Essential Role of Phosphoinositide 3-Kinase δ in Neutrophil Directional Movement

Chanchal Sadhu, Boris Masinovsky, Ken Dick, C. Gregory Sowell and Donald E. Staunton

*J Immunol* 2003; 170:2647-2654; doi: 10.4049/jimmunol.170.5.2647

http://www.jimmunol.org/content/170/5/2647

---

**References**

This article cites 47 articles, 21 of which you can access for free at:

http://www.jimmunol.org/content/170/5/2647.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Essential Role of Phosphoinositide 3-Kinase \( \delta \) in Neutrophil Directional Movement

Chanchal Sadhu,1,2 Boris Masinovsky,1 Ken Dick, C. Gregory Sowell, and Donald E. Staunton

Neutrophil chemotaxis is a critical component of the innate immune response. Neutrophils can sense an extremely shallow gradient of chemotactants and produce relatively robust chemotactic behavior. This directional migration requires cell polarization with actin polymerization occurring predominantly in the leading edge. Synthesis of phosphatidylinositol (3,4,5) trisphosphate (PIP3) by phosphoinositide 3-kinase (PI3K) contributes to asymmetric F-actin synthesis and cell polarization during neutrophil chemotaxis. To determine the contribution of the hemopoietic cell-restricted PI3K\( \delta \) in neutrophil chemotaxis, we have developed a potent and selective PI3K\( \delta \) inhibitor, IC87114. IC87114 inhibited polarized morphology of neutrophils, fMLP-stimulated PIP3 production and chemotaxis. Tracking analysis of IC87114-treated neutrophils indicated that PI3K\( \delta \) activity was required for the directional component of chemotaxis, but not for random movement. Inhibition of PI3K\( \delta \), however, did not block F-actin synthesis or neutrophil adhesion. These results demonstrate that PI3K\( \delta \) can play a selective role in the amplification of PIP3 levels that lead to neutrophil polarization and directional migration. The Journal of Immunology, 2003, 170: 2647–2654.

1 C.S. and B.M. made equal contributions.
2 Address correspondence and reprint requests to Dr. Chanchal Sadhu, ICOS Corporation, 22021 20th Avenue SE, Bothell, WA 98021. E-mail address: csadhu@icos.com
3 Abbreviations used in this paper: PIP3, phosphatidylinositol (3,4,5) trisphosphate; PH, pleckstrin homology; CK1, casein kinase 1; PKB, protein kinase B; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; CHK1, checkpoint kinase 1; PTK, protein tyrosine kinase; SFK, Src family tyrosine kinase; MI, migration index; MAPK, mitogen-activated protein kinase.
variety of inflammatory diseases. In this report, we describe an isoform-specific inhibitor of PI3 kinase that is selective for PI3Kδ. Experiments using the PI3Kδ inhibitor suggest that PI3Kδ plays an important role in the directional migration of neutrophils, but not in random movement.

Materials and Methods

Kinase assay

Various isoforms of PI3K were expressed in SF9 cells as His-tagged proteins using the baculovirus expression system according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). PI3K assay was performed according to Chantry et al. (23) and Volinia et al. (26) with the following modifications. Phosphatidylinositol-(4,5)-bisphosphate (PIP2) containing phospholipid liposomes were prepared using a mini-extruder fitted with an 0.88-μm filter (Avanti Polar Lipids, Alabaster, AL) according to Mayer et al. (27). Briefly, bovine PIP2 and phosphatidylserine (1:2 molar ratio; Avanti Polar Lipids) were vacuum-dried and resuspended at 1 mM PIP2 in 20 mM HEPES-KOH, pH 7.4, 50 mM NaCl, 5 mM EDTA. The lipid suspension was subjected to a brief sonication, followed by 5 freeze-thaw cycles and then 20 extrusion cycles to produce the liposomes. The assay was conducted in 60-μl reaction volumes in 20 mM HEPES, pH 7.4, buffer containing 1 mM PI3K, 1 mM PIP2, 200 μM ATP, 1 μCi [γ-32P]ATP, 5 mM MgCl2, plus 50 μg/ml horse IgG (Pierce, Rockford, IL) as carrier protein. The reaction was incubated for 10 min at room temperature, quenched in 140 μl of 1 M K2PO4, 30 mM EDTA, pH 8.0, captured onto a 96-well polyvinylidene difluoride filter plate (Millipore, Bedford, MA) and washed five times with 1 M K2PO4. The filter was allowed to dry completely, and the bound radioactivity was quantitated. For screening the small molecule compound library, all compound dilutions were assayed in a final concentration of 1% (w/v) DMSO. Details of the synthesis of IC87114 will be published in a patent for which an application, no. 20020161014, has been made.

