Bidirectional regulation of neutrophil migration by mitogen-activated protein kinases

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To kill invading bacteria, neutrophils must interpret spatial cues, migrate and reach target sites. Although the initiation of chemotactic migration has been extensively studied, little is known about its termination. Here we found that two mitogen-activated protein kinases (MAPKs) had opposing roles in neutrophil trafficking. The extracellular signal–regulated kinase Erk potentiated activity of the G protein–coupled receptor kinase GRK2 and inhibited neutrophil migration, whereas the MAPK p38 acted as a noncanonical GRK that phosphorylated the formyl peptide receptor FPR1 and facilitated neutrophil migration by blocking GRK2 function. Therefore, the dynamic balance between Erk and p38 controlled neutrophil ‘stop’ and ‘go’ activity, which ensured that neutrophils reached their final destination as the first line of host defense.

Chemotaxis (the directed migration of cells in response to a gradient of chemoattractant) is essential for lymphocytes to find antigens and for neutrophils to find sites of infection and inflammation. Chemotactic cells such as blood neutrophils and neutrophil-like differentiated human promyelocytic leukemia (HL60) cells respond to chemoattractants such as formyl-Met-Leu-Phe (fMLF) by adopting polarized morphology (polarization) and traveling up the gradient (directional sensing). Extensive studies have been done to elucidate the mechanisms of cell polarization and directional sensing. Neutrophils, for example, use a self-organizing mechanism that diverges from the formyl peptide receptor through different trimeric G proteins to break symmetry and polarize. The GTP-binding Rho-family protein Cdc42 and microtubule pathways are both important for neutrophil directional sensing. In addition to those mechanisms that promote chemotaxis, there are inhibitory mechanisms that ‘instruct’ migrating cells to stop directional movement in the presence of a single attractant and to move in the correct direction when multiple attractants are present. Thus, these inhibitory mechanisms ensure that chemotactic cells reach the correct destination, where they serve bactericidal functions for phagocytes and antigen identification for homing lymphocytes. So far, little is known about the regulatory mechanisms that inhibit cell migration.

In addition to activating the appropriate trimeric G proteins, attractants promote phosphorylation of their receptors by G protein–coupled receptor kinases (GRKs). This phosphorylation enables the receptors to bind arrestins, thereby preventing the receptors from activating G proteins and terminating signaling, a process called ‘desensitization’. The receptor-arrestin complex is subsequently internalized via a clathrin-dependent pathway or a clathrin-independent pathway. Internalized receptors are sorted to be either degraded or sent to recycling compartments. In neutrophils, both internalization of formyl peptide–receptor 1 (FPR1) and uncoupling of G proteins are mediated through GRK2 but do not require arrestin. This GRK-dependent receptor desensitization results in fewer potential active receptors on the cell surface, thereby diminishing the internal signal generated in response to a given concentration of an attractant. However, published studies have reported a negligible chemotaxis deficiency in cells expressing receptors that cannot be desensitized, which suggests that receptor desensitization itself is not required for chemotaxis. The role of receptor desensitization in cell migration remains unclear.

Mitogen-activated protein kinases (MAPKs), including Erk, Jnk and p38, are involved in inflammation, apoptosis and migration. It has been shown that p38 regulates neutrophil chemotaxis both in vivo and in vitro, but the underlying mechanisms of this remain unclear. The roles of Erk and Jnk in chemotaxis have not been fully elucidated. Here we found that p38 and Erk regulated neutrophil migration with opposite effects: p38 counteracted GRK2-mediated receptor desensitization, whereas Erk enhanced it. Furthermore, p38 has been identified as a GRK that binds and phosphorylates FPR1. This p38-mediated phosphorylation prevented binding of GRK2 to the same receptor and thus inhibited GRK2-mediated FPR1 desensitization. Therefore, the p38- and Erk-mediated signals controlled the net chemotactic ‘go’ and ‘stop’ activity, respectively, of migrating...
RESULTS

Opposite roles of Erk and p38 in cell migration

MAPKs have been linked to the regulation of cell migration. However, whether different MAPK isoforms have distinct roles in cell migration and the underlying mechanisms has remained unclear. We treated differentiated HL60 cells with specific inhibitors of MAPKs or knocked down the MAPKs by RNA-mediated interference (RNAi; Supplementary Fig. 1) and characterized migratory activity of these cells in fMLF concentration gradients. Whereas ~80% of control cells migrated up the fMLF gradient (100 nM; n = 109 cells) and reached the top (Fig. 1a,b, Supplementary Table 1 and Supplementary Video 1), only ~20% of the cells treated by RNAi for knockdown of p38α (the predominant isoform expressed in HL60 cells) or the p38 inhibitor SB203580 migrated through and reached the top (n = 147 cells; Fig. 1a,b, Supplementary Table 1 and Supplementary Video 2). The remaining cells were able to polarize and migrate initially in the attractant gradient but quickly lost directionality and ‘wandered’ aimlessly without net forward locomotion, thus ceasing directional migration. The chemotaxis index (CI; the ratio of net migration in the correct direction to total migration distance) was significantly lower in cells treated with RNAi of p38α or with SB203580 than in control cells (0.46 and 0.41 versus 0.72; P < 0.001, Fig. 1c). We obtained similar results in a second chemotaxis assay, in an in vitro transmigration assay and by using human neutrophils (Supplementary Figs. 2 and 3).

