Tissue- and Stimulus-Dependent Role of Phosphatidylinositol 3-Kinase Isoforms for Neutrophil Recruitment Induced by Chemoattractants In Vivo


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Tissue- and Stimulus-Dependent Role of Phosphatidylinositol 3-Kinase Isoforms for Neutrophil Recruitment Induced by Chemoattractants In Vivo


PI3K plays a fundamental role in regulating neutrophil recruitment into sites of inflammation but the role of the different isoforms of PI3K remains unclear. In this study, we evaluated the role of PI3Kγ and PI3Kδ for neutrophil influx induced by the exogenous administration or the endogenous generation of the chemokine CXCL1. Administration of CXCL1 in PI3Kγ−/− or wild-type (WT) mice induced similar increases in leukocyte rolling, adhesion, and emigration in the cremaster muscle when examined by intravital microscopy. The induction of neutrophil recruitment into the pleural cavity or the tibia-femoral joint induced by the injection of CXCL1 was not significantly different in PI3Kγ−/− or WT mice. Neutrophil influx was not altered by treatment of WT mice with a specific PI3Kδ inhibitor, IC87114, or a specific PI3Kγ inhibitor, AS605240. The administration of IC87114 prevented CXCL1-induced neutrophil recruitment only in presence of the PI3Kγ inhibitor or in PI3Kγ−/− mice. Ag challenge of immunized mice induced CXCR2-dependent neutrophil recruitment that was inhibited by wortmannin or by blockade of and PI3Kδ in PI3Kγ−/− mice. Neutrophil recruitment to bronchoalveolar lavage induced by exogenously added or endogenous production of CXCL1 was prevented in PI3Kγ−/− mice. The accumulation of the neutrophils in lung tissues was significantly inhibited only in PI3Kγ−/− mice treated with IC87114. Neutrophil recruitment induced by exogenous administration of C5a or fMLP appeared to rely solely on PI3Kγ. Altogether, our data demonstrate that there is a tissue- and stimulus-dependent role of PI3Kγ and PI3Kδ for neutrophil recruitment induced by different chemotaxants in vivo. The Journal of Immunology, 2007, 179: 7891–7898.

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chemokine-regulated recruitment of neutrophils in vitro (18, 19). Furthermore, PI3K-γ-deficient neutrophils exhibited severe defects in GPCR-induced respiratory burst and produced less PtdIns(3,4,5)P3 after stimulation of cells with C5a, fMLP, and CXCL8 (IL-8) (19). It has been recently suggested that PI3Kδ may also modulate the activity and recruitment of neutrophils in vivo (20, 21). However, the exact extent to which PI3Kγ participates in the process of neutrophil recruitment in vivo is not known.

The present study was conducted initially to investigate the role of PI3Kγ in modulating neutrophil rolling, adhesion, and transmigration induced by the chemokine CXCL1 in the mouse cremaster. However, as PI3Kγ appeared not to be directly involved with CXCL1-induced neutrophil influx, further studies were conducted to investigate whether the enzyme was relevant for the neutrophil recruitment in other sites of inflammation and that induced by more complex inflammatory stimuli, including Ag challenge or bleomycin administration. We also evaluated the relevance of PI3Kγ for neutrophil influx induced by other chemotactant molecules and whether PI3Kδ played a role in CXCL1-induced neutrophil influx. We demonstrate that the involvement of PI3Kγ on neutrophil recruitment depends on the chemotactic stimulus and on the tissue in which the inflammatory stimulation is occurring. PI3Kδ and PI3Kγ have a redundant role in mediating exogenously added or endogenously generated CXCL1-induced neutrophil recruitment.

Materials and Methods

Animals

PI3K-γ-deficient male C57BL/6 × sv129 mice and their wild-type (WT) littermate control mice (+/+ ) (18–22 g) were used throughout these experiments. PI3Kγ−/− mice (in a C57BL/6 × sv129 background) were generated as previously described (18). The mice were a gift of Dr. J. M. Penninger (Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria) and supplied by Taconic Farms. PI3Kγ gene deletion was confirmed by PCR using specific primers (WT primers: sense, 5′-TCAGGC TCGGAGATTAGTA-3′; antisense, 5′-GCAAAATCTGGTGGTAGAAGCT-3′; PI3Kγ−/− primers: sense, 5′-GGACACGGCTTTGATTACAATC-3′; antisense primer, 5′-GGGGTGGGATTAGATAAATG-3′ as previously described (18). Animals were bred and housed in a temperature-controlled room with free access to water and food. All experimental protocols have been subjected to review and approval by the local animal ethics committee.

