Requirement of Myeloperoxidase

for Neutrophil Spatial Sensing

BY

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THESIS

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LIST OF ABBREVIATIONS

MPO	Myeloperoxidase
PMN	Polymorphonuclear Leukocytes
FPR	Formyl Peptide Receptor
ROS	Reactive Oxygen Species
NFAT	Nuclear Factor of Activated T-Cells
Calm	Calmodulin
NF-KappaB	Nuclear Factor-KappaB
I-KappaB	Inhibitor of Kappa Light Chain Gene Enhancer in B-Cells
MAPKs	Mitogen Activated Protein Kinase
ERK1/2	Extracellular Signal-Regulated Kinase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
HL-60	Human promyelocytic leukemia cells
GPCR	G protein coupled receptors
FPR1	Formyl peptide receptor 1
FPR2	Formyl peptide receptor 2
FPR3	Formyl peptide receptor 3
mFpr1	Mouse Formyl peptide receptor 1
mFpr2	Mouse Formyl peptide receptor 2
mFpr-rs1	Mouse Formyl peptide related sequence 1
mFpr-rs3	Mouse Formyl peptide related sequence 3
mFpr-rs4	Mouse Formyl peptide related sequence 4
mFpr-rs5	Mouse Formyl peptide related sequence 5

LIST OF ABBREVIATIONS (continued)

mFpr-rs6	Mouse Formyl peptide related sequence 6
mFpr-rs8	Mouse Formyl peptide related sequence 8
WKYMVm	H-Trp-Lys-Tyr-Met-Val-D-Met-$CONH_2$
fMLP	N-formyl-Met-Leu-Phe
fMIFL	N-formyl-Met-Ile-Phe-Leu
fMIVIL	N-formyl-Met-Ile-Val-Ile-Leu
NLE	N-Formyl-Nle-Leu-Phe-Nle-Tyr-Lys
HPLC	high-pressure liquid chromatography
MS	Mass spectrometry
OPA	o-Phthalaldehyde
E. coli	Escherichia coli
S. aureus	Staphylococcus aureus
L. monocytogenes	Listeria monocytogenes
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
PI3K	Phosphatidylinositide 3-kinases
MAPK	Mitogen-activated protein kinases
PLC	Phospholipase C
DAG	Diacyl-glycerol
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate

LIST OF ABBREVIATIONS (continued)

PKC	Protein kinase C
IP3	Inositol trisphosphate
IP3R	Inositol trisphosphate receptor
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
ERK1/2	Extracellular-signal-regulated kinases 1/2
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
GAP	GTPase activating proteins
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factors
Cdc42	Cell division control protein 42
DOCK2	Dedicator of cytokinesis 2
ATPase	adenosine 5'-triphosphatase
H_20_2	Hydrogen Peroxide
HOCL	Hypochlorous acid
0^{2-}	Superoxide

SUMMARY

Neutrophils are an important factor in host defense. Excessive neutrophil accumulation can lead to deleterious effects on neighboring cells as well as tissue damage. Hence, neutrophil accumulation is tightly regulated and the mechanism is not well known. Understanding mechanisms that contribute to regulation of neutrophil migration and accumulation could lead to novel ways to treat diseases. Directional Sensing is critical during an inflammatory response and is mediated through G protein coupled receptors. These seven-membrane-spanning G-protein coupled receptors are important for detection of the chemoattractant. The ability of a neutrophil to sense a chemoattractant and move in the direction of a chemical cue is termed chemotaxis. This process is not only important for the recruitment of leukocytes to the site of inflammation or infection, but also important during embryogenesis, wound healing, and neuronal patterning (Parent & Devreotes, 1999). Directed migration is maintained via chemotactic factors such as formylated peptides, proteolytic fragments of complement proteins, leukotriene B_4, interleukin 8, RANTES, and chemokines, which evoke various responses from leukocytes (Loitto *et al.*, 2001). The chemotactic factors are initiators not only of leukocyte trafficking but also of the microbiocidal functions of activated leukocytes, such as phagocytosis, degranulation, and oxidative production. Two mechanisms have been proposed to explain directional sensing. The first is temporal sensing, involving sequential measurements of the gradient, and the second, spatial sensing, involving simultaneous measurements. Directional sensing draws on mechanisms related to

intracellular and membrane receptor asymmetry (Loitto *et al.*, 2001). The multistep process of neutrophil chemotaxis is not well defined, but it is our belief that myeloperoxidase plays a critical role in aiding neutrophil chemotaxis. Myeloperoxidase is an enzyme stored in azurophilic granules in the cytoplasm of the neutrophil, and in an activated cell myeloperoxidase is released through degranulation along with other enzymes. Myeloperoxidase's major function is to generate hypochlorous acid and aid in the destruction of foreign particles. In recent years, myeloperoxidase has been shown to attract leukocytes through its electrostatic properties. It also has been shown to aid in the destruction of methionine containing peptides by methionine oxidation. Mice deficient in myeloperoxidase exhibit an obvious defect in migration in the presence of formyl peptides. Our studies demonstrate that MPO is needed at different stages of neutrophil migration. It not only aids in bacterial killing but also is necessary for sustained neutrophil chemotaxis.

LITERATURE REVIEW

1. Cell Migration

Cells have the ability to migrate from one area to another under specific conditions. The phenomenon has raised the question of why cells bother to migrate, how they do so, and what serves as the stirring wheel, engine, wheels, and the GPS system when they decide to go on a road trip. These are questions that have for decades intrigued scientists, who recently have been able to disentangle many of the mechanistic details underlying cell migration. Neutrophils are a subset of migrating cells. They are also key contributors to innate immunity and host defense. The migration mechanism is a vital factor during inflammation and infection. Without migration the cells would never move to the site of infection or inflammation and the body would have no immediate protection from deleterious agents such as pathogens.

2. Innate vs. Adaptive Immunity

The body's ability to fight and clear infectious material depends on innate immunity, and the ability to avoid infection depends on the adaptive immune response. Innate immunity is important in inflammation and is the first line of defense. It recognizes new and foreign material such as bacteria (Zhang *et al.*, 2000). A single bacterium produces multiple offspring, depending on the doubling time.

Therefore, it is critical that the body have a first line of defense in the first couple of hours or day of exposure to protect against infectious material. The innate immune response depends solely on proteins and phagocytic cells to recognize conserved pathogen-associated molecules and label them for destruction. The innate immune response provides an immediate defense and is not specific to a particular pathogen as compared to the adaptive immune response (Zaas & Schwartz, 2005; Grandvaux *et al.*, 2007). Adaptive immune response is not very effective against new pathogens because this response is slow to develop, taking up to a week or so before becoming effective. The adaptive immune response prevents infection by remembering a previous encounter with a specific pathogen (Romani *et al.*, 1996).

3. Inflammation

Inflammation is the biological response to a threat. Inflammation is the body's approach to protecting itself from harmful stimuli by establishing a physical barrier and containing the threat (Kobayashi *et al.*, 2003). It assists in destruction and clearing of pathogens, which all in all leads to repair and healing (Nathan, 2002). The biological response consists of vascular responses, activation, infiltration, migration of leukocytes, and mediator release (Wagner & Roth, 2000). An inflammatory response occurs in the presence of harmful agents. Blood flow increases in the affected area and this extra perfusion aids in increasing the supply of nutrients, fluid, and white blood cells, all of which are necessary for the healing and elimination of the pathogen. The initial inflammatory response is termed acute inflammation and is beneficial to the host, because it wards off the invader (Ward &

Lentsch, 1999), while a prolonged response is termed chronic inflammation and can be deleterious to the host. When the systemic response is prolonged over an extended period, the body starts to harm itself. Then inflammation becomes self-perpetuating and causes uncontrolled inflammation.

Acute inflammation directs resources to the injurious site and can be categorized as a nonspecific response. Acute inflammation eliminates dead tissue, protects against local infections, and allows for the immune system to gain access to the site of injury or attack. Many factors can cause acute inflammation such as thermal injury, mechanical trauma, electrical injury, chemical burns, irradiation injury, and biological factors. The biological factors include fungal, viral, and bacterial infections (Ward & Lentsch, 1999). A principle pertaining to acute inflammation is the increase in blood flow in the small vessels supplying the affected area. The endothelial cells then begin to swell allowing for blood vessel leakiness. Exudates are free to pass through the blood vessel wall, and chemical mediators allow for margination of the neutrophils to the blood vessel walls and transmigration through the blood vessel wall toward the affected area (Hurley & Spector, 1965). During an inflammatory response, the affected area is engulfed with transient acute inflammatory exudates. The components of the exudates are what destroys and eliminates the causative agent. The exudates consist of protein-rich fluid and cells from local blood vessels, which have been carried to the affected area to mediate local defense. The fluid in exudates contains salts and a high concentration of protein and fibrin, and the cellular constituents include neutrophils, macrophages, and lymphocytes. Acute inflammation is controlled by

transitory production of chemical messengers produced by the damaged tissue and the exudates.

The dark side of inflammation is chronic inflammation. When the switch for acute inflammation does not cease, inflammation becomes chronic. It is the inability to switch off acute inflammation that subsequently causes a repeated and prolonged ongoing response. Chronic inflammation is characterized by its persistence and its lack of ability to clear harmful agents in a timely manner.

4. Neutrophil Function

Neutrophils are essential players in the innate immune system. They provide the most assistance during host defense (Nathan, 2006). Neutrophils are also termed PMNs (Polymorphonuclear leukocytes) and are the key leukocytes of the innate immune system in peripheral blood (Simmons *et al.*, 1974). Their counts in the peripheral blood range from 40-70% under normal conditions (Borregaard, 2010). The maturation process for neutrophils takes place in the bone marrow and this process takes approximately 2 weeks. In the first week, PMN precursors undergo five divisions, differentiate into myeloblasts, progress to promyelocytes, and finally reach the neutrophilic myelocytic stage. The latter stage of neutrophil differentiation consists of metamyelocytes, band cells, and segmented cells. During the metamyelocyte stage, tertiary granules and secretory vesicles are formed. The PMN granules are very important during the immune response, because they package the machinery needed for the inflammatory response (Borregaard *et al.*, 1987). There are 3 subsets of PMN granules, categorized as primary, secondary, and

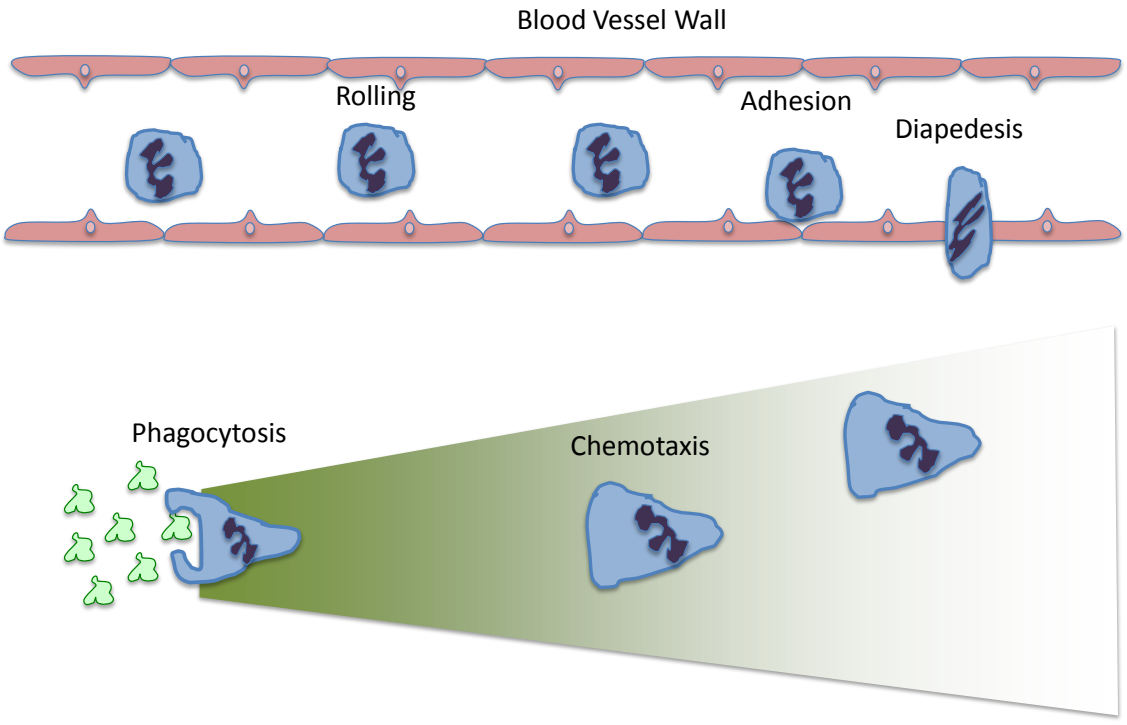


Figure 1. Schematic Illustration of Neutrophil Function. In the inactive state, neutrophils roll along the blood vessel wall, but in the active state, cell surface adhesion molecules such as integrins bind to selectins on the blood vessel wall permitting firm adhesion. The activated neutrophil then enters the tissue by a process termed diapedesis, where it is attracted to invading microbes that are phagocytosed and destroyed

tertiary granules along with secretory vesicles. Approximately 300 proteins are packaged in these granules, which are released during an inflammatory response via a process called degranulation (Borregaard & Cowland, 1997).

