Leukocyte phosphoinositide-3 kinase γ is required for chemokine-induced, sustained adhesion under flow in vivo

David F. Smith,*,[1] Tracy L. Deem,†,[1] Anthony C. Bruce,† Jörg Reutershan,† Daniel Wu,‡ and Klaus Ley†,§,[2]

*Departments of *Molecular Physiology and Biophysics and §Biomedical Engineering, and †Robert M. Berne Cardiovascular Research Center, University of Virginia, Charlottesville, Virginia, USA; and ‡Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, Connecticut, USA*

Abstract: During inflammation, leukocytes roll along the wall of postcapillary venules scanning the surface for immobilized CXCL1, a chemokine that triggers firm adhesion by activating CXCR2 on the neutrophil. PI-3K are signaling molecules important in cellular processes, ranging from cellular differentiation to leukocyte migration. PI-3Kγ can be activated directly by the βγ dimer of heterotrimeric G proteins coupled to CXCR2. Here, we used in vivo and ex vivo intravital microscopy models to test the role of PI-3Kγ in leukocyte arrest. PI-3Kγ null mice showed an 80% decrease in CXCL1-induced leukocyte adhesion in venules of the exteriorized mouse cremaster muscle. In wild-type mice, rolling leukocytes showed rapid and sustained adhesion, but in PI-3Kγ$^{-/-}$ mice, adhesion was not triggered at all or was transient, suggesting that absence of PI-3Kγ interferes with integrin bond strengthening. Wild-type mice reconstituted with PI-3Kγ null bone marrow showed a 50% decrease in CXCL1-induced leukocyte adhesion. In a blood-perfused micro-flow chamber, leukocytes from PI-3Kγ$^{-/-}$ mice showed a defect in adhesion on a P-selectin/ICAM-1/CXCL1 substrate, indicating that leukocyte PI-3Kγ was required for adhesion. The adhesion defect in PI-3Kγ$^{-/-}$ mice was as severe as that in mice lacking LFA-1, the major integrin responsible for neutrophil adhesion. We conclude that the γ isoform of PI-3K must be functional in leukocytes to allow efficient adhesion from rolling in response to chemokine stimulation. *J. Leukoc. Biol.* 80: 1491–1499; 2006.

Key Words: *signal transduction · cell trafficking · inflammation*

INTRODUCTION

Chemokines are small, structurally similar chemoattractant peptides [1] known to regulate cellular processes ranging from inflammation to immune cell differentiation. Some chemokines specifically act as arrest chemokines when presented on the surface of a vessel [2]. CCL2, CCL5, CXCL1, CXCL8, and CXCL12 have all been shown to mediate monocyte arrest [3–6]. CXCL8 [7] and CXCL1-3 [8, 9] can mediate neutrophil arrest. In vivo, one or more ligands of CXCR2 are relevant neutrophil-arrest chemokines [8].

Arrest chemokines bind to heptahelical G protein-coupled receptors to activate downstream signaling pathways that induce firm adhesion. Each G protein is composed of α, β, and γ subunits [10]. Upon chemokine binding, the Gα subunit dissociates from the Gβγ subunit to initiate specific signaling pathways [10]. However, signaling mechanisms downstream of the receptor involved in chemokine-induced leukocyte adhesion have yet to be defined clearly. In human monocytes, CXCL1-induced adhesion was blocked by the PI-3K inhibitors wortmannin and LY294002 [9]. Furthermore, these inhibitors also blocked CXCL12-, CCL19-, and CCL21-induced lymphocyte adhesion [11]. These data suggest that chemokine receptor signaling through PI-3K plays a role in leukocyte adhesion but do not identify the isoform responsible for arrest.

Leukocyte arrest from rolling requires coordinated signaling between the chemokine receptor and the integrin LFA-1($\alpha_L\beta_2$) [12]. LFA-1 is naturally in a folded, bent conformation with low affinity for its ligands such as ICAM-1 [13]. Upon cell activation, the cytoplasmic tails of α_L and β_2 move apart and induce the extended conformation by inside-out signaling [13]. The extended conformation can bind the ligand, which in turn, leads to further integrin activation [14]. LFA-1 binding is stabilized by post-receptor occupancy events such as clustering [11, 15].

PI-3K is a family of enzymes that phosphorylates the 3-hydroxyl group of the inositol head group of D-*myo*-phosphatidylinositol (PI) and its derivative PI 4,5-bisphosphate [16]. PI-3K, present in multiple isoforms, can be divided into three classes [17], categorized based on subunit structure, substrate specificity, and regulation [18]. PI-3Kγ is a Class IB PI-3K composed of a p110γ catalytic subunit and a 101-kDa regulatory subunit, p101 [19, 20]. This PI-3K isoform has been shown to signal downstream of the Gβγ subunit of chemokine receptors [21].

Previous research about PI-3Kγ has focused on its role in cell activation, superoxide production, and migration. This

[1] These authors contributed equally to this work.

[2] Correspondence: University of Virginia, Robert M. Berne Cardiovascular Research Center, MR5 Bldg., Room 1013, P.O. Box 801394, Charlottesville, VA 22908-1394, USA. E-mail: klausley@virginia.edu

Received March 29, 2006; revised July 21, 2006; accepted August 15, 2006
doi: 10.1189/jlb.0306227

work was aided by the generation of PI-3Kγ knockout (KO) mice lacking the p110 catalytic subunit [22–24]. PI-3Kγ was found to be necessary for PI 3,4,5-trisphosphate production, protein kinase B/Akt activation, and superoxide production in fMLP and C5a-treated neutrophils isolated from PI-3Kγ$^{-/-}$ mice [22–24]. Furthermore, these neutrophils had impaired migration in response to fMLP [22–24]. Several reports have identified a role for PI-3Kγ in chemoattractant-directed migration of other leukocyte subsets. Dendritic cells (DC) from PI-3Kγ$^{-/-}$ mice have been shown to have an impaired migratory response to the chemokines CCL3, CCL5, and CCL19 compared with wild-type DC [25]. Macrophage migration to MCP-1 and CCL5 was also impaired in these mice [26, 27]. The possible clinical significance of the role of PI-3Kγ in inflammation is demonstrated by a significant beneficial effect of pharmacological inhibition of PI-3Kγ in experimental arthritis [28]

An inability of leukocyte subsets to migrate into tissue may in part be a result of an inability to adhere to the vessel wall. Blocking PI-3K by pharmalogical inhibitors was shown to block mobility but not affinity changes of integrins on lymphocytes [11], effectively blocking lymphocyte arrest to a low-density ICAM-1 substrate. However, these data did not identify the isoform of PI-3K involved. Gerszten et al. [29] demonstrated that PI-3K was required for MCP-1-stimulated arrest of human monocytes to E-selectin-transfected HUVEC. Lysates from these monocytes were shown to have increased PI-3Kα activity [29]. However, it was not determined if this increased activity mediates human monocyte arrest. In vivo studies using PI-3Kγ$^{-/-}$ mice have demonstrated a role for PI-3Kγ in platelet aggregation in a carotid artery injury model [30] and granulocyte tethering to endothelial cells in TNF-α-treated cremaster venules [31]. However, no studies have addressed whether leukocyte PI-3Kγ is required for leukocyte adhesion.

This study was undertaken to explore the role of leukocyte PI-3Kγ in adhesion in vivo and in an ex vivo, blood-perfused micro-flow chamber with controlled substrates. We discovered a profound adhesion defect in PI-3Kγ$^{-/-}$ mice, similar in severity to that seen in LFA-1$^{-/-}$ mice.

