1C-90 polarization (Figure 4D), further confirming the noninvolvement of these G proteins in integrin-dependent PIP5K1C-90 polarization. As a control, the same pertussis toxin treatment abolished fMLP-induced increases in cytosolic Ca^{2+} concentrations in the neutrophils (data not shown). Thus, vesicle transport may be involved in PIP5K1C-90 polarization.

Phosphorylation of Tyr649 Is Important for PIP5K1C-90 Polarization

The importance of Tyr649, which can be phosphorylated by Src (Ling et al., 2003), in PIP5K1C-90 polarization suggests a possible involvement of Src in the polarization process. We therefore tested a Src inhibitor PP2, which inhibited PIP5K1C-90 polarization on fibrinogen (Figure 4F). Focal adhesion kinase (FAK) was shown to participate in integrin-mediated Src activation and PIP5K1C-90 phosphorylation at Tyr649 (Ling et al., 2003). Expression of two FAK inhibitors, FRNK and Y397F-FAK, inhibited PIP5K1C-90 polarization (Figure 4F). Furthermore, RhoB was shown to be involved in Src activation by integrins (Sandilands et al., 2004). Expression of dnRhoB, but not dnRhoA, inhibited PIP5K1C-90 polarization (Figure 4E). These results together support the conclusion that integrin-mediated Src activation has an important role in PIP5K1C-90 polarization.

Phosphorylation of PIP5K1C-90 at Tyr649 in neutrophils was further confirmed by using an antibody specific for phosphorylated Tyr649. The antibody only recognized PIP5K1C-90 coexpressed with an activated Src mutant, but not Y649F mutant or

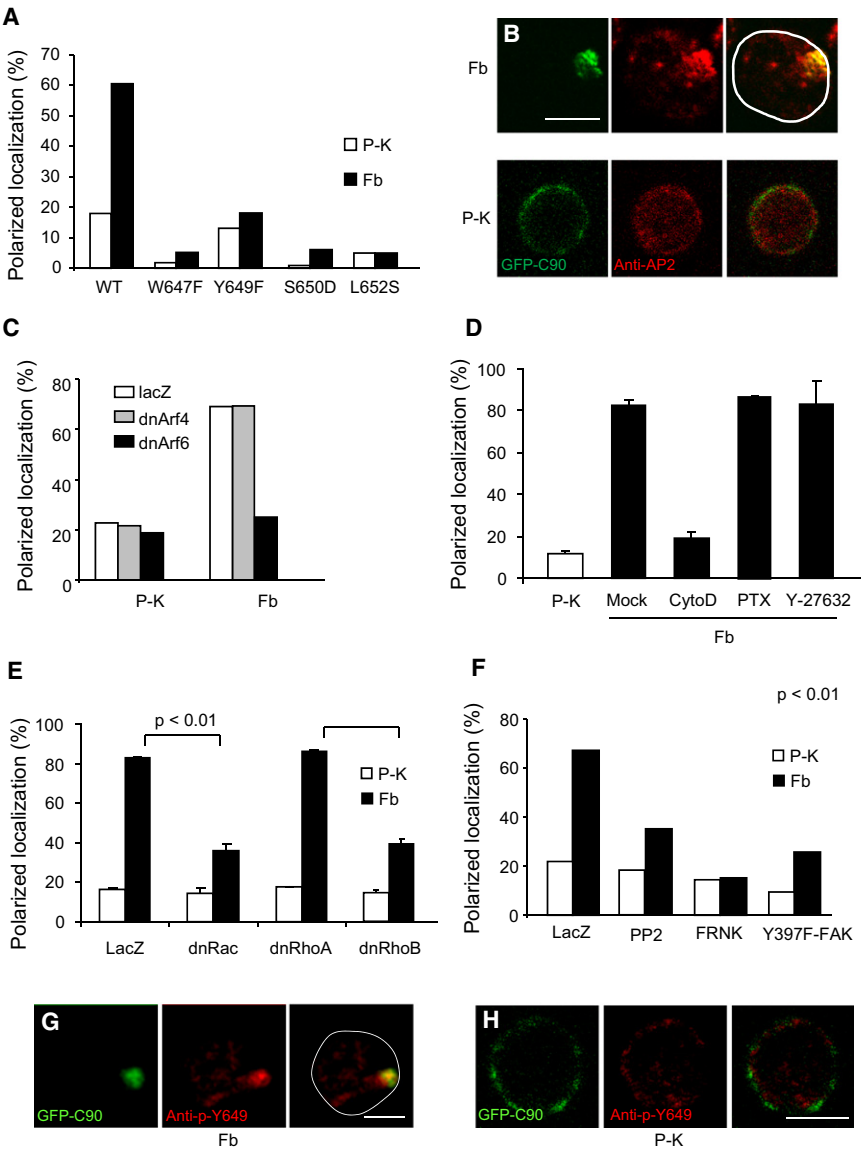

Figure 4. Involvement of Vesicle Transport and Tyr Phosphorylation in Integrin-Induced PIP5K1C-90 Polarization

(A) Quantification of cells showing polarized distribution of GFP-PIP5K1C-90 and its mutants on surfaces coated with polylysine or fibrinogen. Quantification was done in triplicate, and at least 50 cells were examined for each data point (mean ± SD, Student's t test).

