Small-molecule screen identifies reactive oxygen species as key regulators of neutrophil chemotaxis

Hidenori Hattori1,a, Kulandayan K. Subramanian1,a, Jiro Sakai1,a, Yonghui Jia1,a, Yitang Li1,b, Timothy F. Porter1, Fabien Loison2, Bara Sarraj3, Anongnard Kasorn3, Hakryul Jo4, Catlyn Blanchard4, Dorothy Zirkle5, Douglas McDonald6, Sung-Yun Pai7, Charles N. Serhan5, and Hongbo R. Luo1,a,2

Department of Pathology, Harvard Medical School Dana-Farber/Harvard Cancer Center and Department of Laboratory Medicine, Children’s Hospital Boston, Boston, MA 02115; 1Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115; 3Division of Immunology, Children’s Hospital Boston, and Department of Pediatrics, Harvard Medical School, Boston, MA 02115; and 5Combined Department of Pediatric Hematology–Oncology, Children’s Hospital Boston and Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

Edited* by Solomon Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved January 15, 2010 (received for review December 11, 2009)

Neutrophil chemotaxis plays an essential role in innate immunity, but the underlying cellular mechanism is still not fully characterized. Here, using a small-molecule functional screening, we identified NADPH oxidase–dependent reactive oxygen species as key regulators of neutrophil chemotactic migration. Neutrophils with pharmacologically inhibited oxidase, or isolated from chronic granulomatous disease (CGD) patients and mice, formed more frequent multiple pseudopodia and lost their directionality as they migrated up a chemoattractant concentration gradient. Knocking down NADPH oxidase in differentiated neutrophil-like HL60 cells also led to defective chemotaxis. Consistent with the in vitro results, adoptively transferred CGD murine neutrophils showed impaired in vivo recruitment to sites of inflammation. Together, these results present a physiological role for reactive oxygen species in regulating neutrophil functions and shed light on the pathogenesis of CGD.

chronic granulomatous disease | innate immunity | NADPH oxidase

Neutrophils are major players in innate immunity and constitute the first line of host defense against invading bacteria and other pathogens. In response to inflammatory stimuli, neutrophils migrate from the blood to infected tissues, where they protect their host by engulfing, killing, and digesting invading bacterial and fungal pathogens. Conversely, excessive neutrophil accumulation can be detrimental to the system. Hence, neutrophil recruitment in response to inflammatory stimuli needs to be well controlled.

Neutrophils are recruited to the site of infection by responding to a variety of chemokines, leukotrienes, complement peptides, and some chemicals released by bacteria directly, such as peptides bearing the N-formyl group (i.e., formyl-peptides). Neutrophil chemotaxis is mediated by heterotrimeric guanine nucleotide-binding regulatory proteins (G protein)–coupled receptors (GPCRs). One essential downstream target of GPCR is PtdIns (3,4,5)P3. Chemotaxtractants bind receptors on cell membrane and induce the dissociation of a specific G protein into α- and βγ-subunits. Released βγ-subunits initiate accumulation of PtdIns (3,4,5)P3 and subsequent actin polymerization at the leading edge of chemotaxing cells. Earlier studies have suggested that PtdIns (3,4,5)P3 plays the essential role of a cellular compass, localizing to the leading edge of pseudopodia, mediating direction sensing during chemotactic migration and cell polarity (1–4). However, several recent studies have shown that loss of PI3K and reduced PtdIns(3,4,5)P3 level lead to decreased polarity, but does not affect the ability of the cell to sense chemoattractant gradients. In both human neutrophils (5, 6) and Dictostelium (7–9), chemotaxis could occur independently of the PI3K-dependent actin polymerization, although it was somewhat delayed, suggesting extra pathways are required for neutrophil chemotaxis.

To identify these putative signal-induced chemoattractic pathways, we conducted a functional screening for chemical compounds that disrupt neutrophil directionality. We have identified NADPH oxidase dependent reactive oxygen species (ROS) as key regulators of neutrophil chemotaxis. Neutrophils with pharmacologically inhibited NADPH oxidase, or isolated from chronic granulomatous disease (CGD) patients and mice, displayed more frequent multiple pseudopodia formation and impaired directionality during chemotaxis. This finding provides a cellular mechanism for CGD pathogenesis and might lead to development of new therapeutic strategies for this disease.

