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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 chemoattractants. 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 chemoattractant 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...
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 when 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 α-galactoside were purchased from Sigma-Aldrich. Endotoxin-free HBSS (pH 7.4), containing calcium and magnesium and PBS (10X PBS) were acquired from Invitrogen. Ficol-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 Soli Technologies. Rac1 mAb (22AA8) 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 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 pre-warmed Ficol-Paque™ gradient, according to the Ficol-Paque™ manual. After centrifuging at 500 × g for 30 min without braking, human neutrophils were collected, discarding the upper layers of plasma and monocytes. The isolated neutrophils were then briefly exposed to endotoxin-free water to hypotonically lyse remaining RBCs. The isolated neutrophils (up to 98% purity) were then resuspended in HBSS+ buffer (HBSS supplemented with 1 mM α-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 × 10⁻⁶ M fMLP. Superoxide production of stimulated neutrophils was measured continuously for 8 min by cytochrome c assay (26).

For adherent human neutrophils, wells in the 96-well plate were precoated with 50 μg/ml fibronectin for 45 min, then washed three times with 1X PBS. After freshly isolated neutrophils were allowed to adhere to the fibronectin-coated surface for 30 min, the cells were stimulated with different concentrations of fMLP (as indicated), and superoxide production of the adherent neutrophils was measured for 30 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 1X 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 4X 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 micro pipette 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.

Immunochemistry

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. Auto-fluorescence 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 × 10⁻⁶ 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 × 10⁻⁷ or 3 × 10⁻⁵ M fluorescein was recorded. Based on the standard curve of fluorescein, the concentration 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, stimulated adherent 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 1X 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 at the amounts of 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 chemotactic 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 suspended mouse neutrophils were added to 4 × 10⁶ cells/ml with 50 μg/ml fibronectin-coated glass coverslips (22 × 40 mm) at 37°C for 1 h. The coverslip was inverted onto a Zigmold 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-pre-treated human neutrophils in suspension were stimulated by 1 × 10^{-7} M fMLP, and active Rac1-GTP and Rac2-GTP were determined by affinity-based assay (24), as shown in supplemental Fig. S1.4 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 present was verified as in previous studies (25, 30) through the hemagglutinin tag at the N terminus of TAT fusion proteins (data 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} M) of fMLP (Fig. 1A) and the high concentration gradient (10^{-7}-10^{-8} M) of fMLP (Fig. 1B) were able to effectively induce the directional migration of control GFP-TAT-pre-treated neutrophils toward the stimulant 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 either 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
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 chemotaxis. However, Rac2-T17N-pretreated human neutrophils showed normal migration tracks toward the source of fMLP (labeled with asterisk), suggesting that the inhibition of Rac2 did not block neutrophil chemotaxis. However, Rac1-T17N-pretreated human neutrophils showed impaired migration tracks toward the source of fMLP (labeled with asterisk), indicating that the inhibition of Rac1 was able to block neutrophil chemotaxis in a low concentration gradient of fMLP. B, In a high fMLP concentration gradient, both GFP-TAT- and Rac1-T17N-pretreated human neutrophils show normal cell tracks toward the source of fMLP (asterisk), suggesting the inhibition of Rac1 did not block neutrophil chemotaxis. However, Rac2-T17N-pretreated human neutrophils exhibited shorter migration tracks toward the source of fMLP (asterisk), indicating the inhibition of Rac2 was able to block neutrophil chemotaxis in a high fMLP concentration gradient. (Results are representative of three independent experiments.)