Drugs and reagents

Recombinant murine CXCL1 was purchased from PeproTech. CXCL1 was dissolved in water, diluted further in PBS (pH 7.4) containing 0.01% BSA and stored at −70°C until use. OVA was purchased from Sigma-Aldrich and C5a and wortmannin were purchased from Calbiochem. Wortmannin was diluted in DMSO and stored at −70°C until use. Lysozyme (bovine; Bristol-Meyers) was commercially available. The PI3Kδ inhibitor IC75114 was a gift of the ICOS Corporation and stored at −20°C until use. The PI3Kγ inhibitor AS605240 was a gift of Seroano and was stored at −20°C until use.

Intravital microscopy

The mouse cremaster preparation was used to study the behavior of leukocytes in the microcirculation and adjacent connective tissue, as previously described (22). Briefly, an incision was made in the scrotal skin to expose the left cremaster muscle, which was then carefully removed from the associated fascia. A lengthwise incision was made on the ventral surface of the cremaster muscle using a cautery. The testicle and the epididymis were separated from the underlying muscle and were moved into the abdominal cavity. The muscle was then spread out over an optically clear viewing pedestal and was secured along the edges with a 4-0 suture. The exposed tissue was superfused with warm bicarbonate-buffered saline (pH 7.4). An intravital microscope (Olympus BX50F4) with a ×20 objective lens and a ×10 eyepiece was used to examine the cremasteric microcirculation. A video camera (5100 HS; Panasonic) was used to project the images onto a monitor, and the images were recorded for playback analysis using a conventional videocassette recorder.

Single, unbranched cremasteric venules (25–40 μm in diameter) were selected and, to minimize variability, the same section of cremasteric venule was observed throughout the experiment. The number of rolling, adherent, and emigrated leukocytes was determined offline during video playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. The flux of rolling cells was measured as the number of rolling cells passing by a given point in the venule per minute. A leukocyte was considered to be adherent if it remained stationary for at least 30 s, and total leukocyte adhesion was quantified as the number of adherent cells within a 100-μm length of venule. Leukocyte emigration was defined as the number of cells in the extravascular space within an area of 50 μm of distance from the venule. Only cells adjacent to and clearly outside the vessel under study were counted as emigrated.

Experimental protocols

For each experiment, 500 ng of murine rCXCL1 in 0.2 ml of saline was administered locally by s.c. injection beneath the right scrotal skin using a 30-G needle, 2 h before exteriorization. The left cremaster was then prepared for intravital microscopy. Leukocyte kinetics was investigated, as described above, and at the end of each experiment whole blood was drawn by cardiac puncture. Total cell counts were performed in a modified Neubauer chamber using Turk’s stain.

Histology

At the end of each intravital microscopy experiment, the cremaster muscles were removed and fixed in 10% neutral-buffered formalin. The tissues were dehydrated gradually in ethanol, embedded in paraffin, cut into 4-μm sections, stained with H&E, and examined under direct light microscopy.

Sensitization

Animals were immunized with OVA adsorbed to aluminum hydroxide gel as previously described (23). Briefly, mice were injected s.c. on days 1 and 8 with 0.2 ml of a solution containing 100 μg of OVA and 70 μg of aluminum hydroxide (Reheiss).

Leukocyte migration into the pleural cavity induced by Ag and chemotactant agent

Sensitized WT or PI3Kγ−/− mice were challenged by intrapleural (i.pl.) administration of Ag (OVA) or PBS. In addition, to investigate the leukocyte migration into the pleural cavity induced by a chemotactant agent, WT or PI3Kγ−/− mice were injected into the pleural cavity with CXCL1 (30–100 ng/cavity), C5a (100 ng/cavity), and fMLP (200 ng/cavity). These were optimal doses to induce leukocyte recruitment in preliminary experiments (data not shown). The cells present in the pleural cavity were harvested 6 h after Ag challenge or chemotactant agent administration by injecting 2 ml of PBS and total cell counts performed in a modified Neubauer chamber using Turk’s stain. This time was shown to be optimal for neutrophil recruitment in preliminary kinetics experiments. Different cell counts were performed on cytospin preparations (Shandon III) stained with May-Grünwald-Giemsa using standard morphologic criteria to identify cell types. The results are presented as the number of cells per cavity.

