Human Neutrophils Coordinate Chemotaxis by Differential Activation of Rac1 and Rac2

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Human Neutrophils Coordinate Chemotaxis by Differential Activation of Rac1 and Rac2

Hui Zhang,* Chunxiang Sun,† Michael Glogauer,† and Gary M. Bokoch2*

Rac1 and Rac2, members of the small Rho GTPase family, play essential roles in coordinating directional migration and superoxide production during neutrophil responses to chemotactic stimuli. Although earlier studies in Rac1 and Rac2 knockout mice have demonstrated unique roles for each Rac isoform in chemotaxis and NADPH oxidase activation, it is still unclear how human neutrophils use Rac1 and Rac2 to achieve their immunological responses to foreign agent stimulation. In the current study, we used TAT dominant-negative Rac1-T17N and Rac2-T17N fusion proteins to acutely alter the activity of Rac1 and Rac2 individually in human neutrophils. We demonstrate distinct activation kinetics and different roles for Rac1 and Rac2 in response to low vs high concentrations of fMLP. These observations were verified using neutrophils from mice in which Rac1 or Rac2 was genetically absent. Based on these results, we propose a model to explain how human neutrophils kill invading microbes while limiting oxidative damage to the adjacent surrounding healthy tissue through the differential activation of Rac1 and Rac2 in response to different concentrations of chemoattractant. The Journal of Immunology, 2009, 183: 2718–2728.

In response to inflammatory conditions, neutrophils are known to be first-line defenders in the human innate immune response system (1–3). To fulfill this role, human neutrophils carry out two essential biological processes, chemotaxis and the production of reactive oxygen species (ROS).3 In chemotaxis, neutrophils acquire a polarized morphology, cross the blood vessel wall, and migrate through the adjacent epithelial tissues up a gradient of chemoattractants, such as N-formyl peptide products of bacteria, propagated from infectious sites (4). For the production of ROS to kill microbes after their engulfment by phagocytic means, neutrophils initially generate superoxide anion via a membrane-localized NADPH oxidase (5). Interestingly, both chemotaxis and the production of superoxide are often triggered by the same extracellular chemotactic stimuli. However, many earlier studies have also shown these processes to be initiated at different concentrations of N-formyl peptide ligand, with actin polymerization occurring at an ED50 of 0.01 nM and superoxide production occurring with an EC50 of 0.3 nM or greater (6). This has been related to levels of receptor occupancy at various chemoattractant concentrations (6, 7). Because during the neutrophil’s response to invading microbes the leukocytes often are required to travel a relatively long distance across layers of healthy tissues before they eventually reach the infectious sites, controlled regulation of ROS formation during this transit is necessary to avoid causing damage to the healthy tissue by the chemotaxing leukocytes.

Over the past decade, different members of the Rho GTPase superfamily have been recognized as playing critical roles in the multiple biological responses of phagocytic leukocytes (8–10). The Rac GTPases (Rac1 and Rac2) have garnered a great deal of attention due to their roles in reorganizing the actin-myosin cytoskeleton during chemotaxis and in controlling the activity of the NADPH oxidase during the neutrophil response to invading microbes. In human neutrophils, Rac2 is the predominant Rac isoform, making up more than 80–95% of total Rac protein (11, 12). Rac1 and Rac2 have more than 90% homology at the amino acid sequence level and share many similar biochemical properties (13). Several Rac1 and/or Rac2 knockout mouse models have been studied to address the roles of Rac1 and Rac2 in neutrophil functions stimulated by different chemoattractants, including N-formylated peptides such as fMLP (14–21). These studies have established that both Rac1 and Rac2 are necessary for normal neutrophil chemotaxis and motility in response to formyl peptides, whereas only Rac2 is absolutely required for fMLP-stimulated NADPH oxidase activity.

Analysis of the defects in directed migration of neutrophils in Rac1−/− mice has shown that they exhibit the inability to properly orient in a chemotactic gradient, accompanied by the formation of multiple randomly oriented lamellipodia. They exhibit a modest defect in both F-actin assembly and the retraction of the uropod tail during migration (14, 22, 23). The latter has been linked to specific effects of Rac1 to regulate RhoA activation in the uropod to coordinate and promote stable cell polarity during chemotaxis (22). In contrast, Rac2−/− mice exhibit major defects in F-actin assembly and cell migration speed, but are still able to orient to a chemotactic gradient (14). A subsequent study showed Rac1 to control the initial uncapping of existing actin barbed ends, whereas Rac2 regulates the extension of actin filaments via cofilin- and Arp2/3-dependent mechanisms (18).

Although these studies have shed light on the roles of Rac1 and Rac2 during neutrophil immune responses, the exact contributions of Rac1 and Rac2 GTPases to various aspects of chemotaxis and motility remain unclear, and have not been examined in the context

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3 Abbreviations used in this paper: ROS, reactive oxygen species; DAPI, 4′,6-diamidino-2-phenylindole; DIAS, Dynamic Image Analysis System; DIC, differential interference contrast; GEP, guanine nucleotide exchange factor.