Measurement of PI3P3 synthesis by neutrophils

Neutrophils were isolated by density gradient centrifugation on Polymor- phoprep (Accurate Chemical, NY), washed, and resuspended at 107/ml in 10 mM HEPES, pH 7.4, 120 mM NaCl, 10 mM KCl, 1 mM MgCl2, 10 mM CaCl2, and 10 mM HEPES, pH 7.4, buffer containing 1 mM PI3K, 1 mM PIP2, 200 μM ATP, 1 μCi [γ-32P]ATP, 5 mM MgCl2, plus 50 μg/ml horse IgG (Pierce, Rockford, IL) as carrier protein. The reaction was incubated for 10 min at room temperature, quenched in 140 μl of 1 M K2PO4, 30 mM EDTA, pH 8.0, captured onto a 96-well polyvinylidene difluoride filter plate (Millipore, Bedford, MA) and washed five times with 1 M K2PO4. The filter was allowed to dry completely, and the bound radioactivity was quantitated. For screening the small molecule compound library, all compound dilutions were assayed in a final concentration of 1% (w/w) DMSO. Details of the synthesis of IC87114 will be published in a patent for which an application, no. 20020161014, has been made.

Quantitation of F-actin synthesis by flow cytometry

Neutrophils isolated from human blood were treated in suspension with DMSO or IC87114 (5 μM) and then stimulated with fMLP (1 μM) for 30 s at 37°C. The cells were fixed with paraformaldehyde, washed in PBS, permeabilized with 0.1% Triton X-100, and stained with Oregon Green-conjugated phalloidin. The stained neutrophils were washed three times in PBS and analyzed in a FACSCalibur (BD Biosciences, Mountain View, CA).

Visualization of actin redistribution, cell polarization, and spreading

Freshly isolated human neutrophils were pretreated either with DMSO or with IC87114 (final concentration, 5 μM). The neutrophils were then plated on a fibronectin-coated coverslip and allowed to adhere. Subsequently, the neutrophils were stimulated with 1 μM fMLP or with DMSO only. After 1 h, the cells were fixed for 10 min in 4% paraformaldehyde at 4°C, unbound neutrophils were removed by three washes with PBS, and the bound cells were permeabilized with 0.1% Triton X-100. G-actin was stained with Texas Red-conjugated DNase I and F-actin was stained with Oregon Green-conjugated phalloidin (Molecular Probes, Eugene, OR). The actins were visualized by fluorescence microscopy. Dimensions of the neutrophils were measured using the Metamorph program (Gryphon, San Diego, CA).

Measurement of neutrophil adhesion and migration

The adhesion assays were performed by the following modifications of the procedure described by Sadhu et al. (30). Briefly, 96-well plates were coated overnight with 5 μg/ml ICAM-1/Ig, blocked with 1% HSA (no. 12666; Calbiochem, La Jolla, CA) in RPMI 1640, and 2 × 105 neutrophils (untreated or pretreated with the indicated concentration of IC87114) were added in replicate wells. After incubation at 37°C for the desired time, 5% CO2, glutaraldehyde was added to a 1% final concentration. Unbound cells were removed by washing the wells in water and the adherent cells were stained with crystal violet. Cell adhesion was quantified by reading the intensity of crystal violet on Spectra MAX (Molecular Devices, Sunnyvale, CA) at 570 nm.