Neutrophils are the most abundant of the white blood cells and have a shorter half-life than macrophage phagocytes. The average life span of a neutrophil in the blood stream is approximately 5 days and the life span of the neutrophil once it reaches tissue is 1-2 days (Jamuar & Cronkite, 1980). The average diameter of neutrophils is approximately 8-9 μm (Doerschuk, 2000). Neutrophils are a subtype of white blood cells, which play an important role in the acute inflammatory response. They are a part of the leukocyte family, which includes basophils, eosinophils, lymphocytes, and monocytes (Blumenreich, 1990). In their inactive state, neutrophils roll along blood vessel walls. The release of mediators initiates the activation of neutrophils, which adhere to the blood vessel wall and morphologic change results. The neutrophils shed adhesion molecules such as L- selectin, which is involved in rolling and soft adhesion, and integrins traffic to the cell surface. The endothelial counter receptor, ICAM1 (intercellular adhesion molecule 1) is upregulated on the blood vessel wall in response to inflammatory mediators, and this initiates the binding of integrins to ICAM1. Integrin binding to the blood vessel wall causes firm adhesion and initiates diapadesis of the neutrophil through the blood vessel wall (Jannat *et al.*, 2010). After transmigration, the neutrophil then migrates toward the foreign pathogen and phagocytosis subsequently occurs. Phagocytosis is a process by which the foreign pathogen is engulfed, and a phagosome is formed around the pathogen exposing it to reactive oxygen species as

well as antimicrobial molecules released from granules (Figure 1) (Simon *et al.*, 1995; Brinkmann *et al.*, 2004; Jannat *et al.*, 2010).

5. Formyl Peptide Receptor (FPR)

The Formyl Peptide Receptors (FPR) is primarily expressed in monocytes and neutrophils. They are seven - transmembrane – domain -G -protein coupled receptors and are important to the innate immune function of polymorphonuclear and mononuclear monocytes. Binding of the agonist to the receptor initiates cellular responses, which include cytoskeletal reorganization, superoxide generation, degranulation, and calcium mobilization (Le *et al.*, 2002). In the resting state the receptors are distributed uniformly over the cell membrane of the neutrophil. Once the neutrophil is activated, the cell forms a "leading edge," where the majority of the receptors are redistributed. Once the receptor is occupied, it undergoes desensitization and phosphorylation (Van Epps *et al.*, 1990). The receptor is internalized into the phagosome, which eventually fuses with the lysosome, and hydrolytic enzymes are released to aid in the detachment of the ligand from the receptor as well as to target the ligand for destruction. Subsequently, if the receptor is not targeted for destruction in the lysosome, it is then thought to be recycled and to facilitate the reactivation of the receptor (Van Epps *et al.*, 1990). Experimental studies that exposed the FPR receptor to wheat germ agglutinin, as a treatment to block receptor recycling, inhibited chemotaxis, suggesting that recycling is important for sustained chemotaxis of neutrophils (Perez *et al.*, 1986).

Chemotaxis is the ability of a cell to sense and migrate up a chemotactic gradient (Xu *et al.*, 2003). After receptor-ligand interaction, the receptor can take 3 different paths. The first path of the receptor is internalization into the endosome. From there, the receptor can travel to the lysosome to be degraded or travel to the Golgi apparatus to be processed. Lastly, the ligand can dissociate from the receptor and be recycled to the membrane (Steinman *et al.*, 1983).

In humans, FPR1, FPR2, and FPR3 are members of the FPR receptor family, and human neutrophils express FPR1 and FPR2. FPR3 receptors in humans are expressed in monocytes and macrophages (Ye *et al.*, 2009). Mice FPR receptors are far more complicated; the 8 identified members of this family include Fpr1, Fpr2, Fpr-rsl, Fpr-rs3, Fpr-rs4, Fpr-rs5, Fpr-rs6, and Fpr-rs8 (Gao *et al.*, 1998; Ye *et al.*, 2009). mFpr2 and mFpr-rs1 have strong homology to the human FPR2/ALX and FPR3 (Takano *et al.*, 1997). Some members of the murine Fpr family do not encode functional formyl peptide receptors. For example, Fpr-rs5 has been considered to be a pseudogene and Fpr-rs8 is thought to affect the life expectancy of the cell (Tiffany *et al.*, 2011). mFpr-rs3, 4, 5, and 7 have been shown to be expressed by vomeronasal sensory neurons and function as chemoreceptors, while Fpr1, 2, and rs1 are expressed in murine neutrophils and have a high degree of homology to human FPRs at the protein level (Gao *et al.*, 1999; Liberles *et al.*, 2009; Riviere *et al.*, 2009).

The FPR receptor has been described as a functional receptor due to the ability of N formyl peptides to stimulate chemotaxis as well as initiate lysosomal enzyme release in neutrophils (Schiffmann *et al.*, 1975a; Zigmond, 1977). The Fpr receptor was identified as a chemotactic receptor, and during the 1970's

neutrophils were shown to migrate directionally to a point source of N-formylated peptide derived from *E. coli* (Aswanikumar *et al.*, 1977). The fMLP ligand binds with high affinity to the human FPR receptor activating the receptor in the picomolar and low nanomolar concentration ranges, and has been demonstrated in chemotaxis and calcium mobilization assays (He *et al.*, 2000). Other agonists have also been shown to activate the FPR receptor, as for example, the W-Peptide (WKYMVm) that has been shown to bind to FPR at 1 nM, FPRL1 at 1 pM, FPRL2 at 5 nM, mFPR1 at 50 nM, mFPR2 at 1 nM (Le *et al.*, 1999; Karlsson *et al.*, 2006). Fpr-rs8$^{-/-}$ mice displayed a remarkably shorter life span when compared to wild type (Tiffany *et al.*, 2011). Other studies have demonstrated that Fpr1$^{-/-}$ mice are more susceptible to Listeria monocytogenes when compared to wild type. These mice also displayed impairment in chemotaxis in the presence of fMLP, which indicates that the FPR receptor aids in innate host defense. Fpr-rs2 receptors in mouse neutrophils have been shown to be important for protection against Listeria Monocytogenes. Fpr-rs2$^{-/-}$ mice were challenged with Listeria Monocytogenes and none of the mice survived, while in the same experiment, 50% of the WT mice survived (Liu et al., 2012).

6. Formyl Peptides

A. Escherichia coli Derived Formyl Peptides

The observation of a neutrophil's ability to detect bacteria after invasion of tissue led to the discovery of formyl peptides (Harris, 1954). Formyl peptides were initially defined in the 1970's and were discovered by purification from E. *coli*

cultures. It was suggested that the formyl peptides were derived from E. *coli* during protein synthesis and that the resulting peptides induced neutrophil activity. Early studies demonstrated that the formyl peptides are essential mediators during inflammatory reactions and are potent chemoattractants during neutrophil chemotaxis (Schiffmann *et al.*, 1975b).

The derivatives generated by E. Coli displayed chemotactic activity after their purification using High Performance Liquid Chromatography (HPLC). Marasco *et al*. were able to obtain the amino acid sequence N-formyl methionine-leucine-phenylalanine (fMLP) by performing dipeptidyl carboxypeptidase gas chromatography-mass spectrometry. During the HPLC experiment, 5 distinctive peaks were observed that also contained biological and antigenic activity. Subsequently, the 5 peaks were investigated through a purification procedure using carboxypeptidase to digest the peptides after purification from cultures. Amino acid sequences were identified following carboxypeptidase digestion through o-pthaldialdehyde (OPA) derivatization and reverse phase chromatography. This resulted in these 5 peptide sequences: fMet-Ser-Leu, fMet-Phe-Leu, fMet-Met-Ile-Ala, fMet-Gly-Met-Ile, and fMet-Val,-Phe-Ile-Leu-Leu. fMet-Leu-Phe displayed most of the total activity of the mixture and still remains the prototype for formyl peptides (Marasco *et al.*, 1984).

Schiffman *et al.* were able to demonstrate that N-formyl methionine groups are chemotactic when compared to non-formylated methionine groups. It was demonstrated that di- and tri-peptides containing formyl methionine were stronger attractants than formyl methionine by itself (Schiffmann *et al.*, 1975a). It was also

observed that the formyl methionine peptide was derived from the NH_2 –terminal of newly synthesized bacterial proteins. It was this observation that lead to the hypothesis that N-Formyl peptides could activate neutrophil chemotaxis (Schiffmann *et al.*, 1975a).The results suggested that the formylated methionine peptides derived from the amino terminus of bacterial proteins are vital contributors and mediators that are responsible for the leukocyte response to prokaryotic invasion (Marasco *et al.*, 1984).

B. Staphylococcus Derived Formyl Peptides

Chemotactic peptides were also isolated and purified from *Staphylococcus aureus* cultures. The supernatants from S. a*ureus* cultures were analyzed using reverse phase HPLC. The filtrates made from supernatants of cultures displayed 9 distinct activity peaks, none of which seemed to be identical to the *E. coli* derived peptide fMLP (Rot *et al.*, 1986).The initial chemotactic peptide seemed to display equimolar amounts of methionine, leucine, phenylalanine, and isoleucine. This particular peptide was tested on the human FPR and was compared to the properties of fMLP. This peptide displayed higher efficacy and potency for the human FPR. Subsequently, six additional peptides were synthesized and tested for binding, efficacy, and potency for reactive oxygen species (ROS) generation and chemotaxis. 2 of the 6 peptides were stronger activators of the FPR than the remaining 4 peptides. The 2 peptides were the tetrapeptides fMIFL and fMLFI (Rot *et al.*, 1987). From these studies, Rot et al. concluded that tetrapeptides derived from S. *aureus* cultures are the most biologically active peptides (Rot *et al.*, 1989).

C. *Listeria Monocytogenes* Derived Formyl Peptides

Another bacterial source that has been studied for neutrophil activation is the *L. monocytogenes*. It is known that *L. monocytogenes* can adapt to live in intracellular vacuoles and the cytoplasm (Garifulin & Boyartchuk, 2005). L. *monocytogenes* can move from cell to cell without ever being introduced to the extracellular environment. From this information, it is known that neutrophils do not need to detect a formyl peptide in the extracellular environment. This led to the idea that N-formyl peptides derived from *L. monocytogenes* are used as antigens presented by the MHC, also known as human leukocyte antigens (HLA). MHC is the major histocompatibility complex, which mediates interaction of leukocytes with other leukocytes. To be more specific, they are presented by the class 1b molecule, H2-M3, to CD8+ cytolytic T lymphocytes (Pamer *et al.*, 1992). The supernatant from *L. monocytogenes* cultures was analyzed using a mass spectrometer. The peptide fMIVIL was directly released into the supernatant of cultures and characterized using the mass spectrometer (Gulden *et al.*, 1996).

D. Mitochondria Derived Formyl Peptides

Formyl peptides can also be derived from the mitochondria. It is known that the mitochondria originally evolved from a prokaryotic cell, which eventually adapted to thrive inside a eukaryotic cell (McDonald *et al.*, 2010). Because of its prokaryotic origin, the initial step in mitochondrial protein synthesis in a eukaryotic

cell is similar to protein synthesis in prokaryotic cells, suggesting that the machinery for protein synthesis stayed intact. The mitochondria begin protein synthesis with an N-formyl methionine, which serves as a potent chemoattractant for the FPR on neutrophils. These results were impressive, and this led to the discovery that N-formyl peptides produced in the mitochondria can initiate chemotaxis at a site of sterile inflammation and necrosis (Carp, 1982).

7. Formyl Peptide Receptor Signaling for fMLP

N-formylated peptides derived from bacteria and the mitochondria have been shown to be potent agonists for the FPR. Agonist binding and signaling has been extensively studied and has been shown to activate downstream signaling in the neutrophil via the FPR. In an inactive state, the Gαi and the Gβγ subunits are associated with each other. Once the FPR is activated, the Gβγ subunit dissociates from Gαi. The Gβγ subunit then initiates the activation of heterotrimeric G proteins, small GTPases, Protein Kinase C , PI3K, and MAPKs (Haslam *et al.*, 1993; Li *et al.*, 2003; Southgate *et al.*, 2008).