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of the relative concentrations of chemoattractant. The studies presented in this work show that in the low range of stimulatory fMLP concentrations, human neutrophils mainly activate Rac1 to promote initial cell spreading, leading to subsequent directional migration with regular, small lamellipodia at the leading edge and limited production of superoxide. In contrast, at high stimulatory fMLP concentrations, Rac1 is first activated to initiate the formation of lamellipodia, but then the activation of Rac2 is further required for continuous expansion of the large leading edge lamellipodia that drives effective migration, as well as superoxide formation. Our results connect the unique and overlapping roles of Rac1 and Rac2 in coordinating the directional migration of neutrophils with the production of superoxide for bacterial killing in response to changes in the chemoattractant gradient at the infectious site.

**Materials and Methods**

**General materials**

PMA, fMLP, Wortmannin, PP2, PP3, cytochrome c, 4′,6-diamidino-2-phenylindole (DAPI), human fibronectin, fluorescein, and D-glucose were purchased from Sigma-Aldrich. Endotoxin-free HBSS (pH 7.4) containing calcium and magnesium and PBS (10× PBS) were purchased from Invitrogen. Ficoll-Paque™ gradient was purchased from GE Healthcare. Falcon 50-ml and 15-ml sterile tubes were from Fisher Scientific. Micropipette puller P-87 was from Sutter Instruments. Software package of Dynamic Image Analysis System (DIAS) was from Soll Technologies. Rac1 mAb (23A8) was from Upstate Biotechnology-Millipore. Polyclonal Rac2 Ab R786 was generated in the laboratory (12, 24). Phalloidin Alexa-568 was obtained from Sigma-Aldrich. HRP-conjugated secondary Abs were from Thermo Fisher Scientific, whereas Alexa Fluor 488- and Alexa Fluor 563-conjugated secondary Abs were from Molecular Probes.

**TAT protein constructs**

TAT protein constructs were made by fusing the TAT domain from HIV virus with the target genes at the C terminus, and the resultant TAT fusion constructs were expressed in BL21DE3 cells and purified, as described previously (25).

**Isolation and maintenance of human neutrophils**

Fresh human blood was collected through the normal blood donor program at the Scripps Research Institute in accordance with an institutional review board–approved protocol. The blood was drawn by venipuncture, and was subsequently sedimented by 6% Dextran 500 (Pharmacosmos) at room temperature for 45 min. After the top layer of suspended leukocytes and a small amount of remnant RBCs were separated from the majority of sedimented RBCs, this top layer of leukocytes and RBCs was subsequently loaded onto prewarmed Ficoll-Paque™ gradient, according to the Ficoll-Paque™ manual. Briefly, after the proper treatment or stimulation, adherent neutrophils were first incubated in HBSS buffer (HBSS supplemented with 1 mM D-glucose) for experiments.

**Determination of human neutrophil superoxide production**

For human neutrophils in suspension, the cells were first prewarmed for 10 min at 37°C and then stimulated with the final concentration of 5 × 10⁻⁸ M fMLP. Superoxide production of stimulated neutrophils was measured continuously for 8 min by cytochrome c assay (26).

**Chemotaxis assays**

Sterilized coverslips were precoated with 50 μg/ml fibronectin for 45 min at room temperature, then washed three times with 1× PBS. Freshly isolated neutrophils were resuspended at 1 ×10⁶ cells/ml in HBSS* buffer, and allowed to adhere to the fibronectin-coated surface for 30 min before chemotactic assays (with at least 90% of cells adhered).

During a global stimulation assay, adherent human neutrophils were first incubated in HBSS* buffer and recorded through a ×100 objective on the Olympus microscope for 2 min. In the same imaging field, a 4× stock solution of fMLP was added to the experimental chamber to adjust to the final concentration of fMLP for global stimulation, and the neutrophils were recorded for an additional 12 min.

During a directional stimulation assay, adherent human neutrophils were first incubated in HBSS* buffer and recorded through a ×40 objective for 2 min. In the same imaging field, a micropipette filled with different concentrations of fMLP stock solution was lowered into the experimental chamber to create a gradient of fMLP by natural diffusion, and the neutrophils were recorded for an additional 12 min.

The movies recorded from chemotactic experiments were converted into movies with DIAS-specific format and analyzed with the DIAS software package to generate cellular behavioral parameters, such as cell centroid movement, cell tracks, cell speed, and cell area (27). These data were imported into Microsoft Excel for further analysis and graphing. The cell centroid was calculated by the DIAS program from the center of geometric shape of the cell. Centroid speed is the raw measure of cell speed based on the translocation of the cell centroid.

**Immunchemistry**

For optimal preservation of neutrophil morphology and native cytoskeletal structure, a fixation procedure (28, 29) was adapted for human neutrophils. Briefly, after the proper treatment or stimulation, adherent neutrophils were immediately fixed in 0.7% glutaraldehyde in 1× Cramer buffer for 15 min at 0°C, and were permeabilized with 0.1% Nonidet P-40 for 10 min. Autofluorescence was immediately quenched with 1 mg/ml NaBH₄ for 5 min, and nonspecific binding was blocked with 10% goat serum in 1× PBS for 1 h at room temperature. The actin cytoskeleton was stained with polyclonal actin Ab C4. Fixed human neutrophils were first incubated with different primary Abs overnight at 0°C at 1/200 dilution, then with secondary Abs for 90 min. Finally, before the coverslips were mounted, fixed human neutrophils were counterstained with DAPI (1/2000) and phalloidin Alexa Fluor 568 (1/400) for 30 min.