Neutrophil migration under agarose was studied according to a modified protocol described by Nelson et al. (31). Briefly, 6-well plates (no. 3516; Costar, Cambridge, MA) were coated with ICAM-1/Ig (25 μg/ml in bi-carbonate buffer, pH 9.3), overnight at 4°C. After washing, 1% agarose solution (with or without the inhibitor in RPMI 1640 and 0.5% BSA) at 30°C was layered on the ICAM-1-coated surface. Plates were cooled at 4°C before punching holes in the agarose layer to form wells (one central well surrounded by six peripheral wells). Human neutrophils were obtained as described above, and resuspended in 0.5% BSA (in RPMI 1640) at 5 × 106/ml. After combining equal volumes of neutrophil suspension and medium (either with DMSO, or IC87114 in DMSO), 10 μl of the neutrophil suspension were aliquoted into each of the peripheral wells. To the central well 10 μl of fMLP (5 μM) was added. Plates were incubated at 37°C in the presence of 5% CO2 for 2-3 h to allow neutrophil migration. Less than 5% of input cells migrated during the time period of the experiment. Migration was terminated by the addition of glutaraldehyde (1%) in each of the wells. After removing the agarose layer, wells were washed with distilled water and dried. Using a Diaphot inverted microscope (× objective; Nikon, Melville, NY) equipped with a camera and digital processor (Dage-MTI, Michigan City, IN) that was connected to a PowerMac 8500/120 (Apple Computer, Cupertino, CA), three recordings per experimental condition of neutrophil migration were collected using the NIH Image Scion 1.61 program (Bethesda, MD). Using Microsoft Excel and Table Curve 4 (Redmond, WA), data were plotted to obtain migration index (MI) values for each of the conditions. MI was defined as the area under a curve representing the number of neutrophils (OD) vs the distance of migration.

For video recordings of neutrophil migration under agarose, 20× objective with phase contrast was used with the NIH Image Scion 1.61 program. Data were collected at around 2 h from the time of cell addition into the wells. At this time, a sufficient number of cells migrated for subsequent analysis of each experimental condition without interference artifacts from the edge of the well. Positions of cells were recorded every 15 s. A total of 25 consecutive frames were used for the analysis of neutrophil movements per condition. Using the NIH Image Object 1.62p4.1 program, 10 cells that were present in all 25 frames were traced and analyzed with Microsoft Excel. Total distance of movement was calculated using the formula

$$d = \sum_{n=1}^{25} \sqrt{(x_{n+1} - x_n)^2 + (y_{n+1} - y_n)^2},$$

while directional movement was calculated using the formula $X_{25} - X_1$.

Statistics

For data analysis, the Student t test was performed using SigmaStat 2.03 software (SPSS (Jandel Scientific), Chicago, IL).

Results

IC87114 is a selective inhibitor of PI3Kδ

Of the four class I PI3K isoforms, PI3Kδ is preferentially expressed in cells of hematopoietic lineage. To determine the potential role of PI3Kδ in leukocyte migration we identified an inhibitor of this isoform in a screen of our diverse chemical library. IC87114 (Fig. 1a), an analog of the original inhibitor, was synthesized and tested for PI3Kδ selectivity relative to the other class I PI3Ks (Fig.
The IC50 of IC87114 for PI3Kδ inhibition was 0.5 μM whereas the IC50 values for PI3Kα, PI3Kβ, and PI3Kγ were >100, 75, and 29 μM, respectively (Fig. 1b). Thus IC87114 is 58-fold more selective for PI3Kδ relative to PI3Kγ and over 100-fold selective relative to PI3Kα and PI3Kβ. In contrast, the widely used PI3K inhibitor LY294002 demonstrated IC50 values that differed by only ~10-fold among the four class I PI3Ks (Fig. 1c), consistent with earlier reports stating that LY294002 is a nonselective PI3K inhibitor (32). Importantly, there is no concentration of LY294002 that will selectively antagonize any single class I PI3Ks. In contrast, IC87114 selectively antagonizes PI3Kδ over at least a concentration range of 0.3–10 μM.