(B) Localization of endogenous AP2 and GFP-PIP5K1C-90 in mouse neutrophils placed on fibrinogen (top) or polylysine (bottom). Representative cells are shown, n = 15.

(C–F) Effects of chemical inhibitors and dominant-negative mutants on GFP-PIP5K1C-90 polarization. Neutrophils were cotransfected with GFP-PIP5K1C-90 and LacZ or a dominant-negative mutant of Arf4 (dnArf4), Arf6 (dnArf6), Rac1 (dnRac), RhoA (dnRhoA), RhoB (dnRhoB), FRNK, or Y397F-FAK, or treated with Cytochalasin D, PP2, Y-27632, and PTX. Quantification was done in triplicate, and at least 50 cells were examined for each data point (mean ± SD, Student's t test).

(G and H) Localization of GFP-PIP5K1C-90 and anti-phosphorylated PIP5K1C-90 (pTyr649) immunostaining in transfected mouse neutrophils placed on fibrinogen (G) or polylysine (H). Representative cells are shown, n = 15. Scale bars represent 8 μm.

PIP5K1C-87 (Figure S3H). Immunostaining shows that Tyr649-phosphorylated PIP5K1C was detected at the same location as polarized PIP5K1C-90 in neutrophils (Figures 4G and 4H). Because the phospho-mimetic mutation of Tyr649 to Glu showed reduced interaction with AP2-β (Figure S3C), it is possible that Tyr649-phosphoryated PIP5K1C-90 also has a reduced affinity for AP2-β. Thus, Tyr649 phosphorylation may lead to the dissociation of PIP5K1C-90 from AP2 so that AP2 can be recycled while PIP5K1C stays. This idea is consistent with the partial colocalization of AP2 or clathrin with PIP5K1C-90 (Figure 4B; Figures S3E and S3F).

Integrin-Induced PIP5K1C-90 Polarization Is Colocalized with Chemoattractant-Induced Uropod Markers

Because integrins induce PIP5K1C-90 polarization independently of chemoattractants, it would be important to know the relationship of this integrin-induced polarity with the one induced by chemoattractants. As shown in a previous study (Lokuta et al., 2007), GFP-PIP5K1C-90 is colocalized with uropod makers including pERM (Figure S4A) and pMLC (Figure S4B) upon fMLP stimulation. Because fibrinogen alone could not induce polarized distribution of pMLC (Figure S4C) or GFP-moesin (Figure S4D) (the localization of GFP-moesin correlates with phosphorylated ERM localization in neutrophils [Lacalle et al., 2007; Lokuta et al., 2007]), integrin-regulated PIP5K1C-90 polarization does not depend on either pERM or pMLC polarization. These observations are consistent with the fact that neither dnRhoA (Figure 4E) nor the Rho kinase inhibitor Y-27632 (Figure 4D) inhibited PIP5K1C-90 polarization. RhoA, a small GTPase, is localized at the uropod of chemoattractant-polarized neutrophils and regulates actomyosin structure formation and functions including MLC phosphorylation via Rho kinase (Li et al., 2003, 2005; Wheeler and Ridley, 2004; Xu et al., 2003). Thus, although integrin-induced PIP5K1C-90 polarization is colocalized with the uropod markers induced by chemoattractants, it does not depend on these chemoattractant-regulated uropod markers.

PIP5K1C-90 Polarization Determines Initial Neutrophil Polarity and Directional Responses

Knowing that the integrin-induced PIP5K1C-90 polarization is independent of chemoattractant signaling, we investigated the

impact of PIP5K1C-90 polarization on chemoattractant-regulated neutrophil polarization and directional response. Mouse neutrophils expressing RFP-PIP5K1C-90 were placed on fibrinogen, followed by directional stimulation with fMLP. To assess real-time neutrophil responses, the cells were also cotransfected with YFP-actin (Figure 5A). The stimulation was applied either proximally or distally to polarized RFP-PIP5K1C-90 as shown in Figure 5A. Upon stimulation, the cells responded in about 30 s evidenced by polarized distribution of YFP-actin toward the micropipette (Movie S6). This polarized distribution of YFP-actin is presumably the result of rapid formation of F-actin, which primarily occurs at the lamellipodia upon chemoattractant stimulation.