Leukocyte migration into the lung induced by chemotactant agent or bleomycin

WT or PI3Kγ−/− mice were injected intratracheally with 500 ng of murine rCXCL1 or 0.125 U of bleomycin. The stimulus was given in 50 μl of sterile PBS. The control mice received PBS alone. The cells present in the alveolar space and the lung were harvested 6 h after CXCL1 administration or 48 h after bleomycin administration.

Bronchoalveolar lavage (BAL)

BAL was performed to obtain leukocytes in the alveolar spaces. The trachea was exposed and a 1.7-mm outside-diameter polyethylene catheter was inserted. BAL was performed by instilling three 1-ml aliquots of PBS and ~2 ml of fluid was retrieved per mouse. The number of total leukocytes was determined by counting leukocytes in a modified Neubauer chamber after staining with Turk’s solution. Differential counts were obtained from cytospin preparations by evaluating the percentage of each leukocyte on a slide stained with May-Grünwald-Giemsa.

Quantification of neutrophil accumulation in lung tissue by myeloperoxidase (MPO) activity measurement

The extent of neutrophil accumulation in the lung tissue was measured by assaying MPO activity as previously described (24). Using the conditions...
described below, this methodology is very selective for the determination of neutrophils over macrophages (data not shown). Before lung removal, the pulmonary vasculature was perfused with 3 ml of PBS via the right ventricle. Upon thawing, the tissue (0.1 g of tissue per 1.9 ml of buffer) was homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M NaPO4, 0.015 M sodium EDTA), centrifuged at 3000 × g for 10 min and the pellet was subjected to hypotonic lyses (1.5 ml of 0.2% NaCl solution followed 30 s later by addition of an equal volume of a solution containing 1.6% NaCl and 5% glucose). After a further centrifugation, the pellet was resuspended in 0.05 M NaPO4 buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide and rehomogenized. One-milliliter aliquots of the suspension were transferred into 1.5-ml Eppendorf tubes followed by three freeze-thaw cycles using liquid nitrogen. The aliquots were then centrifuged for 15 min at 3000 × g to perform the assay. MPO activity in the resuspended pellet was assayed by measuring the change in OD565 nm using tetramethylbenzidine (1.6 mM) and H2O2 (0.5 mM). The reaction was stopped by adding 100 μl of 4 M H2SO4 and was quantified at 450 nm in a spectrophotometer (Emax; Molecular Devices). The neutrophil content was calculated from a standard curve based on MPO activity expressed as absorbance increase at 450 nm from 5% casein peritoneal-induced neutrophils assayed in parallel. The results were expressed in relative number of neutrophils per milligram of wet tissue.

Leukocyte migration into an articular cavity

The role of PI3Kγ for neutrophil recruitment was also investigated after administration of CXCL1 in articular cavity. WT or PI3Kγ−/− mice were injected into the tibia-femoral articulation with an optimal dose of murine rCXCL1 (30 ng/cavity). The stimulus was given in 5 ml sterile PBS and control mice received PBS alone. The cells present in the tibia-femoral articulation were harvested 6 h after chemoattractant agent administration. Neutrophil recruitment was assessed 6 h after chemokine injection. Results are expressed as the means ± SEM of five mice in each group. * p < 0.01 when compared with PBS-injected mice; # p < 0.01 when compared with vehicle-treated CXCL1-injected mice.

Treatment with inhibitors of PI3K

The role of PI3K on neutrophils recruitment was also investigated by using selective inhibitors of PI3K, wortmannin and LY294002; the selective inhibitor of PI3Kδ, IC87114; and selective inhibitor of PI3Kγ, AS605240. Wortmannin or LY294002 were administered systemically (i.p.) at the dose of 1.0 mg/kg 60 min before the i.pl. administration of OVA or chemoattractant agent. This dose was shown to be effective in other experimental systems (25). Moreover, preliminary experiments showed the dose of 1.0 mg/kg to be maximally effective at inhibiting eosinophil recruitment in a similar model (26). IC87114, a highly selective inhibitor of PI3Kδ (20), was administered orally at the dose of 25 mg/kg 60 min before administration of chemoattractant agents. This dose was shown to be effective and selective in another model of leukocyte influx (21). AS605240, a highly selective inhibitor of PI3Kγ (27), was administered orally at the dose of 50 mg/kg 60 min before administration of chemoattractant agents. This dose was shown to be effective and selective in vivo (27). Wortmannin and LY294002 were dissolved in DMSO and further diluted in PBS. IC87114 and AS605240 was suspended in 0.1% methylcellulose solution and ground in a homogenizer to ensure a uniform suspension. Control animals received drug vehicle.