We also tested the inhibition profile of IC87114 on several protein kinases such as p38 MAPK, CHK1, DNA-PK, and cSrc. IC87114 (100 μM) did not significantly inhibit the activities of any of these kinases (Fig. 1d). Similarly, no significant inhibition of CK1, PKBα (Akt 1), PKCα, and PKCβII by IC87114 was observed (Table I). These results show that IC87114 is a highly selective antagonist of PI3Kδ.

**PI3Kδ-dependent PIP3 biosynthesis in βMLP-stimulated neutrophils**

In neutrophils, chemoattractants can activate both the Gβγ-regulated PI3K (p110γ) and the p85-associated PI3Ks (p110α, p110β, and p110δ) (29, 33–35). The p85-associated PI3Ks can be regulated by protein tyrosine kinases (PTKs) of the Src family (29, 36). We tested the effects of PI3K and PTK inhibitors on fMLP-stimulated PIP3 generation in human neutrophils. Neutrophil suspensions were pretreated with the inhibitor and then exposed to fMLP (1 μM) for 15 s to stimulate fMLP synthesis. Consistent with earlier reports, the pan PI3K inhibitor LY294002 blocked fMLP-stimulated PIP3 biosynthesis by almost 80% (Fig. 2). Similar to

Table I. Effect of IC87114 on kinase activity

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Control (cpm)</th>
<th>IC87114* (cpm)</th>
<th>Activity in the Presence of IC87114 (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK1</td>
<td>34,901</td>
<td>35,669</td>
<td>102 ± 9</td>
</tr>
<tr>
<td>PKBα</td>
<td>57,285</td>
<td>61,903</td>
<td>108 ± 3</td>
</tr>
<tr>
<td>PKCα</td>
<td>19,556</td>
<td>19,734</td>
<td>101 ± 6</td>
</tr>
<tr>
<td>PKCβI</td>
<td>18,967</td>
<td>20,292</td>
<td>107 ± 3</td>
</tr>
</tbody>
</table>

* Kinase activities are tested in the presence of 10 μM IC87114.

**FIGURE 1.** IC87114 inhibits PI3Kδ with high selectivity. Structure of IC87114 (a) and activity profiles of class I PI3Ks in the presence of the PI3K inhibitors IC87114 (b) and LY294002 (c). PI3Ks were assayed in the presence of the inhibitors according to the protocol described in Materials and Methods and the data are presented as the residual activity compared with the activity in the absence of any inhibitor. Representative data from one of five experiments is shown. d, Activity of cSrc, CHK1, DNA-PK, and p38 MAPK.

**FIGURE 2.** IC87114 inhibits fMLP-stimulated PIP3 biosynthesis in neutrophils. Image of the TLC separation of 32P-labeled phospholipids (a) and a histogram (b) displaying relative levels of PIP3 in the neutrophils pretreated with LY294002 (50 μM), IC87114 (10 μM), IC87048 (10 μM), and Genistein (100 μM) for 10 min and stimulated with fMLP (1 μM) in suspension for 15 s.
the report by Ptasznik et al. (29), the broad-spectrum PTK inhibitor genistein also blocked 80% of PIP3 biosynthesis induced by fMLP (Fig. 2b). These observations indicate that the p85-associated PI3Ks contribute significantly in fMLP-stimulated PIP3 biosynthesis in a PTK-dependent manner. Although neutrophils express all three p85-associated PI3Ks as well as PI3Kδ (16), the PI3Kδ-selective inhibitor IC87114 potently inhibited PIP3 biosynthesis by \( \sim 60-65\% \) (Fig. 2). IC87048, an analog of IC87114 and a weak inhibitor of PI3Kγ (IC \( \text{IC}_{50} \approx 50 \mu\text{M} \)), was much less effective in the inhibition of PIP3 biosynthesis. These data suggest a significant role for PI3Kδ in production of PIP3 by neutrophils in response to fMLP stimulation.