To further demonstrate the regulatory effect of p38 on cell migration, we examined migratory activity in p38α-deficient neutrophils obtained from mice with conditional knockout of p38α (ref. 23). We found substantially less transmigration in vivo and chemotaxis in vitro by p38α-deficient neutrophils (Supplementary Fig. 4a–e), consistent with our observations (described above) of cells treated with SB203580 or RNAi.

In contrast to results obtained by inhibition of p38, cells treated with the Erk inhibitor PD98059 or with RNAi of Erk showed more chemotaxis than did untreated control cells, with more than 95% of cells migrating through the entire gradient (n = 331 cells; Fig. 1a,b, Supplementary Table 1 and Supplementary Video 3). The CI of cells treated with RNAi of Erk or with PD98059 was significantly higher than that of untreated control cells (0.88 and 0.92 versus 0.72, P < 0.001; Fig. 1c). We obtained similar results with U0126, another Erk inhibitor (Supplementary Fig. 4). However, inhibition of Jnk with SP600125 has little effect on cell migration (Fig. 1b,c). Neither cell polarization, measured by actin polymerization, nor cell-migration speed, measured before cells lost net forward locomotion, were affected in cells in which p38 or Erk was inhibited (Supplementary Fig. 5 and Fig. 1d). Thus, downregulation of p38 inhibited the directional migration of cells in an fMLF gradient, whereas inhibition of Erk enhanced cell chemotaxis, which suggested that these two MAPKs have opposing functions in neutrophil chemotaxis.

Concentration-dependent cell-activity switch

As high concentrations of chemoattractants are known to inhibit neutrophil orientation, we next characterized the chemotactic activities of differentiated HL60 cells in various fMLF concentration gradients (Fig. 2). In gradients generated by a lower concentration of fMLF (50 nM and 100 nM), the majority of cells (>70%; n = 186) showed continuous directional migration up the gradient field (Fig. 2a,b and Supplementary Table 1). In contrast, in gradients generated by a higher concentration of fMLF (500 nM and 1,000 nM), only ~10% of cells (n = 355) completed migration through the gradient (Fig. 2a,b and Supplementary Table 1). As expected, the CI was significantly lower at fMLF gradients of higher concentration (0.38 and 0.27 for 500 nM and 1,000 nM, respectively; Fig. 2c) than at fMLF gradients of lower concentration (0.66 and 0.72 for 50 nM and 100 nM, respectively; Fig. 2c). We obtained similar results for neutrophil transmigration into the peritoneal cavity of wild-type mice (Fig. 2e), for human neutrophils (Supplementary Fig. 3) and with cells stimulated by uniform concentrations of fMLF (used for assessment of chemokinesis; Supplementary Fig. 2b,d). Thus, the migration of cells in response to higher-concentration fMLF gradients (such as 500 nM) was similar to that of cells treated with SB203580 or with RNAi of p38α in response to fMLF gradients of lower concentration (such as 100 nM).

To address whether p38 or Erk is involved in terminating directional cell migration, we next assessed the activation of both p38 and Erk at various concentrations of fMLF. We found that activation of p38 (measured by its phosphorylation) peaked at an fMLF concentration of 100 nM, then dropped considerably at concentrations of 500 and 1,000 nM (Fig. 3a). The activation of Erk also peaked at 100 nM but plateaued as fMLF concentration was increased further (Fig. 3b). In the activation assays described above, we stimulated cells for 2 min, as phosphorylation of p38 and Erk peaked at 2 min for each concentration of fMLF (Supplementary Fig. 5c). We obtained similar results with human neutrophils (Supplementary Fig. 6).

Figure 1 Erk and p38 have opposite roles in neutrophil chemotaxis. (a) Trajectories of cells left untreated control (Ctrl) or treated with SB203580 (SB; 10 µM), RNAi of p38α, PD98059 (PD; 50 µM) or RNAi of Erk in an fMLF gradient of 100 nM (>30 cells per condition). Each trace represents the trajectory of one cell (throughout). Scale bar, 100 µm. (b) Frequency of cells that migrated through the entire gradient field (Migrate) relative to cells that did not reach the top (Arrest) for cells treated as in a and in cells treated with SP600125 (SP; 10 µM). (c,d) CI (c) and migration speed (d) of cells treated as in b. * P < 0.001, compared with control (Student’s t-test). Data are representative of (a) or are from (b–d) three independent experiments mean and s.e.m. in c,d. 
Figure 2 Concentration-dependent switch for neutrophil chemotaxis and transmigration. (a) Cell trajectories in FMLF gradients of 50 nM, 100 nM, 500 nM and 1,000 nM (n > 30 cells per condition). Scale bar, 100 µm. (b) Quantification of the migration of cells treated as in a (presented as in Fig. 1b). (c) CI (e) and migration speed (d) of cells treated as in a. *P < 0.001, compared with CI at 100 nM FMLF (Student’s t-test). (d) Migration speed of cells treated as in a. *P < 0.001, compared with speed at 100 nM FMLF (Student’s t-test). (e) Migration of neutrophil into the peritoneal cavity of mice (n = 3–4 mice per group) in response to the injection of various concentrations of FMLF. *P < 0.05, compared with basal (Student’s t-test). (f) Receptor internalization at various concentrations of FMLF, presented as mean fluorescence intensity (MFI) of bound ligand. Data are representative of (a) or are from (b–f) three independent experiments (mean and s.e.m. in c–f).