Regardless of the positions of the stimulation, which did not markedly affect the rate of YFP-actin polarization, YFP-actin always started to polarize at the sites opposite to polarized RFP-PIP5K1C-90 (Figure 5A; Figure S4E, Movies S6 and S7). These observations indicate that the initial polarization of YFP-actin or formation of leading edges is determined by the location of PIP5K1C-90 polarization rather than the fMLP gradient. It is also important to note that cells with their PIP5K1C-90-rich structures near the pipette often started with more than one pseudopod pointing to different directions, which were always against the gradient (Figure 5A, lower panels; Figure S4E, lower panels, arrowheads). Although these pseudopods eventually formed consolidated singular lamellipodia, it took significantly longer for them to do so than for those with their PIP5K1C-90-rich structures distal to the pipette (Figure 5B). In a majority of cells that were stimulated by a pipette proximal to the PIP5K1C-90-rich structures, their lamellipodia were able, at the end, to turn toward the stimulation. Thus, we conclude that integrin-induced polarization of PIP5K1C-90 has an important role in regulating neutrophil response to directional chemoattractant stimulation. It determines where the leading edge, whose formation is stimulated by chemoattractants, can be initially formed regardless of the directional cue of a chemoattractant gradient, probably by specifying the uropod.

PIP5K1C Deficiency Impairs Neutrophil Adherence to Endothelial Cells

Knowing that PIP5K1C deficiency does not impede neutrophil chemotaxis, we sought other possible causes for the in vivo infiltration defects of *Pip5k1c*$^{-/-}$ neutrophils observed in Figure 1. We examined the interaction between neutrophils and endothelial cells, the first step in neutrophil infiltration in vivo, via a flow chamber assay. PIP5K1C deficiency significantly reduced the number of neutrophils that can firmly adhere to endothelial cells (Figure S5A). This defect may provide an explanation to the in vivo infiltration defects.

Intravital microscopic examination of the cremaster muscle venules was carried out to confirm the in vivo significance of the adhesion defect. Although PIP5K1C deficiency increased rolling flux, it reduced the number of cells adherent to the endothelium upon the treatment of TNF-α, particularly in the smaller vessels (Figures 6A and 6B). PIP5K1C deficiency had little effects on rolling velocity or rate of emigration (Figures 6C and 6D). These observations are consistent with those observed in the flow chambers and demonstrate that PIP5K1C is important for neutrophil firm adherence to endothelial cells in vivo.

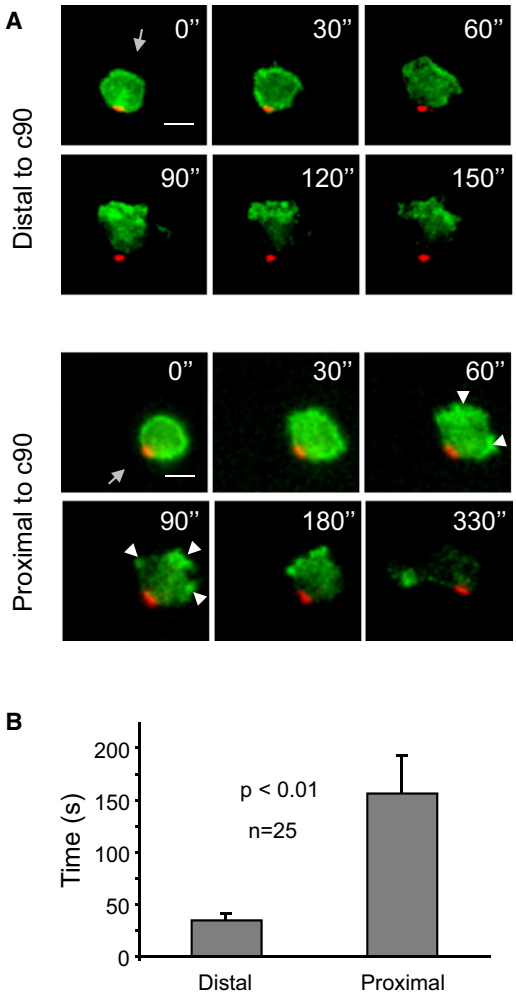

Figure 5. Effect of Integrin-Induced Polarization of PIP5K1C-90 on Neutrophil Responses to Directional Chemoattractant Stimulation

(A) Neutrophils coexpressing RFP-PIP5K1C-90 and YFP-actin were allowed to adhere to fibrinogen-coated coverslips. An fMLP gradient was applied with a micropipette. Images were recorded at 15 s intervals, and selected images from Movies S6 and S7 are shown. Arrows denote the locations of the micropipettes, whereas arrowheads denote the pseudopods. Scale bars represent 8 μm.

(B) The times for neutrophils to form singular consolidated leading edges that point toward the general direction of the micropipette (mean ± SD; Student's t test).

