PI3K accelerates, but is not required for, neutrophil chemotaxis to fMLP

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Summary

PI3K activity, resulting in the accumulation of PIP_3 along the leading edge of a chemotaxing cell, has been proposed to be an indispensable signaling event that is required for cells to undergo chemotaxis to endogenous and exogenous chemoattractants. Some studies have suggested that this might be the case for chemoattractants such as IL8, whereas chemotaxis to other stimuli, such as the bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), might occur normally in the absence of PI3K activity. Herein, we systematically analyze the role of PI3K in mediating chemotaxis to fMLP, both in vitro and in vivo. Using short- and long-term in vitro assays, as well as an in vivo chemotaxis assay, we investigated the importance of PI3K in response to the prototypic chemoattractant fMLP. Exposure of neutrophils to fMLP induced an immediate polarization, which resulted in directional migration towards fMLP within 2-3 minutes. PI3K-inhibited cells also polarized and migrated in a directional fashion towards fMLP; however, this process was delayed by ~15 minutes, demonstrating that PI3K accelerates the initial response to fMLP, but an alternative pathway replaces PI3K over time. By contrast, p38-MAPK-inhibited cells, or cells lacking MK2, were unable to polarize in response to fMLP. Long-term chemotaxis assays using a pan-PI3K inhibitor, a PI3Kδ-specific inhibitor or PI3Kγ-knockout neutrophils, demonstrated no role for PI3K in mediating chemotaxis to fMLP, regardless of the steepness of the fMLP gradient. Similar results were observed in vivo, with PI3Kγ$^{-/-}$ cells displaying a delayed, but otherwise normal, chemotactic response to gradients of fMLP. Together, these data demonstrate that, although PI3K can enhance early responses to the bacterial chemoattractant fMLP, it is not required for migration towards this chemoattractant.

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Introduction

Chemotaxing cells have an amazing ability to detect small changes in the concentration of a chemoattractant, with some cells being able to sense changes as small as a 1% over the length of one cell (8-12 μm) (Song et al., 2006). This ability to detect such small changes in chemoattractant concentration requires some form of gradient amplification within the cell. Several signaling pathways have been proposed to be involved in this gradient-amplification process, the most predominant being the phosphatidylinositol-3 kinase (PI3K) pathway (Li et al., 2000; Sasaki et al., 2000; Stephens et al., 2002). It has been proposed that, upon detecting a chemotactic stimulus, cells will activate PI3K in such a way that PI3K is active along the region of the cell facing the chemoattractant. This results in the accumulation of the product of PI3K, phosphatidylinositol triphosphate (PIP_3), along the leading edge of the cell (Funamoto et al., 2002; Huang et al., 2003; Merlot and Firtel, 2003; Sasaki et al., 2004; Zhelev et al., 2004). This is followed by the accumulation of proteins containing PIP_3-binding domains, thus recruiting the proteins required to form the leading edge of the migrating cell (Kunisaki et al., 2006; Sasaki et al., 2004; Sossey-Alaoui et al., 2005; Van Keymeulen et al., 2006). At the same time, enzymes that mediate the breakdown of PIP_3 will be active on the sides and back of the cell, thus limiting PI3K activity to the front of the cell (Funamoto et al., 2002; Nishio et al., 2007; Wain et al., 2005). This process is believed to be a major mechanism of gradient amplification in migrating cells and has been proposed to be indispensable for chemotaxis to all stimuli.

There are multiple isoforms of PI3K, divided into four classes (Ia, Ib, II and III) (reviewed in Hawkins et al., 2006). Of these four classes, only class Ia and Ib have been implicated in chemotaxis. In particular, one member of the class Ia group (PI3Kδ) and the sole class Ib member (PI3Kγ) have been identified as playing central roles in neutrophil chemotaxis (Ferguson et al., 2007; Ferreira et al., 2006; Sadhu et al., 2003; Sasaki et al., 2000). These two isoforms are activated in fundamentally different ways – class Ia PI3K via binding to phosphorylated motifs, whereas class Ib is activated via direct interactions with the G-proteins associated with chemotactic receptors (Wymann and Pirola, 1998). In line with its direct activation by chemotactic receptors, PI3Kγ was proposed to be a major isoform of PI3K involved in chemotaxis, a conclusion supported by both in vitro and in vivo assays (Ferguson et al., 2007; Hirsch et al., 2000; Li et al., 2000; Naccache et al., 2000; Sasaki et al., 2000). However, recent studies have demonstrated a role for PI3Kδ in mediating the directionality of neutrophil chemotaxis, although the pathway by which PI3Kδ is activated by chemotactic receptors remains unknown (Ferreira et al., 2006; Sadhu et al., 2003).

Although PI3K has been demonstrated to be a central pathway in the chemotaxis of some cell types, it is not clear whether PI3K is a universal pathway for chemotaxis, or a pathway only used by

some cell types and/or some chemoattractants. One example of these conflicting data is neutrophil chemotaxis towards the bacterial peptide formyl-Met-Leu-Phe (fMLP). Some groups have proposed that PI3K activity is an absolute requirement for migration towards fMLP (Sadhu et al., 2003), whereas other groups have identified a partial requirement for PI3K in migration towards fMLP (Boulven et al., 2006; Ferguson et al., 2007), and still other groups have demonstrated that chemotaxis towards fMLP is independent of PI3K (Heit et al., 2002). In addition, recent studies using *Dictyostelium* have demonstrated that chemotaxis, at least under some circumstances, can occur in the absence of both PI3K and its negative regulator PTEN (Andrew and Insall, 2007; Loovers et al., 2006). On the surface, the different conclusions of these studies appear to be unexplainable; however, there are significant temporal and gradient differences in the assay methods used which might explain these differences. Generally speaking, papers that identified a requirement for PI3K used short-term chemotactic assays (15-30 minutes) with steep chemoattractant gradients, whereas papers that identified no role for PI3K generally used long-term assays (2+ hours) with shallow chemoattractant gradients. Long-term assays are unable to detect the minor delays or subtle changes in chemotactic efficiency that can be identified using short-term assays. However, long-term assays might reveal the overall role of PI3K activity in complex biological environments. Of course, in vivo chemotactic responses best reflect the biological importance of PI3K in gradient sensing. However, in vivo chemotactic systems are often difficult to interpret because of the complex nature of the in vivo environment.

Herein, we demonstrate, both in vitro and in vivo, that chemotaxis towards fMLP occurs independently of PI3K. However, when the initial stages of chemotaxis were monitored, it was found that cells deficient in PI3Kγ, or cells treated with either a pan-PI3K inhibitor or a PI3Kδ-specific inhibitor, had a delay in their initial chemotactic response to fMLP, but within 15-20 minutes these cells began migrating normally towards fMLP. Because these in vitro assays have a questionable ability to mimic the in vivo environment, we confirmed these results in PI3Kγ-deficient animals, using an in vivo chemotaxis assay in which we can monitor the migration of cells within tissue. Using this assay, we validated the in vitro results and observed a delayed, but otherwise normal, migration towards fMLP. However, the in vivo assay also demonstrated an important role for PI3K in mediating the emigration of neutrophils out of the blood vessel, explaining why some previous in vivo studies identified chemotactic defects in these animals. Together, these studies demonstrate that PI3K is dispensable for chemotaxis towards fMLP, but can play a role in accelerating chemotaxis to this stimulus.