To test whether the lower p38 activity and sustained Erk activity were responsible for the impaired chemotaxis at high concentrations of FMLF, we treated cells with either anisomycin, which activates p38, or PD98059, which inhibits Erk, and assessed their chemotactic migration in the gradient at 500 nM. Notably, unlike the untreated control cells, most of which ceased directional migration in the middle of the gradient field, ~70% of p38-activated cells (n = 95) migrated through the field (Fig. 3c,d and Supplementary Table 1). The CI of those cells was significantly higher than that of untreated control cells in a 500-nM gradient (0.76 versus 0.37; P < 0.001) but was similar to the CI of control cells migrating in a gradient of 100 nM FMLF (Figs. 3e and 1c). We obtained similar results by inhibiting Erk with PD98059 (Fig. 3c–e). These observations indicated that p38 counteracted the stop signal during cell chemotaxis, whereas Erk enhanced it.

MAPKs regulate FPR1 internalization in different ways

We next explored which signals for the cessation of directional cell migration might be induced by high concentrations of FMLF. Attractants not only activate the appropriate trimeric G proteins but also promote receptor internalization and thus prevent the receptor from activating G proteins. As expected, cells expressing wild-type FPR1 quickly arrested at a uniform concentration of 500 nM FMLF (Fig. 4a). The majority of cells (∼70%; n = 33) lost net locomotion during the 15-minute recording period (Fig. 4b, left). In contrast, only ∼13% of cells expressing FPR1-∆ST (n = 69) arrested during the same recording period (Fig. 4b, right). Those cells also had negligible receptor internalization (Fig. 4a, bottom). On the basis of the trajectories of the two groups of cells (Fig. 4b), the mean migration distance of the wild-type cells was significantly shorter than that of the cells expressing FPR1-∆ST (17.9 µm versus 33.4 µm (P < 0.01); Fig. 4c). Thus, higher concentrations of FMLF enhanced receptor internalization and promoted the cessation of neutrophil migration.

Figure 3 Inhibition of Erk or enhancement of p38 restores cell migration at high concentrations of FMLF. (a,b) Immunoblot analysis (below) of phosphorylated (p-) and total p38 (a) or Erk (b) in cells stimulated for 2 min with concentrations of FMLF, and quantification (above) of phosphorylated p38 and Erk, presented relative to maximum activation at 100 nM FMLF. (c) Trajectories of cells left untreated (Ctrl) or treated with anisomycin (AN; 1 µM) or PD98059 (PD; 50 µM) in an FMLF gradient of 500 nM (n > 30 cells per condition). Scale bar, 100 µm. (d) Quantification of the migration of cells treated as in c (presented as in Fig. 1b). (e) CI of cells treated as in c. *P < 0.001, compared with control (Student’s t-test). Data are from one representative of three independent experiments (a,b, graphs) and mean and s.e.m. in a,b,e).
Prevention of receptor internalization restored cell migration at high concentrations of fMLF, which indicated that more receptor internalization at high concentrations of chemoattractant acted as an essential stop signal for chemotactic neutrophils.

We next determined whether p38 and Erk have opposing effects on receptor internalization. To visualize receptor internalization, we expressed a yellow fluorescent protein (YFP)-tagged FPR1 in HEK293 human embryonic kidney cells, which lack endogenous FPR1. Receptor internalization was evident within 15 min of fMLF stimulation in untreated control cells but was present as early as 3 min after fMLF stimulation in SB203580-treated cells (Fig. 5a). In contrast, much of the FPR1-YFP signal remained at the plasma membrane in PD98059-treated cells after 15 min (Fig. 5a), which indicated that there was little if any receptor internalization. We obtained similar results with HL60 cells (Fig. 5b,c) and human neutrophils (Supplementary Fig. 6e,f). Together, these results showed that Erk and p38 had opposite effects on FPR1 internalization.

**GRK2 mediates the stop signal for chemotaxis**

GRK2 is known to promote FPR1 internalization through phosphorylation13. To determine whether GRK2 mediates the stop signal during chemotaxis, we knocked down GRK2 in HL60 cells by RNAi (Supplementary Fig. 1) and measured migration of these cells in the presence or absence of SB203580. As expected, inhibition of p38 resulted in less directional migration of untreated control cells in an fMLF gradient of 100 nM (Fig. 6a–c and Supplementary Table 1). Knockdown of GRK2 reversed this effect and restored cell migration (Fig. 6a–c and Supplementary Table 1), which suggested that the SB203580-induced cell arrest was mediated through GRK2. That finding led us to test whether p38 and Erk affected GRK2 function. We found more association of GRK2 with the membrane after fMLF stimulation in untreated control cells (Fig. 6d,e). Treatment of the cells with SB203580 further increased the association of GRK2 with the membrane in basal and stimulated states (Fig. 6d,e), which indicated that p38 antagonized recruitment of GRK2 to the membrane. We obtained similar results with human neutrophils (Supplementary Fig. 7). In contrast, PD98059 prevented the recruitment of GRK2 to the membrane (Fig. 6f,g and Supplementary Fig. 7), which suggested that Erk enhanced the activity of GRK2.