**Measurement of fMLP gradients**

A series of uniform fluorescein solutions from 1 × 10⁻⁸ M to 1 × 10⁻⁴ M were imaged to create a standard curve of fluorescein for estimating the concentration of fluorescein in different diffusion fields of mimicked fMLP gradients. The process of fluorescein diffusion from micropipettes loaded with either 3 × 10⁻³ or 3 × 10⁻⁴ M fluorescein was recorded. Based on the standard curve of fluorescein, the concentrations of fluorescein in the diffusion fields were determined using Metamorph software package and Microsoft Excel.

**Rac activation assay**

Rac1 and Rac2 activation was examined, as described previously (24). In brief, freshly isolated neutrophil extract was prepared as follows: after freshly isolated neutrophils were allowed to adhere to 50 μg/ml fibronectin-coated 5-cm plates for 60 min, they were immediately stimulated with uniform fMLP at the final concentration of 1 × 10⁻⁷ M. The stimulation of uniform fMLP was stopped at different time points by removing the stimulation medium, and placing the plates on ice and adding 450 μl of 1× cold lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 30 mM MgCl₂, 1% Nonidet P-40, and 5% glycerol supplemented with protein inhibitors, including 1 mM PMSF, 1 mM aprotinin, 1 μM leupeptin, and 1 μg/ml pepstatin A. A total of 20 μl of each cell lysate at each time point was examined on 12% SDS-PAGE and blotted with the indicated Rac Ab through the levels of Rac1 and Rac2. To determine Rac1 and Rac2, 400 μl of cell lysate was used with 10 μg of PBD (GST-fusion protein containing the Rac/Cdc42 binding domain of PAK1).

**Knockout mouse model and murine neutrophil chemotaxis assay protocols**

All procedures described were performed in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the University of Toronto Animal Care Committee. Rac1-conditional-null and Rac2-null mice were generated according to the protocol described by Glogauer et al. (14). Bone marrow mouse neutrophils were isolated, as described (17), and were resuspended in HBSS and 1% gelatin. The suspensions of mouse neutrophils (1 × 10⁶ cells/ml) were added to 50 μg/ml fibronectin-coated glass coverslips (22 × 40 mm) at 37°C for 1 h. The coverslip was inverted onto a Zigmond chamber, and then 100 μl of HBSS medium containing fMLP (1 × 10⁻⁶ M) was added to the right and
left chambers for stimulation with uniform fMLP. Time-lapse video microscopy (Nikon Eclipse E1000) was used to examine mouse neutrophil behavior in the Zigmond chamber with a ×60 objective, and images were captured at 10-s intervals. Cell-tracking software (Retrac version 2.1.01 Freeware) was used to characterize cell behaviors from the captured images. Image J software was used to quantify the spreading area and centroid movements of each cell at each time point (a total of 30 cells was analyzed for each time point).

Results

Dominant-negative Rac1 and Rac2 TAT fusion proteins independently inhibit Rac1 vs Rac2 activity

Dominant-negative mutants (threonine 17 to asparagine, T17N) of both Rac1 and Rac2 were fused to the C terminus of the TAT domain to construct cell-permeant Rac1 and Rac2 TAT fusion proteins (Rac1-T17N and Rac2-T17N will refer to these dominant-negative TAT fusion proteins). To examine whether Rac1-T17N and Rac2-T17N could specifically and independently inhibit the activation of Rac1 and Rac2, Rac1-T17N- and Rac2-T17N-pretreated human neutrophils in suspension were stimulated by \(1 \times 10^{-7} \text{ M fMLP}\), and active Rac1-GTP and Rac2-GTP were determined by affinity-based assay (24), as shown in supplemental Fig. S1. Rac1-T17N substantially reduced Rac1 activity, except at the maximum activation time point of 0.5 min (Fig. S1A). Given the slight cross-reactivity of the Rac1 and Rac2 Abs, it is likely that some of the activity observed at the 0.5-min maximum was contributed by Rac2. In contrast to Rac1, Rac2 activation was not significantly affected at any time point (Fig. S1A), suggesting that Rac1-T17N treatment specifically and independently inhibits the activation of Rac1 in human neutrophils. Conversely, dominant-negative Rac2-T17N dramatically suppressed Rac2 activity at all time points, while not affecting Rac1 (Fig. S1B), suggesting that Rac2-T17N treatment specifically and independently inhibits the activation of Rac2 in human neutrophils. The transduction of Rac1-T17N and Rac2-T17N into \(~95%\) of the human neutrophils was confirmed as in previous studies (25, 30) through the hemagglutinin tag at the N terminus of TAT fusion proteins (not shown). The above data indicate that Rac1-T17N and Rac2-T17N can be effectively transduced into human neutrophils, where they specifically and independently inhibit the activation of either Rac1 or Rac2, respectively, upon fMLP stimulation.