Results

PI3K is not required for migration to fMLP in long-term chemotaxis assays

To assay long-term migration towards fMLP, we used the under-agarose migration assay, which allows for the establishment of a shallow gradient and for the monitoring of chemotaxis over a period of several hours (Foxman et al., 1997). In the absence of a chemoattractant gradient there is no observable migration in this assay, with an average of 0.2 cells observed outside of a neutrophil-containing well (Heit et al., 2002; Heit and Kubes, 2003). Using this assay, we measured the ability of either untreated cells, cells treated with the pan-PI3K inhibitor LY294002 (30 μM) or cells treated with the PI3Kδ-specific inhibitor IC87114 (10 μM) (Sadhu et al., 2003) to migrate towards fMLP over a 2-hour period. The concentration of inhibitors used was previously demonstrated to inhibit PI3K (Sadhu et al., 2003). These inhibitors had no effect on the number of migrating cells (Fig. 1a), the directionality of cell migration (Fig. 1b), the speed of migration (Fig. 1c) or the distance of the cell front (Fig. 1d) in response to fMLP. It should be noted that a small but consistent decrease in the number of chemotaxing cells (10-25%) was always observed in cells treated with PI3K inhibitors, although this decrease never reached statistical significance.

Another isoform of PI3K, PI3Kγ, has been proposed to be involved in migration towards fMLP (Li et al., 2000). However, no inhibitor specific to this isoform exists. To test the role of this isoform, we isolated neutrophils from wild-type (C57Bl/6) and PI3Kγ$^{-/-}$ mice. Murine neutrophils do not respond as quickly as human neutrophils in the under-agarose assay, so chemotaxis was monitored over a 4-hour period. As expected, no significant difference in the number of migrating neutrophils (Fig. 1e), directionality of migration (Fig. 1f), speed of migration (Fig. 1g) or distance of the cell front (Fig. 1h) was observed between wild-type and PI3Kγ$^{-/-}$ cells, although a small decrease (10-25%) was seen in the number of migrating PI3Kγ$^{-/-}$ cells compared with wild type.

Pan-PI3K inhibitors delay initial responses to fMLP, but block migration to IL8

Although inhibition of PI3K resulted in no significant differences in the number of cells migrating towards fMLP, there was a trend towards fewer migrating cells. This suggests that there might be an impairment or delay at some stage of chemotaxis. Given the proposed role of PI3K in mediating polarization and gradient amplification, we used an Ibidi short-term chemotaxis assay to monitor the initial stages of chemotaxis. This assay places neutrophils in a small volume channel between two fluid reservoirs. A chemoattractant placed in one reservoir will diffuse through the channel, forming a steep and short-lived (~30 minutes) gradient. In the absence of a chemoattractant, cells move less than the length of one cell over the 20-minute observation period (supplementary material Fig. S1a). However, these cells do spread, occasionally taking on a slightly polarized phenotype (supplementary material Fig. S1b). By contrast, uninhibited human neutrophils polarized (Fig. 2a,b) and began moving (Fig. 2c) rapidly in response to fMLP; reaching full polarization and speed within 3 minutes upon addition of fMLP. Cells treated with the pan-PI3K inhibitor LY294002 did not respond as rapidly to fMLP. However, after a 12- to 15-minute delay, these cells reached a normal degree of polarization (Fig. 2a,b) and migratory speed (Fig. 2c) compared to wild-type cells.

Previously, we have reported that, in a long-term chemotaxis assay, chemotaxis to some stimuli, such as the chemokine IL8, is dependent on PI3K (Heit et al., 2002). To determine whether PI3K is also important in mediating the initial responses to IL8, we performed polarization analysis on cells responding to IL8. Unlike fMLP, polarization (Fig. 2d,e) and speed of migration (Fig. 2f) to IL8 were completely inhibited by LY294002. This demonstrates that the PI3K-independent pathway mediating chemotaxis to fMLP is not a universal pathway, and instead is selectively activated by certain chemoattractants.

PI3Kδ accelerates polarization, whereas PI3Kγ accelerates the orientation of neutrophils

It has been proposed that PI3Kγ and PI3Kδ play differential roles in mediating chemotaxis (Reif et al., 2004). Although our long-

term migration assay demonstrated no role for these isoforms in mediating chemotaxis to fMLP, our short-term assay suggests that these isoforms might act to accelerate the initial polarization and orientation of cells in response to fMLP. Using our short-term assay, we first examined the ability of PI3Kδ-inhibited neutrophils to migrate towards fMLP. Interestingly, PI3Kδ-inhibited cells had delayed polarization (Fig. 3a) but there was no significant delay in the percent of polarized cells orientating towards fMLP (Fig. 3b) or the speed of migration (Fig. 3c) in response to fMLP.

We then assayed the initial polarization and acceleration of PI3Kγ$^{-/-}$ neutrophils in response to fMLP. In contrast to PI3Kδ-inhibited neutrophils, PI3Kγ$^{-/-}$ neutrophils polarized in a near-normal fashion (Fig. 3d), but did not orientate themselves towards fMLP as rapidly (Fig. 3e). Like PI3Kδ inhibited cells, PI3Kγ$^{-/-}$ neutrophils had normal migratory speed (Fig. 3f). Together, these results point to distinct roles for PI3Kδ and PI3Kγ in mediating the initial polarization in response to fMLP, with PI3Kδ accelerating the initial polarization of the cells, whereas PI3Kγ aids in orientating the polarized cells towards the source of fMLP.

Interestingly, whereas pan-PI3K inhibition produced a decrease in migration speed (Fig. 2c), neither PI3Kγ$^{-/-}$ cells nor cells treated with the PI3Kδ inhibitor showed a decrease in speed (Fig. 3c,f). One possible explanation for this phenomenon is that another isoform of PI3K is involved in mediating the speed of migration. Alternatively, PI3Kγ and PI3Kδ could be able to maintain full migratory speed independently, and as such changes in speed would only be observed when both isoforms are inhibited. To test these possibilities, we compared the polarization of PI3Kγ$^{-/-}$ cells treated with the PI3Kδ inhibitor (IC87114) to the polarization of uninhibited and pan-PI3K-inhibited (LY294002-treated) murine neutrophils. The same degree of inhibition of polarization (Fig. 3g), orientation (Fig. 3h) and speed (Fig. 3i) was observed between LY294002-treated and PI3Kγ$^{-/-}$/IC87114-treated cells, suggesting that PI3Kδ and PI3Kγ, and not other PI3K isoforms, are responsible for amplifying the initial stages of chemotaxis to fMLP.

One possible explanation as to why there is a delay in the polarization of PI3K-inhibited neutrophils to fMLP is that there is a delay in the regulation of integrins. In particular, neutrophil adhesion via LFA-1 (CD11a-CD18 heterodimer) and MAC-1 (CD11b-CD18 heterodimer) has been demonstrated to require PI3K signaling (Gao et al., 2001; Smith et al., 2006). To test this possibility, we analyzed the adherence of murine neutrophils to ICAM1 under flow before and after the addition of fMLP to the perfusate. Uninhibited neutrophils significantly upregulated their adhesiveness to ICAM1 in response to fMLP, whereas cells treated with a pan-PI3K inhibitor (LY294002) or lacking PI3Kγ were unable to upregulate their adhesion to ICAM1 (Fig. 3j). Neutrophils treated with the PI3Kδ inhibitor IC87114 could upregulate their adhesiveness, but with slower kinetics then uninhibited cells (Fig. 3j).

fMLP and p38 MAPK

Because PI3K does not play an essential role in mediating chemotaxis towards fMLP, we proposed that another pathway was involved in this process. We previously demonstrated that the p38 MAPK pathway is indispensable in mediating the chemotaxis of neutrophils towards fMLP (Heit et al., 2002). To further investigate the underlying mechanism for this observation, we looked at the chemotaxis and