**Blockade of GRK2 function by p38 acting as a GRK**

Erk has been shown to prevent GRK2 degradation26,27. We observed a similar effect in HL60 cells treated with RNAi of Erk, which had much less abundance of GRK2 before and after fMLF stimulation.
Figure 6 GRK2 mediates the stop signal for neutrophil migration. (a) Trajectories of cells left untreated (Ctrl) or treated with SB203580 alone (SB) or SB203580 and RNAi of GRK2 (SB + GRK2i) in an fMLF gradient of 100 nM (n > 30 cells per condition). Scale bar, 100 µm. (b) Quantification of the migration of cells treated as in a (presented as in Fig. 1b). (c) CI of cells treated as in a. *P < 0.001 (Student’s t-test). (d,e) Immunoblot analysis (d) of GRK2 in cells stimulated for various times (above lanes) with fMLF in the presence or absence of SB, assessed in cell pellets or supernatants (sup), and quantification (e) of GRK2 in cells stimulated for various times (above lanes) with fMLF in the presence or absence of PD, assessed as in d. *P < 0.05, compared with control (Student’s t-test). Data are representative of three independent experiments (a,d,f) or three experiments (b,c,e,g; mean and s.e.m. in c,e,g).

(Supplementary Fig. 8a). However, the mechanism by which p38 regulates GRK2 remained unclear. By immunostaining, we examined subcellular localization of p38 and FPR1 in HL60 cells. We found phosphorylated p38 in the pseudopods of polarized cells, whereas total p38 was distributed more uniformly (Fig. 7a). FPR1 localized mainly to the cell membrane at both the leading and trailing edges (Fig. 7b). Thus, phosphorylated p38 might interact with FPR1 in pseudopods. We assessed that possibility by immunoprecipitation of p38 with FPR1 and found that the binding of p38 to FPR1 was greater after fMLF stimulation and was inhibited by SB203580 (Fig. 7c). We obtained similar results with human neutrophils (Supplementary Fig. 7). In contrast, phosphorylated Erk was distributed uniformly and we detected no interaction between Erk and FPR1 (Supplementary Fig. 8b,c).

Because p38 phosphorylates serine and threonine residues and there are 19 serine and/or threonine residues in the intracellular domains of FPR1, including 11 in the carboxyl tail (of a total of...
Data are representative of three independent experiments (mean and s.e.m.).

We further determined whether MAPK phosphatase had a role in the regulation of Erk and p38. We first tested WIP1, a MAPK phosphatase that belongs to the protein phosphatase family 2C and is specific for p38 but not Erk. We tested two dual-specificity MAPK phosphatases: MKP1 and MKP5, which dephosphorylate both p38 and Erk. Knockout of the gene encoding MKP1 or MKP5 in mouse neutrophils resulted in a greater abundance of phosphorylated p38 after stimulation with chemoattractants but did not affect the bell-shaped activation curve of p38 (data not shown). Thus, these phosphatases did not seem to be required for the activation of Erk or p38 under our experimental conditions. It is possible, however, that heterotrimeric G proteins activated differently by one or more formyl peptide receptors could be responsible for the observed differences in the activation of Erk and p38.

**DISCUSSION**

Understanding how chemotaxis is dynamically and precisely regulated is of great importance, given its vital roles in inflammatory cell infiltration, lymphocyte homing, embryonic development, axon guidance and tumor invasion. In addition to triggering polarization and directional sensing, which are important for initiation of neutrophil chemotaxis, chemoattractant stimulation elicits a distinct stop mechanism that negatively regulates directional cell migration and

**Different regulation by G proteins of Erk versus p38**

As shown above, Erk and p38 had opposite roles in cell migration, which raised the possibility that Erk and p38 antagonize each other. However, we found that p38 activation was not altered in cells with RNAi of Erk, and Erk activation was not altered in cells with RNAi of p38 (Fig. 8a,b), which indicated p38 and Erk were independently regulated. That conclusion was further supported by finding that p38 and Erk had distinct activation patterns in response to increasing concentrations of FMLF (Fig. 3 and Supplementary Fig. 6). Whereas the activation of Erk plateaued, the activation curve of p38 was bell-shaped. To determine how regulation of Erk differed from that of p38, we assessed whether various heterotrimeric G proteins activated by FPRs mediated activation of Erk and p38. Inactivation of Gi by pertussis toxin abolished p38 activation, but only partially inhibited Erk activity (~70% inhibition, Fig. 8c,d), which indicated that some activation of Erk was independent of Gi. Because Erk and p38 showed different activation patterns with increasing FMLF concentrations, we assessed the concentration-dependent activation of Erk and p38 in the presence of pertussis toxin. At all concentrations tested, pertussis toxin blocked p38 activation but only partially inhibited Erk activation (Supplementary Fig. 9a). We used RNAi to screen other G proteins that might mediate Erk activation (Supplementary Fig. 1). We found that knockdown of Gq resulted in significantly less FMLF-induced activation of Erk but did not affect p38 activation (Fig. 8e,f).