Results

Screening for Inhibitors of Neutrophil Chemotaxis. The screening was performed using an EZ-TAXIScan chemotaxis device in which a stable chemoattractant gradient was formed in a 260-μm–wide channel (Fig. S1A). Freshly purified human primary neutrophils migrated robustly up the gradient and most cells crossed the channel in 20 min (Fig. S1B). A Tocris screening library containing 386 biologically active compounds was used for screening (Table S1). To achieve the maximal inhibition of each targeted pathway in the primary screening, we treated neutrophils with each drug at a concentration equivalent to 10 times the IC50 of the drug. Although most compounds did not affect neutrophil chemotaxis (Dataset S1), 83 compounds displayed inhibitory effects. (The video files for each compound will be released to a public database after the publication of this article.) The inhibitory effects were elicited via a variety of mechanisms (Table S1), such as induction of cell death (Dataset S2), complete inhibition of polarization and migration, slow migration, and impairment of directionality. Selected compounds from the primary screen were then used at 0.2 to 10 times the IC50 in a secondary screening (Dataset S3). Most positive compounds identified from the primary screening showed the same inhibitory effect at lower concentrations, suggesting that the drug-induced phenotype changes were most likely caused by specific inhibition of each targeted pathway.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through thePNAS open access option.

*H.H., K.K.S., and J.S. contributed equally to this work.

This article contains supporting information online at www.pnas.org/cgi/content/full/0914351107/DCSupplemental.


The authors declare no conflict of interest.

This Direct Submission article had a prearranged editor.

Freely available online through thePNAS open access option.

This article contains supporting information online at www.pnas.org/cgi/content/full/0914351107/DCSupplemental.

The authors declare no conflict of interest.

H.H., K.K.S., and J.S. contributed equally to this work.

To whom correspondence should be addressed. E-mail: hongbo.luo@childrens.harvard.edu

This article contains supporting information online at www.pnas.org/cgi/content/full/0914351107/DCSupplemental.
We focused on the 12 compounds that led to impaired directionality, but did not inhibit neutrophil migration completely (Figs. S2 and S3). Five of these drugs are compounds that inhibit microtubule polymerization, which is consistent with recent reports indicating that microtubules negatively regulate uropod signaling and enhance directional sensing in neutrophils (10, 11). Interestingly, the most dramatic inhibitory effect was induced by diphenyleneiodonium chloride (DPI), a well characterized and commonly used flavoprotein inhibitor that was known to suppress activity of NADPH oxidase and NOS (12–14). As neutrophil chemotaxis was not affected by other NOS inhibitors (e.g., 7-nitroindazole, L-NIO dihydrochloride, 1-[2-(trifluoromethyl) phenyl]imidazole, 2-amino-5,6-dihydro-6-methyl-4H-1,3 thiazine, ethylisothiourea, S-isopropylisothiourea hydrobromide, NG-methyl-L-arginine, Nω-nitro-L-arginine methyl ester, Nω-nitro-L-arginine, and L-canavanine) or NO donors (3-morpholinosydnonimine, s-nitrosoglutathione, and spermine NONOate; Dataset S1), it is most likely that the effect of DPI on neutrophil chemotaxis was mediated by the inhibition of NADPH oxidase, suggesting that chemoattractant elicited ROS production might play a role in regulating neutrophil chemotactic migration.

**ROS Are Physiological Regulators of Neutrophil Chemotactic Migration.** We further investigated the effect of DPI on neutrophil directional migration using a transwell migration system. Cells were plated on transwell filters and induced to migrate in response to...
chemoattractant added to wells beneath the filters. The migration of neutrophils to these lower wells requires 2D chemotaxis on top of the filter (toward the holes), followed by migration through the holes into the bottom well of chemoattractant. The number of cells in the bottom well was then used to calculate percentage of cells migrated. Consistent with the EZ-TAXIScan results, treatment with DPI significantly inhibited neutrophil migration into the lower wells (Fig. 1A). To take a closer look at the morphological changes elicited by DPI treatment, multiple pseudopod formation was measured in chemotaxing neutrophils. We observed that DPI-treated neutrophils showed multiple pseudopodia much more frequently compared with untreated neutrophils, although the migration speed of these two populations was essentially the same (Fig. 1B). The DPI-induced inhibitory effect appeared not to be specific to chemotaxis-elicited by chemoattractant N-formyl-methionyl-leucyl-phenyalanine (fMLP), which was used for the initial screening. Treatment with DPI also significantly inhibited chemotaxis elicited by leukotriene B4 (LTB4) and IL-8, suggesting that DPI might block a general pathway in directional migration (Fig. 1C). Interestingly, IL-8–mediated chemotaxis was more resistant to DPI treatment compared with fMLP and LTB4. It seems that this is not a sensitivity