PIP5K1C-90 Is Involved in Polarized Activation of RhoA and fMLP-Stimulated Integrin Affinity Increases

Because there were no differences in the cell surface expression of CD18 (β2-integrins) between wild-type and *Pip5k1c*$^{-/-}$ cells (Figure S5B), the differences in their adherence to endothelial cells have to be attributed to other factors. RhoA, which is activated by endothelial cell-tethered chemokines, plays an important role in regulating monocyte and T cell adherence to endothelial cells under flow conditions (Giagulli et al., 2004; Honing et al., 2004). We found that PIP5K1C deficiency significantly reduced fMLP-induced activation of RhoA (Figure 7A). Consistent with this result, PIP5K1C deficiency decreased

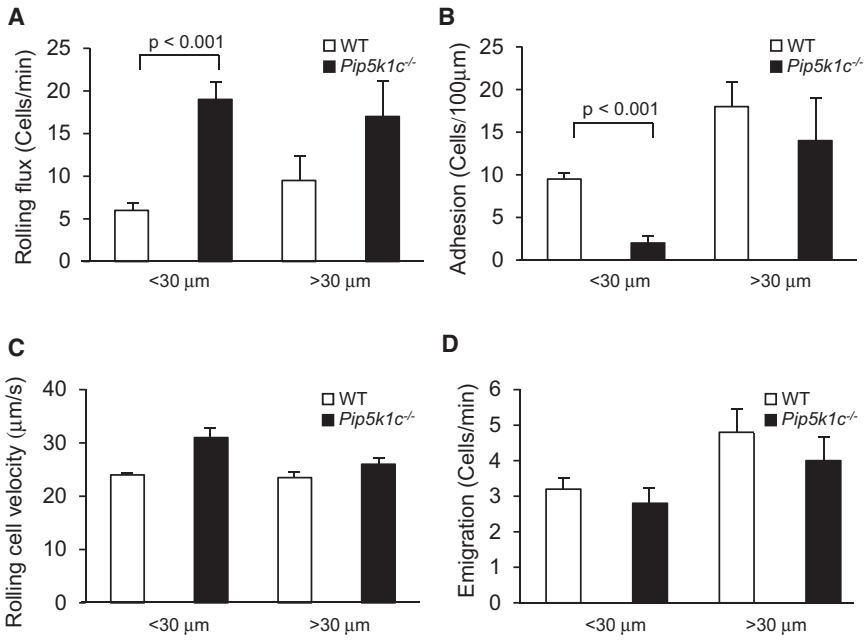

Figure 6. Effect of PIP5K1C Deficiency on Neutrophil-Endothelial Cell Interaction in Inflamed Cremaster Muscle Venues

Rolling flux (A), adhesion (B), rolling cell velocity (C), and emigration of neutrophils (D) were determined after stimulation of the cremaster muscle with TNF-α (0.5 μg) for 4 hr according to different vessel sizes (<30 μm = vessels between 20 and 30 μm of diameter; >30 μm = vessels between 30 and 45 μm of diameter). All values are means of n = 3 animals (with 5 to 7 vessels per animal) ± SEM.

RhoA-dependent phosphorylation of MLC (Figures 7B and 7C). Moreover, PIP5K1C deficiency decreased RBD staining at the uropods of neutrophils stimulated with fMLP (Figure S6A). RBD is a protein domain of rhotekin and preferentially binds to activated RhoA (Ren et al., 1999). All of these results indicate that PIP5K1C has an important role in RhoA activation by fMLP in mouse neutrophils.

RhoA is involved in chemokine-stimulated enhancement of integrin affinity in T lymphocytes (Giagulli et al., 2004). Consistent with the finding, PIP5K1C deficiency attenuated the binding of sICAM-Fc complexed with a Fc antibody (Figure 7D; Figure S6B) or sICAM directly conjugated with a fluorochrome (Figure S6C) to fMLP-stimulated neutrophils, without effects on the expression of cell surface β2-integrins (Figure S5B; data not shown). In addition, dnRhoA expression (Figure 7E) or Y-27632 treatment (Figure S6D) could reduce ICAM binding in response to fMLP. Moreover, PIP5K1C deficiency or inhibition of Rock reduced the ability of neutrophils to retain their adhesion to the endothelial cells under high shear flow (Figure S6E). Together with the lack of obvious effect of PIP5K1C deficiency on integrin clustering or lateral mobility (Figure S6F), we conclude that PIP5K1C may regulate neutrophil firm adhesion primarily through facilitating RhoA activation and integrin affinity increase.