Neutrophils exhibit chemotactic responses differentially dependent on Rac1 and Rac2 upon exposure to different levels of fMLP

Human neutrophil chemotactic responses were examined in response to low vs high concentration gradients of fMLP (as determined in supplemental Fig. S2). We observed that both the low concentration gradient (10^{-8}–10^{-9} \text{ M fMLP} (Fig. 1A) and the high concentration gradient (10^{-7}–10^{-8} \text{ M fMLP} (Fig. 1B) were able to effectively induce the directional migration of control GFP-TAT-pretreated neutrophils toward the stimulus source. We investigated potential differences in the Rac GTPase isoform signaling dependence during chemotaxis, neutrophil chemotactic behaviors in fields of different concentration gradients of fMLP vs fields of different uniform concentrations of fMLP were compared (supplemental Movie 3). As shown in Figs. 2A and 3A, resting adherent neutrophils had a smooth spherical shape before stimulation with fMLP. As the neutrophils were stimulated with a high concentration gradient of fMLP (Fig. 2B) or by a high uniform concentration of fMLP (Fig. 3B), they showed a very similar behavioral pattern. This started with spreading and expansion of the cell periphery, resulting in an up to 3-fold increase in cell area, and was followed by the formation of a large lamellipodium at the leading front of the neutrophil, associated with rapid chemotactic movement (Fig. 2B vs 3B and supplemental Movie 3).

In contrast to this behavior, when human neutrophils were stimulated with either a low concentration gradient of fMLP (Fig. 2A) or a low uniform concentration of fMLP (Fig. 3A), they responded initially with the spreading at the cell periphery, but to a lesser extent than seen in the high concentration of fMLP (Fig. 2A vs 3A and supplemental Movie 3). This was followed with relatively slow migration of the cells in uniform low fMLP that was supported by small lamellipodia that formed randomly around the cell periphery, as shown in the cell tracks of Figs. 2A and 3A. Similarly, the cells in the low fMLP gradient, although they assumed a more elongated appearance as they responded to the chemoattractant gradient, also only extended small lamellipodia at the leading edge (supplemental Movie 3).

Because of the apparent similarity in neutrophil chemotactic behavioral patterns between the fields of uniform fMLP and the fields of fMLP gradients, and the ability to obtain higher magnification images of cells upon uniform fMLP stimulation, this condition was subsequently used for more accurate morphological analysis and evaluation of the relative roles of Rac1 and Rac2 in chemotactic responses.

Formation of the large leading edge lamellipodia requires Rac1 for its initiation and Rac2 for continuous expansion at the leading front

To dissect individual roles of Rac1 and Rac2 for morphological changes at the leading edge of chemotaxing neutrophils, they

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4 The online version of this article contains supplemental material.
were pretreated with dominant-negative Rac1 vs Rac2 TAT fusion proteins, stimulated with a high concentration of uniform fMLP, and then analyzed with DIAS software package for detailed dissection of cellular behavioral changes (supplemental Movie 4). Compared with untreated neutrophils, pretreatment with Rac1-T17N resulted in significant changes of neutrophil
chemotactic behaviors, but did not inhibit overall neutrophil motility, consistent with previous knockout studies (14, 17, 22). At the initial stage, untreated neutrophils experienced rapid cell spreading (stage 1: 0 – 4 min in supplemental Movie 4 and sequential differential interference contrast (DIC) images of Fig. 4A), characterized by an increase of cell area and centroid movement, as shown in Fig. 4A. This initial phase of rapid cell spreading was characteristically missing in Rac1-T17N-treated neutrophils (stage 1 in supplemental Movie 4 and sequential DIC images of Fig. 4B). This was evident also from the quantitative parameters of cell area and centroid movement shown in Fig. 4B.

Following this initial cell spreading, untreated chemotaxing neutrophils started to expand a large lamellipodium at the leading edge to support continuous rapid migration (stage 2: 4 – 6 min in supplemental Movie 4 and sequential DIC images of Fig. 4A), quantitatively characterized by the maintenance of a large cell area and by a further increase of centroid movement (Fig. 4A). Conversely, Rac1-T17N-treated neutrophils during stage 2 showed a 25–40%
decrease in centroid movement compared with control neutrophils (Fig. 4, A vs B) and had a markedly reduced cell area compared with control neutrophils (Fig. 4, A vs B), consistent with the absence of the formation of the large lamellipodium at the leading edge during this time period (supplemental Movie 4 and Fig. 4, B vs A).