**Fig. 2.** Disruption of NADPH oxidase leads to chemotaxis defects. (A–E) Neutrophils from CGD mice do not produce ROS in response to chemoattractant stimulation, display multiple pseudopodia, and show loss of directionality during chemotaxis. (A) ROS production in neutrophils (5 × 10^5) from WT or CGD mice after stimulation with 1 μM fMLP was evaluated by monitoring chemiluminescence (for 1 s) every 7 s for 280 s, in the presence of 50 μM luminol and 0.8 U HRP in a luminometer at 37 °C. Data represents mean ± SD from three wells from one experiment representative of three. (B) Neutrophils from WT and CGD mice (3,000 cells) were plated into the EZ-TAXIScan device and exposed to a shallow chemoattractant gradient generated by addition of 1 μL LTB4 (100 nM). Cell tracks of migrating WT (Left) and CGD (Right) neutrophils (cells that move ≥65 μm from the bottom of the channel; n = 20) were traced from captured images, realigned such that all cells started from the same starting point (0,0) and plotted. Chemoattractant concentration increases in the positive y direction. (C) Representative images of WT (Left) and CGD migrating neutrophils (Right) are also shown; white arrowheads specify pseudopodia. (D) Migrating neutrophils were evaluated (n = 20 cells; *P < 0.05 vs. WT neutrophils) for directionality, upward directionality, percentage of neutrophils displaying multiple pseudopodia, and migration speed as described earlier. (E) Transwell migration of CGD mice neutrophils. WT or CGD murine neutrophils were allowed to migrate in response to the indicated concentration of LTB4. Percentage of cells that migrated into the bottom well was recorded. Data shown are means ± SD from three wells from one experiment representative of three. (*P < 0.05 vs. WT neutrophils) (F–H) Knocking down p22phox via siRNA results in impaired cell migration and chemotaxis. HL60 cells were differentiated with 1.75% DMSO for 1 d, transfected with 1 μM control siRNA or p22phox siRNA, and further differentiated until d 5 or 6. (F) Knockdown of p22phox in dHL60 cells. At d 5 (Left) or d 6 (Right), dHL60 cells were lysed and probed with p22phox antibody to evaluate knockdown and GAPDH antibody to evaluate loading. (G) Decreased ROS production in p22phox knockdown dHL60 cells. ROS production in control siRNA or p22phox siRNA transfected dHL60 cells (2 × 10^5, 5 d of differentiation) after stimulation with 10 nM Csa was evaluated by monitoring chemiluminescence (for 1 s) every 30 s for 300 s, in the presence of 50 μM luminol and 0.8 U HRP in a luminometer at 37 °C. (H) Control siRNA or p22phox siRNA transfected dHL60 cells (d 5) were exposed to a chemoattractant gradient generated by addition of 25 nM or 100 nM C5a (1 μL) in the EZ-TAXIScan device and imaged every 0.5 min for 20 min. Cell tracks of migrating dHL60 cells (n = 15) were traced from the captured images, realigned to start from the same point (0,0), and plotted (Left). Migration paths of the dHL60 cells were evaluated for a 0- to 20-min time frame (n > 15 cells, *P < 0.005 vs. control siRNA dHL60 cells) for directionality, upward directionality, and migration speed as described in Experimental Procedures (Right).
issue, as we checked several chemoattractant concentrations and the effect of DPI treatment on IL-8–induced chemotaxis is always weaker (Fig. S4). This effect might be caused by relatively lower ROS production in IL-8–treated cells. Chemical inhibitors often have multiple targets. DPI may also inhibit other flavin-containing enzymes. To ensure that the DPI-induced neutrophil chemotaxis defect was indeed caused by inhibition of NADPH oxidase, we examined another NADPH oxidase inhibitor, sinomenine (15). Similar to DPI, sinomenine induced multiple pseudopodia in chemotaxing neutrophils and significantly reduced chemotaxis efficiency. Interestingly, two of the compounds identified from the initial screening, EHNA hydrochloride and LE135, previously known as adenosine deaminase inhibitor and retinoic acid antagonist, respectively, also drastically suppressed chemoattractant-elicited NADPH oxidase activation, again indicating the involvement of ROS in chemotactic migration (Fig. 1D and Fig. S5). Further supporting this hypothesis is the observation that DPI-induced chemotaxis defects were partially rescued by including H₂O₂ in chemotaxis buffer (Fig. 1E).