Preparation of bone marrow neutrophils

The femurs and tibias from both hind limbs were removed of mice and freed of soft tissue attachments, and the extreme distal tip of each extremity was cut off. HBSS (without Ca2+ and Mg2+) was forced through the bone by using a 5-ml syringe with a 22-gauge needle. After dispersing cell clumps and removing the debris, the bone marrow cells were suspended in 2 ml of HBSS and laid on top of a two-layer Percoll gradient of 72 and 65% Percoll (Sigma-Aldrich) diluted in HBSS (100% Percoll = nine parts Percoll and one part 10× HBSS) and centrifuged (1200 × g, 30 min, room temperature) without braking. The enriched neutrophil fraction was recovered at the interface between 65 and 72% Percoll. After washing twice with HBSS, 5.0 ± 1.0 × 106 cells were obtained per mouse, 95% neutrophils, identified by staining the nuclei with Turk reagent (Merck).
Neutrophil chemotaxis

A modified Boyden chamber assay to examine the neutrophil chemoattractant response to CXCL-1 and C5a was performed using a 48-well microchamber (Neuro Probe). Murine bone marrow neutrophils were isolated as above described and resuspended in running buffer (1/100 HBSS, 2 mg/ml BSA, 10 mM HEPES, 1 mM CaCl₂, and 1 mM MgCl₂). C5a (10 nM) and recombinant mouse CXCL-1 (20 ng/ml) diluted in running buffer (for wells containing neutrophils) or appropriate buffer control was added to the lower chambers of the apparatus. A 5-micron polycarbonate membrane (Neuro Probe) was placed between the upper and lower chambers, and 5×10⁴ cells in a volume of 50μl were added to the top chambers of the apparatus. Cells were allowed to migrate into the membrane for 1 h per treatment at 37°C with 5% CO₂. Following incubation, the chamber was disassembled and the membrane was scraped and washed three times in PBS to remove nonadherent cells before being fixed in methanol and stained using the Diff-Quik system (Dade Behring). Each well-associated membrane area was scored using light microscopy to count the intact cells present in five random fields. The results are expressed as the number of neutrophils per field.

Statistical analysis

All results are presented as the mean ± SEM. Normalized data were analyzed by one-way ANOVA, and differences between groups were assessed using the Student-Newman-Keuls posttest. A p value <0.05 was considered significant.

Table I. Circulating leukocyte counts and venular diameter in untreated mice and mice treated intrascratably with CXCL1 (500 ng)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Treatment</th>
<th>Circulating Leukocyte (10⁶/ml)</th>
<th>Venular Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>PBS</td>
<td>6.3 ± 2.2</td>
<td>30.9 ± 2.3</td>
</tr>
<tr>
<td>WT</td>
<td>CXCL1 (500 ng)</td>
<td>8.5 ± 2.1</td>
<td>33.4 ± 1.7</td>
</tr>
<tr>
<td>PI3K⁻⁻⁻⁻</td>
<td>PBS</td>
<td>6.8 ± 1.3</td>
<td>33.5 ± 3.0</td>
</tr>
</tbody>
</table>

* Murine rCXCL1 in 0.2 ml of saline was administered locally by s.c. injection beneath the right scrotal skin 2 h before exteriorization of cremaster muscle. The left cremaster was then prepared for intravital microscopy. Venular diameter was verified and circulating leukocyte kinetics were assessed in whole blood drawn by cardiac puncture. Total cell counts were performed in a modified Neubauer chamber using Turk’s stain. Results are expressed as the means ± SEM of five mice in each group.

FIGURE 2. Neutrophil adhesion (A) and emigration (B) in the cremaster muscle of WT and PI3K⁻⁻⁻⁻ mice 2 h after intrascrotal administration of CXCL1 (500 ng/animal). Control mice were injected with PBS. Results are expressed as the means ± SEM of four to five mice in each group. *, p < 0.01 when compared with PBS-injected mice.

