Neutrophil chemotaxis is a critical component in innate immunity. Recently, using a small-molecule functional screening, we identified NADPH oxidase-dependent Reactive Oxygen Species (ROS) as key regulators of neutrophil chemotactic migration. Neutrophils depleted of ROS form more frequent multiple pseudopodia and lost their directionality as they migrate up a chemoattractant concentration gradient. Here, we further studied the role of ROS in neutrophil chemotaxis and found that multiple pseudopodia formation induced by NADPH inhibitor diphenyleneiodonium chloride (DPI) was more prominent in relatively shallow chemoattractant gradient. It was reported that, in shallow chemoattractant gradients, new pseudopods are usually generated when existing ones bifurcate. Directional sensing is mediated by maintaining the most accurate existing pseudopod, and destroying pseudopods facing the wrong direction by actin depolymerization. We propose that NADPH-mediated ROS production may be critical for disruption of misoriented pseudopods in chemotaxing neutrophils. Thus, inhibition of ROS production will lead to formation of multiple pseudopodia.

Chemoattractants elicit a number of changes in neutrophils. These include 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 found that neutrophils with inhibited ROS production, that were isolated from CGD patients/mice or pharmacologically/siRNA treated to inhibit the NADPH oxidase complex, formed more frequent multiple pseudopodia and...
lost their directionality as they migrated up a chemoattractant gradient. Interestingly, the most dramatic multiple pseudopodia formation induced by NADPH inhibitor diphenyleneiodonium chloride (DPI) was observed in the middle part of the device, where the chemoattractant gradient was relatively shallow (Fig. 1A). It is noteworthy that ROS does not appear to be involved in directional sensing per se, since most neutrophils depleted of ROS can still migrate up the chemoattractant gradient. At the lower section of the channel, where the chemoattractant gradient was relatively steep, the DPI-induced multiple pseudopodia formation was less prominent (Fig. 1B). This result suggested that the dependent on ROS in neutrophil chemotaxis may rely on the feature of the gradient.

To further confirm that ROS is dispensable for neutrophil chemotaxis in steep gradient of chemoattractant, we generated a gradient using a micropipette. In this setup, a micropipette (Eppendorf Femtotip with an opening of 0.5 µm) filled with 10 µM fMLP was lowered onto a cover slip plated with neutrophils. Chemoattractant gradient was formed by continuous passive diffusion from the tip of the micropipette. It was well documented that the chemoattractant gradient generated by this device is steepest near the source (Fig. 2A–C). We examined chemotactic behavior of cells within a 50 µm radius. We observed stable formation of single pseudopodia in both untreated and DPI-treated neutrophils, again suggesting that multiple pseudopod formation induced by ROS depletion was less prominent in relatively steep chemoattractant gradient.

Based on these results, we propose that ROS may only be involved in regulating pseudopod formation in neutrophils exposed to shallow chemoattractant gradient. It was recently reported that, in shallow chemoattractant gradients, new pseudopods are usually generated when existing ones bifurcate. The location and direction of pseudopod formation are thought to be random and are not oriented by chemoattractants. Directional sensing is mediated by maintaining the most accurate existing pseudopod, and destroying pseudopods facing the wrong direction by actin depolymerization. NADPH mediated ROS production may be critical for disruption of misoriented pseudopods in chemotaxing neutrophils. Thus, inhibition of ROS production will lead to formation of multiple pseudopodia (Fig. 3).

The mechanism by which ROS regulates pseudopod formation remains elusive. ROS can oxidize thiol (-SH) on protein cysteine residues, leading to reversible protein post-translational modifications such as glutathionylation, disulfide bond formation and sulfenic acid formation. Redox regulation of numerous signaling proteins such as Ras, protein tyrosine kinases (Src kinases), and protein tyrosine phosphatases, have been reported. These modifications often alter functionality/activity of the targeted proteins. ROS can also regulate actin polymerization via modifying G-actin monomers. Thus, they may directly affect actin polymerization/dem polymerization in chemotaxing neutrophils.

**References**

Figure 1. Multiple pseudopodia formation induced by NADPH inhibitor diphenyleneiodonium chloride (DPI) was more prominent in relatively shallow chemoattractant gradient. (A) Relatively shallow chemoattractant gradient was generated in the upper section in the EZ-Taxiscan device. (B) Dependence on ROS in neutrophil chemotaxis relies on the feature of the gradient. Neutrophils were treated with 50 µM DPI for 30 min and chemotaxis was induced by 100 nM fMLP. Neutrophil purification, EZ-taxiscan chemotaxis assay, and analysis of cell tracks and morphology were conducted as previously described. Percentage of cells that display multiple pseudopodia (n = 20 cells, Fisher’s 2 x 2 test, *p < 0.05 versus untreated) during the course of the EZ-taxiscan chemotaxis assay was quantified as described by Hattori et al.¹⁴
Figure 2. Comparison of various chemotaxis assays. (A) Needle Assay. Chemoattractant gradient was formed by continuous passive diffusion of chemoattractant from a micropipette tip. Equations describe the concentration gradient $C(r,t)$ generated in the radial direction (neglecting convection). $D$ denotes the diffusion constant for the chemoattractant (cm$^2$/sec), $q$ denotes the rate at which the chemoattractant is released (mols/sec), $r$ is the radius from the needle tip (cm). (B) EZ-taxiscan chemotaxis device. Gradient is set up by addition of 1 µl chemoattractant to the chemoattractant reservoir, and allowing diffusion towards the cell reservoir. A linear gradient is setup across a 260 µm channel within the time scale of the experiment (30 mins) (as per manufacturer’s description). (C) Comparison of gradients generated by Needle Assay and EZ-taxiscan device. Percentage change in chemoattractant concentration across 10 µm sections are plotted for the steady state gradient in the needle assay ($C \sim 1/r$) and a linear gradient in a EZ-taxiscan device. The chemoattractant gradient is most shallow near the source for the EZ-taxiscan assay and steepest near the source for the needle assay.
Figure 3. Neutrophil chemotaxis in shallow chemoattractant gradient. In shallow chemoattractant gradients, new pseudopods are usually generated when existing ones bifurcate. Their location and direction are random and are not oriented by chemoattractants. Directional sensing is mediated by maintaining the most accurate existing pseudopod, while the ones facing wrong direction need to be quickly destroyed via actin depolymerization. Inhibition of actin depolymerization in misoriented pseudopods should lead to multiple pseudopod formation and reduced chemotaxis efficiency.