After 8 min of fMLP stimulation, untreated neutrophils retracted the single large lamellipodium and started to form much smaller lamellipodia at the leading edge to support and maintain cell migration (stage 3: after 7 min in supplemental Movie 4). This was associated with a decrease in cell area after 8 min (Fig. 4A). However, during stage 3, Rac1-T17N-treated neutrophils started to form a relatively large lamellipodium at the leading edge (stage 3 in supplemental Movie 4 and sequential DIC images in Fig. 4B), demonstrated by the increase of cell area and centroid movement (Fig. 4B), suggesting that there was an abnormal delay in the formation of the large lamellipodium in Rac1-T17N-pretreated neutrophils. In contrast, pretreatment with Rac2-T17N inhibited neutrophil chemotaxis overall (supplemental Movie 4), consistent with prior knockout studies (15, 16, 18, 20). Compared with control neutrophils, Rac2-T17N-treated cells were able to undergo the initial cell-spreading response to fMLP stimulation (stage 1: 0–4 min in supplemental Movie 4 and sequential DIC images of Fig. 4C), demonstrated by the increase of cell area and centroid movement. However, during stages 2 and 3, Rac2-T17N-treated neutrophils had a 30% decrease in centroid movement compared with control cells (Fig. 4, A vs C). They also did not form the large lamellipodium at the leading edge over the time course of imaging (stages 2 and 3 in supplemental Movie 4 and sequential DIC images of Fig. 4C).

Overall, the absence of initial cell spreading in Rac1-T17N-treated neutrophils and the ability of Rac2-T17N-treated neutrophils to spread during stage 1 suggest that the activation of Rac1, but not Rac2, is crucial for early neutrophil spreading. Furthermore, the delayed formation of the large lamellipodium in Rac1-T17N-treated neutrophils and the absence of the large lamellipodium in Rac2-T17N-treated neutrophils imply that the formation of the large lamellipodium in chemotaxing neutrophils during stage 2 may be initially triggered by Rac1 activation. However, the subsequent expansion of the large lamellipodium appears to require the further activation of Rac2.

Verification of the distinct roles of Rac1 vs Rac2 using knockout mice

To verify Rac1 and Rac2 functions during neutrophil chemotactic responses to high concentration of uniform fMLP, neutrophils from conditional Rac1 knockout and Rac2 knockout mice were examined. In the Rac1 conditional knockout mice, we observed that neutrophils were defective in the initial increase of cell area due to cell spreading from 1 to 3 min post-stimulation, but subsequently exhibited a delayed spreading response over 2–5 min that was coupled with a delayed increase in centroid movement, as compared with wild-type mouse neutrophils (Fig. 5, A vs B). These results are similar to our observations in human neutrophils treated with Rac1-T17N (Fig. 4B), although the time course is slightly different. In contrast, in the Rac2 knockout mice, the neutrophils showed an increase of cell area (albeit weaker) due to cell spreading within the first 2–4 min, but exhibited almost no increase in centroid movement during the experimental period, as compared with wild-type neutrophils (Fig. 5, A vs C). The Rac2-deficient cells also never formed a large leading edge lamellipodium and were largely nonresponsive to the chemoattractant. These results confirm our observations with human neutrophils treated with...
Taken together, our observations suggest that Rac1 activation is an important determinant for initiating cell spreading and the initial formation of the lamellipodium, whereas Rac2 activation is required for the continuous expansion and maintenance of the leading edge lamellipodium upon fMLP stimulation.

The activation and redistribution of Rac1 and Rac2 correlate with leading edge morphological changes in chemotaxing neutrophils

To determine whether Rac1 and Rac2 activity and/or subcellular distribution were consistent with the distinct roles of each GTPase in human neutrophil chemotactic behavior, neutrophils were stained with Rac1- and Rac2-selective Abs (Fig. S3) after stimulation with uniform 10^{-7} M fMLP for various times (Fig. 6).

Rac1-T17N (Fig. 4C). Taken together, our observations suggest that Rac1 activation is an important determinant for initiating cell spreading and the initial formation of the lamellipodium, whereas Rac2 activation is required for the continuous expansion and maintenance of the leading edge lamellipodium upon fMLP stimulation.

**FIGURE 4.** Differential Rac GTPase regulation of neutrophil responses to a high concentration of uniform fMLP. **A.** Left, Sequential DIC images of an untreated human neutrophil are shown during stimulation with uniform 10^{-7} M fMLP; right, the measurements of cell area and centroid speed averaged from 30 cells were plotted against the time, in which the time of fMLP addition (zero) is labeled with a red asterisk on the x-axis. **B.** Sequential DIC images of a Rac1-T17N-pretreated human neutrophil are shown during stimulation with uniform 10^{-7} M fMLP (left side of B). At the right side of B, the measurements of cell area and centroid speed averaged from 30 cells were plotted against the time, in which the time of fMLP addition (zero) is labeled with an asterisk on the x-axis. **C.** Sequential DIC images of a Rac2-T17N-pretreated human neutrophil are shown during a uniform 10^{-7} M fMLP stimulation (left side of C). At the right side of C, the measurements of cell area and centroid speed averaged from 30 cells are plotted against the time, in which the time of fMLP addition (zero) is labeled with an asterisk on the x-axis. (Results are collected from three independent experiments.)
The Rac2 distribution pattern during 1 \times 10^{-7} M fMLP stimulation was substantially different from that of Rac1. After 1 \times 10^{-7} M fMLP stimulation, Rac2 changed from entirely cytosolic to association with the region that ultimately initiated expansion of the large lamellipodia at \sim 3 \text{ min} (Fig. 6B). After 3 min, Rac2 continued to accumulate at the front of the large expanding lamellipodium, where it colocalized with F-actin. Such colocalization was maintained until \sim 7 \text{ min}, when the neutrophils began to form smaller lamellipodia (Fig. 6B). Consistent with these observations, the PBD activity assay showed that the activation of Rac2 was initially detected and peaked at 0.5 \text{ min}, and was maintained at an elevated level for 6 –7 \text{ min} (Fig. 7, B and C). Thus, Rac2 activity ended as the expansion of the large lamellipodium was completed.