MATERIALS AND METHODS

Reagents

The murine chemokine CXCL1 [keratinocyte-derived chemokine (KC)] was purchased from PeproTech (Rocky Hill, NJ). Recombinant murine P-selectin and recombinant murine ICAM-1 were purchased from R&D Systems (Minneapolis, MN). fMLP was purchased from Sigma-Aldrich (St. Louis, MO).

Mice

All mouse experiments were performed under a protocol approved by the Animal Care and Use Committee of the University of Virginia (Charlottesville). Animals were housed in a barrier facility under specific pathogen-free conditions, and all experiments were performed on 6- to 20-week-old mice that appeared healthy. Breeder pairs of PI-3Kγ KO mice were provided by Dr. Dianqing Wu at the University of Connecticut (Farmington) [24]. Mice were bred, and deletion of the p110 subunit of PI-3Kγ was determined by PCR. Data from PI-3Kγ null mice were compared with data from littermate-control, wild-type mice. In experiments using the PI-3K inhibitor LY294002, mice were treated for 1 h with 100 mg/kg LY294002 or the vehicle control ethanol followed by intravital microscopy. LFA-1$^{-/-}$ mice were a kind gift of Dr. Christie Ballantyne [32].

Surgical preparation

Mice were anesthetized with an i.p. injection of ketamine hydrochloride (125 mg/kg, Sanofi Winthrop Pharmaceuticals, New York, NY), atropine sulfate (0.025 mg/kg, Fujisawa USA, Inc., Deerfield, IL), and xylazine (12.5 mg/kg, TranquiVed, Phoenix Scientific, Inc., St. Joseph, MO) and placed on a heating pad maintained at 37°C. Polyethylene (PE) 90 tubing [internal diameter (ID) 0.86 mm, outer diameter (OD) 1.27 mm] was used to intubate the trachea of the mouse to allow unobstructed breathing, and PE 10 tubing (ID 0.28 mm, OD 0.61 mm) was used to cannulate the carotid artery. The cannula in the carotid artery was flushed with 10 units/ml heparin sodium in saline before exteriorization of the cremaster. A 50 μl aliquot of blood was drawn from the PE 10 tubing connected to the carotid artery, and differential leukocyte counts were measured from this aliquot using an automated blood cell counter (Hemavet 850FS, CDC Technologies, Oxford, CT).

Intravital microscopy

The cremaster muscle was prepared for intravital microscopy as described previously [33, 34]. The cremaster muscle, epididymis, and testis were exteriorized and pinned to the surgical stage. An incision was made along the length of the cremaster so that the muscle could be splayed. The epididymis and testis were pinned to the side to expose the cremaster muscle microcirculation. During surgery, the cremaster was superfused with bicarbonate-buffered saline (131.9 mM NaCl, 4.7 mM KCl, 18 mM $NaHCO_3$, 1.2 mM $MgCl_2$, and 2.0 mM $CaCl_2{\cdot}2H_2O$) and equilibrated with 5% CO_2 in N_2. In this model, >95% of all adherent and rolling leukocytes are neutrophils [35].

Brightfield intravital microscopy was performed using a Zeiss intravital microscope with a salt water (SW) 40/0.75 saline immersion objective. Three venules per mouse with diameters between 20 μm and 50 μm were recorded using a charged-coupled device (CCD) camera (Model VE-1000CD, Dage-MTI) on a Panasonic S-VHS recorder. Adherent cells were counted in the vessel 1 min before and 1 min after injection of 600 ng CXCL1 or 0.1 μM fMLP i.v. Only the cells that were adherent at the 1 min time-point were included. The number of adherent cells was divided by the surface area of the vessel wall seen in the field of view (FOV). Surface area (S) was calculated for each vessel, using $S = \pi * d * l_v$, where d is the diameter, and l_v is the length of the vessel. Rolling flux was measured as the number of cells that rolled past a line perpendicular to the vessel axis per minute. To compare leukocyte-rolling flux in venules of different diameters, rolling flux was normalized to an average vessel width of 50 μm.

Blood-perfused micro-flow chamber

Micro-flow chambers were constructed from 20 × 200 μm rectangular glass capillaries (VitroCom, Mountain Lake, NJ) as described [36]. Rectangular glass capillaries (30 mm), cut with a capillary cutting stone (Hampton Research, Aliso Viejo, CA), were placed between two plastic microscope coverslips, allowing ~5 mm of the capillary to extend beyond the edge of the coverslips. The slide covers were attached to a glass microscope slide using clear fingernail polish. P-selectin (18 μg/ml), ICAM-1 (15 μg/ml), and CXCL1 (15 μg/ml) were diluted in PBS and allowed to adsorb onto the glass surface for 2 h at room temperature alone or as mixtures. Heparinized PE 50 tubing (ID 0.58 mm, OD 0.965 mm, Becton Dickinson, Sparks, MD), 5 cm in length, was inserted over the end of one side of the capillary tubing, and the joint was sealed with epoxy gel resin (Loctite, Manco Inc., Avon, OH). The chambers were washed with saline and incubated with 10% casein (Pierce Chemicals, Dallas, TX) in PBS for 1 h at room temperature. The free end of the chamber was inserted into a piece of saline-filled PE 50 tubing, and the joint was glued with epoxy gel resin. The saline-filled PE 50 tubing was used to control the wall shear stress of the blood flow. The upstream PE 50 tubing was connected to the PE 10 inserted into the carotid artery of the mouse. Microscopy was conducted using a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY) with a saline immersion objective (SW 20/0.5). Images were recorded with a 3-CCD color video camera (Model DXC-390, Sony Corp., Japan) connected to a Panasonic S-VHS recorder.

Blood was allowed to perfuse through the flow chamber for 6 min. After the 6th min, one FOV was recorded from the center of each capillary chamber for 1 min. Arrested cells were defined as those cells that were adherent for 30 s.

Bone marrow transplants

Male, wild-type mice were irradiated with a split dose of 1200 rads over 3 h. The mice were then reconstituted with 5×10^6 bone marrow cells from female PI-3K$\gamma^{-/-}$ mice by lateral tail vein injections. Reconstitution was verified by RT-PCR using primers for the y-chromosome [37]. The chimeric mice were used after 6 weeks.

Statistics

Data are presented as the mean ± SEM of three to 10 independent experiments. An ANOVA followed by a Tukey's multiple comparisons test was used with the null hypothesis rejected at $P < 0.05$.

RESULTS

PI-3K is required for leukocyte adhesion in cremaster muscle postcapillary venules

To determine if PI-3K is required for leukocyte adhesion in CXCL1-treated cremaster muscle postcapillary venules, wild-type mice were pretreated for 1 h with LY294002 (100 mg/kg), which inhibits all isoforms of PI-3K, or the vehicle control ethanol prior to exteriorization of the cremaster muscle. The venules of the cremaster muscle were then examined by light microscopy. In this model, surgery-induced trauma elicits low levels of background leukocyte adhesion (<6 cells/200 μm; **Fig. 1A**) [37]. i.v. injection of 600 ng CXCL1 leads to rapid immobilization on the endothelial surface and triggers neutrophil adhesion [8]. CXCL1 induced leukocyte adhesion in ethanol-treated control mice (431±39 adherent cells per mm^2 before to 784±54 after). However, in LY294002-treated mice, CXCL1 did not induce significant leukocyte adhesion (138±41 adherent cells per mm^2 before to 231±50 after). These data are in agreement with previous studies that have shown a role for PI-3K in leukocyte adhesion [11].