FIGURE 3. Role of PI3K in neutrophil accumulation induced by Ag challenge of sensitized PI3K⁻⁻⁻⁻ and WT mice. Neutrophil accumulation was assessed after 6 h in immunized WT or PI3K⁻⁻⁻⁻ mice and challenged with an i.pl. injection of OVA (1 μg/cavity) or PBS at the time point described in the figure (A). Effects of the pretreatment with the nonselective PI3K inhibitors (B), wortmannin (1.0 mg/kg), and LY294002 (1.0 mg/kg), and PI3Kα-selective inhibitor (C), IC87114 (25 mg/kg), in the neutrophil accumulation induced by Ag were also verified. Results are expressed as the means ± SEM of five mice in each group. *, p < 0.05 when compared with PBS-injected mice; #, p < 0.05 when compared with vehicle-treated OVA-injected WT mice.
Results

Neutrophil recruitment induced by the i.pl. administration of 100 ng of CXCL1 was similar in PI3K$\gamma^{-/-}$ and WT mice (Fig. 1A). Neutrophil recruitment induced by lower doses (30 ng/cavity) of CXCL1 was also similar in PI3K$\gamma^{-/-}$ and WT mice (WT mice: $3.1 \pm 0.6 \times 10^6$ neutrophils/cavity; PI3K$\gamma^{-/-}$ mice: $2.1 \pm 0.4 \times 10^6$ neutrophils/cavity). Similar results were obtained using intravital microscopy in the CXCL1-treated cremaster muscle sections of both PI3K$\gamma^{-/-}$ and WT mice. The dose of CXCL1 used in these experiments was based in preliminary experiments and previous studies (28). Intrascrotal administration of 500 ng of CXCL1 induced similar increases in leukocyte rolling (WT mice: $50.6 \pm 4.3$ cell/min; PI3K$\gamma^{-/-}$ mice: $75.6 \pm 8.0$ cell/min), adhesion, and emigration in PI3K$\gamma^{-/-}$ or WT mice (Fig. 2). To identify the type of cells that had been recruited to the site of inflammation at this time point, H&E staining was performed on the tissues after the experiment. Neutrophils were the majority of the cells attached to endothelial cells or recruited to the cremaster muscle sections of both PI3K$\gamma^{-/-}$ or WT mice (data not shown). The circulating leukocyte counts were not different in these mice after administration of PBS or CXCL1 (Table I).

The next series of experiments were conducted in the pleural cavity for ease of performance. Despite the similar neutrophil recruitment in PI3K$\gamma^{-/-}$ and WT mice, treatment with the isoform nonselective PI3K inhibitor wortmannin (1.0 mg/kg) inhibited CXCL1-induced neutrophil recruitment both in PI3K$\gamma^{-/-}$ and WT mice (Fig. 1A). The latter results suggested that a PI3K was indeed relevant for chemokine-induced neutrophil influx. Treatment of WT mice with IC87114, a PI3K$\delta$ inhibitor, or AS605240, a PI3K$\gamma$ inhibitor, alone failed to affect the neutrophil recruitment induced by CXCL1 (Fig. 1B). However, the treatment of PI3K$\gamma^{-/-}$ mice with IC87114 and the concomitant treatment with both IC87114 and AS605240 significantly inhibited CXCL1-induced neutrophil recruitment (Fig. 1B). In vitro, the chemotaxis of bone marrow-derived neutrophils obtained from PI3K$\gamma^{-/-}$ mice was decreased in comparison to their WT control (basal chemotaxis: WT, $5.0 \pm 0.4$ neutrophils/field; PI3K$\gamma^{-/-}$, $4.3 \pm 0.5$ neutrophils/field; CXCL1-induced chemotaxis: WT, $18.5 \pm 0.2$ neutrophils/field; PI3K$\gamma^{-/-}$, $10.5 \pm 0.4$ neutrophils/field, $p < 0.05$).

We then examined whether PI3K$\gamma$ deficiency might alter the neutrophil recruitment induced by endogenously generated CXCL1, i.e., after Ag challenge (OVA, 1 $\mu$g/cavity) of sensitized mice. The i.pl. injection of OVA in sensitized WT mice induced a maximal neutrophil recruitment that occurred between 6 and 12 h and had dropped to baseline levels 24 h later (Fig. 3A). The neutrophil accumulation after Ag challenge was not altered in PI3K$\gamma$-deficient mice (Fig. 3A). Treatment of sensitized WT mice with a CXCR2 antagonist greatly reduced OVA-induced neutrophil accumulation (PBS, $1.8 \pm 0.7 \times 10^5$ neutrophil/cavity; OVA, $10.0 \pm 2.6 \times 10^5$ neutrophils/cavity; OVA + CXCR2 antagonist, $2.9 \pm 1.6 \times 10^5$ neutrophils/cavity, $n = 5$, $p < 0.05$), demonstrating that the
neutrophil recruitment induced by Ag challenge of sensitized mice depended mainly on CXC chemokine production and CXCR2 activation. Treatment with wortmannin or LY294002, two structurally distinct PI3K inhibitors, diminished the accumulation of neutrophils induced by Ag challenge (Fig. 3B). Treatment of WT mice with IC87114 did not affect the neutrophil recruitment induced by Ag challenge of sensitized mice. However, the treatment of PI3Kγ−/− mice with IC87114 diminished the influx of neutrophils into the pleural cavity (Fig. 3C).