The fMLP-FPR interaction activates the downstream enzyme PLC (phospholipase C) and phosphoinositide 3 kinase (PI3K). PLC then cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The cleaved product IP3 is then released into the cytosol to bind IP3 receptors

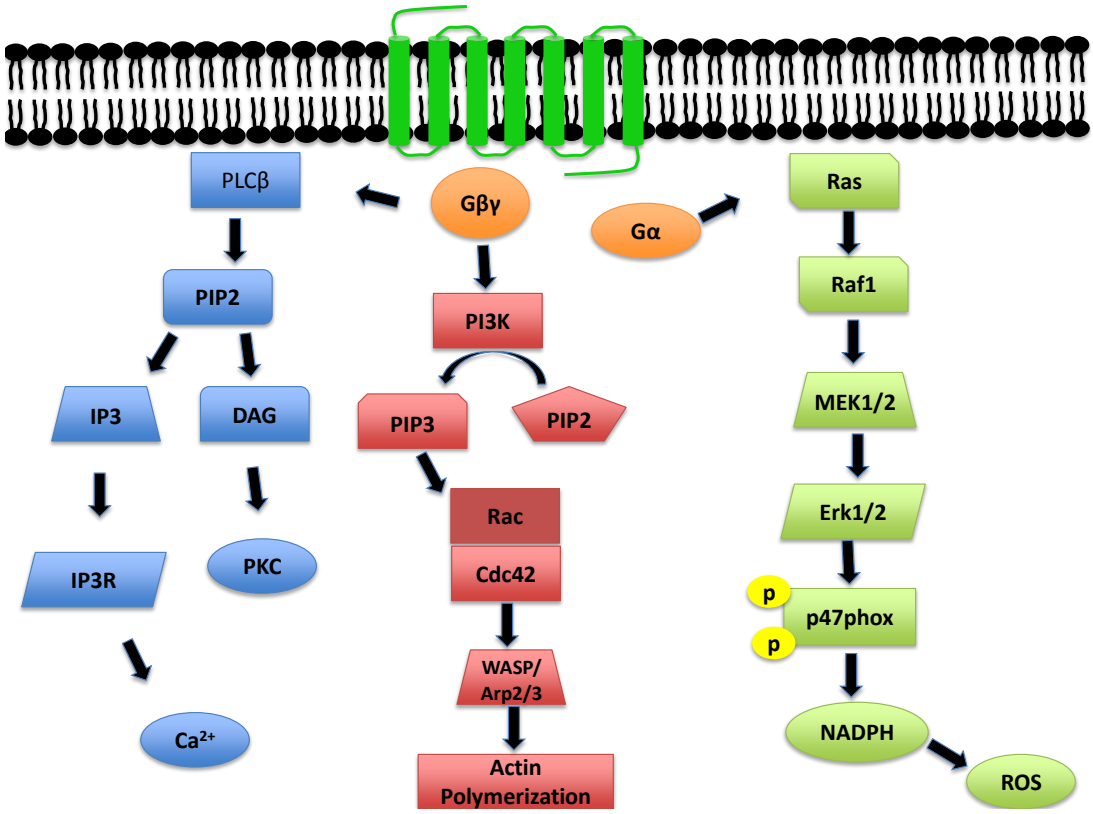


Figure 2. Schematic Representation of the Formyl Peptide Receptor (FPR) in Neutrophils. The FPR is a seven-transmembrane domain receptor. When activated, the Gβγ subunit dissociates from the Gα subunit. This leads to activation of downstream signals. Activation of PLC-Beta leads to the production of the second messengers, DAG and IP3. DAG activates PKC, and IP3 binds to the IP3R, which results in calcium mobilization from intracellular stores. The MAPK cascade is activated as a result of FPR activation. The MAPK cascade leads to Erk1/2 activation, which results in phosphorylation of p47phox . The receptor ligand interaction also activates multiple subunits of NADPH oxidase to produce ROS. The FPR receptor additionally activates Rho GTPase, which induces actin reorganization. PI3K is activated in response to the ligand-receptor interaction. PI3K leads to the production of PIP3, which, in turn, leads to the activation of Rac and Cdc42 resulting in actin polymerization with involvement of WASP and ARP2/3.

(IP3R), resulting in upregulation of intracellular calcium (Bokoch, 1995). The influx of intracellular calcium regulates Calmodulin and Calcineurin (Frohlich *et al.*, 1998). The phosphatase Calcineurin dephosphorylates its substrate protein, transcription factor NFAT, thereby activating its target genes, which include chemokine genes. DAG is compartmentalized in the membrane and works in conjunction with calcium to activate PKC. PKC is then translocated from the cytosol to the plasma membrane as a result of increases in membrane DAG. The kinase PKC phosphorylates IκBα unmasking the nuclear localization signal resulting in posttranslational modification of IκBα, which leads to the activation of NF-kB and the degradation of IκBα (Graham *et al.*, 2007). The MAPK cascade is also activated as a result of activation of the FPR. Activation of MAPK cascades leads to ERK1/2 production, which is probably the most studied pathway in the MAPK system. ERK1/2 phosphorylates p47Phox activating the transcription factor ELK1 and chemokine gene expression. p47phox is an important regulator of NADPH. It is one of the subunits needed for the activation of NADPH oxidase, which produces reactive oxygen species (ROS) (Figure 2)(Zarbock & Ley, 2008; Wong *et al.*, 2010).

8. Two Mechanisms for Detecting a Gradient

It has been suggested that there are two distinct mechanisms by which an organism can detect a gradient. They are termed temporal and spatial sensing. Spatial sensing is characterized by concurrent comparison of the intensity of the activated receptor at different loci on the cell. Temporal sensing is characterized by comparison of the activated receptor at different time points. During spatial sensing

the cells have the ability to detect the gradient while simultaneously directing the organism to move in the correct direction, whereas, in temporal sensing the organism compares the environment surrounding each area it has moved to (Dusenbery, 1998). These sensing mechanisms were originally studied in bacteria and became the foundation for our understanding of the sensory system (Spudich & Koshland, 1975). Evidence identified bacterial receptors as chemoreceptors (Adler, 1969). It was suggested that these receptors sensed the direction of the stimulus and then communicated the information to the flagella (Adler *et al.*, 1973; Aswad & Koshland, 1975). It has been suggested with respect to spatial sensing that it is much easier for a larger organism to detect a gradient , because the receptors are distributed over a larger area of the cell. The receptors on the cells are able to make this comparison because they are further apart, permitting the cell to move at a much faster pace.

Bacteria such as E. *coli* use temporal sensing to decide whether they are moving in the right direction. This phenomenon was tested in neutrophils during the 1970's (Schiffmann *et al.*, 1975a; Schiffmann *et al.*, 1975b). It was suggested that neutrophils use the spatial mechanism in a chemotactic gradient. Unlike bacteria, a neutrophil's receptors do not display asymmetrical morphology, but the majority of them are redistributed to the leading edge of the cell (Serrador *et al.*, 1998). It is believed that neutrophils initiate neutrophil chemotaxis in 3 distinct steps . The first step incorporates the interaction between the ligand and receptor. The second step sends the signal to the motility elements, and the last step is the activation state of the motility elements to produce cell migration (Cicchetti *et al.*, 2002).

9. Neutrophil Chemotaxis

Chemotaxis is defined as directional migration of a cell towards a chemical stimulus in the environment. Neutrophils, which are a type of white blood cell, are the primary leukocytes in the innate immune system. Polymorhponuclear (PMN) leukocytes are also the primary phagocytes. Neutrophils migrate toward a chemical stimulus at rates ranging from 15-30 μm per minute (Schwiebert & Zsembery, 2003; Bokoch, 2005; Abbracchio *et al.*, 2006). They are also able to detect a gradient as shallow as 1% across the length of the cell (Chen *et al.*, 2004). When neutrophils are introduced into a chemoattractant gradient, they produce a bell-shaped dose-response curve. At the peak of the curve is the optimal concentration needed for cell migration (Ye *et al.*, 1992). The general idea behind cell migration is that the receptor senses the external signal and migration proceeds in the direction of the chemical cue. If the receptor becomes saturated or desensitized, then the cell loses responsiveness. Polarization, adhesion, and different signaling pathways also aid in the ability of a neutrophil to migrate (Zigmond, 1980; Stossel, 1994; Janetopoulos & Firtel, 2008). When neutrophils are activated, they polarize forming a distinct front and back. Many studies have characterized actin polymerization as being the reason the cells are able to protrude/extend towards the chemical signal. The small GTPase Rho promotes actin polymerization, which initiates the accumulation of F-actin at the front and contractile actin-myosin at the back (Downey, 1994).

A. Leading Edge

Chemotaxis is initiated when the FPR is activated; the Gβγ subunit then dissociates from the Gαi subunit. The Gβγ subunit mediates downstream signaling

(Rickert *et al.*, 2004) (Parent & Devreotes, 1999). Inhibition of Gβγ impairs neutrophil chemotaxis (Neptune *et al.*, 1999). Studies in D. discoideum showed impairment of directional movement when the Gβγ subunit was genetically deleted. This supported the theory that Gβγ is important for directional sensing. The Gβγ subunit activates downstream signals such as PI3K, heterotrimeric G proteins, small GTPases, PKC, and MAPKs (Haslam *et al.*, 1993; Li *et al.*, 2003). The Gβγ subunit activates PI3K at the leading edge, which has been shown to assist in the progression of actin polymerization. There are 3 classes of PI3K, but class I appears to be the most important for cell adhesion, cytoskeletal rearrangement, cell growth and survival (Stephens *et al.*, 1994; Stephens *et al.*, 1997; Suire *et al.*, 2005). Class I is also thought to be the only class involved in neutrophil migration. Activated PI3K phosphorylates the phospholipids PIP2 and PIP3. The production of PIP3 regulates the signaling pathways that control cell adhesion, growth and survival.

There are 4 isoforms of PI3K, α, β, δ, and γ. PI3Kγ is known to be directly activated by the Gβγ subunit. Experimental studies inhibiting PI3K using Wortmannin and LY294002 demonstrated that degranulation, polarization, and oxidation were affected, but not PLC and the calcium response (Arcaro & Wymann, 1993; Nijhuis *et al.*, 2002). There have been conflicting results about the involvement of PI3K in neutrophil chemotaxis (Niggli & Keller, 1997; Coffer *et al.*, 1998). These reports have demonstrated inhibition of neutrophil chemotaxis when exposed to the inhibitor, while others have shown no effect on chemotaxis. In addition, studies have been done in PI3Kγ deficient mice, which showed a significant reduction in the production of PIP3 (Hirsch *et al.*, 2000; Li *et al.*, 2000). Chemotaxis

assays were also performed in this model, revealing a significant decrease in chemotaxis, but it was not completely abolished, suggesting that PI3K was involved in chemotaxis by mediating adequate organization and orientation of the actin cytoskeleton.

As mentioned above PI3K activates the production of PIP3, which recruits PKD1 and AKT to the plasma membrane. The downstream effectors of PI3K include AKT/PKB, which can be inhibited by treatment with PI3K inhibitors. AKT/PKB production was also studied in PI3K deficient neutrophils. After stimulation, these neutrophils did not activate AKT/PKB (Sasaki *et al.*, 2000). AKT has been used as a marker to determine the localization of PIP3 during neutrophil migration. Transfection studies were carried out in HL-60 cells differentiated into neutrophil like cells. Transfection of PH-AKT-GFP revealed that PIP3 localized at the leading edge. Downstream of PI3K are the guanine nucleotide exchange factors (GEFs) for small guanosine triphosphate (GTP) binding proteins along with the MAPKs.

B. Rho Family of Small GTPase-Binding Proteins

The Rho family of GTPases has been shown to regulate actin polymerization, actin cytoskeleton, and adhesion during neutrophil chemotaxis (Kaibuchi *et al.*, 1999). These small GTP-binding proteins regulate the shift of exchange proteins from an inactive state to an active state and vise versa. These small GTPases are inactive in the guanosine diphosphate (GDP) bound state and active in the GTP-bound state. The switch from GDP to GTP is regulated by GEFs, GTPase-Activating Proteins (GAPs), and guanosine nucleotide dissociation inhibitors (GDIs). GEFs are

responsible for the activation of the Rho GTPases and GAPs are responsible for their inactivation by hydrolyzing the bound GTP. The GDIs act by binding to the GDP-bound form of the small GTPases inhibiting their activation. There are a number of small GTPase-binding proteins in neutrophils, for example, Ras, Rac1, Rac2, Cdc42, and Rho. Some regulators of small GTPase binding proteins include DOCK2, PIX, Vav1/3, Sos, p120-GAP and RhoGDI (Bokoch *et al.*, 1994; Dusi *et al.*, 1996; Zheng *et al.*, 1997; Stephens *et al.*, 2008). Among these, Rac, Cdc42, and Rho have been thoroughly investigated during neutrophil chemotaxis. Studies have shown that in an activated neutrophil Rac and Cdc42 localize to the plasma membrane, where they are then activated by GEFs (Quinn *et al.*, 1993; Philips *et al.*, 1995).