As chemokines mediate the transition from leukocyte rolling to adhesion, and PI-3Kγ has been shown to signal downstream of G protein-coupled chemokine receptors [21], we next determined if PI-3Kγ is required for leukocyte adhesion in the cremaster muscle venules. CXCL1 induced leukocyte adhesion in wild-type mice (279±50 adherent cells per mm^2 before to 583±98 after; Fig. 1B); however, in PI-3Kγ null mice, CXCL1-induced adhesion was reduced drastically (66±27 adherent cells per mm^2 before to 162±55 after; Fig. 1B). Representative video micrographs of wild-type and PI-3K$\gamma^{-/-}$ mice before and after CXCL1 treatment are shown in Figure 1D. It is important to note that trauma-induced adhesion in PI-3Kγ null mice was significantly less than in wild-type mice, suggesting that PI-3Kγ signaling is important for this adhesion, even without chemokines added exogenously. Furthermore, this defect in adhesion is likely a result of signaling, as neutrophils from wild-type and PI-3Kγ do not differ in adhe-

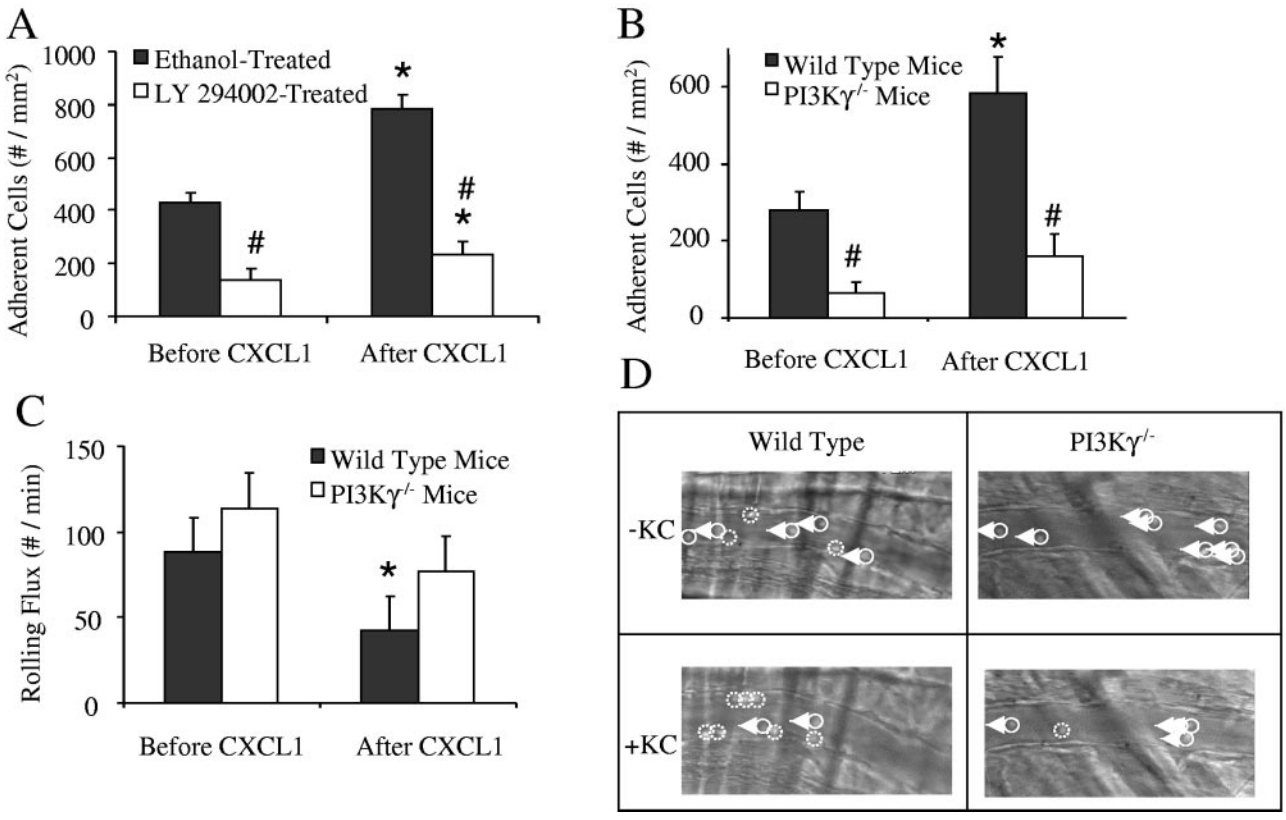

Fig. 1. Rolling and adhesion in cremaster muscle postcapillary venules of PI-3K$\gamma^{-/-}$ and LY294002-treated mice. (A) Adherent leukocytes in LY294002-treated mice (open bars) and vehicle control-treated mice (solid bars) after injection of 600 ng CXCL1. (B) Adherent leukocytes or (C) rolling flux in PI-3K$\gamma^{-/-}$ mice (open bars) and littermate control wild-type mice (solid bars) after injection of 600 ng CXCL1. Data presented are the mean ± SEM from at least 10 vessels from three mice. *, $P < 0.05$, compared with vessels prior to CXCL1 injection; #, $P < 0.05$, compared with littermate control wild-type mice. (D) Representative pictures of cremaster muscle postcapillary venules in wild-type and PI-3K$\gamma^{-/-}$ mice before and 1 min after CXCL1 injection.

sion molecule and chemokine expression (data not shown). As expected, rolling flux was decreased in wild-type mice upon chemokine injection. This effect was less pronounced in PI-3K$\gamma^{-/-}$ mice (Fig. 1C). A decrease in rolling is often seen when more cells adhere [39]. These data indicate that PI-3K$\gamma^{-/-}$ is required for CXCL1-dependent leukocyte adhesion.

PI-3Kγ-deficient leukocytes adhere transiently but cannot remain attached

In wild-type mice, leukocytes adhered rapidly to the vessel wall after injection of CXCL1 and remained attached over time (**Fig. 2A**). However, in PI-3K$\gamma^{-/-}$ mice, leukocytes did not attach or attached transiently (Fig. 2B). This was illustrated further when individual leukocytes were tracked within a given vessel. Individual leukocytes within a vessel of a CXCL1-treated wild-type mouse adhered rapidly and remained attached to the vessel (Fig. 2C). In contrast, individual leukocytes within a vessel of a CXCL1-treated PI-3Kγ null mouse adhered rapidly but only ~50% remained firmly attached over time (Fig. 2D). This suggests that firm adhesion is defective in these mice.

Leukocyte PI-3Kγ is required for adhesion in an auto-perfused micro-flow chamber

Diacovo and colleagues [40] have reported that endothelial PI-3K may play a role in mediating leukocyte capture. Therefore, we designed two sets of experiments to determine if the adhesion defect we found in vivo was a result of endothelial or leukocyte PI-3Kγ. Blood-perfused micro-flow chambers [36] were coated with P-selectin (20 μg/ml) and ICAM (15 μg/ml), with or without CXCL1 (15 μg/ml), and connected to the carotid artery of PI-3K$\gamma^{-/-}$ or wild-type mice. This combination of adhesion molecules and chemokines has been demonstrated previously to mediate leukocyte adhesion [41]. In