PIP5K1C-Mediated RhoA Activation Polarization Has Biological Significance

The next key question is whether PIP5K1C polarization has biological significance. We addressed this question by comparing the two PIP5K1C isoforms: PIP5K1C-90 that can be polarized by integrins and PIP5K1C-87 that cannot. Expression of RFP-PIP5K1C-90 in *Pip5k1c*$^{-/-}$ neutrophils restored polarized localization of PLC δ-PH-GFP or PtdIns(4,5)P2 production at uropods (Figure S6G), whereas PLC δ-PH-GFP showed even distribution in cells expressing RFP-PIP5K1C-87 (Figure S6G). Upon fMLP stimulation, expression of either GFP-PIP5K1C-90 or -87 increased pMLC staining, a surrogate marker for RhoA activity (Figure 7F), which was only weakly detected in *Pip5k1c*$^{-/-}$neutrophils (Figure 7C). Expression of a kinase-dead form of GFP-PIP5KIC-87 led to little increases in pMLC staining in *Pip5k1c*$^{-/-}$ neutrophils (Figure S6H), suggesting that the lipid kinase activity or PtdIns(4,5)P2 may be responsible for the increase in pMLC and RhoA activity. However, there were two important distinctions between cells expressing GFP-PIP5K1C-90 and those expressing GFP-PIP5K1C-87. First, pMLC staining in GFP-PIP5K1C-90-expressing cells was highly polarized and concentrated at the vicinity of polarized GFP-PIP5K1C-90 (Figure 7F), whereas in cells expressing GFP-PIP5K1C-87, pMLC staining was broadly distributed similarly to the distribution of GFP-PIP5K1C-87 (Figure 7F). Second and more importantly, neutrophils expressing GFP-PIP5K1C-87 failed to form F-actin-rich lamellipodia, whereas those expressing GFP-PIP5K1C-90 did, in response to fMLP (Figure 7F). F-actin detected in GFP-PIP5K1C-87-expressing neutrophils was colocalized with GFP-PIP5K1C-87 and pMLC and appeared to resemble cortical actin. Expression of the kinase-dead form of GFP-PIP5K1C-87 did not affect the formation of F-actin-rich lamellipodia (Figure S6H), indicating that the effect of PIP5K1C-87 expression on lamellipodium formation depends on PtdIns(4,5)P_2. These results collectively suggest that although expression of PIP5K1C was able to restore fMLP-induced phosphorylation of MLC and probably RhoA activation, polarized localization of PIP5K1C-90, which is expressed much more than PIP5K1C-87 in mouse neutrophils, is required for polarized activation of RhoA. Failure to do so as in the case of expression of the nonpolarizable PIP5K1C-87 isoform would result in broad activation of RhoA and the inability to form lamellipodia. Consistent with the idea that lamellipodia are required for neutrophils to undergo transendothelial migration, neutrophils expressing PIP5K1C-87 showed markedly attenuated ability to migrate across a layer of endothelial cells in response to fMLP (Figure 7G). Therefore, polarized localization of PIP5K1C, manifested by integrin-induced PIP5K1C-90 polarization (Figure S6I), has important roles in regulating neutrophil polarization and infiltration.

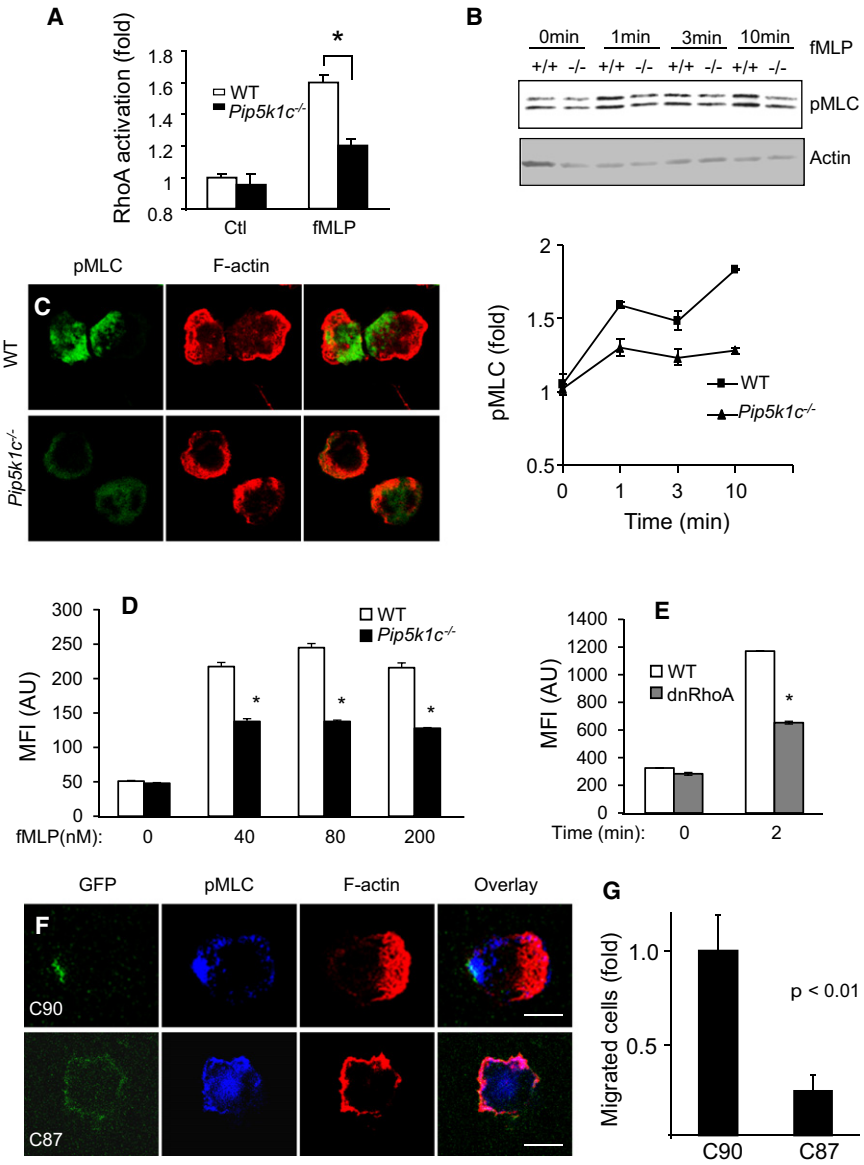

Figure 7. Effect of PIP5K1C Deficiency on Endothelial Adhesion and RhoA Regulation