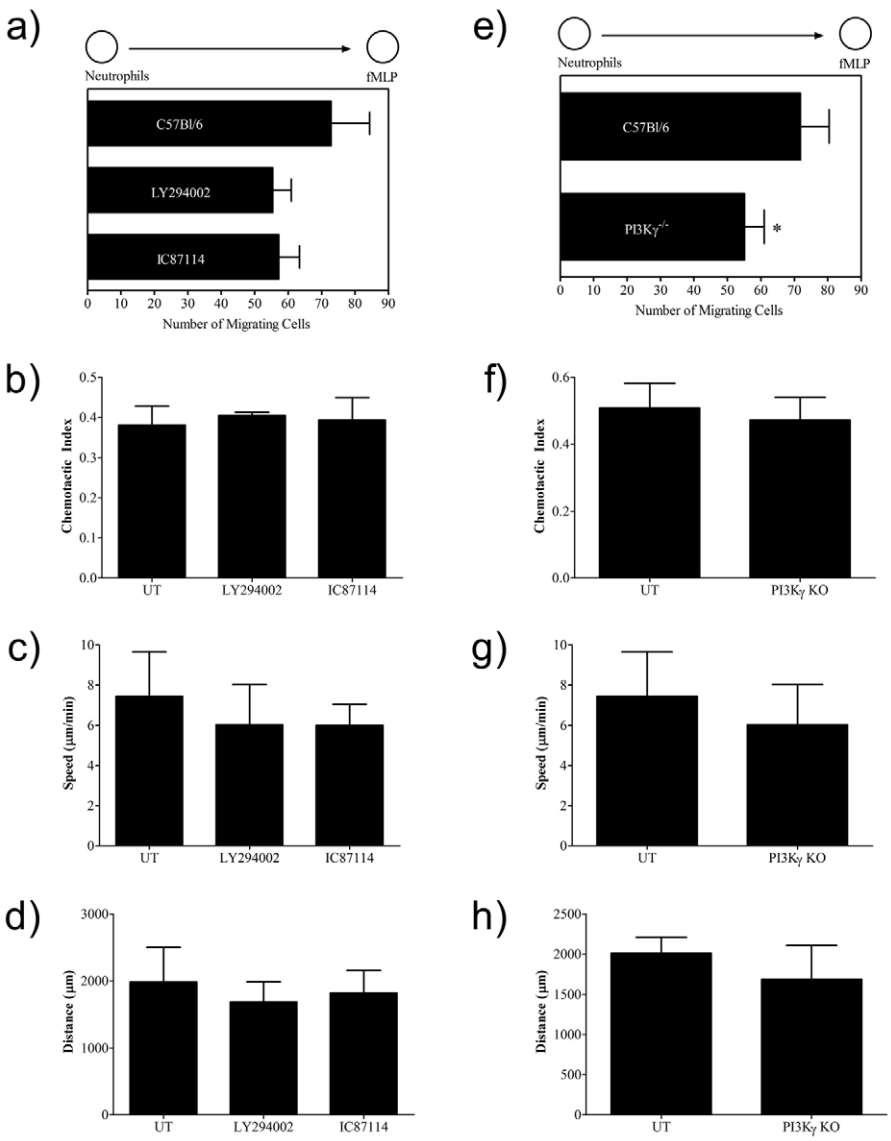

Fig. 1. The role of PI3K in mediating long-term migration to fMLP. Neutrophils were either uninhibited (UT, C57Bl/6), pan-PI3K-inhibited (LY294002), PI3Kδ inhibited (IC87114), wild type (C57Bl/6) or PI3Kγ$^{-/-}$. (a,e) Number of migrating human (a) or murine (e) neutrophils to fMLP. (b,f) Directionality of human (b) or murine (f) neutrophil migration to fMLP. (c,g) Speed of human (c) or murine (g) neutrophil migration to fMLP. (d) Migratory distance of (d) human neutrophil over 2 hours or (h) murine neutrophils over 4 hours to fMLP. $n>4$; $^*P<0.05$, ANOVA with Bonferroni correction.

polarization of human and murine cells treated with the p38 MAPK inhibitor SB203580, as well as murine neutrophils from mice deficient in MAPKAPK2 (MK2), an effector kinase found downstream of p38 MAPK. p38-MAPK-inhibited cells, and cells lacking MK2, did not migrate towards fMLP in a long-term chemotactic assay (Fig. 4a,b). p38-MAPK-inhibited or MK2$^{-/-}$ murine neutrophils did not polarize in response to fMLP in a short-term chemotaxis assay (Fig. 4c).

PI3K accelerates in vivo chemotaxis to fMLP

Because in vitro models of chemotaxis cannot completely replicate the in vivo environment, we assayed the chemotaxis of PI3K$\gamma^{-/-}$ leukocytes within the cremaster muscle in response to a gradient of fMLP. Although this assay allows for the observation of the early and late stages of chemotaxis, the analysis of chemotaxis is complicated by the fact that the presence of neutrophils in this assay requires neutrophils to roll, adhere and emigrate out of the vasculature (reviewed in Kubes, 2002). Each of these events is a completely separate process from chemotaxis and, as such, defects in any of these parameters do not necessarily translate to an equivalent defect in the subsequent chemotaxis. Indeed, PI3K$\gamma^{-/-}$ mice did not recruit leukocytes efficiently in response to fMLP stimulation, with no significant decreases in the number of rolling cells after 30-90 minutes (Fig. 5a), but significant decreases in the number of adherent (Fig. 5b) and emigrating (Fig. 5c) cells observed in the knockout animals.

Even though the recruitment of PI3K$^{-/-}$ leukocytes was drastically reduced, those cells that did emigrate out of the blood vessel migrated in a near-normal fashion, with the same degree of directionality (Fig. 5d) and slightly enhanced migratory speed (Fig. 5e) compared to wild-type cells. Because our in vitro models predicted an initial delay in the migration of PI3K-deficient cells, we measured the time from when a cell first appeared entirely outside the lumen of a blood vessel until that cell was observed to migrate a distance of at least 10 μm (~one-cell length). Wild-type cells rapidly began to move after emigration, whereas PI3K$\gamma^{-/-}$ cells had a significant delay in initiation of migration (Fig. 5f), consistent with our in vitro results.

It is very important to note that, although chemotaxis of individual cells was normal in PI3K$\gamma^{-/-}$ mice, the number of cells that emigrated out of the blood vessel into the tissue was drastically reduced in these animals compared with wild-type animals (Fig. 5c). These data clearly demonstrate that adhesion, emigration and chemotaxis to the same stimuli can occur via different signaling pathways, with PI3Kγ playing an indispensable role in the recruitment of neutrophils, but not in the chemotaxis of neutrophils, in response to fMLP.

These in vivo results might be explained by two possibilities. First, neutrophils within the blood vessels might not be responding directly to fMLP and rather might be responding to a secondary chemoattractant (i.e. CXCL1 and CXCL2, hereafter referred to as KC and MIP-2, respectively), produced by stromal cells or the vascular endothelium, after those cells have been activated by fMLP. This is important because adhesion, emigration and chemotaxis to endogenous chemokines is predominantly dependent on PI3K (Heit et al., 2002; Liu et al., 2007). To test this possibility, we looked at fMLP-induced recruitment and intravascular crawling of neutrophils in CXCR2$^{-/-}$ (IL8RB$^{-/-}$) mice. These mice lack the CXCR2 receptor, which is essential for the recruitment of neutrophils in response to endogenous chemokines such as MIP-2 and KC. Recruitment in response to fMLP was not impaired in these mice (Fig. 6a-d).