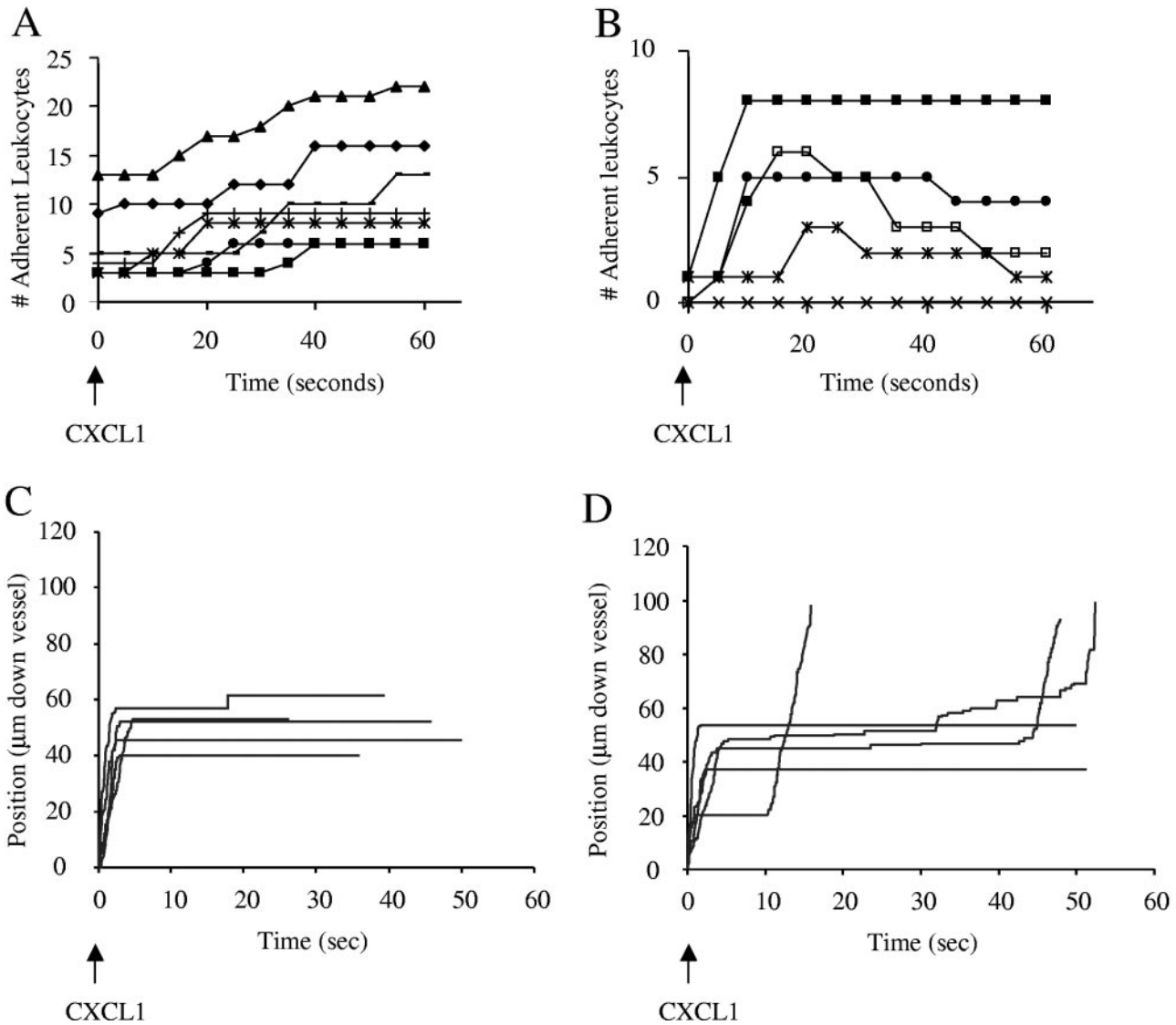

Fig. 2. Tracking of leukocytes in postcapillary venules of littermate-control wild-type and PI-3K$\gamma^{-/-}$ mice after injection of CXCL1. The change in the number of adherent leukocytes in postcapillary venules of (A) littermate-control wild-type mice and (B) PI-3K$\gamma^{-/-}$ mice tracked every 5 s. Time (0) begins when soluble CXCL1 was injected into the carotid artery of each mouse. Each symbol represents one venule. Length-of-time adherent leukocytes remained bound to the endothelium in one representative postcapillary venule of (C) a wild-type and (D) a PI-3K$\gamma^{-/-}$ mouse. Horizontal lines indicate no displacement = adhesion.

matched littermate wild-type control mice, CXCL1-coated flow chambers increased leukocyte adhesion significantly compared with flow chambers coated without CXCL1. However, in PI-3Kγ null mice, CXCL1 did not induce leukocyte adhesion (**Fig. 3B**), although the number of cells rolling on P-selectin was similar to wild-type mice (Fig. 3A). To ensure that rolling and adhesion were not a result of nonspecific interactions, control chambers were coated with P-selectin, ICAM-1, P-selectin and CXCL1 or ICAM-1 and CXCL1. As expected, chambers with P-selectin supported rolling but not adhesion, and chambers coated with ICAM-1 did not support rolling or adhesion (Fig. 3, A and B). Therefore, these data indicate that leukocyte PI-3Kγ is required for chemokine-triggered adhesion in flow chambers with a defined substrate.

PI-3Kγ-deficient leukocytes show an adhesion defect to PI-3Kγ expressing endothelial cells in response to CXCL1 in postcapillary venules of the cremaster muscle

The blood-perfused micro-flow chamber data suggest that leukocyte PI-3Kγ is required for CXCL1-induced adhesion. However, in vivo, we found a reduced number of adherent leukocytes in vessels prior to CXCL1 stimulation. To address if endothelial cell PI-3Kγ plays a role in trauma-induced adhesion, bone marrow from PI-3Kγ null mice was transplanted into lethally irradiated wild-type mice. Leukocyte adherence in response to CXCL1 was examined in postcapillary venules of the cremaster muscle 6 weeks later. Complete bone marrow reconstitution was verified by RT-PCR (data not shown). CXCL1 induced leukocyte adhesion in wild-type mice (279±50 adherent cells per mm^2 before to 583±98 after); however, in wild-type mice with PI-3Kγ-deficient leukocytes, CXCL1-induced adhesion was reduced significantly (86±34 adherent cells per mm^2 before to 288±74 after; **Fig. 4A**). This reduction in adhesion is statistically significant from wild-type mice but not as severe as in PI-3Kγ null mice, suggesting that there may also be an endothelial cell component to CXCL1-induced adhesion. Similar to what was seen in PI-3K$\gamma^{-/-}$ mice, baseline leukocyte adhesion was also reduced significantly from 279 ± 50 to 86 ± 34 cells per mm^2. It is interesting that the reduction in leukocyte rolling upon CXCL1 injection was similar in PI-3Kγ chimeric mice (66±11 before compared with 25±5 after) and wild-type mice (62±7 compared with 29±7; Fig. 4B), suggesting that leukocyte PI-3Kγ is not required for reduced rolling after chemokine exposure. Taken together, these data show that leukocyte PI-3Kγ signaling is important for trauma- and CXCL1-induced adhesion.

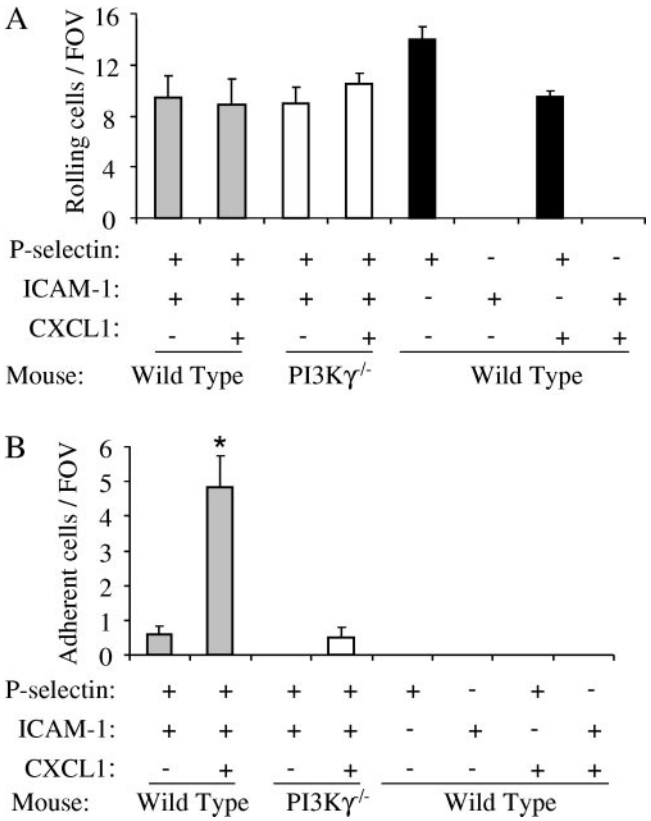

Fig. 3. The number of rolling and adherent leukocytes from wild-type and PI-3K$\gamma^{-/-}$ mice in a blood-perfused micro-flow chamber. (A) Rolling and (B) adherent leukocytes in an auto-perfused flow chamber (2.5 dynes/cm^2) coated with P-selectin (20 μg/ml) and ICAM-1 (15 μg/ml), with or without CXCL1 (15 μg/ml). Data presented are the mean ± SEM from at least nine flow chambers. *, $P < 0.05$, compared with flow chambers coated with P-selectin and ICAM-1 alone.