**Requirement of PI3Kδ activity in neutrophil spreading and polarization**

To determine the requirement of PI3Kδ in fMLP-induced F-actin synthesis, untreated or IC87114-treated neutrophils were stimulated with fMLP and the changes in F-actin content were quantitated by flow cytometry. Stimulation of neutrophils with fMLP resulted in a \( \sim 3\text{-fold} \) increase in F-actin content per cell. Pretreatment of the cells with IC87114 (5 \( \mu\text{M} \)) resulted only in a minor reduction of F-actin. Pairwise comparison shows that the differences in the mean fluorescence intensities between the untreated and fMLP-treated neutrophils \( (p < 0.01) \) and that of the untreated and IC87114 + fMLP-treated neutrophils \( (p < 0.001) \) are significant. However, the difference in mean fluorescence intensities between fMLP-treated and IC87114 + fMLP-treated samples was not significant. These results suggest that PI3Kδ does not play a major role in fMLP-induced F-actin synthesis (Fig. 3).

Neutrophils spread and polarize even when exposed to a uniform concentration of fMLP or C5a (11). Asymmetric localization of F-actin in the leading edge is a hallmark of cell polarization that requires synthesis of PIP3. We determined the effect of IC87114 on actin reorganization in neutrophils polarized in response to a uniform concentration of chemoattractant. Neutrophils were allowed to adhere to a fibrinogen-coated surface through the CD18 integrin α\( \text{M} \)β\( 2 \) and then were stimulated with a uniform concentration of fMLP. In the unstimulated population, the majority of the adherent neutrophils possessed a rounded morphology (mean length 9 ± 2 \( \mu\text{m} \)) and demonstrated colocalization (yellow-orange fluorescence) of G- (red fluorescence) and F- (green fluorescence) actin with an occasional spreading of cells and actin redistribution (Fig. 4a). In the fMLP-treated population, cells had a highly elongated morphology (mean length 17.7 ± 5 \( \mu\text{m} \)) with extensions of lamellipodia. In these cells, there was a distinct separation between the F- and G-actins (Fig. 4b). F-actin was localized almost exclusively at the lamellipodia while G-actin was concentrated in the cell body. Cells pretreated with the PI3Kδ inhibitor IC87114 showed a more rounded morphology (mean length 10 ± 2 \( \mu\text{m} \)) with markedly few lamellipodia but demonstrated occasional long,

**FIGURE 3.** IC87114 has minimal effect of F-actin synthesis. Untreated or IC87114- (5 \( \mu\text{M} \)) treated neutrophils were stimulated with fMLP (1 \( \mu\text{M} \)) for 15 s and stained with Oregon Green-conjugated phalloidin for quantitation of F-actin by flow cytometric analysis. Histograms display the relative amount of F-actin.

**FIGURE 4.** IC87114 inhibits neutrophil spreading and polarization. Fluorescence images of neutrophils displaying cell morphology and distribution of G-actin (red) and F-actin (green) in untreated (a), fMLP- (1 \( \mu\text{M} \)) treated (b), or IC87114 (5 \( \mu\text{M} \)) and fMLP-treated (c) neutrophils adhered to fibrinogen-coated surface. Polarization is evident in fMLP-treated neutrophils from the distinct separation of G- and F-actins (b) whereas colocalization of the actins (as evident from the yellow color) is predominant in untreated neutrophils (a); scale bar = 10 \( \mu\text{m} \).
spindly uropods containing F-actin (Fig. 4c). However, the sequestration of F- and G-actins was still observed in these cells. Quantitation of the spread of the neutrophils shows that the untreated cells had a mean area of $52.16 \pm 18 \mu m^2$. Treatment of the neutrophils with fMLP resulted in a significant increase in the mean surface area to $81.38 \pm 17 \mu m^2$ ($p < 0.01$). However, prior exposure of the fMLP-treated cells to IC87114 reduced their mean surface area to $50.65 \pm 14 \mu m^2$ which is similar to the values obtained with the untreated cells ($p = 0.84$). These results suggest that although PI3Kδ does not significantly inhibit F-actin synthesis and reorganization, it plays an important role in fMLP-stimulated neutrophil spreading and polarization.