The second possible explanation for why PI3K$\gamma^{-/-}$ neutrophils emigrated poorly out of the vasculature in response to fMLP, even though they chemotaxed normally, is that PI3Kγ disruption could result in the disruption of neutrophil recruitment; either by impairing endothelial function or by impairing the ability of neutrophils to transmigrate through endothelial junctions (Phillipson et al., 2006). To test this possibility, we tracked the intraluminal crawling of neutrophils in vivo. Although PI3K$\gamma^{-/-}$ neutrophils adhered to some

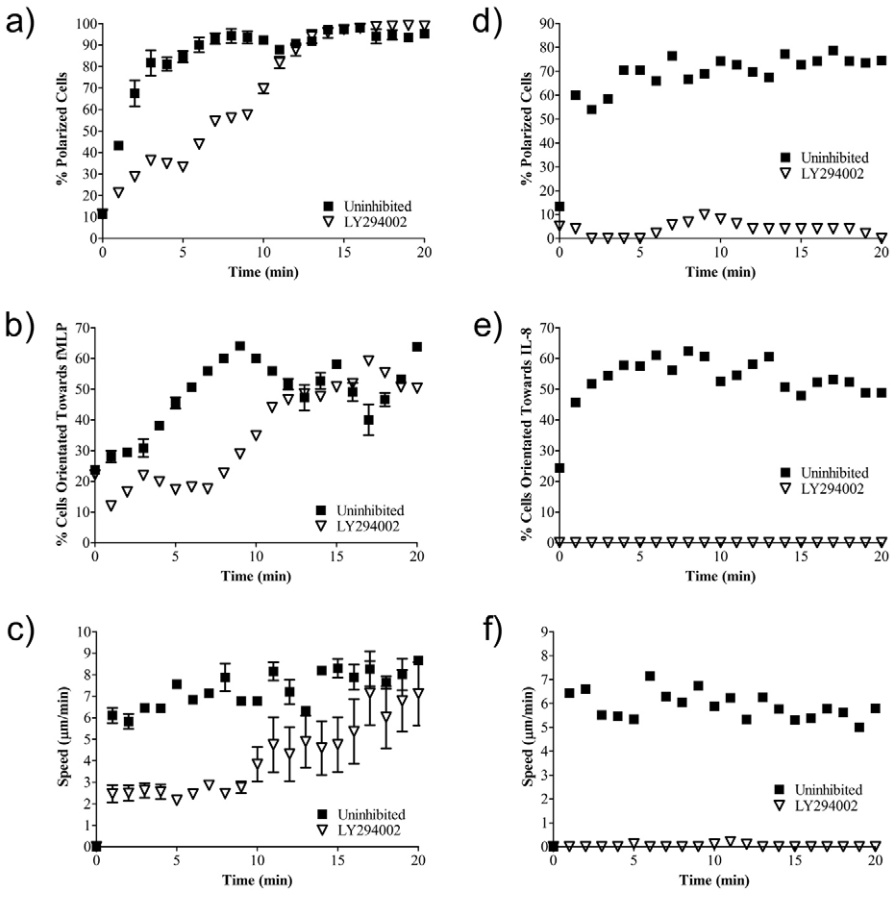

Fig. 2. The role of PI3K in mediating initial responses to fMLP and IL8. Human neutrophils were either uninhibited or treated with a pan-PI3K inhibitor (LY294002). fMLP or IL8 was added at time=0 and the cells monitored for 20 minutes. (a) Percentage of cells that polarized in response to fMLP. (b) Percentage of cells orientated towards the source of fMLP. (c) Speed of migration in response to fMLP. (d) Percentage of cells polarized in response to IL8. (e) Percentage of cells orientated towards the source of IL8. (f) Speed of migration in response to IL8. *n*=4 for all data.

extent (~50% that of wild-type, Fig. 5b), the emigration of PI3K$\gamma^{-/-}$ neutrophils was reduced approximately 50% compared with wild-type neutrophils, with many adherent PI3K$\gamma^{-/-}$ neutrophils eventually detaching from the endothelium rather then emigrating out of the vasculature (Fig. 7). This demonstrates that PI3K$\gamma^{-/-}$ cells were unable to efficiently emigrate out of the vasculature and into the tissues, thus explaining the low level of recruitment observed in these animals. Importantly, these data also suggest that the mechanisms that mediate the chemotaxis of cells in the tissue are different then those that mediate emigration out of the vessel, thus providing an explanation as to why some in vivo studies observed defective recruitment in PI3K-disrupted mice in response to PI3K-independent chemoattractants (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000).

Discussion

In this study, using both in vitro and in vivo models of neutrophil chemotaxis, we demonstrate that PI3K plays a minor role in the chemotaxis of neutrophils towards fMLP. Specifically, PI3K accelerates the initial polarization and chemotaxis to fMLP, but plays no further role in maintaining the directionality and speed of chemotaxis. We observed a similar delay in chemotaxis in both human and murine neutrophils – in cells treated with PI3K inhibitors and in cells lacking the p110γ isoform of PI3K (PI3K$\gamma^{-/-}$). We also identified that the p38 MAPK pathway is key for chemotaxis to fMLP, and contributes to the initial polarization and motility towards fMLP. Lastly, we demonstrated that PI3K plays a role in recruitment out of the blood vessel in response to fMLP, but not in chemotaxis in tissue to fMLP, potentially explaining why some in vivo models of recruitment/chemotaxis observed large decreases in neutrophil accumulation in the involved tissues when PI3K was disrupted (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000).

Short-term chemotaxis assays – such as pipette assays, Boyden chambers, Dunn chambers, Trans-well chambers and the Ibidi chambers used in this study – rely on free diffusion of the chemoattractant through aqueous media to generate gradients and as a result tend to create short-lived 'steep' gradients wherein cells experience a large change in chemoattractant concentration across the cell body (Shutt et al., 1998). By contrast, long-term chemotaxis assays such as our under-agarose assay and in vivo approaches tend to generate 'shallow' chemoattractant gradients in which the cells experience a very small change in concentration over the length of the cell body (Foxman et al., 1997). The steepness of these gradients plays a major role in both the movement of the cells, as well as in the signaling pathways responsible for the resulting cell movement. Indeed, there is growing evidence that the role of PI3K varies greatly depending on the steepness of the gradient. For example, using a system that generates steep gradients, several groups demonstrated that PI3K was required for