Activation of Rac and Cdc42 differ from that of PI3K in that activation is initiated by the Gαi subunit rather than the Gβγ subunit (Li *et al.*, 2000). Rac and Cdc42 activation were investigated using PI3K inhibitors, which did not completely inhibit activation of the two, indicating that there are PI3K dependent and independent pathways for activating Cdc42 and Rac (Benard *et al.*, 1999). In PI3Kγ deficient mice, Rac2 activity was not altered suggesting there is a PI3Kγ independent pathway for activation of Rac2 (Li *et al.*, 2000). Cdc42 has been thought to regulate actin polymerization by Arp2/3, an actin nucleating protein. Experiments were done in neutrophil lysates to investigate whether activation of Cdc42 induced actin nucleation and F-actin polymerization. These studies demonstrated that activation of Cdc42 did indeed induce actin nucleation and F-actin polymerization (Zigmond *et al.*, 1997; Machesky & Insall, 1998; Machesky & Gould, 1999).

There are 2 isoforms of Rac, Rac1 and Rac2, which have been identified in neutrophils. Rac2 seems to be the more abundant of the 2 and has been extensively studied in neutrophils (Quinn *et al.*, 1993). Experimental studies in Rac2 deficient mice showed inhibition of chemotaxis and actin polymerization after chemotactic stimulation (Roberts *et al.*, 1999). In contrast Rho has been shown to be important for the uropod or the back of the cell during neutrophil migration. It functions to form the uropod and to release it from the substratum.

Experimental studies with C3 exoenzyme to inhibit Rho impaired neutrophil chemotaxis, but initiation of actin polymerization was not altered (Stasia *et al.*, 1991; Ehrengruber *et al.*, 1995; Niggli, 1999; Yoshinaga-Ohara *et al.*, 2002). This inhibition does not affect the protrusion of the cell, but the tails of the neutrophil seem to strongly adhere to the substratum, causing the cell body to become elongated. ROCK and atypical PKCδ isoform have been implicated as downstream effectors of Rho. ROCK inhibition had similar effects to Rho inhibition on neutrophils, suggesting that ROCK mediates and modulates myosin II in the generation of actin-myosin contractility during neutrophil chemotaxis (Kent *et al.*, 1996; Laudanna *et al.*, 1998; Kawaguchi *et al.*, 2000).

As mentioned previously, Rac, Rho, and Cdc42 regulate neutrophil chemotaxis. Rac and Cdc42 regulate chemotaxis by regulating actin polymerization and lamellipodia formation, while Rho regulates the actin-myosin contractile machinery and uropod release from the substratum. The enzyme PAK links the two different pathways. This enzyme inactivates MLCK to induce relaxation at the

uropod. The two pathways work in concert so that protrusion is followed by retraction (Dharmawardhane *et al.*, 1999; Sanders *et al.*, 1999).

C. Mitogen-Activated Protein Kinase (MAPK)

There are a number of MAPK enzymes that regulate motility in neutrophils, the most extensively studied being (ERK1/2), c-Jun amino-terminal kinases 1 to 3 (JNK1 to -3), and p38 (α, β, γ, and δ). The small GTP-binding protein, Ras, is important for the activation of the MAPK cascade and is activated in neutrophils upon N-formyl peptide stimulation by Gi-protein dependent pathways (Worthen *et al.*, 1994; Knall *et al.*, 1996). The fact that Ras can be activated in the presence of inhibitors of tyrosine kinases, PI3K, or PKC, rules out the possibility that these molecules are acting upstream of Ras activation. In other cell systems, Ras functions as an initiator of the mitogen-activated protein kinase (MAPK) cascade, which has been considered to be the same in neutrophils. Inhibition of MEK1/2, the ERK1/2 module, fails to inhibit neutrophil chemotaxis when stimulated with N-formyl peptide, suggesting that this pathway is not essential for the migratory function of neutrophils (Coffer *et al.*, 1998; Zu *et al.*, 1998). In contrast, inhibitors of p38 MAP kinase impair neutrophil chemotaxis to N-formyl peptide (Knall *et al.*, 1997; Zu *et al.*, 1998). The mechanism proposed is that p38MAP kinase activated by N-formyl peptide relieves Hsp27-mediated inhibition of actin polymerization.

10. Degranulation

Neutrophils contain granules that are important in the elimination of foreign particles during host defense (Baggiolini *et al.*, 1985). The granule content is released into the phagosome to aid in microbicidal activity (Chertov *et al.*, 2000). PMN granules are released in a hierarchical order (Pryzwansky & Breton-Gorius, 1985). Secretory vesicles are released immediately upon contact between the PMN and the endothelium. Tertiary granules are mobilized once the PMN transmigrates, and the primary and secondary granules are released in the inflamed site. These proteins are liberated when needed, during PMN to phagocytosis and bacterial killing. These proteins are released into the extracellular space and can functionally affect inflammatory cells in their vicinity (Bentwood & Henson, 1980; Sengelov *et al.*, 1993; Borregaard & Cowland, 1997).

A. Secretory Vesicles

Secretory vesicles include a pool of membrane-associated receptors, which are incorporated into the plasma membrane after the release of vesicles. HBP, FPR, CR1, CD16, CD14, B_2 Integrin's, and the metalloproteinase leukolysin all can be found in the secretory vesicles. Once the receptors are incorporated into the membrane, the phenotype of the PMN changes. Therefore, an inactive PMN rolling along the endothelium is not the same as an active cell, which is capable of interacting with the endothelium, monocytes, and dendritic cells to gain the information needed from the extracellular environment (Tapper *et al.*, 2002).

B. Secondary and Tertiary Granules

The secondary and tertiary granules are also known as specific granules. They have overlapping content but can be differentiated by the difference in their buoyancy when centrifuged in density gradient. It is thought that these granules play an important role in the initiation of the inflammatory response (Nicolaides, 2005). These granules include lactoferrin and matrix proteins along with other substances. When compared to secondary granules, tertiary granules are more easily exocytosed, which indicates the importance of these granules as a reservoir for matrix degrading enzymes, while the secondary granule content contributes mainly to antimicrobial activities (Jesaitis *et al.*, 1990; Kjeldsen *et al.*, 1992; Mollinedo *et al.*, 1997).

C. Primary Granules

Primary Granules are also known as azurophilc granules, and have been characterized by their lysosomal content that is important in the digestion of phagocytosed material (Rice *et al.*, 1987). They are termed primary granules because they are the first granules produced during the development of neutrophils. The contents of azurophilic granules include myeloperoxidase, phospholipase A2, acid hydrolases, elastase, defensins, neutral serine proteases, bactericidal/permeability-increasing protein, lysozyme, cathepsin G, proteinase 3, and proteoglycans. These granules are characterized by their arsenal of molecules needed for antimicrobial activity in the phagosome (Pryzwansky & Breton-Gorius, 1985; Sinha *et al.*, 1987).

11. Phagocytosis

Activated neutrophils are attracted to a particle such as a bacterium via chemical attractants. They migrate to the particle; engulf it, and target it for destruction. This process is initiated through receptor ligand interactions, which leads to internalization, surface remodeling, and lipid metabolism (Niedergang & Chavrier, 2004). The phagosome is formed by endocytosis of the foreign particle (Mukherjee *et al.*, 1997). To engulf the foreign material, the cell has to change its shape. The cell sends out projections called pseudopodia. These projections then make contact with the particle to ingest it. Thereafter, the pseudopodia form around the foreign particle and capture it when the plasma membrane surrounds and isolates the foreign particle from the extracellular environment (Underhill & Ozinsky, 2002). The pathogen is initially internalized and sequestered into a compartment that originated from the plasma membrane called the phagosome. The foreign particle is then compartmentalized and transported to the lysosome where it fuses with the lysosome to target the foreign particle for destruction.

The endosome differs from the phagosome in that it is formed from liquid material or much smaller particles. The endosome is also formed by endocytosis. The endocytosed material is compartmentalized and travels deeper in the cell. This compartment is unable to perform the activity of a mature phagosome and, therefore, must undergo a maturation process. While traveling deeper into the cell, the compartment becomes more acidic and matures into a late endosome. The late

endosome then fuses with the lysosome to target the foreign material for degradation (Mellman, 1992; Rabinowitz *et al.*, 1992; Vieira *et al.*, 2002).

These compartments utilize microtubules and move along them where they then recognize and begin to fuse with endocytic organelles (Blocker *et al.*, 1997; Desjardins & Descoteaux, 1997; Jahraus *et al.*, 1998). The V-ATPase pump is required for acidification of most intracellular compartments such as the endosome, lysosome, and golgi derived vesicles (Grinstein *et al.*, 1992). This pump is thought to be the main source of acidification of the phagosome in leukocytes. The V-ATPase pump contributes to the destruction of the pathogen and also prevents further infection. The V-ATPase along with hydrolytic enzymes and NADPH oxidase contribute to the microbicidal activity of the lysosome (Mellman, 1992; Claus *et al.*, 1998)

12. Respiratory Burst

After membrane sealing, the phagosome does not have any bactericidal activity. It is not until it fuses with the lysosome that harm comes to the foreign particle. The phagosome fused with lysosome has a very low pH and contains hydrolytic enzymes, bactericidal peptides, and can generate superoxide (Vieira *et al.*, 2002). The microbicidal activity depends on the activation of NADPH oxidase (Werner, 2004). NADPH oxidase is able to generate superoxide anion through a Rac dependent pathway (Bokoch & Diebold, 2002). NADPH oxidase produces reactive oxygen species in the phagosome, which contribute to the regulation of signal

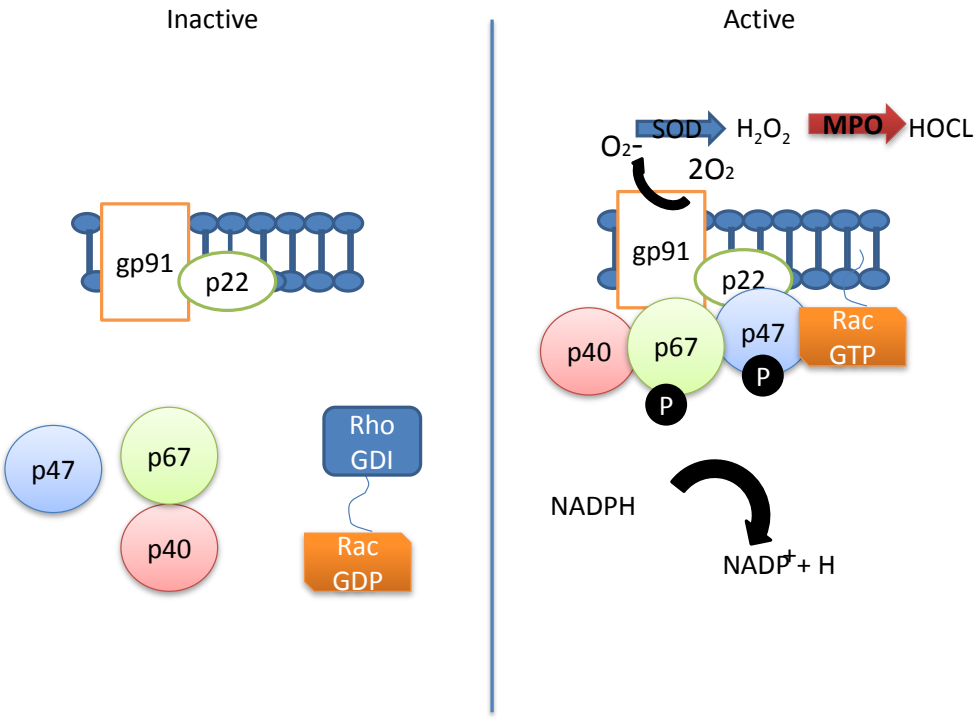


Figure 3. Schematic Representation of the NADPH Oxidase during a "Respiratory Burst." In the inactive state, the NADPH oxidase of the phagocyte consists of two membrane subunits (p22phox and gp91phox) and four cytosolic subunits (p67phox, p47phox, p40phox, and Rac-GTPase). In the active state, p47phox is phosphorylated, and the cytoplasmic subunits are translocated to the cell membrane, where the functional NADPH oxidase enzyme system is assembled from the membrane and cytosolic subunits. Activated NADPH oxidase catalyzes the reduction of molecular oxygen O_2 into O_2^-. Superoxide is dismutated by SOD (superoxide dismutase) to form hydrogen peroxide. MPO then catalyzes the synthesis of HOCl (hypochlorous acid) from hydrogen peroxide and chloride.

transduction, bacterial killing, and oxidative damage. NADPH oxidase is a 5-component complex that includes gp91phox, p22phox, p40phox, p47phox, p67phox, and the inactive form of Rac. In an inactivated neutrophil, gp91phox and p22phox are located on the membrane while p67phox, p47phox, p40phox, and Rac GTPase are found in the cytosol (Dinauer *et al.*, 1990; Sheppard *et al.*, 2005). Once the cell is stimulated, the cytosolic components p47 and p67 become phosphorylated, and nucleotide exchange converting Rac GDP (inactive) to Rac GTP (active) allows for translocation of the cytosolic components to the cell membrane (Heyworth *et al.*, 1994). This complex then produces superoxide by transferring electrons from NADPH across the membrane where the electrons couple with molecular oxygen to form superoxide anions. Superoxide is considered to be a primary reactive oxygen species and is produced in large amounts to kill invading pathogen. Superoxide dismutase then allows for the dismutation of superoxide producing hydrogen peroxide. In the presence of hydrogen peroxide and chloride, MPO generates a cytotoxic reactive oxygen species called hypochlorous acid (Figure3) (Floris & Wever, 1992; Hampton *et al.*, 1998; Furtmuller *et al.*, 2000). Hypochlorous acid can react with other radicals amplifying the effects of reactive oxygen species and ultimately causing tissue damage (Floris & Wever, 1992).