**PI3Kδ activity regulates neutrophil chemotaxis but not random locomotion**

Because cell polarization is an essential element of migration against a concentration gradient of stimulus, we determined the effect of IC87114 on neutrophil migration in the “under-agarose” assay (31, 37). This technique, together with video recording and computer analysis, permitted us to quantify the resulting cell movements. As shown in Fig. 5a, the number of neutrophils migrated on ICAM-1-coated plates in 3 h was higher in the untreated sample, compared with the IC87114- (1 μM) treated sample. Not only were the total number of migrating neutrophils reduced, but the distance of migration was also diminished. We quantified the effect of IC87114 on neutrophil migration.

**FIGURE 5.** IC87114 inhibited human neutrophil migration. **a**, Distribution of neutrophils after 3 h of migration on ICAM-1 under agarose toward an fMLP gradient. The left side of each figure corresponds to the edge of the well from which neutrophils migrated, while the width of the rectangle corresponds to the diameter of the well. Arrows indicate the direction of increasing fMLP concentration. **b**, Graphical representation of the effect of IC87114 on neutrophil migration as shown in (a). The MI of the untreated sample was 152,672, while that of the IC87114-treated sample was 37,578. **c**, Effects of IC87114 on neutrophil migration and adhesion. MIs are from analysis of data similar to the ones presented in **a** and **b**. The average and SD (bars) of MIs for each concentration of IC87114 are shown ($n = 3$). Neutrophil adhesion to ICAM-1 was conducted in the presence of 20 nM fMLP.
migration using a MI, which integrates both the number of cells and their migration distances. Thus, neutrophil migration at 1 μM IC87114 was inhibited by 75% as compared with the control (MIcontrol = 157, 672; MIcon87114 = 37, 578; Fig. 5b). Using MI values from different experiments we show that the PI3Kδ inhibitor IC87114 blocked fMLP-induced neutrophil chemotaxis on ICAM-1 in a concentration-dependent manner (Fig. 5c). The EC50 value for IC87114-mediated inhibition of chemotaxis was 0.375 ± 0.072 μM (n = 4).

In our experimental conditions, neutrophil migration required adhesion to ICAM-1 through the integrins α<sub>6</sub>β<sub>2</sub> and α<sub>4</sub>β<sub>2</sub> (data not shown). To determine whether the effect of IC87114 on neutrophil migration is due to inhibition of integrin binding to ICAM-1, neutrophils were allowed to adhere to ICAM-1-coated substrate in the presence of fMLP. Neutrophil adhesion was then measured at 30-min intervals. As shown in Fig. 5c, after 2 h, IC87114, at concentrations as high as 20 μM, did not inhibit fMLP-stimulated neutrophil adhesion to ICAM-1. Similar results were obtained at other time points (data not shown), suggesting that neutrophil migration was at least 40-fold more sensitive to IC87114 relative to adhesion. These results demonstrate that under our experimental conditions the effect of IC87114 on migration was not a result of blocking neutrophil adhesion.