**NADPH Oxidase Is Required for Efficient Neutrophil Chemotaxis.** Although DPI is a well known and commonly used NADPH oxidase inhibitor, its effect on cell migration may be mediated by other undefined mechanisms. To definitely prove that the DPI-induced neutrophil chemotaxis defect is at least partially caused by inhibition of NADPH oxidase, we next explored neutrophil chemotaxis using a CGD mouse in which gp91 subunit of NADPH oxidase holoenzyme was deleted and thus chemoattractant-elicited superoxide production was completely abolished (Fig. 2A). Similar to chemical inhibition, neutrophils isolated from these mice displayed multiple pseudopods (Fig. 2C) and reduced chemotaxis efficiency (Fig. 2B and D). Consistently, neutrophils isolated from the CGD mice also displayed a migration defect in the transwell assay compared with WT neutrophils (Fig. 2E). Finally, we investigated the chemotaxis of neutrophil-like differential HL60 cells in which the p22phox subunit of NADPH oxidase was knocked down by a specific siRNA, and essentially the same results were observed (Fig. 2F–H). Collectively, all these results suggest that signal-induced NADPH oxidase-mediated ROS production plays an essential role in regulating neutrophil chemotaxis.

**Neutrophils Isolated from CGD Patients Also Show Severe Chemotaxis Defect.** To further explore the physiological and clinical significance of the regulation of neutrophil migration by ROS, we examined chemotaxis behaviors of neutrophils isolated from a CGD patient that contain mutated alleles of the gene encoding gp91phox. As expected, neutrophils from the CGD patient displayed impaired chemoattractant-elicited ROS production in comparison with neutrophils from a healthy volunteer (Fig. 3A). The ROS peak in these neutrophils is significantly smaller than that in the WT neutrophils. Adhesion-induced ROS production in the absence of chemoattractant was also abolished in the CGD neutrophils. The CGD neutrophils displayed a striking chemotaxis defect, showing lack of directionality, more frequent formation of multiple pseudopodia, and slow migration toward the direction of higher chemoattractant (Fig. 3B). It is noteworthy that the CGD patient in this study was receiving IFN-γ treatment. Nevertheless, it is unlikely that the observed neutrophil chemotaxis defect was a result of this treatment, as IFN-γ–treated WT neutrophils showed normal directionality during chemotaxis. Collectively, these results suggest that the defective neutrophil chemotaxis might be contributive to the compromised bactericidal activity in CGD patients, providing a cellular mechanism for CGD pathogenesis.

**CGD Mice Display Impaired in Vivo Neutrophil Recruitment.** Our in vitro experiments showed that neutrophils depleted of ROS display reduced chemotaxis efficiency. We next investigated whether this defect in chemotaxis will lead to impaired neutrophil recruitment to sites of inflammation in live mice using a murine acute peritoneal inflammation (i.e., peritonitis) model. To compare neutrophil recruitment under exactly the same environment, an adoptive transfer experiment was conducted (Fig. S6). We labeled in vitro purified CGD neutrophils with intracellular fluorescent dyes 5–(and 6–) carboxyfluorescein diacetate succinimidyl esters (CFSE; green) and WT neutrophils with another dye, 5– (and 6–) chloromethyl SNARF-1 acetate (red), or vice versa. The mixed (1:1) population was i.v. injected into a WT recipient mouse 2.5 h after the i.p. thioglycollate (TG) injection. By doing this, variability caused by difference in inflammatory environment in each individual recipient mouse will be eliminated. CGD (green) and WT (red) neutrophils

*Fig. 3. Neutrophils isolated from the CGD patient are defective in ROS production and chemotactic migration. (A) Decreased ROS production in neutrophils from a CGD patient. ROS production in neutrophils (5 x 10⁶) from a CGD patient or a healthy volunteer after addition of HBSS (Top) or 100 nM FMLP (Bottom) was evaluated by monitoring chemiluminescence (for 1 sec) every 20 s for 360 s, in the presence of 50 μM isoluminol and 0.8 U HRP in a luminometer at 37 °C. Data represent mean ± SD from three wells. (B) Neutrophils from the CGD patient or healthy volunteer were exposed to a chemoattractant gradient generated by addition of 100 nM FMLP (1 μM) in the EZ-TAXIScan device and imaged every 0.5 min for 20 min. Cell tracks of migrating neutrophils (n = 20) were traced from the captured images, realigned to start from the same point (0,0), and plotted (Top). Images of chemotaxing neutrophils from the CGD patient or healthy volunteer are shown (Middle). White arrowheads specify pseudopodia in the cells. Neutrophils were evaluated (n = 20 cells,* P < 0.01 vs. WT neutrophils) for directionality, upward directionality, percentage of neutrophils displaying multiple pseudopodia, and migration speed as described earlier (Bottom)."
were identified by their unique fluorescent labels. As we measured neutrophil numbers at 4 h after the TG injection, when neutrophil death is minimal, the ratio of CGD neutrophils to WT neutrophils most likely reflected their relative capability to migrate to the inflamed peritoneal cavity. Consistent with the in vitro results, we detected a much reduced peritoneal recruitment of CGD neutrophils compared with WT neutrophils (Fig. 4A). A similar effect was also detected in a murine air pouch model in which recruitment of adoptively transferred neutrophils to a preformed air pouch was induced by TNF-α (Fig. 4B). These results further support the conclusion that ROS generated by NADPH oxidase are key physiological regulators of actin dynamics in neutrophils.