Leukocyte adhesion in cremaster muscle postcapillary venules is LFA-1-dependent

As chemokine signaling induces firm adhesion through activation of integrins [10], and β_2 integrins are important for leukocyte adhesion [42], we wanted to determine if the defect in LFA-1 null mice was similar to PI-3Kγ null mice. Therefore, leukocyte adhesion in wild-type and LFA-1 null mice [32] was analyzed by intravital microscopy. Similar to the PI-3Kγ null mice, LFA-1 null mice had decreased trauma-induced (148±49 compared with 279±50) and CXCL1-induced adhesion significantly (213±70 compared with 583±98; **Fig. 5A**); however, LFA-1 deficiency did not totally abolish adhesion in these mice, suggesting that LFA-1-independent mechanisms for adhesion exist. In most LFA-1$^{-/-}$ mice, adherent cells did not accumulate over time (Fig. 5B), underscoring the importance of LFA-1 in CXCL1-induced adhesion. To test whether LFA-1 is involved in the residual leukocyte adhesion in PI-3K$\gamma^{-/-}$ mice, we treated these mice with a function-blocking mAb to LFA-1. This treatment reduced neutrophil adhesion to baseline (Fig. 5C).

PI-3K-γ null mice cannot induce leukocyte adhesion in postcapillary venules of the cremaster muscle in response to fMLP

As PI-3Kγ null mice have a defect in CXCL1-induced adhesion, we tested whether PI-3Kγ may be a signaling molecule for leukocyte adhesion triggered by other chemoattractants. To address this, leukocyte attachment in PI-3Kγ null mice was analyzed using intravital microscopy. fMLP induced leukocyte adhesion in wild-type mice (232±68 adherent cells per mm^2 before to 633±126 after; **Fig. 6A**); however, in PI-3Kγ null mice, fMLP-induced adhesion was reduced drastically (51±17 adherent cells per mm^2 before to 92±27 after; Fig. 6A). Examination of leukocyte attachment showed that increasing

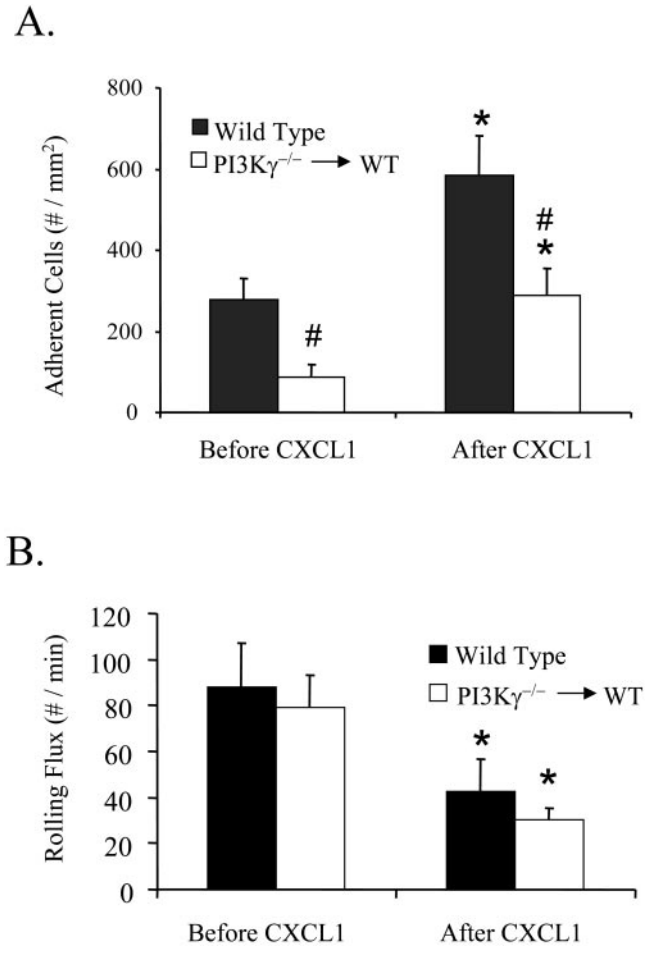

Fig. 4. Rolling and adhesion in cremaster muscle postcapillary venules of wild-type mice and wild-type mice transplanted with bone marrow from PI-3K$\gamma^{-/-}$ mice. (A) Adherent leukocytes or (B) rolling flux (cells/min in a 50 μm wide vessel) of leukocytes in wild-type (solid bars) or irradiated, wild-type mice transplanted with bone marrow from PI-3K$\gamma^{-/-}$ mice (open bars) after injection of 600 ng CXCL1. Data presented are the mean ± SEM from at least nine vessels from three mice. *, $P < 0.05$, compared with vessels prior to CXCL1 injection; #, $P < 0.05$, compared with wild-type mice.

numbers of leukocytes in wild-type mice adhered firmly after fMLP injection (Fig. 6B). In contrast, leukocytes in vessels of fMLP-treated PI-3K$\gamma^{-/-}$ mice only attached transiently (Fig. 6C). These data suggest that PI-3Kγ is also required for fMLP-induced leukocyte adhesion.

DISCUSSION

Although PI-3Kγ has been implicated previously in neutrophil migration and superoxide production [22–24], its role in adhesion of rolling neutrophils has not been investigated. Furthermore, as chemokine signaling through G protein-coupled receptors induces leukocyte adhesion, and PI-3Kγ is activated downstream of the βγ subunit of G protein-coupled receptors, we hypothesized that PI-3Kγ-deficient mice would have a defect in chemokine-induced leukocyte adhesion. Using intravital microscopy to examine leukocyte adhesion to the microvessels of the cremaster muscle, we demonstrate that a lack