FIGURE 1. The inhibition of Rac1 or Rac2 by TAT fusion proteins, Rac1-T17N or Rac2-T17N, resulted in different chemotactic responses in human neutrophils in high and low concentration fMLP gradients. A, In a low fMLP concentration gradient, both GFP- and Rac2-T17N-pretreated human neutrophils showed normal migration tracks toward the source of fMLP (labeled with asterisk), suggesting that the inhibition of Rac2 did not block neutrophil chemotaxis. However, Rac1-T17N-pretreated human neutrophils showed impaired migration tracks toward the source of fMLP (labeled with asterisk), indicating that the inhibition of Rac1 was able to block neutrophil chemotaxis in a low concentration gradient of fMLP. B, In a high fMLP concentration gradient, both GFP-TAT- and Rac1-T17N-pretreated human neutrophils show normal cell tracks toward the source of fMLP (asterisk), suggesting the inhibition of Rac1 did not block neutrophil chemotaxis. However, Rac2-T17N-pretreated human neutrophils exhibited shorter migration tracks toward the source of fMLP (asterisk), indicating the inhibition of Rac2 was able to block neutrophil chemotaxis in a high fMLP concentration gradient. (Results are representative of three independent experiments.)
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 cell area and centroid movement compared to untreated neutrophils.

### Table I. Rac1 and Rac2 inhibitions differentially affect neutrophil chemotaxis in either high fMLP concentration or low fMLP concentration gradients

<table>
<thead>
<tr>
<th>Gradient Type</th>
<th>Pretreatment</th>
<th>Rac1-T17N</th>
<th>Rac2-T17N</th>
<th>GFP-TAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High fMLP concentration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell number</td>
<td>39</td>
<td>28</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Average chemotactic index</td>
<td>0.069</td>
<td>0.043</td>
<td>0.089</td>
<td></td>
</tr>
<tr>
<td>Student’s t test (vs GFP-TAT)</td>
<td>0.358 (Nonsignificant)</td>
<td>0.007 (Significant)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of positive chemotactic index</td>
<td>100%</td>
<td>71%</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td><strong>Low fMLP concentration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell number</td>
<td>36</td>
<td>39</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Average chemotactic index</td>
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<td>0.093</td>
<td>0.089</td>
<td></td>
</tr>
<tr>
<td>Student’s t test (vs GFP-TAT)</td>
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<td>0.899 (Nonsignificant)</td>
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<td></td>
</tr>
<tr>
<td>Percentage of positive chemotactic index</td>
<td>69%</td>
<td>95%</td>
<td>87%</td>
<td></td>
</tr>
</tbody>
</table>

*Chemotactic index of an individual cell was calculated by dividing net translocation distance of the cell (to the source (positive number), and away from the source (negative number)) with total translocation distance of the cell. Therefore, positive chemotactic index indicates that the cell has the ability to chemotax toward a chemotaxant source. Percentage of positive chemotactic index was calculated by dividing the number of the cells with positive chemotactic index with the number of total cells, indicating whether a group of cells still has the ability to chemotax to a chemotaxant source (results are collected from three independent experiments).
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. 4 A). 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, Rac2-T17N-treated neutrophils 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 in 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 was initially observed throughout the cytoplasm, but became colocalized with F-actin in the cell periphery at 0.5 min after stimulation with $10^{-7}$ M fMLP (Fig. 6A). At this time, the stimulated neutrophils began to spread, as shown in the corresponding DIC-DAPI overlaid image of Fig. 6A (see also Fig. 4). Following this initial morphological change over the course of the first 2–3 min, the colocalization of Rac1 and F-actin was maintained, but as the chemotaxing neutrophil started to form the large lamellipodium at the leading edge, Rac1 accumulation disappeared from the expanding lamellipodium, which was marked by the presence of strong F-actin staining (between 3 and 5–6 min in Fig. 6A). Colocalization between Rac1 and F-actin did not reappear at the leading edge until the small lamellipodia became the dominant force for cell migration again (at 7 min in Fig. 6A). Consistent with these observations, the activation pattern of Rac1 detected by PBD pull-down assay showed that the activation of Rac1 peaked by 0.5 min, remained elevated throughout first 4-min period of observation, but was inactivated from 4 to 7 min, the period during which the expansion of the large lamellipodium occurred (Fig. 7, A and C). Rac1 activity was observed to be increased again after 7 min (Fig. 7, A and C), consistent with the formation of smaller lamellipodium at this time and the localization of Rac1 to these structures (Fig. 6A).