**Discussion**

In this study, using an unbiased screening approach, we have identified ROSs as essential players for modulating neutrophil chemotaxis. Chemotaxis is a process in which cells sense and move up a gradient of molecules (chemoattractants). It plays a central role in the regulation of host defense and inflammatory reactions by recruiting circulating effector leukocytes, including neutrophils, monocytes, and effector T cells, to the sites of injury or infection. During chemotaxis, chemoattractants elicit a number of changes in neutrophils. These include a localized polymerization of F-actin at the site of cell cortex closest to the chemoattractant source, a morphological change characterized by cell elongation, the formation of new lamellipodia or pseudopods at the leading edge, and the forward protrusion of the leading edge followed by retraction of posterior of the cell. We have found that neutrophils with inhibited ROS production that were isolated from CGD patients or mice or pharmacologically/siRNA–treated to inhibit the NADPH oxidase complex displayed defective migration. These neutrophils formed more frequent multiple pseudopodia and lost their directionality as they migrated up a chemoattractant concentration gradient.

CGD is an inherited disorder characterized by recurrent bouts of infection as well as chronic inflammation with granuloma formation. Consistently, neutrophil recruitment to sites of inflammation is dramatically elevated in the CGD mice (16). This hyperinflammatory phenotype is likely caused by dysfunctional kynurenine pathway of tryptophan catabolism (17) and suppression of ROS-induced deactivation of proinflammatory chemokines such as C5a, fMLP (18), LTB4 (19), and IL8 (20). At the sites of infection, the inability of CGD neutrophils to produce ROS in response to chemoattractant stimulation may contribute to the impaired bactericidal activity of these cells. Our discovery that depletion of signal-elicited ROS production in fact inhibits neutrophil chemotactic migration provides another cellular mechanism for CGD pathogenesis and might lead to development of new therapeutic strategies for this disease.

How is chemotactic migration regulated by ROSs? ROSs have been identified as important second messengers that can regulate intracellular signal transduction under a variety of physiological and pathophysiological conditions. This has been shown to occur predominantly via oxidation of thiols (-SH) on protein cysteine...
residues, resulting in reversible protein posttranslational modifications such as glutathionylation, disulfide bond formation, and sulfenic acid formation. Many of these modifications control signal transduction by altering functionality/activity of the protein involved. Redox regulation of numerous proteins such as Ras, protein tyrosine kinases (Src kinases), and protein tyrosine phosphatases has been reported and are known to alter protein functions. PTEN has also been identified as a target of ROSs (21–23). ROSs can also directly activate actin polymerization via modifying G-actin monomers (21–23). In addition, the NADPH oxidase is also essential for chemoattractant-elicted depolarization of membrane potential and can regulate Ca2+ and K+ homeostasis in neutrophils (24). This may contribute to directional sensing in an indirect way. The exact mechanism by which ROS regulate neutrophil chemotaxis needs to be further investigated. During chemotaxis, many signaling events take place locally within the cell. For example, Rac-related signaling and actin polymerization are detected at the leading edge of chemotaxing cells, whereas RhoA activation and contractile actin–myosin complexes appear only at the back of the cells. Interestingly, we found NADPH oxidase was highly enriched near the leading edge of migrating neutrophils (Fig. S7). This specific localization is essential for chemotaxis needs to be further investigated.

Experimental Procedures

Neutrophil Purification and Functional Assays. Human blood neutrophil purification, murine bone marrow neutrophil purification, quantification of F-actin levels by phalloloid labeling, measurement of superoxide production by luminol chemiluminescence, micropipette chemotaxis, and transwell migration assays were performed as described previously (25–27). Peripheral blood was obtained from a human CGD patient and healthy volunteer, with informed consent. EZ-TAXIscan chemotaxis assay, analysis of cell tracks and morphology, siRNA knockdown, and other related assays are described in SI Materials and Methods.