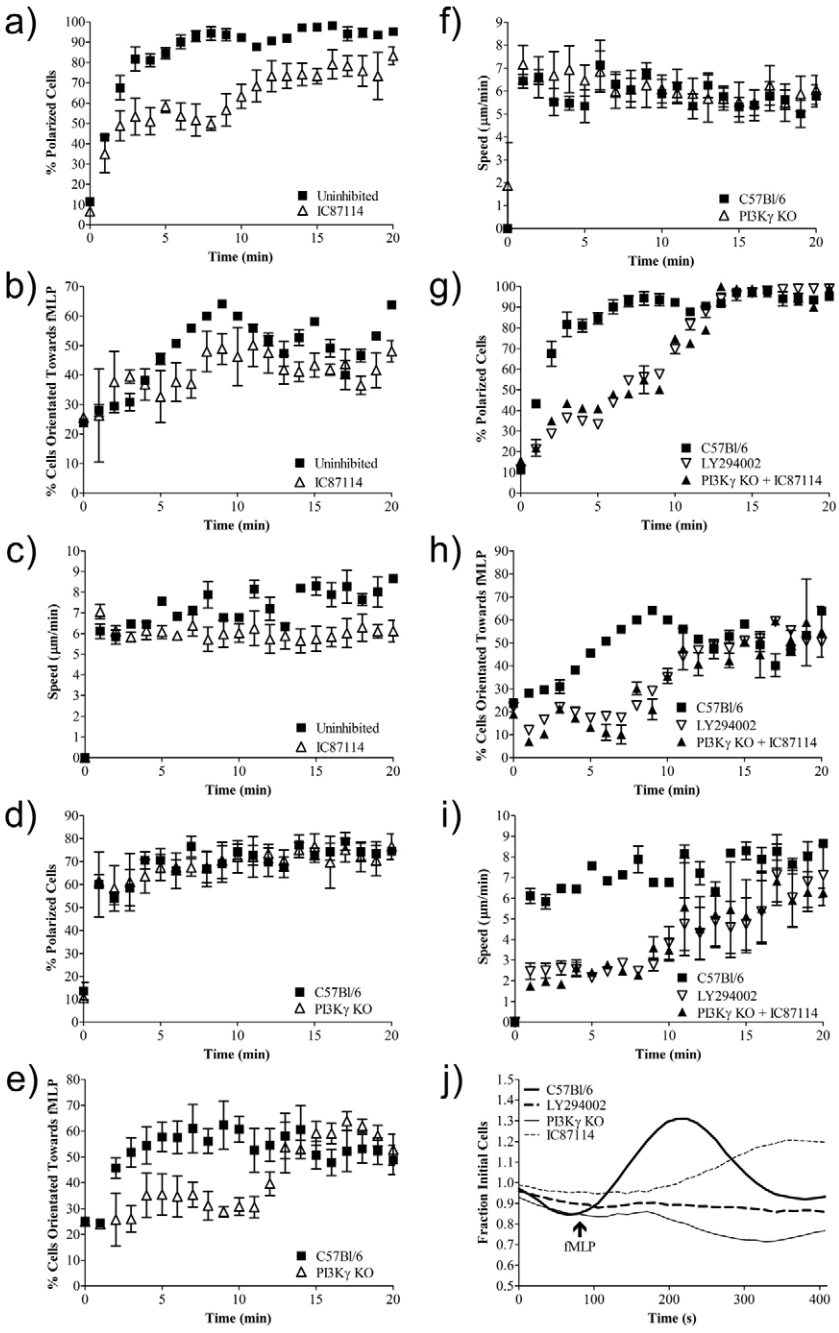

Fig. 3. The role of PI3Kδ and PI3Kγ in mediating initial responses to fMLP. Neutrophils were either uninhibited human cells (uninhibited), uninhibited murine cells (C57Bl/6), treated with a PI3Kδ-specific inhibitor (IC87114) or isolated from PI3K$\gamma^{-/-}$ mice. fMLP was added at time=0 and the cells monitored for 20 minutes. (a) Percentage of human cells that polarized in response to fMLP. (b) Percentage of human neutrophils orientated towards fMLP. (c) Speed of human neutrophil migration in response to fMLP. (d) Percentage of murine cells that polarized in response to fMLP. (e) Percentage of murine neutrophils orientated towards the source of fMLP. (f) Speed of murine neutrophil migration in response to fMLP. (g) Percentage of murine cells that polarized in response to fMLP. (h) Percentage of murine neutrophils orientated towards the source of fMLP. (i) Speed of murine neutrophil migration in response to fMLP. (j) Adhesion of murine neutrophils to ICAM1 pre- and post-fMLP stimulation. *n*=4 for all data.

the chemotaxis of *Dictyostelium* towards cAMP, and that PI3K played a major role in the formation of the leading edge, extension of pseudopods and maintenance of cell polarity (Funamoto et al., 2002; Huang et al., 2003; Iijima et al., 2004; Janetopoulos et al., 2004). However, using the same organism and chemoattractant, Andrew and Insall have demonstrated that, in the presence of shallow gradients, PI3K is not involved in the directional migration of *Dictyostelium*, but rather directional migration is mediated by a different chemotaxis process wherein directional migration is maintained by an initial bifurcation of the pseudopod, followed by retraction of the pseudopod, which detects the lowest chemoattractant concentration (Andrew and Insall, 2007). In this 'alternate' form of chemotaxis, PI3K plays only a minor role, with its activity limited to accelerating the rate in which pseudopods are extended. Our data suggest that mammalian cells (human and murine) can undergo a similar process and that chemotaxis occurs via a variety of mechanisms. Moreover, we demonstrate that different experimental systems might inadvertently bias experiments towards one form of chemotaxis verses another.

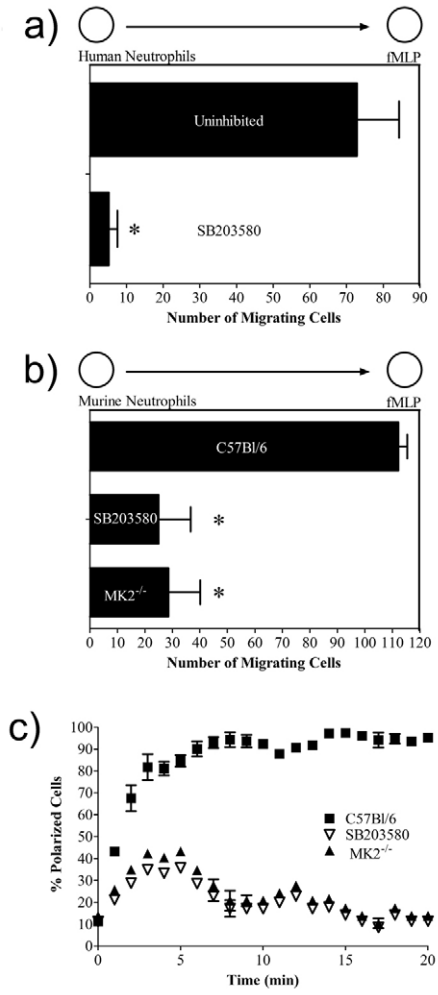

Fig. 4. The role of p38 MAPK during neutrophil chemotaxis to fMLP. (a) Human neutrophils treated with the p38 MAPK inhibitor SB20580 did not chemotax to fMLP in a long-term chemotactic assay. (b) Murine neutrophils treated with the p38 MAPK inhibitor SB203580, or deficient in MK2, did not chemotax to fMLP in a long-term chemotactic assay. (c) Murine neutrophils treated with the p38 MAPK inhibitor SB203580, or deficient in MK2, did not polarize in response to fMLP. n=4 for all data; *P<0.05, ANOVA with Bonferroni's correction.

In addition to the steepness of the gradient, the context in which a cell perceives a chemoattractant gradient can have a profound effect on their response to the chemotactic stimulus. For example, both our group (Heit et al., 2005) and others (Ferguson et al., 2007) have demonstrated that neutrophil chemotaxis to fMLP is profoundly impacted by the makeup of the substratum upon which the cell is crawling. For example, ligands for LFA-1, MAC-1 and VLA-4 need to be present to get a full chemotactic response to fMLP (Heit et al., 2005). Moreover, Ferguson et al. have demonstrated profound differences in the chemotaxis of neutrophils to fMLP on glass verses protein substrata (Ferguson et al., 2007). These profound differences in the behavior of neutrophils, based on the substratum they are crawling upon, demonstrate that careful selection of the substratum is an important factor when selecting or designing chemotactic assays, especially in mammalian systems. In this regard, we have used a multi-protein substratum (serum) in vitro and the physiological extracellular matrix in vivo to study chemotaxis. Our data demonstrate that the multi-protein substratum used in our in vitro assays accurately reflects the in vivo environment.

This context-dependent migration might also have profound implications in vivo. First, recent work has demonstrated that cells crawling within blood vessels do so on adhesion molecules such as ICAM1 and VCAM1 (Phillipson et al., 2006), whereas other studies have demonstrated that the adhesion to these vascular ligands is dependent on PI3K activity (Reinhardt et al., 1997; Smith et al., 2006). This is in stark contrast to cells migrating in the extravascular space, in which the cells crawl on extracellular matrix proteins such as collagen, laminin and fibronectin (Kuntz and Saltzman, 1997). Indeed, in this study, we demonstrated that PI3K inhibition or deficiency altered the kinetics of integrin adhesion to ICAM1 under flow (Fig. 3j), demonstrating that, at least in the vasculature, PI3K deficiency can directly impact fMLP-mediated adhesion. However, similar effects were not observed on multi-protein substrata (Figs 1-4), or outside of the vasculature in the in vivo environment (Fig. 5). These differences in the migratory ligands might explain the vastly different effect of PI3Kγ deficiency observed in neutrophil recruitment and crawling in the blood vessel verses neutrophil chemotaxis in the tissue and in our in vitro assay systems.

Because of the differences induced by the steepness of the chemoattractant gradient and the makeup of the substratum, it is very difficult to evaluate chemotactic systems in regard to their ability to reproduce the in vivo environment seen within mammals. As such, we have begun to study chemotaxis using an in vivo model, in which cells crawl within the in vivo environment and respond in part to endogenously produced chemoattractant gradients. In agreement with a growing number of in vitro studies, as well as our previous work, our in vivo model of chemotaxis demonstrated that chemotaxis to fMLP occurs independently of PI3K (Chodniewicz and Zhelev, 2003a; Chodniewicz and Zhelev, 2003b; Ferguson et al., 2007; Heit et al., 2002; Zhelev et al., 2004). Previous work from our laboratory demonstrates that PI3K activity in neutrophils will be inhibited in the presence of fMLP. As such, the majority of neutrophils will be responding directly to fMLP, even though numerous endogenous mediators will be released in response to the fMLP gradient (Heit et al., 2002; Khan et al., 2005).