Using an in vitro chemotaxis assay, it has been previously shown that C5a-induced chemotaxis is impaired in neutrophils from PI3Kγ−/− mice (19). In experiments with bone marrow-derived neutrophils, C5a-induced chemotaxis was partially diminished in PI3Kγ−/− mice vs WT (basal chemotaxis: WT, 5.0 ± 0.4 neutrophils/field; PI3Kγ−/−, 4.3 ± 0.5 neutrophils/field; C5a-induced chemotaxis: WT, 17.8 ± 1.2 neutrophils/field; PI3Kγ−/−, 11.4 ± 1.2 neutrophils/field, p < 0.05). In our in vivo experiments, the recruitment of neutrophils induced by i.pl. administration of C5a was abrogated in PI3Kγ−/− mice (Fig. 4A). Similarly, neutrophil recruitment induced by the administration of the chemotactant molecule fMLP was abrogated in PI3Kγ−/− mice as compared with their WT controls (Fig. 4B).

The recruitment of neutrophils to lung tissue and BAL fluid was evaluated after instillation of CXCL1 or bleomycin. Neutrophil influx to alveolar spaces was totally prevented in PI3Kγ−/− mice (Fig. 5A). In contrast, the deficiency of PI3Kγ had no effect on CXCL1-induced recruitment of neutrophils to lung tissues of mice, as assessed by measuring tissue MPO content (Fig. 5B). Treatment of WT mice with IC87114 failed to affect CXCL1-induced recruitment of neutrophils to lung tissues (Fig. 5C). However, the amount of neutrophils in lung tissues was significantly inhibited in PI3Kγ−/− mice treated with IC87114 (Fig. 5C).

Bleomycin-induced neutrophil influx depends of the release of CXCL1 into the alveolar space and activation of CXCR2 receptors (R. de Castro Russo and M. M. Teixeira, unpublished data). In agreement with the findings after CXCL1 administration, neutrophil influx to alveolar space after bleomycin administration was markedly diminished in PI3Kγ−/− as compared with WT mice (Fig. 6A) and the recruitment of neutrophils to lung tissues was unaltered in PI3Kγ−/− mice given bleomycin (Fig. 6B).

Furthermore, the role of PI3Kγ in neutrophil recruitment induced by CXCL1 was further confirmed in another model of neutrophil recruitment using the tibia-femoral joint. Neutrophil influx into the tibia-femoral joint was not significantly affected in PI3Kγ-deficient mice (number of neutrophils × 10⁶/cavity: WT mice: PBS, 1.2 ± 0.9; CXCL1, 12.8 ± 5.9; PI3Kγ−/− mice: PBS, 0.2 ± 0.1; CXCL1, 12.4 ± 6.4, n = 5, p > 0.05 when comparing WT vs PI3Kγ−/− mice).

Discussion

The present study investigated the role of PI3Kγ and PI3Kδ for neutrophil influx induced by the exogenous administration of the chemokine CXCL1. Overall, administration of CXCL1 induced leukocyte migration that was similar in PI3Kγ−/− or WT mice, or in mice treated with a specific PI3Kδ inhibitor, IC87114, or a specific PI3Kγ inhibitor, AS605240. CXCR2-dependent neutrophil recruitment was only inhibited when there was concomitant inhibition of both PI3Kδ and PI3Kγ. In contrast, neutrophil recruitment into alveolar space or in response to exogenous administration of C5a or fMLP appeared to rely solely on PI3Kγ.