Recruitment of Adoptively Transferred Neutrophils in TG-Induced Peritonitis. Peritonitis was induced as previously described (26). Neutrophils isolated from WT and CGD mice were labeled with intracellular fluorescent dye CFSE (final concentration, 1 μM; Molecular Probes) or 5- (and 6-) chloromethyl SNARF-1 acetate (final concentration, 1 μM; Molecular Probes) at 37 °C for 10 min. Labeled cells were mixed as indicated in Fig. S6 then injected i.v. (via tail vein) into WT mice that have been challenged with 1 mL 3% TG for 2.5 h. Mice were euthanized by CO2 inhalation 1.5 h after the injection of cell mixture (4 h after TG injection) and peritoneal exudate cells were recovered by peritoneal lavage with 10 mL of ice-cold PBS solution containing 5 mM EDTA. The amount of adoptively transferred neutrophils recruited to the peritoneal cavity was analyzed using a BD FACSCanto II flow cytometer (Becton Dickinson) and BD FACSDiva software. Relative recruitment of neutrophil was calculated as the ratio of indicated populations in the peritoneal cavity.

Recruitment of Adoptively Transferred Neutrophils in a Murine Dorsal Air Pouch Model. A dorsal air pouch was created by injecting mice with 5 mL of air s.c. on the back at d 0. On 3 and 5, the pouches were reinflated with 2 mL of air. At 6 d after the initial air injection, TNF-α (in 0.5 mL sterile 0.9% saline solution) was directly injected into the pouch. Four hours after TNF-α injection, mice were anesthetized, and the pouch was flushed with 2 mL saline solution. The relative recruitment of WT and CGD neutrophils was calculated as described earlier.

Statistics. Analysis of statistical significance for indicated data sets was performed using the Student t test capability on Microsoft Excel.

ACKNOWLEDGMENTS. The authors thank Leslie Silberstein, John Manis, Li Cai, and Narayanawamy Ramesh for helpful discussions; and Dan Stevens from Hirata Corp for assistance with the EZ-TAXIscan device. B.S. was supported by National Institutes of Health (NIH) training Grant HL06987. H. L. was supported by NIH Grants HL085100, A076471, HL092020, and GM076084, and a Research Scholar Grant from the American Cancer Society.


Supporting Information

Hattori et al. 10.1073/pnas.0914351107

SI Methods

Analysis of Cell Tracks and Morphology. (x,y) coordinates of migrating neutrophils (i.e., neutrophils that cross >65 μm from the starting line) were tracked from sequential images using DIAS imaging software (Solltech). Cell tracks were then realigned such that all of the cells started from the same starting point (0,0) and plotted using Matlab. Migration parameters are described in detail in Fig. S3. Directionality and upward directionality were calculated as the straight-line migration distance from the origin divided by total migration length and straight-line distance migrated in the upward direction divided by total migration length (Fig. S3). Migration speed (in μm/min) was calculated as average of cell speeds (migration distance between the current frame and the previous frame divided by the time between sequential frames, 0.5 min) at each captured frame. All parameters were calculated only for migrating cells in the 5 min to 15 min time frame of each movie. Percentage of neutrophils with multiple pseudopodia during the course of the 20-min video was evaluated from images of migrating cells. Fisher exact test (2 × 2) was performed to evaluate significant difference from untreated or WT neutrophils.

EZ-TAXIScan Chemotaxis Assay. The EZ-TAXIScan chamber (Effector Cell Institute) was assembled with a 260-μm-wide × 4-μm-thick silicon chip on a 2-mm untreated glass base, as described by the manufacturer and filled with RPMI/0.1% BSA. Inhibitor-treated (or carrier-treated) neutrophils (1 μL, 3 × 10⁶/mL) were added to the lower reservoir of each of the six channels and allowed to line up by removing 18 μL of buffer from the upper reservoir. RPMI/0.1% BSA (15 μL with the appropriate pharmacological inhibitor) was then added to fill both reservoirs to the brim. One microliter of chemoattractant (fMLP, C5a, or LTB4) was then added to the upper reservoir and neutrophil migration (at 37 °C) in each of the channels was captured sequentially every 30 s for 20 min using a 10× lens on a Discovery Screening System (Universal Imaging). Pharmacological inhibitors (from Toecris Bioscience library) along with 200 nM of pan-PI3K inhibitor wortmannin (used to reduce random migration) were added directly to human neutrophils (100 μL, 3 × 10⁶/mL) in RPMI/0.1% BSA and incubated in a 37 °C, 5% CO₂ chamber for 30 min before the chemotaxis assay.

CGD Mice. X-linked CGD mice (1) that contain disrupted alleles of the gene encoding gp91phox (B6.129S6-Cybbtm1Din/J; strain, C57BL/6) were purchased from Jackson Laboratories. In all of the experiments performed with the CDG mice, we used C57BL/6 mice of the same age as WT controls. All procedures involving mice were approved and monitored by the Animal Care and Use Committee of Children’s Hospital Boston.