13. Introduction to Myeloperoxidase (MP0)

Agner first described myeloperoxidase approximately 70 years ago. It was first described in patients with tuberculosis empyema: purification revealed an intensely green product, which was initially named verdoperoxidase (Agner, 1947),

because *verida* means green in Spanish. Verdoperoxidase was later changed to myeloperoxidase when it was discovered that expression of this peroxidase is restricted to cells of the myeloid lineage (Agner, 1958; Schultz, 1958). Approximately 30 years thereafter, the functional role of myeloperoxidase was explored by Klebanoff. (Klebanoff, 1968, 1970). He demonstrated that MPO in combination with its substrates, hydrogen peroxide and a halide, constitute a potent antimicrobial system.

MPO belongs to a superfamily of mammalian heme peroxidase enzymes, which include eosinophil peroxidase (EPO) and lactoperoxidase (LPO) (O'Brien, 2000; Furtmuller *et al.*, 2006). MPO is a key enzyme in polymorphonuclear leukocytes (PMN) and monocytes; however, polymorphonuclear leukocytes contain much more of the enzyme than monocytes. MPO was highly purified in the 1940s and was shown to be present in neutrophils at a concentration of 1-2% of their dry weight (Schultz & Kaminker, 1962; Schultz & Shmukler, 1964; Schultz *et al.*, 1965; Floris & Wever, 1992; Hampton *et al.*, 1998). Another study was done and reported MPO concentration to be 5% of the dry weight (Kinkade *et al.*, 1983). MPO has a very distinctive green color and is what gives pus its color. MPO is released from azurophilic granules upon activation of the neutrophil. In conjunction with NADPH oxidase, MPO generates reactive oxygen species within the phagosome (Kinkade *et al.*, 1983).

A. Myeloperoxidase Properties

MPO is released from azurophilic granules and matures in these granules during cell differentiation (DeLeo *et al.*, 1998). In the bone marrow, synthesis of MPO primarily occurs during the promyelocytic stage, which results in an inactive precursor, apopro–MPO. During maturation, apopro–MPO has to go through a process of binding to chaperone proteins such as calrecticulin and calnexin, which allow for the incorporation of heme. Mature MPO is predominately expressed in neutrophils, whereas levels in monocytes are much lower. In humans, MPO levels range from 2-5% of cellular protein, and the main cellular source of this protein is in neutrophils (Klebanoff, 2005). The main function of these short-lived cells is to migrate, ingest, and destroy invading pathogens, such as bacteria, fungi, and protozoa (Nathan, 2006; Dale *et al.*, 2008). The nonspecific neutrophil response usually is accompanied by tissue damage, but they are also recognized for their importance in initiating and shaping the immune response and tissue repair (Nathan, 2006). After migration and ingestion, neutrophils form phagolysosomal compartments and due the phagosome fuses with intracellular granules, one of which contains the MPO protein (Faurschou & Borregaard, 2003). During degranulation of primary, secondary, and tertiary granules, various bactericidal components are released into the phagosome. These granules contain material that aids in the destruction of foreign particles. In conjunction with the acidic pH that is maintained in the granules, this creates a bactericidal environment (Borregaard & Cowland, 1997; Borregaard *et al.*, 2007). At the same time, the neutrophilic NADPH complex is activated and recruited to the phogolysosome as well as to the plasma

membrane. The activation of this complex produces superoxide anions that are used as a substrate for H_2O_2. MPO then uses H_2O_2 as a substrate to produce hypochlorous acid. All of the components previously mentioned are necessary to produce the bactericidal environment to destroy foreign pathogens (Babior *et al.*, 2002; Babior, 2004).

B. Enzymatic Properties of Myeloperoxidase

The binding of heme causes a conformational change in the protein, yielding pro-MPO, which is considered to be enzymatically active. In its native form, the heme center is in the ferric state, but in the presence of H_2O_2, the redox intermediate, compound I, is formed. Compound I readily oxidizes halides, such as bromide, chloride, and iodide. When Compound I reacts with these halides, it is reduced back to its ferric state. Compound I is transformed into Compound II due to oxidation of organic and inorganic substrates (Davies *et al.*, 2008). Compound III can also be formed through reaction with the native enzyme, superoxide or through reactions with Compound I and III by one-electron reduction.

An excess of active MPO can be deleterious to tissue during chronic inflammation. An uncontrollable inflammatory response leads to the release of MPO into the extracellular milieu, which exposes neighboring cells to its toxic effects and oxidizes tissues and proteins. This oxidative stress is due to the overproduction of ROS and impairment of the capacity of the biological system to detoxify the reactive intermediates (Edwards *et al.*, 1987; King *et al.*, 1997). MPO can be inactivated by products of the respiratory burst and can be cleared from the extracellular

environment by macrophages (Edwards *et al.*, 1987; Shepherd & Hoidal, 1990). MPO is very basic and therefore binds to negatively charged surfaces. Not only does MPO contribute to bactericidal activity, but also to enhance association of macrophages with coated cells, to which MPO adheres strongly, once it is released into the extracellular milieu.

C. Properties of Myeloperoxidase in Modulating Immune Responses and Inflammation

The over production of MPO and other peroxidases can lead to tissue damage in many diseases. Evidence suggests that enzymatically active MPO together with 3-chlorotryrosine, a tissue marker for HOCl mediated damage, has been detected in diseased tissues (Witko-Sarsat & Descamps-Latscha, 1994). In addition to its toxic effects, it is also known that MPO can modulate the activity and function of an array of immune cells. *In vitro* in T and B cells, it was shown that the product of the MPO system HOCL can activate nuclear factor–κB and tyrosine phosphorylation, thus leading to increased calcium mobilization, increased production of tumor necrosis factor α, reduction of mitogen–induced proliferation, and a decrease in cytotoxic activity (el-Hag *et al.*, 1986; el-Hag & Clark, 1987; Schoonbroodt *et al.*, 1997; Schieven *et al.*, 2002). This information demonstrates that MPO derived oxidants can modulate immune responses as well as inflammatory reactions.

In recent studies, it has become clear that MPO plays a role beyond its enzymatic activity. Lefkowitz et al. provides evidence that MPO modulates the immune response via activation of macrophages. Exposure of macrophages to MPO

leads to the release of TNF-α and interferon -γ (IFN-γ) in *in vitro* studies. In *in vivo* studies, mice were intravenously injected with MPO resulting in a similar response, namely an increase in the circulating levels of TNF-α and interferon-γ (Lefkowitz *et al.*, 1992; Lefkowitz *et al.*, 1993; Lefkowitz *et al.*, 1999). Furthermore, there is evidence that introduction of MPO to macrophages increases micro- and bactericidal activity in these cells (Lincoln *et al.*, 1995; Lefkowitz *et al.*, 1996).

Other functions of MPO include its role in cell-cell interactions and adhesion of leukocytes. The evidence for this possibility was obtained in dHL-60 cells and peripheral human leukocytes. When these cells were placed on a substratum containing MPO, adhesion increased, and this effect was not blocked by mannan, an inhibitor of peroxidase activity. However, when monoclonal antibodies against CD11b and CD18 were used to inhibit these integrins, adhesion to MPO was inhibited, suggesting that MPO plays a vital role in integrin mediated adhesion. This evidence also suggests that MPO may be essential for leukocyte migration and infiltration during an inflammatory response.

14. Conclusion

MPO has been shown to play a role in both health and disease. In health, MPO is an important component present in neutrophils and macrophages. MPO catalyzes the production of very reactive halides derived from oxidants and aids in the annihilation of digested phagocytosed pathogens. In diseases characterized by the development of inflammation and oxidative stress, MPO is involved in the initiation

and continuation of the inflammatory response. These responses suggest that MPO may be a therapeutic target. Using 4-ABH as a selective inhibitor of MPO, it has been demonstrated that pathogens are capable of thriving in cells (Kettle *et al.*, 1995; Burner *et al.*, 1999). Also, patients deficient in the MPO enzyme proved to be more susceptible to bacterial infection. They are able to fight off the infection, but at a slower rate compared to an individual who is not deficient in the enzyme (Klebanoff, 2005).

However, it would be great to see if the development of MPO inhibitors would be beneficial in situations in which inflammation becomes self-perpetuating and chronic. Does MPO have other functions that could be beneficial to the host? Our lab proposes that MPO is involved in the regulation of chemotaxis through inactivation of the ligand on the receptor. It is our belief that unoccupied receptors act as the "eyes" of the cell. When the eyes are closed, the cell is unable to determine its direction. The same applies to neutrophils: when the receptors are occupied, it is as if the ligand has closed the eyes of the neutrophils, thus making it hard for them to determine their direction. It is our hypothesis that MPO helps to maintain a reasonable population of unoccupied receptors. It does so by inactivating the methionine-containing agonist peptide and changing the strength with which the ligand binds. This frees the receptor from the ligand, or by analogy, reopens the eyes of the cell.

MATERIAL AND METHODS

Reagents and Mice.

Reagents. Human fibronectin was purchased from BD BioSciences. *N*-formyl-Met-Ile-Phe-Leu (fMIFL) was synthesized at the Protein Research Laboratory at the University of Illinois, purified to ≥90% homogeneity, and identified using mass spectrometry. fMLF (≥90% purity) was purchased from Sigma-Aldrich. Calcein AM was purchased from Invitrogen. FLIPR calcium reagents were purchased from Molecular Devices. Percoll was purchased from GE Healthcare. Wild-type C57BL/6 mice were from Charles River Laboratories. $MPO^{-/-}$ mice were from Jackson Laboratory. Mice were bred and housed in a pathogen-free environment with free access to food and water at the University of Illinois at Chicago Animal Care Facility. All experimental procedures complied with University of Illinois at Chicago and National Institutes of Health guidelines for animal use.

Isolation of Mouse Neutrophils

C57BL/6 and $MPO^{-/-}$ mice were used in all experiments. Mice of 8–10 wk of age were euthanized by CO_2 inhalation, followed by cervical dislocation. Femurs and tibias were removed and then rinsed in HBSS-prep (Ca2+/Mg2+ with 20 mM HEPES, 0.2% BSA). The bones were then flushed with HBSS-prep using a 25G 5/8“ needle. Subsequently after flushing, marrow was passed through a 70-μm cell strainer to remove aggregated cells. Cells were pelleted at 1500 rpm for 5 min and then re-suspended and washed in 50 ml of HBSS-Prep. Cells were pelleted again and re-

suspended in 3 ml of HBSS-Prep. The recovered bone marrow was layered onto a discontinuous gradient consisting of 3 ml of 1.077 Nycoprep, which in turn was layered over 3 ml of 72% Percoll, and then centrifuged at 2400 rpm for 25 min at room temperature with no acceleration and no brake. Neutrophils were removed from the Nycoprep/Percoll interface and washed twice with HBSS. After the final wash, cells were resuspended in the appropriate assay buffer for further use. All experiments involving the use of mice were conducted according to protocols approved by the Institutional Animal Care and Use Committee at University of Illinois, Chicago.

Micropipette Assay

The micropipette assay was used to determine real time chemotaxis in HL-60 cells. Coverslips were pretreated with 50 μg/ml fibronectin for 1h at room temperature. Control or 4-ABH treated HL-60 cells were added to fibronectin (50 μg/ml) pretreated coverslips for 5 min. Non-adherent cells were washed away with HBSS. Chemoattractant (fMLP) was loaded into the micropipette at a concentration of 100 μM. A chemoattractant gradient was formed by slow release of the fMLP from the tip of the micropipette into the medium. Cell migration was observed using a light microscope with a 40X objective. Frames were taken every 10 seconds for 30 min. All migration images were collected with CarlZeiss AxioCam and analyzed by ImageJ software. Directionality was calculated as net migration distance from origin divided by total length of the migration path. Migration speed was calculated from migration distance divided by elapsed time.