We demonstrated that PI3Kγ deficiency did not interfere significantly with CXCL1-induced rolling, adhesion, and transmigration of neutrophil in cremaster muscle. Recently, it was shown that PI3Kδ-deficient mice presented a decrease in CXCL1-induced leukocyte adhesion in venules of the exteriorized mouse cremaster muscle (29). However, the experimental conditions used by the authors differ from ours in an important point. Although the chemotactic stimulus was injected i.v. and leukocyte adhesion was counted 1 min after the administration of CXCL1, CXCL1 was given locally (i.e., into the tissue) in our experiments and rolling, adhesion, and emigration were evaluated 2 h later. Thus, whereas in our experiments the inflammatory stimulus was present in the tissues where they are usually generated, in the previous study (29), the chemokine was given i.v. In the latter case, there may be stimulation (activation) of leukocytes before they migrate into tissue permitting leukocyte adherence in vessels but not their migration into tissue. Leukocyte activation may mimic the situation of chemotaxis in vitro and neutrophil chemotaxis induced by chemotactic agents is dependent on PI3Kγ (Ref. 30 and our results). In contrast, injection of stimulus in the tissue was PI3Kγ independent and correlated better with the PI3Kγ-independent chemokinetic activity of neutrophils (30, 31). More recently, Liu et al. (38) showed that neutrophil emigration in response to CXCL1 was PI3Kγ dependent at least up to 90 min after the addition of stimulus and was PI3Kγ independent thereafter. The results in later time points are consistent with the PI3Kγ-independent neutrophil influx in response to Ag and CXCL1 in our systems. Further studies are certainly needed to understand in greater detail the relevance of these in vitro phenomena (chemotaxis vs chemokinesis) for neutrophil recruitment in vivo and how the whole process is modulated by PI3Kγ.
Although CXCL1-induced accumulation of neutrophils into the pleural cavity was similar in PI3Kγ-deficient and WT mice, treatment with an isoform nonselective PI3K inhibitor, wortmannin, inhibited neutrophil accumulation induced by CXCL1. Wortmannin was capable of preventing neutrophil influx both in WT and PI3Kγ−/− mice. Previous studies have demonstrated that activation of PI3K/Akt is required for the induction of human neutrophil migration in response to CXCL8 (a homolog of murine CXCL1) (6, 32). Thus, although CXC chemokine-induced neutrophil recruitment appears to be PI3K dependent, it appears that sole blockade (or absence) of PI3Kγ is not sufficient to prevent neutrophil influx induced by CXCL1. In contrast to our findings, Hirsch et al. (19) demonstrated that neutrophil influx induced by IL-8 was partially inhibited in PI3Kγ−/− mice. One possibility to explain the discrepancies between those and our findings could be the need for the stimulation of intermediate cells and molecules for human IL-8 to induce recruitment of neutrophils in rodents (33, 34).

Recent reports have also suggested a role for PI3Kδ in promoting neutrophil trafficking in a mouse model of TNF-α-induced inflammation and in a model of acute pulmonary inflammation induced by LPS in rats (20, 21). Treatment with a PI3Kδ inhibitor, IC87114, did not affect CXCL1-induced neutrophil recruitment into the pleural cavity of WT mice at a dose previously shown to be effective in vivo (21). However, the data with wortmannin still suggested that the recruitment of neutrophils induced by CXCL1 would still depend on the activation of other PI3K isoforms or of the joint action of diverse isoforms. Sadhu et al. (15) have proposed that different PI3K isoforms may be activated in response to in vitro stimulation of GPCRs leading to human neutrophil migration. In our experiments, we demonstrated that inhibition of PI3Kδ in PI3Kγ−/− mice or treatment with inhibitors of both enzymes was accompanied by significant inhibition of neutrophil migration induced by exogenous administration of CXCL1. This result suggests that neutrophil recruitment following triggering of CXCR2 depends on the function of both PI3Kγ and PI3Kδ. In human neutrophils, PI3Kγ and PI3Kδ act together to amplify PIP3 production leading to cell polarization and chemotaxis (15). In accordance with the latter finding, PI3Kγ/PI3Kδ−/− mice have a profound reduction of T cell development (35).

It has been shown that PI3Kγ deficiency affected the ability of the endothelium to adhere neutrophils, most likely due to a reduction in E-selectin-mediated rolling (36). However, deficiency in p110γ and p110δ catalytic subunits of PI3K in the venular endothelium had an additive effect in the ability of this cell type to recruit neutrophils in response to TNF-α stimulation (36). More recent studies from the same group show that PI3Kγ and PI3Kδ have temporarily distinct roles in mediating leukocyte recruitment in vivo (31). Thus, PI3Kγ and PI3Kδ mediate neutrophil influx in a kinetic- (31), tissue-, and stimulus- (this study) dependent manner.

A series of experiments were then conducted to examine the role of PI3Kγ and PI3Kδ for the recruitment of neutrophils induced by administration of Ag to immunized mice. In this model, we show that neutrophil recruitment is totally dependent on the endogenous release of CXCR2-acting chemokines. As such, Ag-induced neutrophil recruitment was unchanged in PI3Kγ-deficient mice but was abrogated by treatment with PI3K-nonselective inhibitors. Administration of the PI3Kδ inhibitor failed to alter neutrophil recruitment in WT mice. In contrast, the PI3Kδ inhibitor greatly prevented neutrophil recruitment in Ag-challenged PI3Kγ-deficient mice. Altogether, the latter results demonstrate that endogenous CXCR2-acting chemokines, such as CXCL1, are necessary for Ag-induced neutrophil influx and work via activation of both PI3Kγ and PI3Kδ.