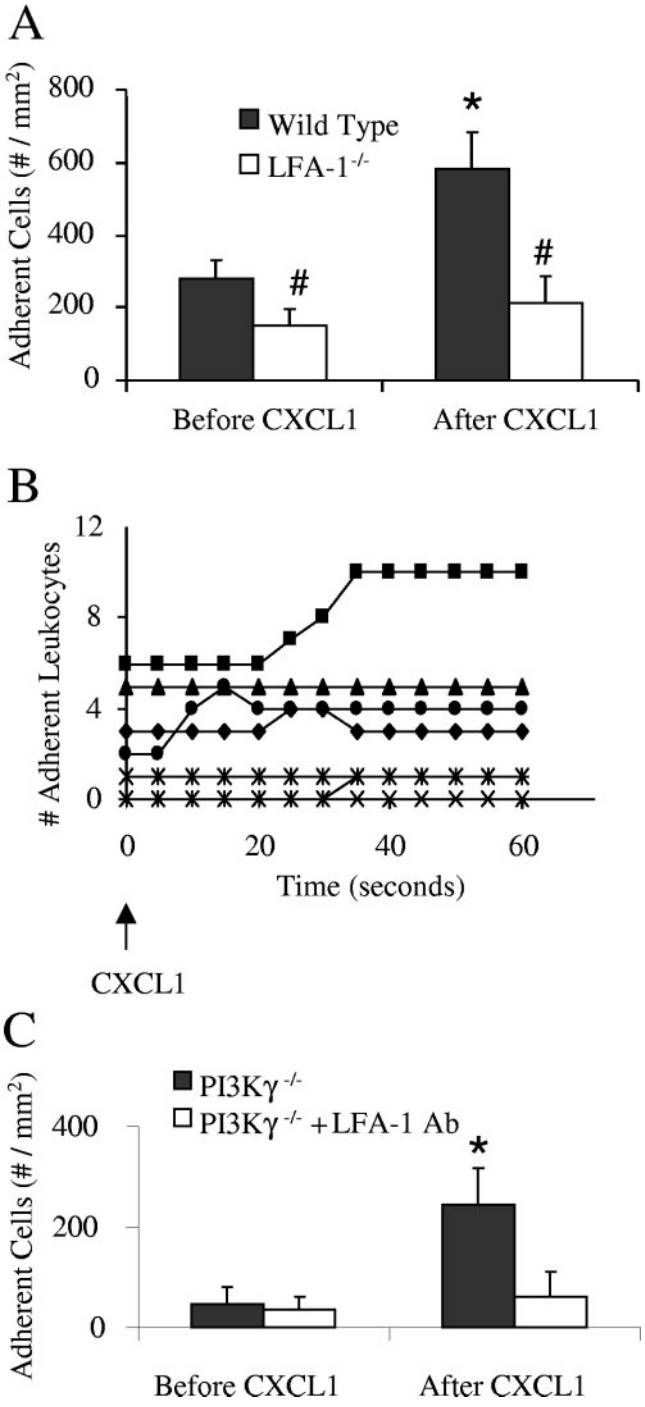

Fig. 5. Rolling and adhesion in cremaster muscle postcapillary venules of LFA-1$^{-/-}$ and wild-type mice. (A) Adherent leukocytes in LFA-1$^{-/-}$ (open bars) and wild-type mice (solid bars) after injection of 600 ng CXCL1. Data presented are the mean ± SEM from at least 10 vessels from three mice. *, $P < 0.05$, compared with vessels prior to CXCL1 injection; #, $P < 0.05$, compared with wild-type mice. (B) The change in the number of adherent leukocytes in postcapillary venules of LFA-1$^{-/-}$ mice tracked every 5 s. Time (0) begins when soluble CXCL1 was injected into the carotid artery of each mouse. Each symbol represents one venule. (C) A blocking antibody to LFA-1 was injected into P13K$\gamma^{-/-}$ mice (1 μg/g body weight; open bars). After 30 min when the antibody had time to bind, and the effects of injected CXCL1 were gone, another 600 ng CXCL1 was administered. The number of leukocytes adhered 1 min before and 1 min after was recorded in the same vessel visualized previously. Data presented are the mean ± SEM from five vessels from five mice. *, $P < 0.05$, compared with vessels prior to CXCL1 injection.

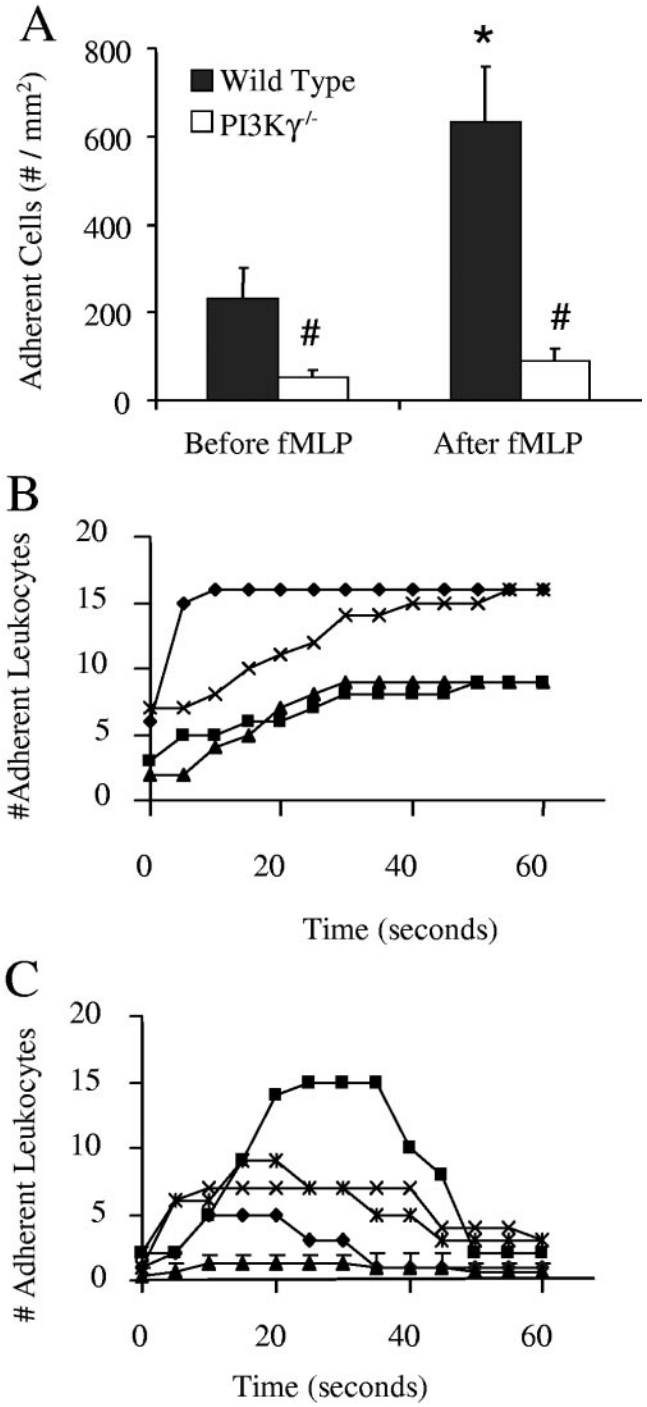

Fig. 6. Rolling and adhesion in cremaster muscle postcapillary venules of PI-3K$\gamma^{-/-}$ and wild-type mice treated with fMLP. (A) Adherent leukocytes in PI-3K$\gamma^{-/-}$ mice (open bars) and wild-type mice (solid bars) after injection of 0.1 μM fMLP. Data presented are the mean ± SEM from at least six vessels from three mice. *, $P < 0.05$, compared with vessels prior to fMLP injection; #, $P < 0.05$, compared with wild-type mice. The change in the number of adherent leukocytes in postcapillary venules of (B) wild-type mice or (C) PI-3K$\gamma^{-/-}$ mice tracked every 5 s. Time (0) begins when soluble fMLP was injected into the carotid artery of each mouse. Each symbol represents one venule.

of PI-3Kγ in mice blocked trauma-, CXCL1-, and fMLP-induced adherence of leukocytes to the vessel wall. Furthermore, using a blood-perfused micro-flow chamber and bone marrow transfer experiments, we demonstrate that the inability of the leukocytes to adhere to the vessel wall is a result of a signaling defect within the leukocyte.