The initial rapid burst of Rac2 activation observed at 0.5 \text{ min} was most likely required for supporting superoxide production, which we observed to peak at 0.5–2 \text{ min} poststimulation (data not shown). Rac2 is known to be required for fMLP-induced NADPH oxidase activation (5, 15, 31).

These activation and distribution patterns of Rac1 and Rac2 are thus consistent with a role for Rac1 activation in the initiation of cell spreading, perhaps the initiation of the large lamellipodium, and the appearance of smaller lamellipodia. In contrast, Rac2 seems to be important for extending the Rac1-initiated lamellipodium to form a large lamellipodia at the leading edge, which supports rapid migration to the chemotactic source.

**Discussion**

We report in this study that Rac1 and Rac2 play distinct roles in regulating human neutrophil morphological responses to stimulation with different concentrations of chemotactant. This was determined using TAT dominant-negative Rac1-T17N or Rac2-T17N fusion proteins to acutely inhibit fMLP-stimulated changes in Rac1 or Rac2 activity, and was verified using mouse neutrophils in which either Rac1 or Rac2 was genetically deleted.

In steep gradients of chemoattractant, neutrophils can move strongly up-gradient by extending a major pseudopod in the direction of the chemoattractant source (32). This behavior appears very similar to what we have shown to be regulated by Rac2. Conversely, in shallow gradients of chemoattractant, neutrophils extend multiple small pseudopodia, then steer up-gradient by favoring the correctly oriented pseudopod. This is reminiscent of the Rac1 phenotype that we have observed.

The differential changes in neutrophil responses observed in the presence of the TAT-Rac1-T17N or the TAT-Rac2-T17N were confirmed in neutrophils derived from the Rac1 and Rac2 knockout mice. Chemotactic defects have been reported previously in both the Rac2 (16, 20, 33) and Rac1 (14, 17, 18, 33) knockout mice. Rac1 deficiency was characterized by normal cell motility, but inability to sense a chemotactic gradient (14). In the absence of Rac2, orientation to the gradient was normal, but the cells failed to...
migrate efficiently (16, 18, 20, 33). In the current study, we observed that TAT-Rac1-T17N-mediated inhibition resulted in a loss of the initial spreading response to chemoattractant, as well as a delay in formation of the large lamellipodium at the leading edge (Fig. 4B and supplemental Movie 4). In contrast, the initial spreading response was intact in Rac2-inhibited cells, but the leading lamellipodium did not form at all when Rac2 was inhibited (Fig. 4C and supplemental Movie 4). These results appear consistent with a prior study showing that Rac1 induces the uncapping of actin filaments to drive rapid formation of free actin barbed ends, whereas Rac2 produces a slower and more sustained activation of actin filaments to drive rapid formation of free actin barbed ends, and maintenance of a single large lamellipodium at the leading front and efficient chemotactic motility (Figs. 6B and 7C). The timing of Rac1 and Rac2 activation we observed matched very well with changes in intracellular distribution of Rac1 vs Rac2 in the stimulated neutrophils (Fig. 6).

It remains unclear exactly why Rac1 does not support NADPH oxidase activity. We, and others, have shown that Rac1 is much less abundant than is Rac2 in human neutrophils. Dinauer and colleagues (20, 35) have reported that Rac levels alone did not explain the differences in activity, however. They specifically examined oxidase activation by Rac1 vs Rac2 and reported that a chimeric Rac1 protein in which the Rac1 C-terminal polybasic domain was replaced with that of Rac2 did not. Thus, the polybasic domain seems to be sufficient for determining Rac isoform specificity in the production of superoxide in murine neutrophils in vivo, and this may be related to differences in activity, however. They specifically examined oxidase activation by Rac1 vs Rac2 and reported that a chimeric Rac1 protein in which the Rac1 C-terminal polybasic domain was replaced with that of the human Rac2 polybasic domain containing only three basic residues reconstituted superoxide production, whereas expression of a Rac2 derivative in which the polybasic domain was replaced with that of Rac1 did not. Thus, the composition of the polybasic domain seems to be sufficient for determining Rac isoform specificity in the production of superoxide in murine neutrophils in vivo, and this may be related to differential GTPase localization (as observed in this study; Fig. 6) and/or protein binding of Rac1 vs Rac2 in the neutrophil.
In the current study, we established that human neutrophils respond to increasing concentrations of fMLP through the differential activation of Rac1 and Rac2 (Fig. 1). In response to a low concentration gradient of fMLP, neutrophils will activate Rac1 as the predominant form of Rac for initiating chemotaxis and directional migration. As neutrophils move toward the infectious sites, they experience a higher concentration of fMLP and activate both Rac1 and Rac2 to support both chemotaxis and inflammatory activity, such as superoxide production. Once neutrophils eventually reach the infectious site, under the high concentration of fMLP, only Rac2 is activated and the cells stop moving, allowing the neutrophils to stay at the infectious site for performing their inflammatory functions.