The observed inhibition of neutrophil chemotaxis by IC87114 could be due to a disruption of either the basic cell movement machinery, or of the components involved specifically in the directional migration. To distinguish between these two possibilities, we analyzed both directional (chemotaxis toward fMLP) and random movement (chemokinesis). After neutrophils were exposed to a gradient of fMLP for 2 h, cell movements were video recorded (see Materials and Methods), and the individual neutrophil paths were traced and analyzed. An example of such analyses is shown in Fig. 6a. In this particular example, the average speed of neutrophils either in the absence of any inhibitor, or in the presence of 1 μM of the inhibitor was almost identical (2000 ± 310 μm/h vs 2130 ± 180 μm/h, respectively). However, the average speed of neutrophil directional movement in the presence of IC87114 was greatly reduced compared with the control cells. (Fig. 6a). The speed of directional migration of untreated cells was 1530 ± 360 μm/h, while that of IC87114-treated cells was 920 ± 1000 μm/h. (The apparently large SD in the latter case is due to the net negative migration of 4 of 10 cells.) Such analyses showed that IC87114 did not affect chemokinesis, although it significantly inhibited directional movement (EC50 = 1 μM; Fig. 6b). These observations clearly demonstrate that rather than affecting the processes underlying adhesion and basic movement, PI3Kδ plays a significant and selective role in chemotaxis by influencing cell polarization and directional movement.

Discussion

Data presented in this study show that IC87114 is a highly selective inhibitor of PI3Kδ. To our knowledge, this is the first selective small molecule inhibitor of a PI3K isofrom. Selective inhibition of PI3Kδ activity demonstrates that this target is responsible for a significant portion of fMLP-induced PIP3 generation in neutrophils, and that it is critical to chemotaxis, but not adhesion. Inhibition of PI3Kδ resulted in an ~60–65% reduction of PIP3 synthesis when neutrophils are exposed to fMLP in suspension. This is consistent with the finding that PI3Kγ can be directly activated by Gβγ, and hence produce the initial burst of PIP3 (33, 34, 38). A role for PI3Kγ in fMLP-induced neutrophil PIP3 generation has been further documented recently by Cadwallader et al. (39). However, inhibition of PIP3 generation by the tyrosine kinase inhibitors genistein and radicicol have also been demonstrated (29, 39) suggesting a role for the p85-associated PI3Ks as well in this process.

The role of PI3Ks in cell polarization and chemotaxis has been demonstrated in diverse systems such as neutrophils and D. discoideum (9, 10, 14, 15). Similarly using the broad-spectrum PI3K inhibitor LY294002, a role of the class I PI3Ks in cell spreading has been demonstrated (40). Our data using IC87114 suggest that PI3Kδ plays an important role in neutrophil spreading.

![Figure 6](http://www.jimmunol.org/). IC87114 inhibits neutrophil chemotactic migration, but not random locomotion. a, Tracings of neutrophil paths during movement on ICAM-1 against a concentration gradient of fMLP. This is an example of two data sets (Control and 1 μM IC87114). For each experiment, five data sets (DMSO, 0.1 μM, 0.3 μM, 1 μM, 3 μM IC87114) were collected and used for further analysis shown in b. The figure shows paths of 10 individual neutrophils. The larger symbol of each trace indicates the starting point. The arrow indicates direction of increasing fMLP concentration. During the course of recording, no neutrophil in the untreated sample moved in the direction opposite to the source of fMLP, while in the presence of IC87114 (1 μM) 4 of 10 cells showed a net negative directional movement. b, Two-component analysis of the effect of IC87114 treatment on neutrophil movements. Each point represents an average and SE (bar) of five experiments. Significant differences (Student’s t test) between total and directional movement are shown (*, p < 0.001).
In comparison to its effect on the initial level of fMLP-stimulated PIP3 production in suspension, the effect of IC87114 on neutrophil migration is much more pronounced. This indicates a specific critical requirement of PI3Kδ in PIP3 synthesis during neutrophil migration. Previously, using isoform-specific Abs, PI3Kδ activity was shown to be important for chemotaxis of macrophage-like cells in response to CSF1 (21). These and the data presented in this study suggest that PI3Kδ probably plays an important role in chemotaxis in a variety of hemopoietic cells. We have further studied the role of PI3Kδ in the two components of chemotaxis, random migration and directed movement. Our analyses, using IC87114 as a selective probe, demonstrate that during fMLP-induced chemotaxis PI3Kδ activity is not required for random locomotion; however, it appears to be essential for cell polarization and CD18 integrin-dependent chemotactic migration. Thus, PI3Kδ plays a unique role in neutrophil chemotaxis by selectively influencing the directional component.