Knockdown of p22phox. A predesigned duplexed Stealth siRNA (Invitrogen) that targets p22phox (CYBA gene; accession no., NM_000101.2) was used to knock down p22phox in dHL60 cells (sense sequence, ACU AUG UUC GGG CCG UCC UGC AUC U; antisense sequence, AGA UGC AGG ACG GCC CGA ACA UAG U). The negative control used was an ON-TARGETplus nontargeting siRNA 2 (Dharmacon). For gene silencing HL60 cells were differentiated for 1 d with 1.75% DMSO. On d 1, 2 × 10⁶ cells were resuspended in 100 μL Cell Line V nucleofector solution, mixed with 1 μM siRNA (negative control or p22phox), and nucleofected using the T-019 program as per manufacturer instructions. Cells were then cultured in 2 mL IMDM plus 20% FBS plus 1.75% DMSO for 4 to 5 d, and then harvested for Western blotting, ROS production, and chemotaxis assays. Lysates of differentiated HL60 cells were probed for p22phox expression using an anti-rabbit p22phox antibody (Santa Cruz Biotechnology) and loading was evaluated using a GAPDH antibody (Encor Biotechnology). ROS production assays and EZ-TAXIScan chemotaxis assays were performed as described earlier.

Fig. S1. Screening for pharmacological agents that modulate actin dynamics during neutrophil chemotaxis. (A) Cross-sectional schematic view of one of the six channels in EZ-TAXIScan device. The apparatus consists of a six-channel silicon chip (with two holes for each channel) pressed on top of a glass surface using a rubber gasket and metal holder. Cells (1 μL) are loaded to the bottom of one reservoir and chemoattractant (1 μL) is added to the other reservoir. This sets up a chemoattractant gradient between the reservoirs, driving cells to migrate toward the chemoattractant reservoir through a narrow gap (4 μm height, 260 μm length) between the silicon chip and glass surface. Images of migrating cells in each of the six channels are captured in parallel using a 10× lens coupled to a CCD camera. (B) Chemotaxis of human neutrophils in response to chemoattractant fMLP. Human neutrophils (3,000 cells) were plated into the neutrophil reservoir (bottom well) of the EZ-TAXIScan device and exposed to no chemoattractant (Left) or to a chemoattractant gradient (Right) generated by addition of 100 nM fMLP (1 μL) into the chemoattractant reservoir (top well). Images of neutrophils are shown at 0 min, 10 min, and 20 min after chemoattractant addition.
Fig. S2. Biologically active pharmacological agents that decrease chemotaxis efficiency of human neutrophils. Neutrophils were incubated at 37 °C for 30 min without any treatment or with the following pharmacological agents: 2,4-diamino-6-hydroxypyrimidine (DAHP; 300 μM), GF 109203X (58 μM), dibutyryl-cAMP (1 mM), SKF 96365 hydrochloride (56 μM), nocodazole (16 μM), vinblastine sulfate (1.78 μM), colchicine (10 μM), D-6413 (0.74 μM), EHNA hydrochloride, 2-methoxyestradiol (19 μM), and LE 135 (14 μM). They were then exposed to a chemoattractant gradient generated by addition of 100 nM fMLP (1 μL) in the EZ-TAXIscan device and imaged every 0.5 min for 20 min. (A) Cell tracks of untreated and drug treated migrating neutrophils (cells that move ≥65 μm from the bottom of the channel; n = 20) were traced from the captured images, realigned such that all cells started from the same starting point (0,0), and plotted. Chemoattractant concentration increases in the positive y direction. (B and C) Cell tracks of migrating neutrophils in the 5 min to 15 min time frame were analyzed as described in Experimental Procedures to determine directionality (0–1) (B), defined as straight-line migration distance from the origin divided by the total migration length; and upward directionality (−1 to 1) (C), defined as straight-line distance migrated in the upward direction divided by total migration length (Fig. S3). Data in B and C are represented as mean ± SD for 20 cells. (*P < 0.05 versus untreated neutrophils.)
Fig. S3. Chemotaxis parameters for neutrophil migration in response to a chemoattractant gradient. If \( n \) is the number of successive frames analyzed and \((x_n, y_n, t_n)\) denotes the position of the neutrophil \((x_n, y_n)\) at any time \( t_n \), the chemotaxis parameters can be calculated as follows: directionality \((0-1)\) is \( \frac{d_S}{d_1 + d_2 + \ldots + d_f} \), speed \((\mu \text{m/min})\) is \( \frac{d_1}{t_1 - t_0} + \frac{d_2}{t_2 - t_1} + \ldots + \frac{d_f}{t_f - t_3} \), and upward directionality \((-1\) to \(1)\) is \( \frac{d_U}{d_1 + d_2 + \ldots + d_f} \). Here, “f” denotes the final position of the cell, “0” denotes the initial position, \( d_n \) is the distance migrated between two successive frames \((x_n, y_n)\) and \((x_{n-1}, y_{n-1})\), \( d_S \) is the straight-line migration distance, i.e., distance between \((x_0, y_0)\) and \((x_f, y_f)\), and \( d_U \) is the straight-line migration distance in the direction of the chemoattractant gradient (upward), i.e., distance between \((x_0, y_0)\) and \((x_0, y_f)\), \( y_f - y_0 \).