**A model for differential regulation of neutrophil response to infection by Rac1 vs Rac2**

During the immunological responses of human neutrophils after their initial emigration out of the blood vessel, they encounter an increasing gradient of chemotactic signals diffused from the infectious sites (2). In vivo and vitro, isolated human neutrophils have been shown to be responsive to stimulation with fMLP in a range of $1 \times 10^{-7}$ M to $1 \times 10^{-7}$ M (36–38). In the current study, we establish that human neutrophils respond to increasing concentrations of fMLP through the differential activation of Rac1 and Rac2 (Fig. 1). In response to a low concentration gradient of fMLP (ranging from $10^{-9}$ M to $10^{-8}$ M), which mimics the situation at the time of neutrophil’s emigration from the blood vessel, we found that inhibition of Rac1, but not Rac2, by dominant-negative Rac1 TAT protein was able to impede neutrophil chemotaxis (Fig. 1B). The activation of Rac1 correlates well with the low concentrations of N-formyl peptides that initiate chemotaxis (6). A morphological characteristic in neutrophils responding to such a low concentration fMLP gradient is the relatively small and short lamellipodia at the leading edge. These are likely to be supported by the uncapping of existing actin-free barbed ends through Rac1 activation, as reported (18). This low range of fMLP concentration elicits only very limited production of superoxide, less than one-tenth of Rac2-dependent superoxide production observed at $10^{-7}$ M fMLP (data not shown). At this stage, such a limited amount of superoxide formation will cause minimal damage to the surrounding healthy tissue (see model, Fig. 8).

In response to the high concentration fMLP gradient, which mimics the situation at the vicinity of infectious sites, we suggest that human neutrophils initially elicit Rac1 activation as part of sensing the chemotactant gradient (Fig. 1A) and to initiate chemotactic responses such as cell spreading and the early increase in cell motility. This is evidenced by the lack of these responses in Rac1T17N-pretreated neutrophils and in Rac1 conditional knockout mouse neutrophils. Following these initial responses, we suggest that human neutrophils then rely on the activation of Rac2 to support efficient rapid migration toward the gradient source. The latter is supported by Rac2-regulated Arp2/3- and coflin-mediated actin polymerization for continuous expansion of the leading lamellipodium. This stage would also enable the cells to respond with a burst of superoxide formation (see model, Fig. 8). The differential responses of neutrophils to low vs high concentrations of fMLP may relate to the levels of receptor occupancy, as previously described (6, 7). These studies showed that whereas chemotactic responses occurred at low levels of occupied fMLP receptor, the activation of NADPH oxidase required much higher receptor occupancy by ligand.

Our in vivo model provides a very nice behavioral explanation for the results obtained in an earlier study by Glogauer and colleagues (23) using an acute pulmonary inflammation model in which either *Escherichia coli* or the chemotactant KC were delivered intratracheally to wild-type or to Rac1-null or Rac2-null mice. They observed that there was a modest chemotactic defect in pulmonary recruitment of the Rac1-null neutrophils over a 4-h time period, whereas the Rac2 knockout mice exhibited a substantially more severe defect. This may be explained by our current data in which Rac1 participates in the initial cell responses at low fMLP and in slow chemotactic migration, resulting in less effective, but still functional, chemotaxis when Rac1 is depleted. In contrast, we have shown that Rac2 is necessary for maintenance of the primary pseudopod and for rapid migration, especially in high concentrations of fMLP, and chemotaxis cannot be maintained in its absence. Unlike these observations with chemotaxis, NADPH oxidase-dependent myeloperoxidase release and activity in the lung infection model were observed to be severely compromised in the Rac2 knockout, although being only minimally reduced in the absence of Rac1. These in vivo studies are very consistent overall with what our current results would predict.

**Differential regulation of Rac1 vs Rac2**

The differential effects of the dominant-negative Rac1 vs Rac2 proteins, which are believed to act by sequestering guanine nucleotide exchange factors (GEFs) involved in GTPase activation, suggest that these two Rac isoforms respond to distinct GEFs during chemotaxis. Indeed, several Rac-specific GEFs have been implicated in fMLP-stimulated neutrophil activation, including Vav1 (39, 40), P-Rex1 (41–43), and Dock2 (44). Selectivity for Rac2 has been reported for Vav1 (45) and P-Rex1 (41). Because the studies reported in this work only made use of a single chemotactant, fMLP, we cannot conclusively extend our results to all chemotactants. It has been reported that fMLP differs from a number of other chemotactant agents in its preference for the p38 MAPK pathway for the induction of chemotaxis, as opposed to the PI3K pathway (46). However, we note that under our assay conditions, chemotaxis to fMLP was sensitive to 100 nM PI3K inhibitor wortmannin (data not shown).