EZ-TAXIScan Chemotaxis Assay

The EZ-taxiscan was used to visualize and research real time chemotaxis in mouse neutrophils. The EZ-taxiscan consists of two compartments, a silicone substrate and a glass plate. Coverslips were pretreated with 50 µg/ml of fibrinogen and placed on the glass plate of the lower compartment. The chamber was assembled with a 260 or 130 µm wide, 4-µm thick silicon chip on a 2 mm^2 treated glass base. Purified bone marrow derived WT (C57BL/6) or MPO $^{-/-}$ PMNs were added to the lower reservoir of each of the 6 channels. The cells were lined up on the chip by removing approximately 100 µl of buffer from the upper reservoir. At the top of the individual channels, 1 µl of 10 nM fMIFL was added in the upper reservoir. Chemotaxis was recorded at 30-sec intervals at 37° C for 30 min. Data were analyzed by ImageJ software.

Peritoneal Transmigration

For analysis of peritoneal transmigration of neutrophils, wild type (C57BL/6) and MPO $^{-/-}$ mice were injected intraperitoneally with 100 µl of 10 nM fMIFL for 4 hr. The mice were then anesthetized and the peritoneal cavity flushed. Neutrophils were recovered via peritoneal lavage. Total leukocytes were counted with a HEMA VET. Results are presented relative to the total population.

Neutrophil Adhesion to Endothelial Cells

Mouse lung vascular endothelial cells were isolated and grown to confluence in 96-well, gelatin-coated plates. Isolated bone marrow neutrophils were resuspended at a density of $1X10^7$/ml and labeled by incubation with calcein AM for 30 min. After this incubation, cells were washed in PBS to reduce background signal from the fluorescent dye and resuspended in PBS containing 0.1 % BSA. 100 µl of the cells were added to the endothelial monolayers. Adhesion to the endothelial cells was induced by stimulation with 10 nM fMIFL for 4h. Cells were washed 1-7 times with PBS to remove non-adherent cells. Adhesion was determined at excitation and emission maxima of 494 nm and 517, respectively.

Neutrophil Adhesion to Substratum/Fibrinogen

Isolated bone marrow neutrophils were resuspended at a density of $1X10^7$ /ml. Freshly isolated PMNs from wild type (C57BL/6) and MPO $^{-/-}$ mice were loaded with calcein AM for 30 min. After this incubation, the cells were washed in PBS to reduce background signal produced by the fluorescent dye and resuspended in PBS containing 0.1 % BSA. Cells were added to fibronectin (50 µg/ml) pretreated 96 well plates for 10 minutes. Cells were washed 1-7 times and read at excitation and emission maxima of 494 nm and 517, respectively.

Neutrophil Lung Sequestration

Wild type (C57BL/6) and MPO $^{-/-}$ mice were anesthetized using ketamine (100 mg/kg) and xylazine (2.5 mg/kg). Surgical procedures were performed after

verifying deep anesthesia. Mice received positive pressure ventilation via a tracheal cannula. PMNs were labeled with indium ^{111}In-oxine for 30 min. Cells were washed, centrifuged, and re-suspended in HBSS to minimize background radioactivity. . The right jugular vein was surgically exposed. 1 x 10^{6} ^{111} Indium-oxine labeled cells were injected into the jugular vain. Mice simultaneously received 10 nM fMIFL i.t. to induce lung PMN sequestration. 4 hr post injection, mice were euthanized and sacrificed. BAL fluid was collected; lungs were cleared of vascular tracer and then excised. Lung tissue and lavage fluid samples were counted with the aid of a gamma counter. PMN uptake into the lung was expressed as a fraction of total injected cpm.

Calcium Mobilization Assay

Black/clear-bottom 96-well assay plates were coated with 50 μg/ml of fibrinogen for 1 hr at room temperature. Mouse bone marrow PMN were suspended at 5 × 10^6 cells/ml in HBSS containing 0.5% BSA, and 80 μl of the cell suspension was added to each well. Cells were loaded for 1 hr at 37°C with FLIPR calcium sensitive dye, according to the manufacturer's protocol. Addition of agonist was robotically controlled, and samples were read on a FlexStation (Molecular Devices). Cells were excited at 485 nm, and Ca2+ fluorescence was detected at an emission wavelength of 525 nm. All Ca2+ mobilization assays were conducted with the use of a FLIPR Calcium Plus Kit (Molecular Devices).

Competitive Binding Assay ($I^{[125]}$ fMIFL)

The assay is based on the ability of unlabeled ligand (1nM -100μM fMIFL) to compete with labeled ligand $^{[125]}$I-fMIFL (specific activity 200 μg/μCi) at the FPR receptor. To do this, labeled ligand was added to isolated PMNs from wild type (C57BL/6) and MPO $^{-/-}$ mice. Subsequently, unlabeled ligand was added to the cells at various concentrations from 1 nM to 100 μM. The assay was performed at 4° C under conditions permitting rapid addition of the unlabeled and labeled ligand. After addition of the ligands, the tubes were mixed by very slow inversion. The cells were incubated at 4° C for 30 min. Following this incubation, the cells were washed to remove free unbound ligand. The cells were then pelleted and read using a gamma counter. Agonist competition curves were constructed by plotting specific binding of the radiolabeled agonist against log concentration of the competing ligand. Nonspecific binding was taken as the cell-associated counts in the presence of excess (10^{-4} M) unlabeled ligand. IC50 values were extracted from the raw data with the aid of Prism 6 software.

Methionine Oxidation Analysis

Isolated PMNs were challenged with the chemoattractant fMIFL for 30 min at room temperature. The cells were pelleted and resulting supernatant read via the Mass Spectrometer. An Agilent Technologies (Sanat Clara, CA) 1260 HPLC system coupled to an Agilent 6410 triple quadrupole was employed. Chromatographic separation was carried out using a Waters Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm, Waters, Milford, MA), which was maintained at 40 °C within

the column oven. A linear gradient consisting of acetonitrile and 0.1% formic acid (5:95, v/v) to (95:5, v/v) over 8 min was used, at a flow rate of 300 µL/min and an injection volume of 10 µL. The total run time with column re-equilibration was 15 min. This method employs electrospray ionization in positive ion mode, operated in multiple ion monitoring (MRM) mode at unit resolution, and uses Nitrogen as the bath gas. The optimal MS parameters obtained were as follows: capillary voltage at +4. kV, gas temp 350 °C, nebulizing gas pressure 40 psi, gas flow 12.0 L/min. Specific transitions were selected for the singly protonated peptide at *m/z* 551.3 to 86.0 and 551.2 to 120.2 each with a collision energy set to 30 V and fragmentor set to 10 V. The oxidized peptide species was identified within sample sets as the *m/z* 567.3 to 567.3 (collision energy 0 V and fragmentor set to 10 V). Mass Hunter version B.0.3 was used for instrument control and data analysis.

Statistical Analysis.

Statistical comparisons were made with the two-tailed Student *t*-test. All results represent at least 3 independent experiments. Statistical significance was defined at $P<0.05$. Error bars in the figures represent standard error of the mean.

RESULTS

Myeloperoxidase is Essential for Sustained Neutrophil Migration

Innate immunity is important in the protection of the host from microbial infection. In the host, inactive neutrophils roll along the blood vessel wall until activated by a chemoattractant. The active cells become firmly adherent to the endothelial cell surface and transmigration occurs. Once through the blood vessel wall, the cells are able to migrate toward the invading microorganisms, because the microbes release chemoattractants to form a gradient that the neutrophil can detect and follow to the source.

We wanted to determine the effects of MPO deficiency on neutrophil transmigration *in vivo*. WT and $MPO^{-/-}$ mice aged 8-10 weeks received an intraperitoneal injection of 10 nM fMIFL, a peptide chemoattractant released by *Staphylococcus aureus*. Five hr post injection, neutrophils attracted to the peritoneal cavity were recovered and quantified as described in Materials and Methods. Neutrophil transmigration from the bloodstream into the peritoneal cavity was significantly decreased in $MPO^{-/-}$ mice when compared to WT (Figure 4).

Next, we asked whether this phenotype is due to a defect in the adhesive properties of neutrophils. We first carried out an *ex vivo* adhesion assay (see Materials and Methods) to quantify PMN adhesion to cultured endothelial monolayers. MPO deficiency had no significant effect on PMN adhesion to the endothelial monolayer relative to the WT control (Figure 5). Thus, the observed

inhibition of migration of MPO $^{-/-}$ neutrophils was probably not the result of decreased neutrophil binding to the endothelium.

To evaluate the *in vivo* relevance of these observations, we compared binding of WT and MPO $^{-/-}$ PMNs to intact mouse lung microvessels. WT or MPO deficient PMNs were labeled with ^{111}Indium oxine and injected into the jugular vein of WT mice as described in the Materials and Methods section. At approximately the same time, the chemoattractant fMIFL was introduced into the lung via the trachea. Bronchioalveolar lavage fluid (BALF) was collected from the mice 4 hr later and counted for gamma-radioactivity. The results showed significantly lower sequestration of MPO$^{-/-}$ neutrophils in the lung compared to WT ($P < 0.05$ vs. WT neutrophils; Figure 6A). To show that the observed reduction in PMN sequestration was not due to a defect in the neutrophil's ability to adhere to the endothelium, we assessed binding of labeled PMNs to perfused lung microvessels, as described in Materials and Methods. The results showed no significant difference between WT and MPO deficient PMNs in their ability to adhere to the lung (Figure 6B).

Inactivation of Myeloperoxidase Activity Impairs Neutrophil Chemotaxis

The generally recognized physiological function of MPO is to aid in the destruction of foreign microorganisms during a respiratory burst. However, its function during neutrophil chemotaxis is not well understood. Our goal was to investigate the function of MPO during chemotaxis. Human promyelocytic leukemia cells (HL-60) were induced to differentiate into neutrophils to create a model system for studying the function of MPO in migrating cells. Spontaneous

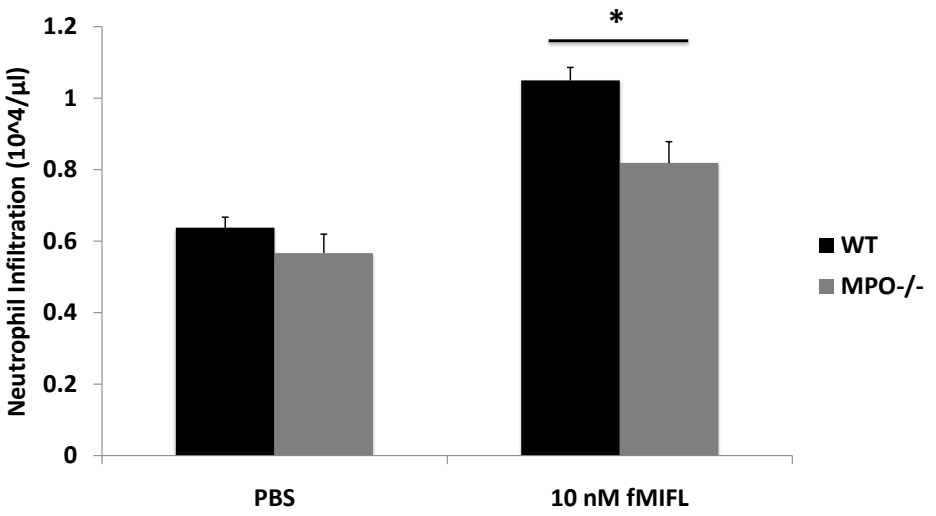


Figure 4. Comparison of fMIFL-Induced Peritoneal Sequestration of PMNs in WT and MPO$^{-/-}$ Mice. WT or MPO$^{-/-}$ mice were injected intraperitoneally with 100 µl of 10 nM fMIFL or with an equivalent volume of PBS (control). 4 hr post-injection, sequestration of PMNs was quantified by peritoneal lavage with the aid of the HEMAVET. * indicates significant reduction in PMN sequestration ($p < 0.05$; $n = 7$ per group).

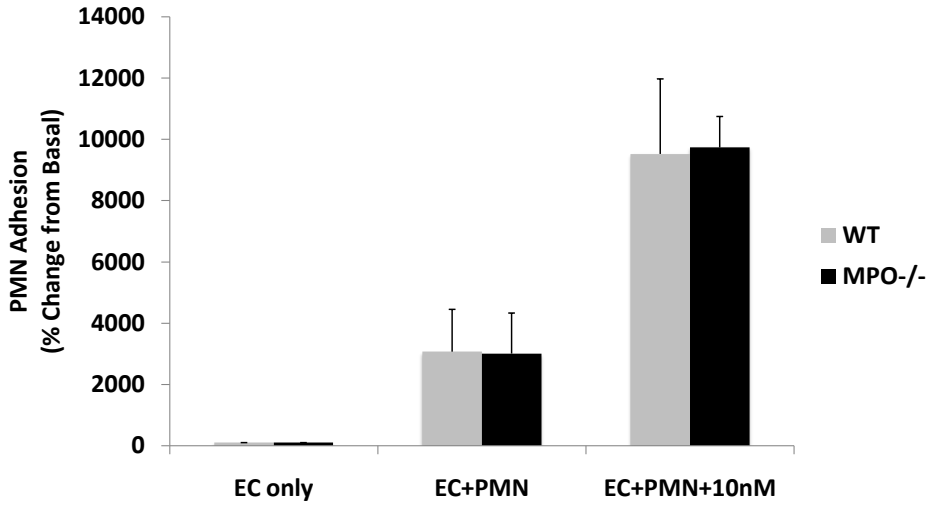


Figure 5. Lack of Influence of Myeloperoxidase Deletion on PMN Adhesion to Lung Endothelial Cell Monolayers. Mouse endothelial cells were first isolated and cultured as monolayers on a 96-well plate. PMNs were isolated from WT or MPO $^{-/-}$ mice. To quantify PMN adherence, the PMNs were fluorescently labeled with calcein AM. Then, labeled PMNs were layered onto the monolayers (10^6 PMNs per well), treated with or without 10 nM fMIFL, and allowed to adhere for 4 hr. Non-adherent cells were washed away and fluorescence of adherent cells read at excitation and emission wavelengths of 494 and 517 nm, respectively. Bar graph shows background fluorescence (1st pair of bars), adhesion of unstimulated PMNs to monolayer (2nd pair), and adhesion of fMIFL-stimulated PMNs to monolayer (3rd pair). Note that MPO genetic deletion has no effect on PMN adhesion. N=3 per group.