Importantly, we observed that the chemotaxis of cells to fMLP was delayed in vivo, and in vitro, suggesting that PI3K acts to

accelerate the initial stages of chemotaxis to fMLP but is not required for chemotaxis to fMLP. The mechanism underlying this delay has not been elucidated, but multiple mechanisms have been identified that might play a role. One such mechanism is the polymerization of F-actin in chemotaxing cells. Two studies, one in human neutrophils (Chodniewicz and Zhelev, 2003b), the second in *Dictyostelium* (Loovers et al., 2006), have demonstrated that there are at least two different phases of actin polymerization in response to a chemotactic stimulus and that one of those two phases is independent of PI3K. Both of these studies demonstrated that chemotaxis could occur independently of the PI3K-dependent actin polymerization, although it was somewhat delayed. This is in direct agreement with our in vitro and in vivo observations, in which we identified a delayed but otherwise normal chemotactic response to fMLP when PI3K was inhibited, or in murine neutrophils lacking the PI3Kγ isoform.

The above delay in actin polymerization is much shorter then the 5- to 10-minute delay we observe in our assays, suggesting that other mechanisms might also be involved. A second potential mechanism resulting in this delay is an alteration in the activation patterns of integrins (Reinhardt et al., 1997; Smith et al., 2006). Indeed, we observed significant changes in the adhesion of fMLP-stimulated neutrophils to the β_2-integrin ligand ICAM1 (Fig. 3j). In addition to impairing chemotaxis, this defect in integrin function could also explain the poor adhesion and emigration observed in these animals (Fig. 5a-c). Indeed, the process of neutrophil migration from blood to tissue is dramatically different from chemotaxis, and requires the close coordination of selectins, integrins and endogenous inflammatory mediators to mediate efficient recruitment (reviewed in Kubes, 2002). PI3K inhibition/deficiency resulted in a significant decrease in adhesion, crawling and emigration during recruitment, consistent with the role of integrins in mediating these portions of the recruitment cascade (Henderson et al., 2001; Sadowska et al., 2004). Interestingly, our recent studies suggest that the function of PI3K in mediating recruitment can be replaced over time by other pathways (Liu et al., 2007), suggesting that, like in chemotaxis, PI3K might mediate early, but not late, recruitment responses. Together, these data suggest that, in the in vivo environment, chemotaxis in tissue can occur independently of PI3K, albeit in a delayed fashion, whereas adhesion and emigration are partially dependent on PI3K.

It is important to note that this PI3K-independent chemotaxis probably occurs for only some types of chemoattractants. Indeed, using our long-term under-agarose assay we demonstrated that chemotaxis to multiple stimuli, including IL8, MIP-2 and LTB_4, is absolutely dependent on PI3K, both in vitro and in vivo (Heit et al., 2002; Khan et al., 2005), and herein we demonstrate that polarization and early chemotaxis in response to IL8 is defective in the presence of a pan-PI3K inhibitor (Fig. 2d-f). This is in agreement with multiple in vitro and in vivo studies in which chemotaxis to chemoattractants such as IL8 (Knall et al., 1996; Knall et al., 1997), LTB_4 (Heit et al., 2002) and MIP-2 (Heit et al., 2005; Khan et al., 2005) was found to be PI3K-dependent.

Previous studies have indicated that p38 MAPK is required for mediating chemotaxis to some stimuli (Heit et al., 2002), although the mechanism by which this occurs is not understood. However, a growing body of evidence has identified potential effector molecules, such as MK2 and heat-shock-protein 27 (HSP27; HSPB1), through which p38 MAPK appears to mediate chemotaxis (Jog et al., 2007; Pichon et al., 2004; Wu et al., 2004). Although these p38-MAPK-dependent pathways need to be elucidated, these studies clearly indicate that chemotaxis can occur in a p38-MAPK-dependent, PI3K-independent, manner.

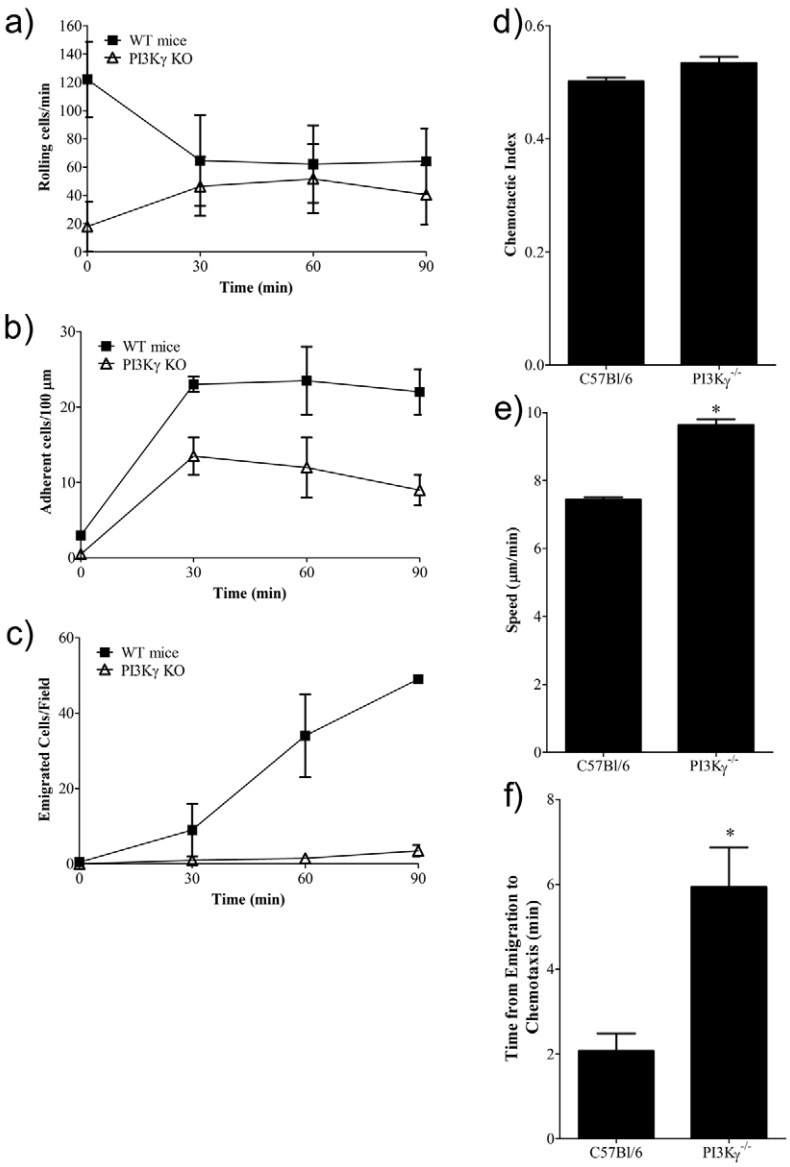

Fig. 5. The role of PI3Kγ in mediating in vivo recruitment and chemotaxis in response to fMLP. Mice are either wild type (C57Bl/6) or PI3K$\gamma^{-/-}$ (PI3Kγ KO). (a) Number of rolling cells passing through the blood vessel per minute. (b) Number of neutrophils adhering to the endothelium per 100 μm of blood vessel. (c) Number of neutrophils emigrating from a single blood vessel, within one field of view. (d) Directionality (C.I.) of in vivo neutrophil migration to fMLP. (e) Speed of in vivo neutrophil migration to fMLP. (f) Delay between emigration and initiation of chemotaxis in vivo in response to fMLP. $n>4$ for all data; *$P<0.05$, Student's t-test.