Fig. S4. Sensitivity to chemoattractant: Quantification of ruffling response and pseudopod formation. Murine neutrophils \((0.5 \times 10^4 \text{ cells in } 50 \mu\text{L})\) were pretreated with 30 \( \mu\text{M} \) DPI or not pretreated and then stimulated with 50 \( \mu\text{L} \) of 2x concentrated fMLP (or not stimulated) for 4 min. Cells were then fixed with 100 \( \mu\text{L} \) 4% formaldehyde in PBS solution, plated onto coverslips, and imaged. Percentage of neutrophils with ruffles or extended pseudopods were quantified from the images (1). More than 100 neutrophils were evaluated for each data point. Data are mean ± SD from three stimulations, from one experiment representative of three. (\( P > 0.05 \) vs. untreated.)

**Fig. S5.** Chemoattractant induced ROS production is suppressed by specific pharmacological inhibitors (raw data for Fig. 1D in the main text). Human blood neutrophils ($5 \times 10^5$) were left untreated or treated with 40 μM EHNA hydrochloride (A), 14 μM LE 135 (B), 50 μM DPI (C), or 10 μM sinomenine (D) for 30 min at 37 °C. Cells were then stimulated with 100 nM fMLP, and ROS production was monitored in the presence of 50 μM luminol and 0.8 U of HRP in a luminometer at 37 °C. Chemiluminescence (in arbitrary light units) was recorded (for 1 sec) at indicated time points. Data are mean ± SD ($n = 3$) from one experiment representative of three. Assays for A–D were conducted on different days with blood neutrophils from different patients.
Fig. S6. Illustration of strategy used for adoptive transfer experiments. Bone marrow neutrophils from WT and CGD mice were labeled with different colors (SNARF1- or CFSE-labeled), mixed 1:1 and then i.v. injected into WT recipient mice that have been challenged with TG (intraperitoneally injected) or TNF-α (injected into an artificially generated dorsal air pouch). After 90 min, cells were collected from the peritoneal cavity or air pouch. The relative recruitment of CGD and WT neutrophils in the WT recipient was evaluated by FACS analysis.

Fig. S7. NADPH oxidase is enriched near the leading edge of chemotaxing neutrophils. Purified human neutrophils were washed once with HBSS with calcium and magnesium, and were plated onto glass coverslips coated with 10 μg/ml fibronectin for 10 min at 37 °C. Neutrophils were then stimulated with 100 nM fMLP for indicated time, fixed with 6% paraformaldehyde/PBS solution for 5 min at room temperature, and washed three times with PBS solution. Neutrophils were then permeabilized with 0.2% Triton-X in PBS solution for 10 min at room temperature and then preblocked with PBS solution/1% BSA blocking buffer for 15 min at room temperature. Primary antibody (1:1,000; rabbit anti-p22 subunit of NADPH oxidase) was then added to the fixed cells in the blocking buffer for 1 h, washed three times with PBS solution, followed by incubation with secondary antibodies (1:1,000; Alexa 488-conjugated anti-rabbit IgG) for 30 min and three washes with PBS solution. The distribution of p22 was imaged in the FITC channel.
Table S1. Summary of the primary screening

<table>
<thead>
<tr>
<th>Drug effect</th>
<th>No. of compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>No effect</td>
<td>303</td>
</tr>
<tr>
<td>Cell death (lysed cells)</td>
<td>15</td>
</tr>
<tr>
<td>Complete inhibition of polarization &amp; migration (intact but round cells)</td>
<td>18</td>
</tr>
<tr>
<td>Slow and/or “long-tailed” migration</td>
<td>38</td>
</tr>
<tr>
<td>Directionality defect and/or Multiple pseudopods</td>
<td>12</td>
</tr>
<tr>
<td>Total number</td>
<td>386</td>
</tr>
</tbody>
</table>

Other Supporting Information Files

- Dataset S1 (XLS)
- Dataset S2 (XLS)
- Dataset S3 (XLS)