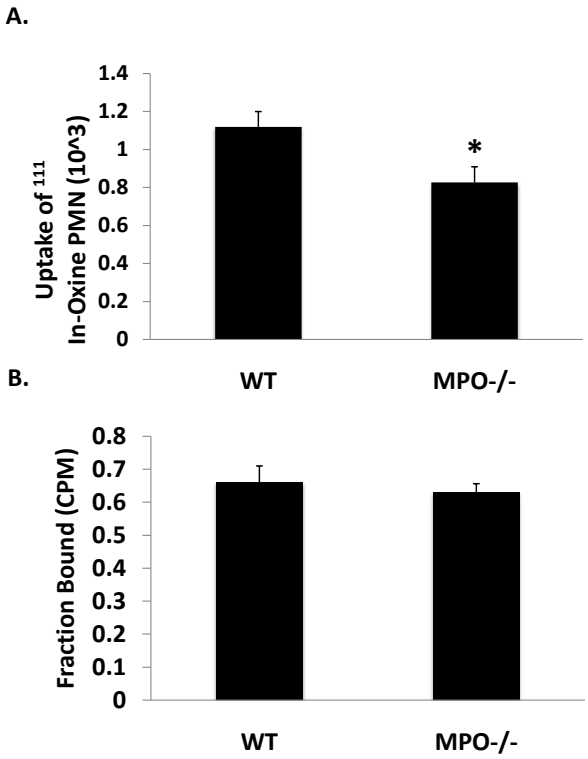


Figure 6. Influence of Myeloperoxidase Expression on Binding and Transmigration of fMIFL Stimulated PMNs in the Lung. PMNs either from WT or $MPO^{-/-}$ mice were isolated or radiolabeled with ^{111}In-oxine. Under anesthesia, mice received an injection of 10^6 Indium-labeled PMNs via the jugular vein. Then, i.t.(intratracheal) fMIFL (50 µl, 10 nM) was immediately administered by instillation. 4 hr post injection, a blood sample was withdrawn, and the lung was purged of unbound PMNs. Radioactivity in the airway was recovered in BALF (3 flushes with 1 ml PBS). The radioactivity in the various samples and lung tissue was counted with the aid of a gamma counter. A: Bar graph showing that MPO deletion significantly reduces PMN transmigration. The number of transmigrating PMNs was computed as the fraction of total injected counts recovered in BALF times 10^6. *, significant reduction in transmigration ($p < 0.05$; $n = 5$ per group). B: Bar graph showing that MPO deletion has no effect on binding of radiolabeled PMNs to lung microvessels. The fraction of bound PMNs was obtained from the ratio of tissue counts to total counts injected. 3 independent experiments were conducted per group.

differentiation of the HL-60 cells into granulocytes was obtained with the addition of 1.3 % DMSO into HL-60 cell culture. Over a period of 6-8 days the cells developed a mature neutrophil morphology.

4-ABH, a specific inhibitor of MPO, was used to block MPO activity in differentiated HL-60 cells (dHL-60). Chemotaxis was monitored using the micropipette assay in dHL-60 cells treated with or without 4-ABH. The micropipette contained fMLP at a concentration of 100μM, which when lowered into the medium, generated a gradient emanating from the micropipette tip. fMLP is an E. coli derived tetrapeptide shown to be a potent agonist for the FPR in dHL-60 cells. Untreated dHL-60 cells rapidly migrated to the point source of fMLP. By contrast, 4-ABH treated dHL-60 cells were disoriented in the fMLP gradient. Unlike control HL-60 cells, the 4-ABH treated cells did not display directed cell movement (Figure 7A), thus indicating that MPO inhibition influences chemotaxis. Additional experiments were conducted to help pinpoint the defect caused by loss of MPO activity. These cells were able to polarize but were unable to sense the fMLP gradient and home to the point source. The Chemotaxis Index was significantly lower in cells treated with the inhibitor ($P < 0.05$; Figure 7B), but there was no significant difference in speed (Figure 7C). To evaluate the orientation of the cells in relation to the micropipette tip, their leading edge in (Figure 8A) was traced in red and the trailing edge traced in green. Three min after placement of the pipette, the orientation of the cells was quantified as the percentage of cells whose leading edge was directed toward the point source. In the presence of 4-ABH, approximately 40% of the cells were

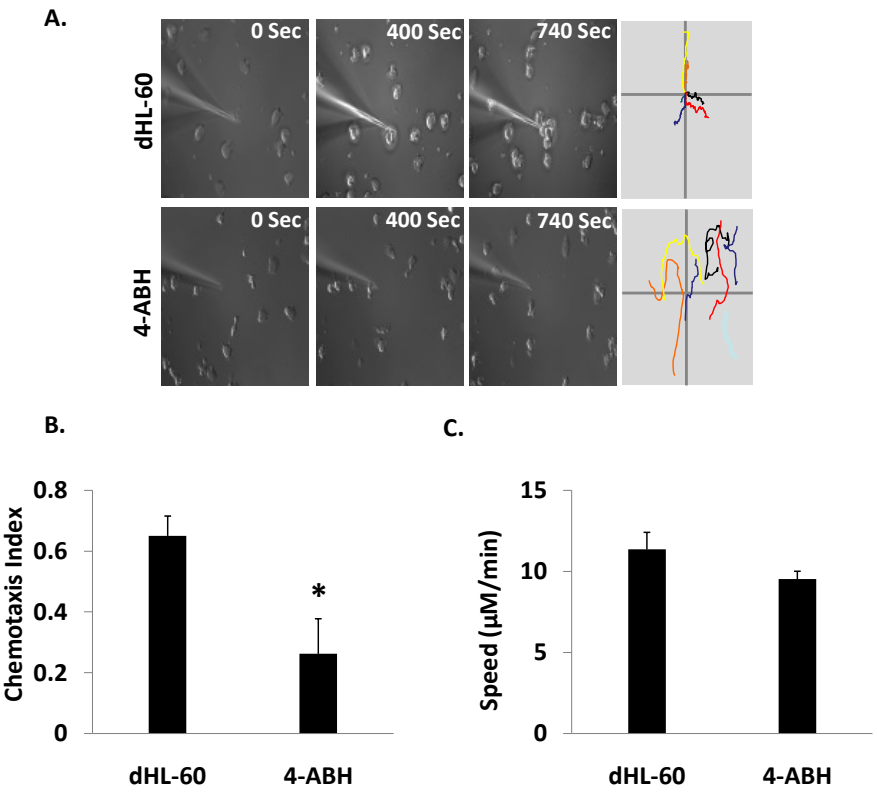

A.
dHL-60
4-ABH
0 Sec
400 Sec
740 Sec
B.
Chemotaxis Index
0.8
0.6
0.4
0.2
0
*
dHL-60
4-ABH
C.
Speed (μM/min)
15
10
5
0
dHL-60
4-ABH

Figure 7. Influence of Myeloperoxidase Inhibition on Neutrophil Chemotaxis and Migration Speed in Differentiated HL-60 Cells. HL-60 cells were differentiated into neutrophils as described in Materials and Methods. Micropipette assay (see Materials and Methods) was performed to determine chemotaxis and migration speed of the differentiated cells. The cells were pretreated for a period of 30 min with the MPO inhibitor 4-ABH (50 μg/ml) or vehicle (control). Then, they were plated on fibronectin (50 μg/ml) treated coverslips and allowed to adhere for 5 min. Next, non-adherent cells were washed away, and the micropipette containing 100 μM fMLP as chemoattractant was introduced into the medium. A chemotactic gradient formed as a result of slow release of fMLP from the tip of the micropipette. Cell migration was observed using a light microscope with a 40X objective. A, Top row: time series following exposure of untreated HL-60 cells to chemotaxin from micropipette. Note clustering of cells around pipette tip in the 740 sec frame. Bottom row: time series following exposure of 4-ABH treated HL-60 cells to chemotaxin from micropipette. Note that treated cells fail to migrate to the pipette tip. Color tracings show the trajectory of individual cells. The intersection of the orthogonal lines represents the location of the pipette tip. B: Chemotactic index for fMLP stimulated cells in the presence and absence of 4-ABH. CI is the ratio of the net displacement of a migrating cell to the length of its actual path. The 4-ABH treated HL-60 cells showed a significant decrease in the CI when compared to control ($p<0.05$; $n=3$). C: Migration speed of fMLP stimulated cells in the presence or absence of 4-ABH. Speed is calculated as the total migration distance divided by elapsed time. 4-ABH treated HL-60 cells exhibited no difference in speed compared to controls ($n=3$).

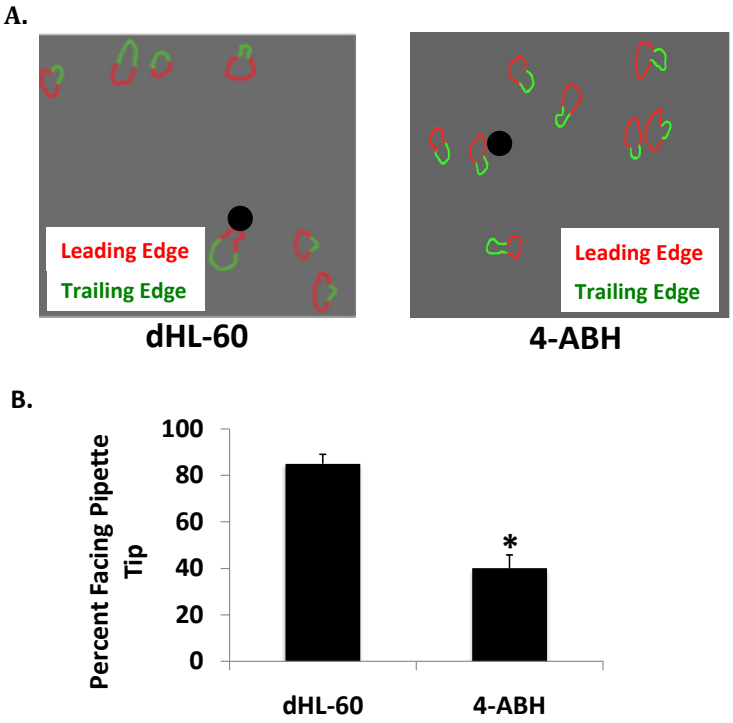


Figure 8. Influence of Myeloperoxidase Inhibition on Neutrophil Orientation in a Chemotactic Gradient. HL-60 cells were differentiated into neutrophils as described in Materials and Methods. Micropipette assay (see Materials and Methods) was performed to determine chemotaxis and migration speed of the differentiated cells. The cells were pretreated for a period of 30 min with the MPO inhibitor 4-ABH (50 µg/ml) or vehicle (control). Then, they were plated on fibronectin (50 µg/ml) treated coverslips and allowed to adhere for 5 min. Next, non-adherent cells were washed away, and the micropipette containing 100 µM fMLP as chemoattractant was introduced into the medium. A chemotactic gradient formed as a result of slow release of fMLP from the tip of the micropipette. Cell migration was observed for a 3-minute period using a light microscope with a 40X objective, and cell orientation was noted. A: Illustration of cell orientation. Leading and trailing edges of the PMNs shown are traced in red and green, respectively, and the point source of chemoattractant is represented by the black dot. Note that the majority of leading edges of MPO impaired cells are not oriented toward the micropipette. B: Quantification of cell orientation as the percentage of cells oriented toward the point source of chemoattractant. MPO blockade caused a significant decrease in the percentage of cells oriented toward the micropipette at the 3 min time point ($p<0.05$; $n=3$).