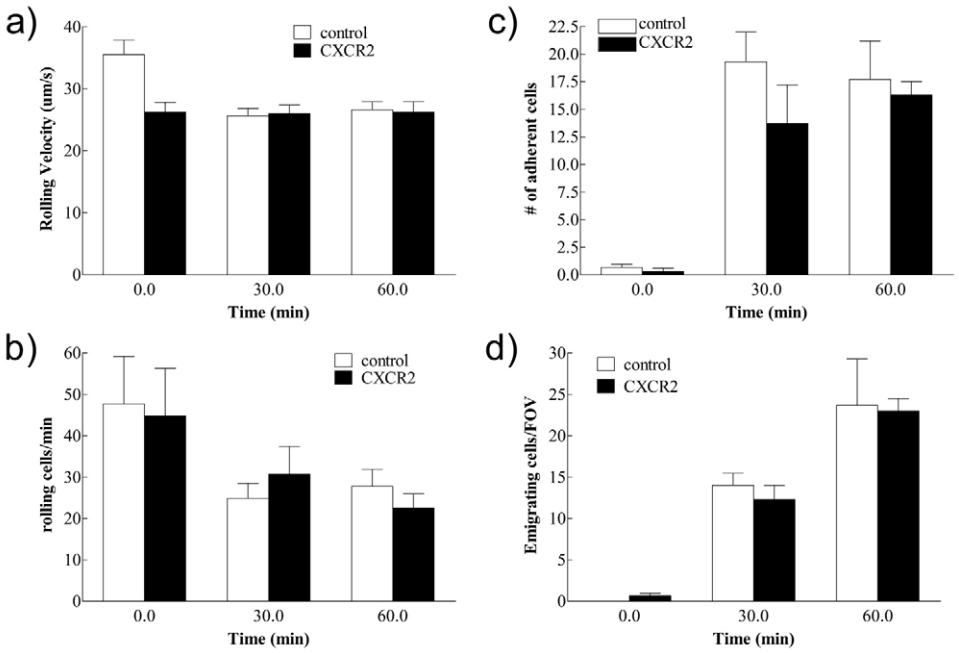

Fig. 6. In vivo recruitment in CXCR2$^{-/-}$ mice in response to fMLP. (a) Velocity of rolling cells. (b) Number of rolling cells passing through the blood vessel per minute. (c) Number of neutrophils adhering to the endothelium per 100 μm of blood vessel. (d) Number of neutrophils emigrating from a single blood vessel, within one field of view. n=3 for all data; no significant difference, ANOVA with Bonferroni's correction.

PI3K appears to have a divergent role during chemotaxis in tissues, verses during the recruitment process wherein neutrophils migrate from the blood into the tissue. Recruitment can be broadly divided into three steps – an initial tethering and rolling of the leukocytes along the vascular endothelium, firm adhesion of the leukocyte to the vascular endothelium, and emigration of the leukocyte through the endothelium and into the surrounding tissue (reviewed in Kubes, 2002). Recently, we identified distinct roles for PI3Kγ and PI3Kδ in the in vivo recruitment of cells in response to MIP-2 and KC (Liu et al., 2007). Significantly, PI3Kγ played an essential role for the early phase of recruitment, whereas PI3Kδ played an essential role in later phases of recruitment in response to MIP-2 and KC. In fact, these two isoforms of PI3K were entirely responsible for all recruitment in response to MIP-2 and KC. In the present study, we identified a similar role for PI3Kγ in mediating recruitment in response to fMLP – specifically that PI3Kγ is required for effective adhesion and emigration of neutrophils (Fig. 5). This work clearly highlights that there are profound differences in the signaling pathways cells use to chemotax to different types of chemoattractants.

In conclusion, our data suggest that chemotaxis to some chemoattractants, notably fMLP, can occur independently of PI3K, and relies instead on p38 MAPK. This is markedly different from the classical PI3K-dependent pathways believed to mediate chemotaxis. Importantly, this PI3K-independent chemotaxis appears to be involved in mediating chemotaxis to only a subset of chemoattractants, suggesting that this alternative chemotactic pathway might be involved in the discrimination between different types of chemoattractants. Finally, our study demonstrates the importance of selecting appropriate chemotactic assays, because certain assays might inadvertently bias the signaling pathways activated by the chemotactic stimulus.

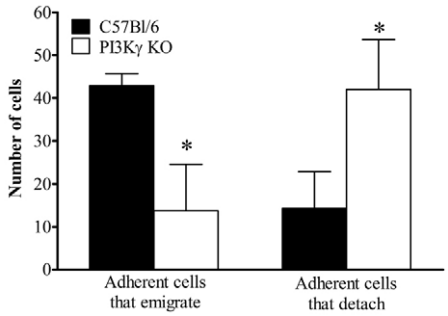

Fig. 7. Number of adhering cells that emigrate or detach in wild-type and PI3Kγ$^{-/-}$ neutrophils in response to fMLP. Animals were either wild type (C57Bl/6) or PI3Kγ$^{-/-}$ (PI3K KO). Data are expressed as the percentage of adherent cells and are presented as mean ± s.e.m. n=3 for all data; *P<0.05 compared to wild-type, Student's t-test.

Materials and Methods

Human neutrophil isolation

Blood was collected from healthy human donors into ACD-containing tubes and erythrocytes removed using dextran sedimentation (6% dextran/0.9% NaCl) followed by two rounds of hypotonic lysis using ddH_2O. Neutrophils were isolated from the resulting cell suspension using Ficol-Histopaque density centrifugation. The entire isolation was done at 4°C. Purified neutrophils were suspended in HBSS at a concentration of 1.0×10^7 cells/ml and were kept on ice until needed.

Murine neutrophil isolation

Mice were euthanized and the femurs and tibias removed. The ends of the bones were resected and the bone marrow was removed by perfusion of 5 ml ice-cold PBS. The bone marrow was then suspended by drawing it through a 20-gauge needle. Marrow cells were then pelleted in a centrifuge (250 ***g***, 4°C, 12 minutes) and resuspended in 2 ml PBS. The cell solution was placed over a discontinuous Percoll gradient consisting of a stock Percoll solution (90 ml Percoll, 10 ml 10× HBSS) diluted to 72, 64 and 52% in PBS. The cell solution was spun at 1100 ***g***, 4°C, for 30 minutes. Purified murine neutrophils localized to a band between the 72 and 64% layers. This band was removed with a transfer pipette, washed in PBS and suspended in HBSS + 10% murine plasma at 1.0×10^7 cells/ml.

Chemoattractants and inhibitors

To induce the chemotaxis of human neutrophils, the bacterial formylpeptide fMLP and the chemokine IL8 were used. Because murine neutrophils do not respond well to this formylpeptide, and a natural ligand for the murine formylpeptide receptor has not been identified, we used the well-characterized synthetic peptide Trp-Lys-Tyr-Met-Val-D-Met-NH2 (WKYMVdM) in lieu of a natural ligand. For simplicity, the

term 'fMLP' is used to indicate fMLP in human experiments and WKYMVdM in murine experiments. The pan-PI3K inhibiter (LY294002) and the p38 MAPK inhibitor (SB203580) were used at the manufacturer's recommended dose (30 μM and 10 μM, respectively), whereas the PI3Kδ-specific inhibitor IC87114 was used at 10 μM, which was previously shown to optimally inhibit PI3Kδ without non-specific effects on other PI3K isoforms (Sadhu et al., 2003).