**Figure 8** Differences in the activation of Erk and p38. (a,b) Immunoblot analysis (below) and quantification (above) of phosphorylated (p-) and total p38 (a) or Erk (b) in cells left untreated (Ctrl) or treated by RNAi of Erk (a) or p38a (b) and stimulated for 0 or 2 min with 100 nM FMLF; results above are relative to those of control cells at 2 min. (c,d) Immunoblot analysis (below) and quantification (above) of phosphorylated and total p38 (c) or Erk (d) in cells left untreated (Ctrl) or treated with pertussis toxin (PTX; 1 µg/ml) and stimulated for 0 or 2 min with 100 nM FMLF (presented as in a,b). (e,f) Immunoblot analysis (below) and quantification (above) of phosphorylated and total p38 (e) or Erk (f) in cells left untreated (Ctrl) or treated with RNAi for G12, G13 or Gq and stimulated for 0 or 2 min with 100 nM FMLF (presented as in a,b). *P < 0.05, compared with control (Student's t-test).
brings chemotactic cells to a state similar to that of unstimulated cells. This mechanism is represented by the incremental amount of membrane association of GRK2 and internalization of FPR1. Our results have demonstrated that, to achieve efficient and precise directional migration, Erk and p38 exerted opposite effects on the stop mechanism. Inhibition of p38 activity enhanced the stop signal and arrested migrating cells, whereas enhancement of the activity of p38 or inhibition of the activity of Erk seemed to overcome the stop signal and ensured directed cell migration, even at concentrations of IMLF that would normally induce termination of cell chemotaxis. Our results have shown that Erk-GRK2 functioned as a mechanism for the termination of chemotaxis and have demonstrated that constant suppression (by p38) of the stop mechanism was required for sustained chemotaxis and for migrating cells to reach their final destinations.

Although p38 has an important role in neutrophil chemotaxis\textsuperscript{18–21}, the underlying mechanisms have not been clear. Here we have demonstrated a previously unknown function of p38 as a GRK that phosphorylates a chemoattractant receptor and blocks the function of the classical GRK2. Different phosphorylation patterns of G protein–coupled receptors may ‘instruct’ different downstream partners of the classical GRK2. Different phosphorylation patterns on FPR1 by p38 and GRK2 achieved opposite functions downstream of the receptor, which indicated the subtlety of the regulation of neutrophil migration by p38.

Studies have reported negligible chemotaxis deficiencies in cells expressing receptors that cannot be desensitized, which suggests that receptor desensitization is not required for chemotaxis\textsuperscript{15}. Our findings, however, have indicated that GRK2-mediated receptor internalization and desensitization had an essential role in regulating cell migration and was responsible for the termination of cell migration at high concentrations of attractant. Thus, it was the enhancement of receptor desensitization, not its inhibition, that blocked neutrophil chemotaxis. Moreover, our findings have shown that sufficient protection of the receptor from desensitization was required for sustained cell migration and that overprotection of receptor from desensitization led to nonstop migration. Therefore, the precise navigation of migrating cells required a balance between the acceleration and deceleration of receptor desensitization, which were mediated by GRK2 and p38, respectively. In other words, signals that control receptor desensitization have a central role in determining the final destination of migrating neutrophils.

Our results have shown that different heterotrimeric G protein signals downstream of the formyl peptide receptor were responsible for the regulation of Erk and p38. Activation of p38 was dependent on Gi, whereas Erk was activated by both Gi and Gq signals. In neutrophils, there are two formyl peptide receptor isoforms: FPR1 is the high-affinity receptor for IMLF (with a dissociation constant of \( \approx 10 \) mM) and activates Gi; FPR2 is the low-affinity receptor for IMLF (dissociation constant \( \approx 1 \) μM) and activates Gq\textsuperscript{22}. Thus, at lower concentrations of IMLF, FPR1 activated Gi, which led to the activation of both p38 and Erk; when IMLF concentration increased, FPR2 was activated, thus activating Gq, which was responsible for sustained Erk activation. At the same time, p38 activity decreased as more FPR1 was internalized under exposure to high concentrations of IMLF. We suggest that the different regulatory functions of these two MAPKs provide bidirectional control of GRK2 function at different stages of directional cell migration, thus allowing migrating cells to accurately reach their destinations.

To reach sites of infection or inflammation, circulating neutrophils must first attach to the endothelial cells lining the blood vessels, then transmigrate into tissue. The mechanisms responsible for the initial arrest of neutrophils in the endothelium are mediated mainly by the interaction of β\textsubscript{2} integrins with their endothelial ligand, ICAM-1 (refs. 33–35). Whether this initial arrest mechanism also has a role in terminating neutrophil migration remains unclear. Likewise, it is unknown whether MAPKs or GRKs terminate directional cell migration by regulating integrin activation. Animals such as fruit flies have a concentration-dependent activity switch mediated by differences in the regulation of neural circuits in response to odor stimulation\textsuperscript{36}. Such a concentration-dependent switch can also be observed at the cellular level as a bell-shaped dose-response curve during cell migration. Directed cell migration initially increases in response to an increase in attractant concentration, but peaks and then gradually decreases as attractant concentration is further increased. Therefore, studies at the cellular level may provide further molecular mechanisms and insights for understanding activity switches in more complex organisms.