In summary, our studies of human and mouse neutrophils in response to different concentrations of fMLP suggest that during chemotaxis from blood vessel to infectious sites, neutrophils initiate different chemotactic responses through the differential activation of Rac1 and Rac2 (Fig. 8). Over the low concentration range, neutrophils activate Rac1 to sustain directional migration to the source, but without the triggering of the superoxide burst that is detrimental to the surrounding healthy tissue. As human neutrophils approach the infectious sites, where they experience high concentrations of chemotactant, the activation of Rac2 at the
leading front is required for maintaining a continuous fast expansion
the large leading edge lamellipodium, as well as supporting
NADPH oxidase activation in a timely manner. Distinct Rho
GTPase GEFs may be essential factors in controlling this differen-
tial activation of Rac1 and Rac2 by chemottractants.

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Supplemental Materials

Supplemental Figure 1 (Figure S1) Rac1-T17N and Rac2-T17N specifically inhibit the activation of Rac1 and Rac2. (A) 1 μM Rac1-T17N pretreated human neutrophils in suspension were stimulated by 1x10^{-7} M fMLP. Active Rac1 and Rac2 were detected by PBD pull-down assay (see Materials and Methods). The ratio of active Rac at each time point as compared to time zero was described as [Active Rac (time X)/Total Rac (time X)] / [Active Rac (0 min)/Total Rac (0 min)]. Therefore, if the ratio of active Rac to the zero time is 1, it means no activation of Rac. If the ratio is more than 1, it means activation of Rac, and if the ratio is less than 1, it means inhibition of Rac activation. Rac1-T17N treatment decreased Rac1 activation but not Rac2, suggesting Rac1-T17N specifically inhibits Rac1 activation in human neutrophils. (B) 2 μM Rac2-T17N pretreated human neutrophils in suspension were stimulated by 1x10^{-7} M fMLP. The active Rac1 or Rac2 were detected by PBD pull-down assay, and presented as described in (A) above. Rac2-T17N treatment decreased Rac2 activation significantly, but not Rac1 activity, suggesting Rac2-T17N specifically inhibits Rac2 activation in human neutrophils.  These concentrations of Tat-GTPases were shown to give optimal inhibition in preliminary dose-response studies, and were used in all chemotaxis experiments. (The result is the representative of two independent experiments)

Supplemental Figure 2 (Figure S2) Determination of fMLP gradient fields. (A) A series of fluorescein solutions of known concentration were imaged. (B) Based on the linear relationship between the concentrations of fluorescein and the fluorescence intensity, a standard curve of fluorescein concentration vs fluorescence intensities was constructed. (C) A sample image of the gradient field from the micropipette containing 1x10^{-4} M fMLP is shown at 5 min after the addition to the chamber at the top of the panel, and a line scan across the gradient field, as indicated by the red arrow, is shown at the bottom of the panel. (D) A sample image of the gradient field from the micropipette containing 1x10^{-5} M fMLP is shown at 5 min after the addition to the chamber at the top of the panel, and a line scan across the gradient field, as indicated by the red arrow, is shown at the bottom of the panel.

Supplemental Figure 3 (Figure S3) The specificity of the Rac1 and Rac2 antibodies. 10 ng of recombinant Rac1 or Rac2 were run in separate lanes on a 12% SDS-PAGE gel and then immunoblotted using either Rac1-selective antibody 23A8 (Upstate) or Rac2-specific antibody R786 (in house). (The result is the representative of two independent experiments)

Supplemental Movie 1-Human neutrophils in high fMLP gradient Control, Rac1-T17N and Rac2 T17N treated neutrophils were stimulated with a high fMLP gradient created by a micropipette containing 3x10^{-4} M fMLP. The movie was recorded at 20 s interval.
Note: The micropipette was added at 2 min.

Supplemental Movie 2-Human neutrophils in low fMLP gradient Control, Rac1-T17N and Rac2 T17N treated neutrophils were stimulated with a low
fMLP gradient created by a micropipette containing 3x10⁻⁵ M fMLP. The movie was recorded at 20 s interval. 
Note: The micropipette was added at 2 min.

**Supplemental Movie 3 – Behavioral similarity of human neutrophils in gradients of fMLP and uniform fMLP**
The morphological changes of sample human neutrophils, which were subjected to the stimulation of either gradients of fMLP or uniform fMLP, were animated and overlaid with their cell tracks by using DIAS.

**Supplemental Movie 4 – Morphological changes of control, Rac1-T17N and Rac2-T17N treated neutrophils in high uniform concentration fMLP**
The morphological changes of control and Rac1-T17N or Rac2-T17N treated neutrophils in high uniform concentration of fMLP were animated and overlaid with their cell tracks by using DIAS.
A

Different Concentrations of Fluorescein's Panel

B

Fluorescein Standard Curve

C

The distribution of fluorescein concentration from micropipette with 3x10^{-6} M FMLP

D

The distribution of fluorescein concentration from micropipette with 3x10^{-7} M FMLP
Mouse 23A8 Ab against Rac1

Rabbit R786 Ab against Rac2

Rac2     Rac1

Rac1     Rac2