Under-agarose assay

The under-agarose assay was performed as described previously (Heit et al., 2002; Heit and Kubes, 2003). Falcon Petri dishes (35×10 mm) were filled with 3 ml of a 0.45% agarose solution containing 50% H_2CO_3-buffered HBSS (Sigma-Aldrich, St Louis, MO) and 50% RPMI +20% FCS culture medium. After the agarose solidified, three wells, 3.5 mm in diameter and 2.4 mm apart, were cut in a straight line into the gel. The gels were equilibrated for 1 hour in a 37°C/5% CO_2 incubator. Then, 10 μl of chemoattractant (0.1 μM fMLP or 1.0 μM of either IL8 or MIP-2) or 1.0×10^5 neutrophils were loaded into the outer wells of the gel. Gels were incubated for 4 hours (murine cells) or 2 hours (human cells) in a 37°C/5% CO_2 incubator. During this period of time the neutrophils migrated towards the chemoattractant-containing well. Results were recorded at 20× magnification using a video camera attached to a Zeiss Axiovert 135 microscope (Zeiss, Thornwood, NY). The magnitude of the neutrophil migration was ascertained by recording the area between the cell-containing wells and the chemoattractant-containing wells, and the number of cells migrating in the region counted. In addition, the distance to the cell front (the distance from the cell-containing well to the cell closest to the chemoattractant-containing well) was measured.

Cell tracking

The under-agarose assay was used to track neutrophil migration, as described previously (Heit et al., 2005). Briefly, the under-agarose assay was prepared as described above and the gels incubated for 1.5 hours to allow the neutrophils to migrate into the target region. At this point, the gel was transferred to an Olympus IX70 inverted microscope equipped with a heated enclosure. The enclosure was maintained at 37°C and the gel perfused with humidified air plus 5% CO_2. The migration of the cells was recorded at 10×/0.30 NA magnification, using Openlab software, with images taken every 20 seconds over a period of 20 minutes. The background was removed, the image binarized and noise removed using the despeckle command. The neutrophil trajectories were then determined using the MTrack2 plugin, and the data were exported to Excel for further analysis.

To quantify the directionality of migration, the chemotactic index (C.I.) was calculated [supplementary material Fig. S2 (Heit et al., 2005)]. C.I. is calculated by dividing the distance the cell moved towards the chemoattractant (Δ_x) by the total distance the cell moved (Σ_{dist}). Previously, we determine that randomly moving cells have a C.I. of –0.20 to 0.20, whereas directionally moving cells have a C.I. of 0.20 to 1.0 (Heit et al., 2005). The speed of cell migration was also calculated by dividing Σ_{dist} by the time of the experiment in minutes [supplementary material Fig. S2 (Heit et al., 2005)].

Cell polarization

Because it is not possible to monitor the initial stages of chemotaxis using the under-agarose assay, we used a short-term chemotactic assay to monitor the early stages of chemotaxis using an Ibidi μ-Slide VI microfluidic chamber. These chambers consist of a small channel (0.4×17×3.8 mm) with a reservoir (~80 μl each) located at each end of the channel. The Ibidi chambers were coated with a solution of 10% human or murine plasma for 30 minutes. The chamber was then washed 3× with HBSS and 30 μl of 1×10^6 neutrophils/ml in HBSS was loaded into the channel. 50 μl of HBSS was then added to each input port. The chamber was then transferred to an Olympus IX70 inverted microscope equipped with a heated enclosure maintained at 37°C. The chamber was given 5 minutes to equilibrate and then 10 μl of 1×10^{-6} M fMLP was placed into one randomly selected input port; the second input port was used as a sink and therefore nothing was added to that port. This generates a stable gradient that maintains chemotaxis up to 30 minutes (data not shown). Although this gradient is quite steep in comparison to the under-agarose assay, it is probably not as steep as the gradients formed in assays with extremely short diffusion distances (i.e. micropipette assays). The polarization and migration of the cells was recorded at 20×/0.30 NA magnification, using Openlab software, with images taken every 20 seconds over a period of 20 minutes. The resulting images were imported into ImageJ and processed as per the cell-tracking protocol.

The speed of cell migration was determined as per the cell-tracking protocol. The polarization of the cells was determined by measuring the eccentricity of the cell, which is equal to the ratio of the major axis of the cell (longest straight line that can be drawn across the cell) and the minor axis (longest straight line that can be drawn across the cell at 90° to the major axis). 95% of unstimulated cells have an eccentricity <0.2, whereas less then 2.5% of fMLP-stimulated cells have an eccentricity of <0.2 (supplementary material Fig. S1b). As such, cells with an eccentricity ⩾ 1.2 (i.e. at least 20% longer then wide) were scored as being polarized. To determine the percentage of cells that were orientated towards the chemoattractant, the angle of the long axis of each polarized cell was determined relative to the chemoattractant gradient (supplementary material Fig. S2). Polarized cells that were orientated within ±45° of the gradient were scored as being orientated towards the chemoattractant.

Adhesion of murine neutrophils under flow

Adhesion to ICAM1 was assayed using a parallel-plate flow chamber, described previously (Reinhardt et al., 1997). Briefly, a coverslip was coated in 100 nM murine ICAM1 (R&D Systems) overnight at 4°C. After coating, the coverslips were washed 3× in PBS and 1×10^6 isolated neutrophils placed onto the coverslip. The coverslip was then inserted into the flow chamber and HBSS profused at a shear of 2 dynes. The assay was recorded for 2 minutes and then HBSS $+1\times10^{-8}$ M fMLP was profused for an additional 7 minutes. The resulting video was then time-lapsed 420× (1 frame=10 seconds) and the number of adherent cells counted for each frame. The resulting data was then graphed as the fraction of adherent cells observed prior to starting flow. In some cases, neutrophils that had detached outside of the field of view would re-adhere within the field of view, thus resulting in an increase in the number of adherent cells. Due to the high variation of this system, the data was smoothed using a 9-point weighted mean.

In vivo recruitment of neutrophils

Intravital microscopy and cell tracking were preformed as previously described (Liu et al., 2005; Liu et al., 2007). Briefly, mice were anesthetized and the cremaster muscle was dissected free of tissues and exteriorized onto an optically clear viewing pedestal. The muscle was cut longitudinally with a cautery and held flat against the pedestal by attaching silk sutures to the corners of the tissue. The muscle was then superfused with 37°C bicarbonate-buffered saline. The muscle was then transilluminated and viewed using a microscope (Axioskop; Carl Zeiss) with a 25× objective lens (Weltzlar L25/0.35; E. Leitz, Munich, Germany). Results were recorded using a video camera (Panasonic 5100 HS; Osaka, Japan) and video recorder for playback analysis. 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. An agarose gel containing 0.1 μM fMLP was placed ~350 μm from the venule under observation. The number of rolling and adherent leukocytes was determined off-line during video-playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. Leukocyte rolling velocity was determined by measuring the time required for a leukocyte to roll along a 100-μm length of venule. Rolling velocity was determined for 20 leukocytes at each time interval. Leukocytes were considered adherent to the venular endothelium if they remained stationary for 30 seconds or longer. Leukocyte emigration was defined as the number of extravascular leukocytes per microscopic field of view (×25 objective lens) adjacent to the selected postcapillary venule. Venular diameter (Dv) was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX).

In vivo chemotaxis of neutrophils

The cremaster muscle was prepared for viewing as described above. However, instead of recording in real-time, the preparation was recorded using a 72× time-lapse video recorder. The time-lapse video was then imported into a computer, time-lapsed to a total of 900× (1 frame=30 seconds) and imported into ImageJ. The migration of each cell was monitored using the manual tracking plugin of ImageJ and the data analyzed using the same parameters as the in vitro tracking data. Owing to the thinness of the cremaster muscle (<100 μm), the narrow depth-of-focus of the lens (5-8 μm) and the optical difficulties of performing optical sectioning of the tissue, all migration was assumed to be occurring in two dimensions. An additional parameter, the delay from emigration to migration, was also measured. This was defined as the time from when a cell completely appeared on the outside of the venule to the time the cell had moved one-cell length from the venule wall.

Statistics

All data is presented as mean ± s.e.m. and analyzed using Graphpad Prism 4 software (Graphpad Software). Unless otherwise noted, results were analyzed using ANOVA with Bonferroni's correction.

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