(A) Effect of PIP5K1C deficiency on fMLP-induced activation of RhoA. Wild-type and PIP5K1C null neutrophils were stimulated with 4 μM fMLP. n = 4, *p < 0.05 (Student's t test).

(B and C) Effect of PIP5K1C deficiency on fMLP-induced MLC phosphorylation. Wild-type and PIP5K1C neutrophils were stimulated with 4 μM fMLP. Phosphorylated MLC levels were analyzed by western blotting, quantified by densitometry, and normalized against the actin levels (B; n = 2, means ± standard errors) or immunostaining by an anti-pSer^{19} MLC antibody (C).

(D) Effect of PIP5K1C deficiency on fMLP-stimulated enhancement in ICAM binding. Wild-type and PIP5K1C-deficient neutrophils were stimulated with fMLP for 2 min and stained with recombinant ICAM-Fc complexed with Alexa-633-anti-human Fc secondary antibody. Cells were then analyzed by a flow cytometer and mean fluorescence intensity (MFI) is shown. The experiments were carried out in triplicates and the data are presented as means ± SEM. *p < 0.01 versus controls (Student's t test).

(E) Effect of RhoA dominant-negative mutant on fMLP-stimulated enhancement in ICAM binding. Wild-type neutrophils were transfected with GFP-dnRhoA and stimulated with fMLP for 2 min, followed by staining and analysis as in (D). MFI of ICAM staining of GFP-positive neutrophils were compared with those of GFP-negative ones. The experiments were carried out in triplicates, and the data are presented as means ± SEM. *p < 0.01 versus WT controls (Student's t test).

(F) Effects of expression of PIP5K1C isoforms on lamellipodia formation and MLC phosphorylation in PIP5K1C null neutrophils stimulated with 4 μM fMLP. PIP5K1C null neutrophils were transfected with GFP-PIP5K1C-90 or GFP-PIP5K1C-87 and placed on fibrinogen. They were stained with an antibody specific to pSer^{19} MLC, followed with a TRITC-conjugated secondary antibody and Alexa-633 phalloidin. The cells were then examined with a confocal microscope. Images are presented with pseudocolors. n > 8 for each observation, and representative images are shown. Scale bars represent 8 μm.

(G) Effect of GFP-PIP5K1C-87 expression on transendothelial migration. Neutrophils were transfected with GFP-PIP5K1C-90 or RFP-PIP5K1C-87. Equal numbers of transfected cells were tested for their ability to transmigrate through endothelial cells under an fMLP gradient in a transwell chamber assay.

DISCUSSION

Here we showed that integrins can confer a polarity to neutrophils by inducing polarized localization of PIP5K1C-90 independently of chemoattractants. This integrin-induced polarity has important roles in neutrophil polarization and infiltration. Although integrins have been shown to regulate cell motility, they are not known to regulate neutrophil polarization or directionality. Thus, our findings have revealed previously underappreciated roles of integrin signaling in regulating neutrophil functions.

The evidence presented in this report implicated an important role of vesicle transport in integrin-induced PIP5K1C-90 polarization. The evidence includes an impediment of PIP5K1C-90 polarization by AP2 interaction mutations on PIP5K1C-90, polarized distributions of AP2 and clathrin by integrin engagement and their colocalization with PIP5K1C-90, and inhibition of the polarization by dnArf6. Because the vesicle transport may be a continuous process, clathrin and AP2 may have to be recycled. Thus, there should be a mechanism for the dissociation of PIP5K1C-90 from AP2. Tyr649 phosphorylation of PIP5K1C-90, which was probably carried out by Src and Fak and detected in polarized PIP5K1C-90 structure, may provide such a mechanism because the phospho-mimetic mutation of Tyr649 reduces the interaction with AP2. Based on these data, we propose a model to suggest that integrin engagement may stimulate

Arf6-dependent vesicle transport, which brings AP2-associated PIP5K1C-90 to one side of a cell. The directionality of the transport may be determined by the cell movement direction prior to its arrest. Fak- and Src-mediated phosphorylation may subsequently result in the dissociation of PIP5K1C-90 from AP2. AP2 might be recycled, whereas PIP5K1C-90 stayed. It remains unknown how cell movement direction determines the directionality of vesicle transport and the location of PIP5K1C-90 polarization and whether additional modifications of PIP5K1C-90 or its interaction with other molecules are required for its final, more consolidated localization. It is also not known how integrin engagement triggers the polarization process. Engaged integrins may either accelerate basal endocytic or endosomal vesicle trafficking or are the cargos that initiate the trafficking.