Our results suggest a model for neutrophil migration in which the dynamic balance of GRK2 and two MAPKs regulates go and stop activity at the receptor level. Our model indicates a mechanism for a concentration-dependent switch for the cell to continue or halt its movement during directional cell migration. The model of stop and go signals provides the opportunity for pharmacological intervention in one or more of the specific pathway(s). Such an intervention would enable appropriate infiltration of phagocytes into inflammatory sites while minimizing neutrophil-mediated tissue injury.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

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AUTHOR CONTRIBUTIONS

X.L., B.M., Y.L., R.D.Y. and J.X. designed the research; X.L., B.M., H.T., T.Y., B.S. and G.W. did the experiments; X.L., B.M. and J.X. analyzed data; A.B.M., R.D.M. and Y.Z. contributed new reagents and tools; and X.L., B.M., A.B.M., Y.L., R.D.Y. and J.X. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Devreotes, P.N. & Zigmond, S.H. Chemotaxis in eukaryotic cells: a focus on leukocytes and Dictyostelium. *Annu. Rev. Cell Biol.* 4, 649–686 (1988).
2. Ridley, A.J. et al. Cell migration: integrating signals from front to back. *Science* **302**, 1704–1709 (2003).
3. Swaney, K.F., Huang, C.H. & Devreotes, P.N. Eukaryotic chemotaxis: a network of signaling pathways controls motility, directional sensing, and polarity. *Annu. Rev. Biophys.* **39**, 265–289 (2010).
4. Janetopoulos, C. & Firtel, R.A. Directional sensing during chemotaxis. *FEBS Lett.* **582**, 2075–2085 (2008).
5. Xu, J. et al. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. Cell 114, 201–214 (2003).

6. Srinivasan, S. et al. Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. J. Cell Biol. 160, 375–385 (2003).

7. Li, Z. et al. Directional sensing requires Gβγ-mediated Pak1 and PI(3,5)P2-dependent activation of Cdc42. Cell 114, 215–227 (2003).

8. Xu, J., Wang, F., Van Keymeulen, A., Rentel, M. & Bourne, H.R. Neutrophil microtubules suppress polarity and enhance directional migration. Proc. Natl. Acad. Sci. USA 102, 6884–6889 (2005).

9. Xu, J. et al. Polarity reveals intrinsic cell chirality. Proc. Natl. Acad. Sci. USA 104, 9296–9300 (2007).

10. Foxman, E.F., Campbell, J.J. & Butcher, E.C. Multistep navigation and the combinatorial control of leukocyte chemotaxis. J. Cell Biol. 139, 1349–1360 (1997).

11. Lefkowitz, R.J. G protein–coupled receptors, III. New roles for receptor kinases and β-arrestins in receptor signaling and desensitization. J. Biol. Chem. 273, 18677–18680 (1998).

12. McLeish, K.R., Gierschik, P. & Jakobs, K.H. Desensitization uncouples the formyl peptide receptor–guanine nucleotide-binding protein interaction in HL60 cells. Mol. Pharmacol. 36, 384–390 (1989).

13. Prossnitz, E.R., Kim, C.M., Benovic, J.L. & Ye, R.D. Phosphorylation of the N-formyl peptide receptor carboxyl terminus by the G protein–coupled receptor kinase, GRK2. J. Biol. Chem. 270, 1130–1137 (1995).

14. Moore, C.A., Milano, S.K. & Benovic, J.L. Regulation of receptor trafficking by GRKs and arrestins. Annu. Rev. Physiol. 69, 451–482 (2007).

15. Hsu, M.H., Chiang, S.C., Ye, R.D. & Prossnitz, E.R. Phosphorylation of the N-formyl peptide receptor is required for receptor internalization but not chemotaxis. J. Biol. Chem. 272, 29426–29429 (1997).

16. Johnson, G.L. & Lapadat, R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298, 1911–1912 (2002).

17. Huang, C., Jacobson, K. & Schaller, M.D. MAP kinases and cell migration. J. Cell Sci. 117, 4619–4628 (2004).

18. Nick, J.A. et al. Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. J. Clin. Invest. 99, 975–986 (1997).

19. Cara, D.C., Kaur, J., Forster, M., McCafferty, D.M. & Kubes, P. Role of p38 mitogen-activated protein kinase in chemokine-induced emigration and chemotaxis in vivo. J. Immunol. 167, 6552–6558 (2001).

20. Heit, B., Tavener, S., Raharjo, E. & Kubes, P. An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. J. Cell Biol. 159, 91–102 (2002).

21. Zu, Y.L. et al. p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-α or FMLP stimulation. J. Immunol. 160, 1982–1989 (1998).

22. Hele, K.K., Trottling, D., Rihanek, M. & Manthey, C.L. Differential expression and activation of p38 mitogen-activated protein kinase α, β, γ, and δ in inflammatory cell lineages. J. Immunol. 162, 4246–4252 (1999).

23. Nishida, K. et al. p38α mitogen-activated protein kinase plays a critical role in cardiomyocyte survival but not in cardiac hypertrophic growth in response to pressure overload. Mol. Cell Biol. 24, 10611–10620 (2004).

24. Sullivan, S.J. & Zigmund, S.H. Chemotactic peptide receptor modulation in polymorphonuclear leukocytes. J. Cell Biol. 85, 703–711 (1980).