In p110γ−/− mice, neutrophils are severely defective in fMLP-stimulated PIP3 biosynthesis, suggesting a critical requirement of PI3Kδ in the response of neutrophils to fMLP (17–19). However, the p110γ null neutrophils are still able to migrate although at a slower rate (18). Recently it has been demonstrated that the defect in p110γ null mice is primarily at the level of chemotaxis rather than chemokinesis (20). We propose that PI3Kγ plays an important role during the initial burst of fMLP-induced PIP3 biosynthesis, and that PI3Kδ plays a critical role in amplification of PIP3 production leading to polarization and chemotaxis. This is consistent with the observation that exogenous PIP3 can induce endogenous PIP3 production that is necessary for neutrophil polarization (14). The initial burst of PIP3 production could activate GTP exchange factors of the Rho/Rac family of small GTPases that can turn activate PI3Kδ. In particular, PI3Kδ could be activated by Rac via the recently identified GTP exchange factor, P-Rex1, that is simultaneously activated by PI3P and Gβγ (41). Stimulation of neutrophils by fMLP is also known to activate Ras (42) as well as the Src family tyrosine kinases (SFKs) such as Fgr (43, 44). Upon activation, Rac can activate PI3Kδ by direct association with the p110δ catalytic subunit (24). It has been shown that Hck, another member of SFKs, can also be activated by Gao (45). Fgr and Hck can activate PI3Kδ via tyrosine phosphorylation of the associated p85 subunit (36). Thus, in fMLP-stimulated neutrophils PI3Kδ can be activated by the components of heterotrimeric G proteins (Gao and Gβγ), the Ras superfamily of G proteins as well as SFKs. These signaling pathways leading to activation of PI3Kδ and amplification of the PIP3 level are schematically presented in Fig. 7. In our model, PI3Kγ activity is an important initial source of PIP3 in response to stimulation of G protein-coupled receptors. Signal- ing through these receptors leads to the activation of the Ras superfamily of GTPases and SFKs that in turn activate PI3Kδ leading to a positive feedback loop amplifying PIP3 levels. Chan et al. (46) have demonstrated the existence of a motif on the p85 subunit that can mediate activation of the associated PI3Ks by the small GTPases H-Ras and Rac1 as well as Src, which further supports our model.

Production of PIP3 can also be induced in neutrophils following engagement of the CD18 integrins. Ligation of CD18 integrins αβ2 and αmβ2 can activate SFKs like Fgr and Hck (36, 44, 47) which, as discussed above, can activate PI3Kδ via the p85 subunit. Thus, through both chemoattractant receptors and integrin engagement pathways, PI3Kδ can serve as a signal amplifier for neutrophil chemotaxis by further enhancing or maintaining the PIP3 level beyond that produced by PI3Kγ.

Acknowledgments

We thank Dr. Sam Lee for purified PI3K preparations and Kelly Hensley and Janine Harrison for help with microscopy. We also thank Drs. Vince Florio, David Chantry, Linda Mackeen, and David Crowe for critically reading the manuscript, and Alice Dersham for expert assistance in the preparation of the manuscript. We gratefully acknowledge help from Jim Ward for statistical analysis, Jeff Dantzler for the p38 MAPK assay, and Adam Kashishian for the CHK1 assay.

References


FIGURE 7. Model of signaling pathways leading to activation of PI3Kδ by SFKs and heterotrimeric, as well as nonomeric, G proteins resulting in sustained PIP3 generation and cell polarization in fMLP-stimulated neutrophils.