Current concepts regard chemoattractants as the sole regulators of polarization and directionality in chemotaxis. It is also believed that chemoattractants polarize the cell by specifying the "front" and "back" through polarized localization and/or activation of signaling and structural molecules at both leading edges and uropods. The results of this study extend the concepts to suggest that signaling other than elicited by chemoattractants can also break the symmetry and polarize the neutrophils. In this case, integrin-induced polarization specifies the back without mobilizing the front signaling molecules. Importantly, this integrin-regulated back signaling is dominant enough to determine the initial polarity, along which chemoattractants have to polarize their front and back signals. Our results also confirm that chemoattractant-controlled fronts, once formed, become more dominant, which can lead a direction change if the cell polarity is not aligned with the chemoattractant gradient. Therefore, chemotactic directionality determination may be the result of the summation of signaling inputs of multiple pathways in a context-dependent manner. On a different note, this effect of integrin-PIP5K1C-90-regulated polarization on initial neutrophil directionality provides an explanation to the poor initial directionality on fibrinogen as well as the heterogeneity in neutrophil chemotactic directionality often observed in many of the in vitro assays. In these assays, the neutrophils may have taken an initial polarity because of integrin signaling, which is random to the chemoattractant gradient applied afterward. The more integrin activation as in the case of fibrinogen coating may cause more cells to take up the initial polarity and thus higher directional errors.

The integrin-PIP5K1C-90-regulated polarization intersects chemoattractant-regulated polarity at RhoA regulation. Chemoattractants are long known to activate RhoA at the uropods, but the mechanisms for such polarized activation remain unclear. PDZ-RhoGEF, which contains a PH domain, was found to be localized at uropods and appeared to regulate uropod RhoA activation in neutrophil-like HL-60 cells (Wong et al., 2007; Xu et al., 2003). We are currently investigating whether PIP5K1C-90 and PtdIns(4,5)P_2 can regulate PDZ-RhoGEF localization or activity.

Our data also showed an incomplete abrogation of RhoA activation by PIP5K1C deficiency. This may be attributed to the existence of other RhoA activation mechanisms and/or PIP5K1 isoforms. Human PIP5K1B is also polarized at the uropod upon chemoattractant stimulation in neutrophil-like HL-60 cells (Lacalle et al., 2007). However, our analysis of neutrophils isolated from mice lacking human PIP5K1B ortholog PIP5K1A (Sasaki et al., 2005) revealed no chemotactic, RhoA activation or adhesion defects compared to the wild-type neutrophils (data not shown). It also remains unclear whether the kinase activity of PIP5K1C is regulated during the polarization process. Both Arf6 and RhoA have been shown to activate its activity (Bolomini-Vittori et al., 2009; Honda et al., 1999; Krauss et al., 2003). It is possible that RhoA and PIP5K1C constitute a positive feed-forward mechanism for the production of PtdIns(4,5)P_2 at uropods.

The results in this study suggest that the biological significance of PIP5K1C and its polarization upon integrin engagement may not lie in its regulation of neutrophil chemotaxis but rather its infiltration through the endothelium. On one hand, PIP5K1C facilitates RhoA activation, which leads to the increase in integrin affinity required for neutrophil firm adhesion to the endothelium. In contrast, PIP5K1C-90 polarization is required for polarized RhoA activation. RhoA is known to antagonize Rac, which is required for the formation of F-actin and hence lamellipodium. Without lamellipodia, cells cannot undergo migration. Thus, two competing activities (RhoA-mediated firm adhesion and Rac-mediated formation of lamellipodia for cell migration) have to occur in the same cell for successful neutrophil infiltration. Polarized activation of RhoA through integrin-induced PIP5K1C-90 polarization provides a solution for these two competing biological activities to occur concomitantly in the same cell. Thus, PIP5K1C has two critical roles in regulating neutrophil infiltration in vivo; whereas its role in facilitating RhoA activation by chemoattractants regulates endothelial cell adhesion, its role in polarizing RhoA activation helps evade the suppressive effect of RhoA activation on the formation of lamellipodia that is required for neutrophil extravasation and migration.

EXPERIMENTAL PROCEDURES

Reagents and Constructs

Wild-type GFP-PIP5K1C-90 and the antibody specific for PIP5K1C-90 (Di Paolo et al., 2002) were kindly provided by P. De Camilli. Plasmids encoding FRNK and Y397-FAK were gifts from J.L. Guan. The antibody specific for Tyr649 of PIP5K1C-90 was generated by AbMax (Beijing, China). The PIP5K1C mutants were generated by PCR-based mutagenesis and verified by nucleotide sequencing. GFP-PH-PLCδ was a gift from A. Smrcka. Talin antibody was purchased from Sigma. Phospho-MLC antibody was obtained from Cell Signaling. Anti-mouse β2 integrin blocking antibody (GAME-46) was purchased from BD Biosciences PharMingen. Blocking antibodies to α_M (Clone M1/70) and α_L (Clone M17/4) integrins were purchased from eBiosciences. YFP-β-actin was described previously (Kress et al., 2009).