25. Barros, L.F., Young, M., Saklatvala, J. & Baldwin, S.A. Evidence of two mechanisms for the activation of the glucose transporter GLUT1 by anisomycin: p38(MAP kinase) activation and protein synthesis inhibition in mammalian cells. J. Physiol. (Lond.) 504, 517–525 (1997).

26. Theilade, J., Hansen, J.L., Haunso, S. & Sheikh, S.P. MAP kinase protects G protein–coupled receptor kinase 2 from proteasomal degradation. Biochem. Biophys. Res. Commun. 330, 685–689 (2005).

27. Theilade, J., Lerche Hansen, J., Haunso, S. & Sheikh, S.P. Extracellular signal-regulated kinases control expression of G protein–coupled receptor kinase 2 (GRK2). FEBS Lett. 518, 195–199 (2002).

28. Ono, K. & Han, J. The p38 signal transduction pathway: activation and function. Cell. Signal. 12, 1–13 (2000).

29. Takekawa, M. et al. p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK-p53 signaling in response to UV radiation. EMBO J. 19, 6517–6526 (2000).

30. Dickinson, R.J. & Keyse, S.M. Diverse physiological functions for dual-specificity MAP kinase phosphatases. J. Cell Sci. 119, 4607–4615 (2006).

31. Zidar, D.A., Violin, J.D., Whalen, E.J. & Lefkowitz, R.J. Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. Proc. Natl. Acad. Sci. USA 106, 9649–9654 (2009).

32. Ye, R.D. et al. International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. Pharmacol. Rev. 61, 119–161 (2009).

33. Butcher, E.C. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 67, 1033–1036 (1991).

34. Springer, T.A. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76, 301–314 (1994).

35. Xu, J. et al. Nonmuscle myosin light-chain kinase mediates neutrophil transmigration in sepsis-induced lung inflammation by activating J2 integrins. Nat. Immunol. 9, 880–886 (2008).

36. Semmelhack, J.L. & Wang, J.W. Select Drosophila glomeruli mediate innate olfactory attraction and aversion. Nature 459, 218–223 (2009).
ONLINE METHODS

Antibodies, reagents and mice. Rabbit polyclonal antibody to GRK2 (anti-
GRK2; sc-562) and anti-FPR1 (sc-30016) and lentiviral particles containing short
hairpin RNA (shRNA) specific for human GRK2 or Erk1 and Erk2 were from
Santa Cruz Biotechnology. Mouse polyclonal anti-p38 (9228), rabbit monoclonal
antibody to p38 phosphorylated at Thr180 and Tyr182 (9215) or to the MAPK
p44/p42 phosphorylated at Thr202 and Tyr204 (9101) were from Cell Signaling
Technology. SB203580 was from Calbiochem. Human fibronectin was from BD
BioSciences. Human albumin (low endotoxin), FMLP, protease inhibitors and
phosphatase inhibitor ‘cocktails’, okadaic acid and phallolidin–tetramethylrhodos-
amine B isothiocyanate were from Sigma. Fluorescein conjugate of the hexapep-
tide formyl-Neu-Phe-Neu-Tyr-Lys was from Invitrogen.

Wild-type C57BL/6 mice were from Charles River Laboratories; mice with
lox-flanked alleles encoding p38α (Mapk14) were from the Riken BioResource
Center, with permission from K. Otsu (noted in Acknowledgments); and B6.129 P2-Lyz2tm1Ser/lj mice were from the Jackson Laboratory (strains were
crossed to generate conditional knockout mice for p38α in myeloid cells).
Mice were bred and housed in pathogen-free conditions with access to food and
water ad libitum in the animal care facility of the University of Illinois at
Chicago. All experimental procedures complied with University of Illinois at
Chicago and National Institutes of Health guidelines for animal use.

DNA constructs and knockdown via RNAi. The cDNA encoding FPR1, p38α or
GRK2 was cloned by RT-PCR, then inserted into pEYFP and Flag-tagged vectors.
FPR1–AST mutant has been described.15 GST-tagged FPR1 loop 1, loop 2 and loop 3
were constructed by pairwise annealing of single DNA strands as follows: 95 °C
for 5 min, 70 °C for 10 min, and cooling at 25 °C for 12 h. The fragments were
inserted into eukaryotic expression plasmid pGEX-4T-2. The following sequences
were used (from 5’ to 3’): Loop1-1: GAT CCG CGG TAT TCC GGA CAC
ACA CAG TCA CCA CCT GAC; Loop1-2: TCG AGT CAG GTG GTG ACT GTG
TGT GTC ATC CGG AAT CCA GCG; Loop2-1: GAT CCG ACC GCT GTG TTT
GCC TGC ATC TAC CCT GGA CCC AGA ACC ACC GCA CCG TGA
GCT GAC; Loop2-2: TCG AGT CAG CTC ACG GTG CGG TGG TTC TGG GTC
CAG CAG TGA TGG AGG ACA CAA CAG CCG TCG; Loop3-1: GAT
CCA AGA ACC GAG AAT CGG ATG TTA GGA ATT CAA GCC GTC
TAC GAG CCT GAC; Loop3-2: TCG AGT CAG ACC GGT AAG GGA CGA
GTG CAG TTA ATC AAG CCT TGT TGG TGG ATC TGG.