Neutrophil infiltration into BAL fluid after intranasal administration of CXCL1 was greatly diminished in PI3Kγ-deficient mice, an effect consistent with other studies (37). However, neutrophil influx into pulmonary tissue was not dependent on PI3Kγ. It has been demonstrated that intravascular, interstitial, and intra-alveolar accumulation of neutrophils may be differentially regulated; indeed, polymorphonuclear neutrophils can enter the pulmonary interstitium without advancing to the alveolar airway space and crossing the epithelial barrier (38). Thus, the differential mechanisms used by neutrophils to cross endothelial and epithelial cells may explain the differential use of PI3Kγ for neutrophil influx into lung tissues and alveolar spaces. However, the latter possibility needs to be investigated in more detail.

We also demonstrated in a model of bleomycin-induced pulmonary injury that deficiency of PI3Kγ inhibited neutrophil influx into the alveolar space but did not interfere with neutrophil recruitment to lung tissue. In this model of pulmonary inflammation, neutrophil recruitment is associated with endogenous production and action of CXCL1 on CXCR2 receptors (R. de Castro Russo and M. M. Teixeira, unpublished data). Therefore, CXCL1-dependent neutrophil transmigration through lung endothelial cells depends on the cooperative action of both PI3Kγ and PI3Kδ. In contrast, migration through epithelial cells is totally dependent on PI3Kγ.

There is evidence that GPCRs are able to activated class IA PI3K (39, 40). How might PI3Kδ be activated by GPCRs? It is proposed that an initial burst of PI3P induced by PI3Kγ activation could activate Rac and Src family tyrosine kinases that could then activate PI3Kδ (15). It is not clear whether the latter hypothesis would be functional in our experiments, as one would expect complete blockade of migration once PI3Kγ or PI3Kδ were blocked. Additional experiments are necessary to understand the interplay (or functional relationships) between these two isoforms in vivo.

We also evaluated the importance of PI3Kγ for neutrophil recruitment to the pleural cavity induced by the complement fragment C5a and the bacterial-derived chemotactic peptide fMLP. C5a is a potent chemoattractant for neutrophils, basophils, eosinophils, monocytes, and macrophages and is released after complement activation at sites of inflammation (41, 42). The role of PI3Kγ to C5a-induced neutrophil migration is supported by previous studies demonstrating the inhibition of PtdIns(3,4,5)P3 production and chemotaxis after in vitro stimulation of neutrophil with C5a in PI3Kγ−/− neutrophils (19). It is well-known also that neutrophil recruitment in response to Gram-negative infection involves bacterial-derived chemotactic factors such as N-formyl peptides (fMLP) and that, in the absence of PI3K neutrophils, cannot migrate toward fMLP (5, 19, 43, 44). In agreement with these previous data, the recruitment of neutrophils induced by C5a and fMLP was prevented in PI3Kγ−/− mice.

In conclusion, we demonstrate there is differential usage of PI3Kγ when neutrophil recruitment is induced by different chemommmvers (CXCL1, C5a, and fMLP) and whether recruitment occurs across endothelial or epithelial barriers. Whereas C5a and fMLP appear to rely entirely on PI3Kγ for induction of neutrophil influx, CXCL1-mediated effects largely depend on the redundant role of PI3Kγ and PI3Kδ. Neutrophil emigration across the epithelium also relies on PI3Kγ expression. The redundant role of and cooperation between signaling molecules and enzymes may be fundamental in providing additional specificity and complexity to signals initiated after seven-transmembrane receptor activation.

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Disclosures
Joel S. Hayflick is an employee of ICOS Corporation and Christian Rom- nel and Thomas Ruckle are employees of Merck Serono. These companies have commercial interest in developing PI3K-selective inhibitors for the treatment of inflammatory disease. The other authors declare no financial conflict of interest.

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The second author’s name should have been published as Remo Castro Russo.


In **Materials and Methods**, the accession number should have been included on page 631 as the last sentence at the end of the paragraph under the heading **Microarrays analysis**. The omitted sentence should read, “The microarray data were deposited in the public Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/) under accession no. GSE11115.”