Rac2-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.
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 $\sim 3$ 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 $\sim 7$ 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 min, and was maintained at an elevated level for 6–7 min (Fig. 7, B and C). Thus, Rac2 activity ended as the expansion of the large lamellipodium was completed.

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 chemoattractant source.

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**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 chemoattractant. 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 chemoattractant 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, which is consistent with Rac1 promoting the production of superoxide (34), and then remained elevated over the baseline until 7–8 min, when it dropped to unstimulated levels. (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 active activation of Rac, and if the ratio is less than 1, it means inhibition of Rac activation. The activation of Rac1 was increased throughout the first 4 min of stimulation, then dropped sharply between 4 and 6 min, but increased again after 7 min. Conversely, the activation of Rac2 was increased throughout the first 7 min, then decreased sharply after this time. (The ratio is representative of two independent experiments.)

FIGURE 6. Adherent human neutrophils stimulated by $1 \times 10^{-7}$ M fMLP show a differential subcellular distribution of Rac1 and Rac2 with time of stimulation. A, Freshly prepared human neutrophils were allowed to adhere to fibronectin-coated surface for 1 h, stimulated with the final concentration of $1 \times 10^{-7}$ M fMLP, and immunostained for Rac1 (green) and F-actin (red) at 0, 0.5, 1, 3, 5, 7, and 9 min with Rac1-specific Ab (Upstate Biotechnology; 23A8) and phalloidin. B, Freshly prepared human neutrophils were allowed to adhere to fibronectin-coated surface for 1 h, stimulated with the final concentration of $1 \times 10^{-7}$ M fMLP, and immunostained for Rac2 (green) and F-actin (red) at 0, 0.5, 1, 3, 5, 7, and 9 min with Rac2-specific Ab (R786) and phalloidin. In both A and B, the morphological changes were monitored through the corresponding DIC-DAPI images (top panels). (The result is representative of two independent experiments.)

FIGURE 7. Rac1 and Rac2 are differently activated by uniform fMLP stimulation in human neutrophils. A, Freshly prepared human neutrophils were allowed to adhere to fibronectin-coated surface for 1 h and were stimulated with the final concentration of $1 \times 10^{-7}$ M fMLP for the time period indicated, and active Rac1 was detected by affinity-based PBD pull-down assay, as described in Materials and Methods. B, Adherent human neutrophils were stimulated by $1 \times 10^{-7}$ M fMLP for the times indicated. Active Rac2 was detected by affinity-based PBD pull-down assay. C, The ratio of active Rac at each time point as compared with time zero was described as follows: (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. The activation of Rac1 was increased throughout the first 4 min of stimulation, then dropped sharply between 4 and 6 min, but increased again after 7 min. Conversely, the activation of Rac2 was increased throughout the first 7 min, then decreased sharply after this time. (The result is representative of two independent experiments.)

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 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.
FIGURE 8. Model proposed to explain the regulation of Rac1 and Rac2 in a chemoattractant gradient from blood vessels to infectious sites. In this model, the x-axis represents the distance from the emigration sites, where neutrophils migrate out of the blood vessels, to the infectious sites, where neutrophils exhibit inflammatory responses, such as superoxide production. The y-axis represents the concentration range of fMLP (in log scale) over the distance from the emigration sites, where the fMLP concentration is lowest (1 × 10^{-7} M), to the infectious sites, where the fMLP concentration is highest (1 × 10^{-3} M). At the emigration site, where the concentration of fMLP is low, 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 × 10^{-7} M to 1 × 10^{-3} 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 actin-free barbed ends through Rac1 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.

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 chemoattractant, the activation of Rac2 at the lowest (1 × 10^{-6} M) to 1 × 10^{-3} 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 actin-free barbed ends through Rac1 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.

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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 Rac GTPase GEFs may be essential factors in controlling this differential activation of Rac1 and Rac2 by chemoattractants.

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Disclosures
The authors have no financial conflict of interest.