Leukocyte adhesion occurs by multiple sequential events. A leukocyte must first be captured from free flow, roll, and then loosely attach to the endothelium. After loosely attaching, the leukocyte strengthens its adhesion by outside-in signals that mediate cytoskeletal rearrangements, resulting in integrin clustering. As rolling leukocytes in PI-3K$\gamma^{-/-}$ and LY294002-treated mice show transient adhesion that can be blocked by anti-LFA-1 antibodies, it is unlikely that PI-3Kγ is involved in LFA-1 activation by conformational change. Rather, the role of leukocyte PI-3Kγ seems to be involved in strengthening the adhesion and keeping the leukocytes in place under flow. The transient adhesion seen in PI-3K$\gamma^{-/-}$ mice is similar to what has been shown for Vav1/3 null mice, which have impaired activation of Rho family GTPases [43]. In PI-3Kγ and Vav KO mice, the adhesion defect is likely a result of the lack of cytoskeletal rearrangements and thus, inhibition of integrin mobility. Ma et al. [44] demonstrated that transfected COS cells induced fMLP-stimulated actin cytoskeleton restructuring through PI-3Kγ and Vav signaling downstream of the βγ subunit of the G protein-coupled fMLP receptor.

Studies using different densities of the LFA-1 ligand ICAM-1 also confirm a role for PI-3Kγ in interin clustering. Constantin and co-workers [11] demonstrated that inhibition of PI-3K blocked lymphocyte adhesion on low-site densities of ICAM-1; however, high-site densities of ICAM-1 could overcome the requirement for PI-3K. These data are in agreement with the data presented here in neutrophils. The present dataset identifies PI-3Kγ as the isoform responsible for this effect. In our studies, the PI-3K$\gamma^{-/-}$ mice are not treated with proinflammatory cytokines, and consequently, ICAM-1 expression is at baseline levels, and PI-3Kγ is required for firm adhesion. Given these data, it is likely that the leukocytes in the PI-3Kγ null mice cannot adhere firmly to the endothelial cells because of their inability to redistribute integins properly on the cell surface.

A phenotype similar to that described here in PI-3K$\gamma^{-/-}$ mice was described recently in mice lacking the src family members *hck* and *fgr* [44]. Although all chemoattractant-induced neutrophil function tested was normal in these mice, including arrest on fibrinogen, adherent *hck*$^{-/-}$ *fgr*$^{-/-}$ neutrophils failed to show sustained adhesion. However, the time course of adhesion failure was different: *hck*$^{-/-}$ *fgr*$^{-/-}$ neutrophils detached after 4–6 min of chemoattractant activation. PI-3K$\gamma^{-/-}$ neutrophils detached within 1–2 min of chemokine. This difference suggests that PI-3Kγ may be upstream of *hck* and *fgr* signaling. Indeed, *hck* and *fgr* are known to be involved in postadhesion, β_2-integrin-induced, outside-in signaling [45]. Conclusive comparison of the roles of PI-3Kγ and src kinases in adhesion will require side-by-side testing.

Although little is known about endothelial cell function in leukocyte arrest, some data suggest that the PI-3K family may play a role. It has been shown previously that endothelial PI-3Kδ and PI-3Kγ are required for leukocyte capture in TNF-α-stimulated microvessels of the cremaster muscle of mice [31, 40]. In these studies, the authors failed to find

a role for leukocyte PI-3Kγ. Our flow chamber data and bone marrow transplant experiments show unequivocally that PI-3Kγ null leukocytes have a significant and previously unidentified adhesion defect. However, the adhesion defect in chimeric mice is not as dramatic as in PI-3Kγ$^{-/-}$ mice, which confirms a role of endothelial cell PI-3Kγ as proposed by Puri et al. [40]. Leukocyte PI-3Kδ and PI-3Kγ are not required for P- and E-selectin-dependent capture in flow chambers [40], which agrees with our flow chamber data that PI-3Kγ-deficient leukocytes roll equally as well as wild-type leukocytes.

In summary, this report furthers our understanding of signaling mechanisms required for leukocyte adhesion. This paper identifies a key role of leukocyte PI-3Kγ in mediating CXCL1- and fMLP-induced leukocyte adhesion in vivo. Future studies will be important to determine if this is a universal signaling pathway for adhesion of all leukocyte subsets and if all arrest chemokines signal through PI-3Kγ.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health HL58108.