For RNAi knockdown of p38α, two pairs of sequences (1 and
Supplementary Table 2) were used to make an shRNA expression
vector, which was cloned into the BamHI and XhoI restriction enzyme sites of
the pEN_kH1C plasmid (Invitrogen). Through the use of an LR recombination
reaction, the shRNA expression cassette was inserted into lentiviral shRNA
expression plasmid pDSLp fgUGIP, followed by transfection together with
pspAX2 and pMD2.G into the 293FT cell line for packaging of lentivirus
particles and reactions were quenched by the addition of ten volumes of ice-cold
HBSS. Cells were allowed to migrate for 30 min, and images were recorded
by an EZ-TAXIScan device (Effector Cell Institute) or micropipette. For
micropipette assays, gradients were generated by a point source of chemooat-
tractant from a micropipette containing 10 μM FMLP. Time-lapse video
microscopy was done as described15Cell migration was recorded and analyzed by ImageJ.
For analysis of the peritoneal transmigration of neutrophils, mice were
injected intraperitoneally with 100 μl of saline containing various concentra-
tions of FMLP (1 nM, 10 nM, 100 nM or 1,000 nM). After 4 h, peritoneal
cavities of anesthetized mice were lavaged and leukocytes were recovered. Total
leukocytes was counted with a hemocytometer and neutrophil counts were
determined on 100-μl cytopsins stained with Diff-Quik; results are presented
as relative to the total population.

Immunoprecipitation and immunofluorescence. Immunoprecipitation
and immunofluorescence were done as described15. The intensity of bands on auto-
radiograms (densitometry) was assessed on scanned X-ray films with ImageJ.

In vitro assay of p38 phosphorylation. First, active p38α (40 ng/μl) was incu-
bated for 15 min with the substrate FPR1-C in 1× assay buffer together
with 250 μM ATP and [γ-32P]ATP (0.16 µCi/μl), then reactions were terminated by
the addition of 25 μl of 2× sample buffer and boiling for 10 min, followed by
SDS-PAGE and analysis with a FujiFilm Fluorescent and Radiosotope Science
Imaging System FLA-7000.

Surface binding assay of FMLP. Differentiated HL60 cells were given no pretreat-
ment or were pretreated for 30 min at 37 °C with 10 μM SB203580 or 50 μM
PD98059, then were washed with RPMI-1640 medium and Hanks’ balanced-salt
solution (HBSS). Cells were resuspended in HBSS and then stimulated with
MLLP, and reactions were quenched by the addition of ten volumes of ice-cold
HBSS. Cells were then washed extensively and incubated with 10 μM N-formyl-Nleu-
Leu-Phe-Nleu-Tyr-Lys–fluorescein before analysis by flow cytometry. Unlabeled
cells stimulated with 10 μM FMLP were used as a negative control.

Membrane-bound GRK2 assay. Differentiated HL60 cells in suspension
were preincubated for 30 min in RPMI-1640 medium with or without inhibitors
(10 μM SB203580 or 50 μM PD98059). Cells were then collected and suspended in
modi-
fied HBSS (1.26 mM calcium and 0.9 mM magnesium without phenol red; 1 × 107
cells in 0.5 ml), then were stimulated with 100 nM FMLP and reactions were
quenched by the addition of ten volumes of ice-cold HBSS. Cells were then
washed extensively and incubated with 10 μM N-formyl-Nleu-Leu-Phe-Nleu-Tyr-
Ly-32P]ATP (0.16 µCi/μl), then reactions were terminated by
the addition of 25 μl of 2× sample buffer and boiling for 10 min, followed by
SDS-PAGE and analysis with a FujiFilm Fluorescent and Radiosotope Science
Imaging System FLA-7000.

Surface binding assay of FMLP. Differentiated HL60 cells were given no pretreat-
ment or were pretreated for 30 min at 37 °C with 10 μM SB203580 or 50 μM
PD98059, then were washed with RPMI-1640 medium and Hanks’ balanced-salt
solution (HBSS). Cells were resuspended in HBSS and then stimulated with
MLLP, and reactions were quenched by the addition of ten volumes of ice-cold
HBSS. Cells were then washed extensively and incubated with 10 μM N-formyl-Nleu-
Leu-Phe-Nleu-Tyr-Lys–fluorescein before analysis by flow cytometry. Unlabeled
cells stimulated with 10 μM FMLP were used as a negative control.

Membrane-bound GRK2 assay. Differentiated HL60 cells in suspension
were preincubated for 30 min in RPMI-1640 medium with or without inhibitors
(10 μM SB203580 or 50 μM PD98059). Cells were then collected and suspended in
modi-
fied HBSS (1.26 mM calcium and 0.9 mM magnesium without phenol red; 1 × 107
cells in 0.5 ml), then were stimulated with 100 nM FMLP in suspension. The stimu-
lation was stopped by the addition of 0.5 ml of saline buffer and boiling for 10 min, followed by
SDS-PAGE and analysis with a FujiFilm Fluorescent and Radiosotope Science
Imaging System FLA-7000.

Statistical analysis. Statistical comparisons were made with a two-tailed
Student’s t-test. Differences in mean values were considered significant at a
P value of less than 0.05.