Mice and Bone Marrow Transplantation

PIP5K1C-deficient mice have been previously described (Di Paolo et al., 2004). Liver cells (2 million) from neonatal wild-type or PIP5K1C null mice were transplanted into wild-type recipient mice that had been subjected to 1000 cGy X-ray irradiation. Eight weeks later, the transplanted mice were used for neutrophil preparation. All mouse work described in this study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University prior to the commencement of the work.

Neutrophil Isolation, Transfection, Staining, and Migration and Flow Chamber Assays

Bone marrow neutrophils were purified from mouse bone marrow, transfected, stained, and assayed for its migration in Dunn chambers and transwell plates as described previously (Zhang et al., 2010). The detailed protocols are also described in the Supplemental Experimental Procedures.

PIP5K1C Polarization Assay

Coverslips were coated with 1 mM polylysine or 100 μg/ml fibrinogen at 37°C for 1 hr. Neutrophils were plated onto the coverslips for 10 min. In some experiment, neutrophils were pretreated with PTX (1 μg/ml for 2 hr at 37°C), PP2 (10 μM for 15 min), Y-27632 (10 μM for 15 min), or Cytochalasin D (10 μM for 15 min). The cells were then fixed with 4% paraformaldehyde and examined by using Leica SP5 confocal microscope.

Flow Chamber Assay

Mouse endothelial cells (Wang et al., 2007b) were cultured to confluency on 10 μg/ml fibronectin-coated coverslips and treated with 50 ng/ml TNF-α for 4 hr. The coverslips containing the endothelial cell layer were washed with PBS and placed in a flow chamber apparatus (GlycoTech). Purified wild-type and PIP5K1C null neutrophils were labeled with CFSE and FarRed SSAO SE. Dye, respectively, at 37°C for 15 min and then mixed at a 1:1 ratio. The mixed neutrophils were placed on top of the endothelial cells and subjected to sheer flow of 1 dyne/cm^2 for 1 min. The cells were then fixed, and the number of neutrophils adhering to the endothelial cells was counted with a fluorescence microscope. For examining directionality, neutrophils transiently transfected with GFP-PIP5K1C-90 were flowed through the chamber coated with a monolayer of mouse endothelial cells as described above.

To test the adherence under high sheer stress, wild-type and PIP5K1C null or Y-27632-treated neutrophils labeled with CFSE and FarRed SSAO SE. Dye, respectively, or vice versa were allowed to sediment to the monolayer endothelial cells for 10 min in the chamber. The sheer stress was gradually ramped up to 4 dyn/cm^2 in 10 min. Images sequences were taken at 15 s intervals. The numbers of wild-type and PIP5K1C null cells or Y-27632-treated WT cells attached to the endothelium at first min were confirmed to be no more than 2.5% different. The numbers of untreated wild-type cells adhered to endothelial at the end of recording are taken as 1.

Determination of Active RhoA Level and ICAM-Binding Assay

The levels of active GTP-bound RhoA were determined with a G-LISA RhoA Activation Assay kit (Cytoskeleton, Inc). One million of wild-type or PIP5K1C null neutrophils were stimulated with mock or 4 μM fMLP for 3 min before the assay. The ICAM binding assay was carried out as previously described (Konstandin et al., 2006). Detailed protocols for ICAM binding and integrin clustering are described in the Supplemental Experimental Procedures.

In Vivo Neutrophil Infiltration Assays and Intravital Microscopy

Neutrophil recruitment to the peritonitis and MSU-induced gout was carried out as described (Di Lorenzo et al., 2009; Jia et al., 2007). Neutrophil infiltration in the mouse cremaster muscle venules was performed as previously described (Liu et al., 2005). The detailed protocols are also described in the Supplemental Experimental Procedures.

RT-PCR

Total RNA was isolated from purified wild-type or PIP5K1C null neutrophils via TRIzol. The cDNA was synthesized with iScript cDNA synthesis kit (Bio-Rad), and qPCR was carried out with PIP51A-, 1B-, and 1C-specific oligos.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and seven movies and can be found with this article online at doi:10.1016/j.immuni.2010.08.015.

ACKNOWLEDGMENTS

We thank M. Orsulak and other members of the D.W. lab for various assistances, P. De Camilli for the *pip5k1c* mutant mice, plasmids, and reagents, and D. Calderwood for plasmids. This work was supported in part by NIH grants (HL080706, HL070694, 1U54 RR022232-01, NS36251, DA018343), by German Academy of Sciences Leopoldina (BMBF-LPD 9901/8-162) (H.K.), and by AHA and NSFC30800587, 30971521, 2010CB529704, 09QA1401900, 06DZ22923 (P.W.).

Received: March 4, 2010
Revised: July 19, 2010
Accepted: August 11, 2010
Published online: September 16, 2010

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