REFERENCES

1. Wells, T. N., Proudfoot, A. E. (1999) Chemokine receptors and their antagonists in allergic lung disease. *Inflamm. Res.* **48,** 353–362.
2. Ley, K. (2003) Arrest chemokines. *Microcirculation* **10,** 289–295.
3. Gerszten, R. E., Garcia-Zepeda, E. A., Lim, Y. C., Yoshida, M., Ding, H. A., Gimbrone Jr., M. A., Luster, A. D., Luscinskas, F. W., Rosenzweig, A. (1999) MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* **398,** 718–723.
4. Chan, J. R., Hyduk, S. J., Cybulsky, M. I. (2001) Chemoattractants induce a rapid and transient upregulation of monocyte α4 integrin affinity for vascular cell adhesion molecule 1 which mediates arrest: an early step in the process of emigration. *J. Exp. Med.* **193,** 1149–1158.
5. Huo, Y., Weber, C., Forlow, S. B., Sperandio, M., Thatte, J., Mack, M., Jung, S., Littman, D. R., Ley, K. (2001) The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *J. Clin. Invest.* **108,** 1307–1314.
6. Weber, K. S., von Hundelshausen, P., Clark-Lewis, I., Weber, P. C., Weber, C. (1999) Differential immobilization and hierarchical involvement of chemokines in monocyte arrest and transmigration on inflamed endothelium in shear flow. *Eur. J. Immunol.* **29,** 700–712.
7. Rainger, G. E., Fisher, A. C., Nash, G. B. (1997) Endothelial-borne platelet-activating factor and interleukin-8 rapidly immobilize rolling neutrophils. *Am. J. Physiol.* **272,** H114–H122.
8. Smith, M. L., Olson, T. S., Ley, K. (2004) CXCR2- and E-selectin-induced neutrophil arrest during inflammation in vivo. *J. Exp. Med.* **200,** 935–939.
9. Smith, D. F., Galkina, E., Ley, K., Huo, Y. (2005) GRO family chemokines are specialized for monocyte arrest from flow. *Am. J. Physiol. Heart Circ. Physiol.* **289,** H1976–H1984.
10. Horuk, R. (2001) Chemokine receptors. *Cytokine Growth Factor Rev.* **12,** 313–335.
11. Constantin, G., Majeed, M., Giagulli, C., Piccio, L., Kim, J. Y., Butcher, E. C., Laudanna, C. (2000) Chemokines trigger immediate β2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* **13,** 759–769.
12. Cinamon, G., Grabovsky, V., Winter, E., Franitza, S., Feigelson, S., Shamri, R., Dwir, O., Alon, R. (2001) Novel chemokine functions in lymphocyte migration through vascular endothelium under shear flow. *J. Leukoc. Biol.* **69,** 860–866.
13. Kim, M., Carman, C. V., Yang, W., Salas, A., Springer, T. A. (2004) The primacy of affinity over clustering in regulation of adhesiveness of the integrin αLβ2. *J. Cell Biol.* **167,** 1241–1253.
14. Shamri, R., Grabovsky, V., Gauguet, J. M., Feigelson, S., Manevich, E., Kolanus, W., Robinson, M. K., Staunton, D. E., von Andrian, U. H., Alon, R. (2005) Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat. Immunol.* **6,** 497–506.
15. Hogg, N., Henderson, R., Leitinger, B., McDowall, A., Porter, J., Stanley, P. (2002) Mechanisms contributing to the activity of integrins on leukocytes. *Immunol. Rev.* **186,** 164–171.
16. Deane, J. A., Fruman, D. A. (2004) Phosphoinositide 3-kinase: diverse roles in immune cell activation. *Annu. Rev. Immunol.* **22,** 563–598.
17. Vanhaesebroeck, B., Leevers, S. J., Panayotou, G., Waterfield, M. D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem. Sci.* **22,** 267–272.
18. Fruman, D. A., Meyers, R. E., Cantley, L. C. (1998) Phosphoinositide kinases. *Annu. Rev. Biochem.* **67,** 481–507.
19. Stephens, L. R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A. S., Thelen, M., Cadwallader, K., Tempst, P., Hawkins, P. T. (1997) The G β γ sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell* **89,** 105–114.
20. Vanhaesebroeck, B., Leevers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J., Waterfield, M. D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **70,** 535–602.
21. Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweis, P. C., Hawkins, P. T. (1994) A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein β γ subunits. *Cell* **77,** 83–93.
22. Sasaki, T., Irie-Sasaki, J., Jones, R. G., Oliveira-dos-Santos, A. J., Stanford, W. L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., Joza, N., Mak, T. W., Ohashi, S., Suzuki, A., Penninger, J. M. (2000) Function of PI3Kγ in thymocyte development, T cell activation, and neutrophil migration. *Science* **287,** 1040–1046.
23. Hirsch, E., Katanaev, V. L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F., Wymann, M. P. (2000) Central role for G protein-coupled phosphoinositide 3-kinase γ in inflammation. *Science* **287,** 1049–1053.
24. Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A. V., Wu, D. (2000) Roles of PLC-β2 and -β3 and PI3Kγ in chemoattractant-mediated signal transduction. *Science* **287,** 1046–1049.
25. Del Prete, A., Vermi, W., Dander, E., Otero, K., Barberis, L., Luini, W., Bernasconi, S., Sironi, M., Santoro, A., Garlanda, C., Facchetti, F., Wymann, M. P., Vecchi, A., Hirsch, E., Mantovani, A., Sozzani, S. (2004) Defective dendritic cell migration and activation of adaptive immunity in PI3Kγ-deficient mice. *EMBO J.* **23,** 3505–3515.
26. Jones, G. E., Prigmore, E., Calvez, R., Hogan, C., Dunn, G. A., Hirsch, E., Wymann, M. P., Ridley, A. J. (2003) Requirement for PI 3-kinase γ in macrophage migration to MCP-1 and CSF-1. *Exp. Cell Res.* **290,** 120–131.
27. Weiss-Haljiti, C., Pasquali, C., Ji, H., Gillieron, C., Chabert, C., Curchod, M. L., Hirsch, E., Ridley, A. J., van Huijsduijnen, R. H., Camps, M., Rommel, C. (2004) Involvement of phosphoinositide 3-kinase γ, Rac, and PAK signaling in chemokine-induced macrophage migration. *J. Biol. Chem.* **279,** 43273–43284.
28. Camps, M., Ruckle, T., Ji, H., Ardissone, V., Rintelen, F., Shaw, J., Ferrandi, C., Chabert, C., Gillieron, C., Francon, B., Martin, T., Gretener, D., Perrin, D., Leroy, D., Vitte, P. A., Hirsch, E., Wymann, M. P., Cirillo, R., Schwarz, M. K., Rommel, C. (2005) Blockade of PI3Kγ suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat. Med.* **11,** 936–943.
29. Gerszten, R. E., Friedrich, E. B., Matsui, T., Hung, R. R., Li, L., Force, T., Rosenzweig, A. (2001) Role of phosphoinositide 3-kinase in monocyte recruitment under flow conditions. *J. Biol. Chem.* **276,** 26846–26851.
30. Lian, L., Wang, Y., Draznin, J., Eslin, D., Bennett, J. S., Poncz, M., Wu, D., Abrams, C. S. (2005) The relative role of PLCβ and PI3Kγ in platelet activation. *Blood* **106,** 110–117.
31. Puri, K. D., Doggett, T. A., Douangpanya, J., Hou, Y., Tino, W. T., Wilson, T., Graf, T., Clayton, E., Turner, M., Hayflick, J. S., Diacovo, T. G. (2004) Mechanisms and implications of phosphoinositide 3-kinase δ in promoting neutrophil trafficking into inflamed tissue. *Blood* **103,** 3448–3456.
32. Ding, Z. M., Babensee, J. E., Simon, S. I., Lu, H. F., Perrard, J. L., Bullard, D. C., Dai, X. Y., Bromley, S. K., Dustin, M. L., Entman, M. L., Smith, C. W., Ballantyne, C. M. (1999) Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. *J. Immunol.* **163,** 5029–5038.
33. Baez, S. (1973) An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. *Microvasc. Res.* **5,** 384–394.

34. Ley, K., Bullard, D. C., Arbones, M. L., Bosse, R., Vestweber, D., Tedder, T. F., Beaudet, A. L. (1995) Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *J. Exp. Med.* **181,** 669–675.
35. Jung, U., Norman, K. E., Scharffetter-Kochanek, K., Beaudet, A. L., Ley, K. (1998) Transit time of leukocytes rolling through venules controls cytokine-induced inflammatory cell recruitment in vivo. *J. Clin. Invest.* **102,** 1526–1533.
36. Chesnutt, B. C., Smith, D. F., Smith, M. L., White, E. J., Ley, K. (2006) Induction of LFA-1-dependent neutrophil rolling on ICAM-1 by engagement of E-selectin. *Microcirculation* **13,** 99–109.
37. Peters, S. O., Bauermeister, K., Simon, J. P., Branke, B., Wagner, T. (2002) Quantitative polymerase chain reaction-based assay with fluorogenic Y-chromosome specific probes to measure bone marrow chimerism in mice. *J. Immunol. Methods* **260,** 109–116.
38. Fiebig, E., Ley, K., Arfors, K. E. (1991) Rapid leukocyte accumulation by "spontaneous" rolling and adhesion in the exteriorized rabbit mesentery. *Int. J. Microcirc. Clin. Exp.* **10,** 127–144.
39. Ley, K., Baker, J. B., Cybulsky, M. I., Gimbrone Jr., M. A., Luscinskas, F. W. (1993) Intravenous interleukin-8 inhibits granulocyte emigration from rabbit mesenteric venules without altering L-selectin expression or leukocyte rolling. *J. Immunol.* **151,** 6347–6357.
40. Puri, K. D., Doggett, T. A., Huang, C. Y., Douangpanya, J., Hayflick, J. S., Turner, M., Penninger, J., Diacovo, T. G. (2005) The role of endothelial PI3Kγ activity in neutrophil trafficking. *Blood* **106,** 150–157.
41. Smith, M. L., Sperandio, M., Galkina, E. V., Ley, K. (2004) Autoperfused mouse flow chamber reveals synergistic neutrophil accumulation through P-selectin and E-selectin. *J. Leukoc. Biol.* **76,** 985–993.
42. Springer, T. A. (1995) Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu. Rev. Physiol.* **57,** 827–872.
43. Gakidis, M. A., Cullere, X., Olson, T., Wilsbacher, J. L., Zhang, B., Moores, S. L., Ley, K., Swat, W., Mayadas, T., Brugge, J. S. (2004) Vav GEFs are required for β2 integrin-dependent functions of neutrophils. *J. Cell Biol.* **166,** 273–282.
44. Ma, A. D., Metjian, A., Bagrodia, S., Taylor, S., Abrams, C. S. (1998) Cytoskeletal reorganization by G protein-coupled receptors is dependent on phosphoinositide 3-kinase γ, a Rac guanosine exchange factor, and Rac. *Mol. Cell. Biol.* **18,** 4744–4751.
45. Giagulli, C., Ottoboni, L., Caveggion, E., Rossi, B., Lowell, C., Constantin, G., Laudanna, C., Berton, G. (2006) The src family kinases hck and fgr are dispensable for inside-out, chemoattractant-induced signaling regulating β2 integrin affinity and valency in neutrophils, but are involved in sustained adhesion. *J. Immunol.* **177,** 604–611.