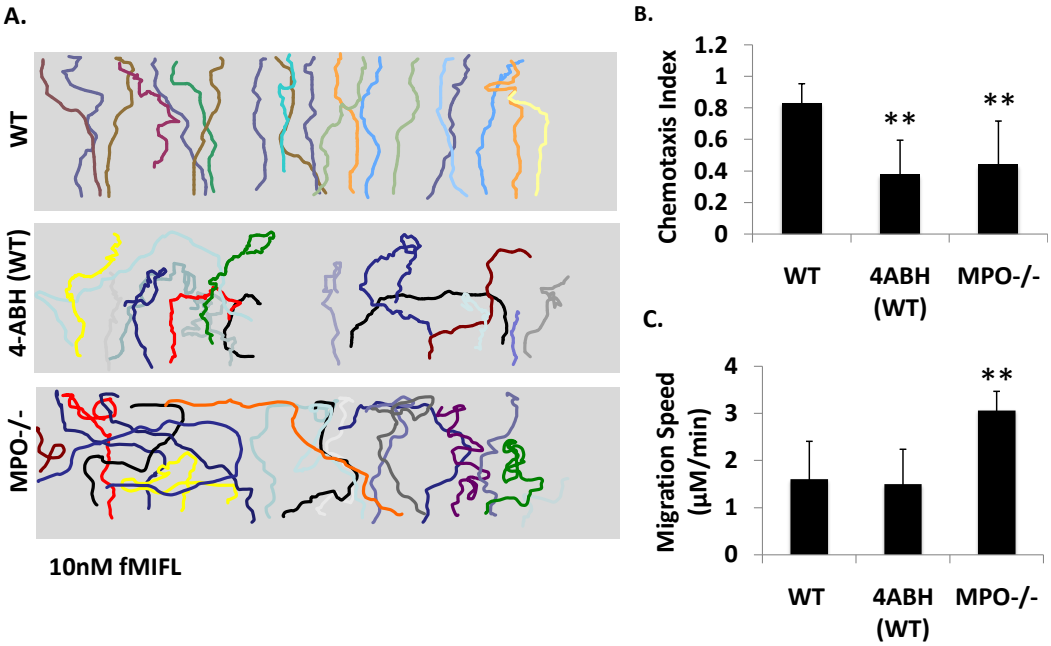


Figure 9. Influence of Myeloperoxidase Deletion or Blockade on Chemotactic Index and Migration Speed in Isolated Mouse PMNs Placed in an fMIFL Gradient. PMNs were isolated from WT and MPO$^{-/-}$ mice as described in Materials and Methods. EZTAXIScan (see Materials and Methods) was performed to determine the chemotactic index and migration speed. WT or MPO$^{-/-}$ PMNs were added to the lower reservoir and fMIFL (10 nM) added to the upper reservoir to produce a chemotactic gradient. A: Individual trajectories of untreated and 4-ABH treated WT PMNs (1st and 2nd rows) and MPO$^{-/-}$ PMNs (3rd row). Note that 4-ABH treated and MPO$^{-/-}$ cells did not migrate to the upper reservoir and stop. B: Chemotactic index for fMIFL stimulated cells in untreated and 4-ABH treated WT PMNs and MPO$^{-/-}$ PMNs. CI is the ratio of the net displacement of a migrating cell to the length of its actual path. The 4-ABH treated WT and MPO$^{-/-}$ cells showed a significant decrease in the CI when compared to WT controls ($p<0.05$; n=3). C: Migration speed of fMIFL stimulated cells in untreated and 4-ABH treated WT PMNs and MPO$^{-/-}$ PMNs. Speed is calculated as the total migration distance divided by elapsed time. 4-ABH treated WT cells showed no significant difference in speed from controls (n=3), but there was a significant increase in speed in MPO$^{-/-}$ PMNs ($p<0.05$; n=3).

oriented toward the fMLP point source (Figure 8B). This value may be compared to a control value of 90%.

Myeloperoxidase is Required for Neutrophil Migration

In innate immunity the ability of a neutrophil to detect a chemotactic gradient and polarize is an important initial step during cell migration. To evaluate whether $MPO^{-/-}$ and 4-ABH treated PMNs display a similar phenotype as the HL-60 cells, we investigated the impact of deficient MPO expression or inhibition of MPO activity on the ability of the PMN to detect a gradient when challenged with fMIFL. The EZ-TAXIScan Scan was used to determine the migratory characteristics of murine PMNs. Bone marrow derived PMNs have been shown to have similar physiological and functional characteristics as the circulating PMNs in the blood stream, and is therefore a good working model with which to study PMN function (Boxio *et al.*, 2004). $MPO^{-/-}$ and WT PMN were loaded at the bottom of the reservoir and baited with a continuous gradient of the chemoattractant fMIFL as described in Materials and Methods (Figure 9A). $MPO^{-/-}$ and 4-ABH treated PMNs displayed impairment of their directional orientation when compared to WT. WT PMNs possessed a normal phenotype whereby they were able to migrate to the highest concentration on the gradient and stop. The Chemotaxis index was significantly lower than control in cells treated with the inhibitor ($P< 0.05$; Figure 9B), but there was no significant difference in speed (Figure 9C). $MPO^{-/-}$ and 4-ABH treated PMNs differed from WT in that the MPO impaired cells migrated in different directions and never came to a complete stop at the concentration maximum on the gradient,

suggesting that MPO is not only important in the respiratory burst but also during chemotaxis.

The Effect of Myeloperoxidase on Cell-PMN Adhesion

Adhesion is an important factor in cell migration. Adhesion to the endothelium is due to the up regulation of selectins on endothelial cells lining the blood vessel wall and up regulation of integrins on the neutrophil, but once the PMN has transmigrated through the endothelium, cell adhesion depends on an underlying layer termed the substratum. PMN spread more extensively during chemotaxis and generate tension against the underlying substrate. The cell must possess the ability to extend lamellipodia and grab hold of the substrate, and the trailing edge must be released to ensure appropriate cell migration. In our experiments the substratum that we chose was fibrinogen, which we applied at a concentration of 50 μg/ml. There was no significant difference in adhesion between $MPO^{-/-}$ and WT PMN (Figure 10).

Effect of Non -Methionine Containing Peptide's on PMN Migration.

Previous studies have shown that formyl peptides are potent agonists for the FPR receptor, and the initial step in protein synthesis in bacteria usually begins with a formyl methionine. Supporting research has shown that the MPO system is required for methionine oxidation, which aids in the process of bacterial killing. The MPO system oxidizes the methionine residues on the peptide to methionine sulfoxide. This process is necessary for bactericidal activity. The NLE peptide was

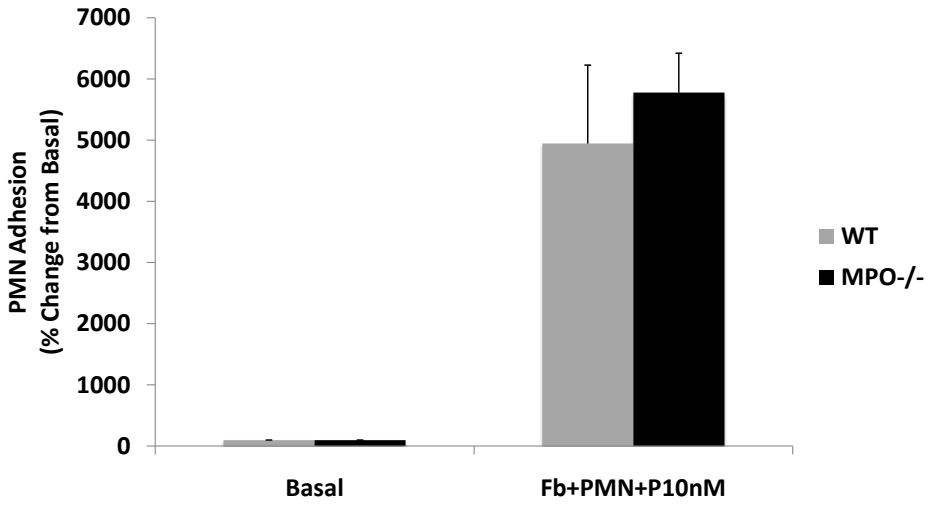


Figure 10. Influence of Myeloperoxidase Genetic Deletion on Cell Adhesion to a Fibrinogen Matrix. 96 well plates were pretreated with 50 μg/ml fibrinogen for 1 hr at room temperature. Subsequently, PMNs were isolated from WT or $MPO^{-/-}$ mice. To quantify PMN adhesion, the PMNs were fluorescently labeled with calcein AM. Then, labeled PMNs were plated onto the fibrinogen treated plates (10^6 PMNs per well), treated with or without 10 nM fMIFL, and allowed to adhere for 10 min. Non-adherent cells were washed away and their fluorescence read at excitation and emission wavelengths of 494 and 517 nm, respectively. Bar graph represents background fluorescence (1st pair of bars) and adhesion of fMIFL-stimulated PMNs to fibrinogen (2nd pair). Note that MPO genetic deletion has no significant effect on PMN adhesion. N=3 per group.

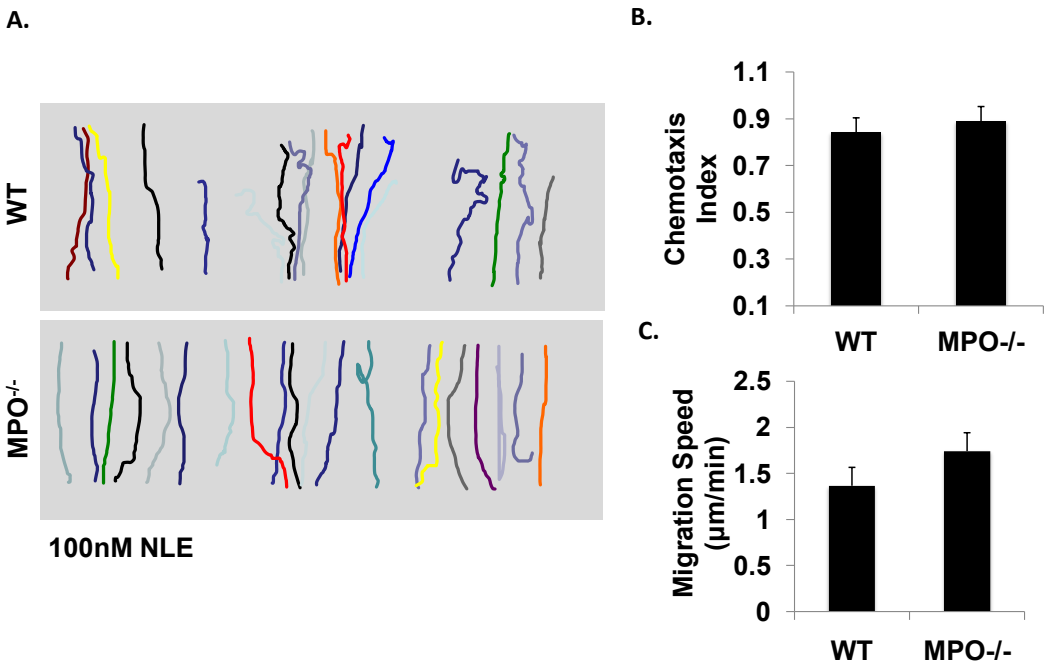


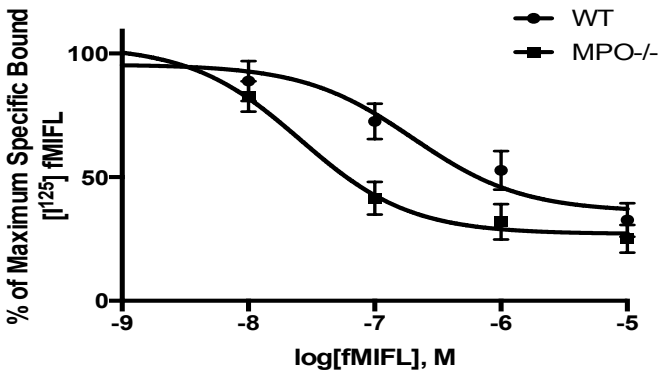
Figure 11. Effect of Non-Methionine Containing Peptide (NLE) on Chemotactic Index and Migration Speed in Mouse MPO$^{-/-}$ Cells. PMNs were isolated from WT and MPO$^{-/-}$ mice as described in Materials and Methods. EZTAXIScan (see Materials and Methods) was performed to determine chemotaxis and migration speed. WT or MPO$^{-/-}$ PMNs were added to the lower reservoir and the chemoattractant NLE (100 nM) added to the upper reservoir to generate a chemotactic gradient. A: Individual trajectories for NLE stimulated WT (1st row) and MPO$^{-/-}$ PMNs (2nd row). B: Chemotactic index for NLE stimulated WT and MPO$^{-/-}$ PMNs. CI is the ratio of the net displacement of a migrating cell to the length of its actual path. The NLE stimulated MPO$^{-/-}$ PMNs showed no significant difference in the CI compared to WT (n=3). C: Migration speed of NLE stimulated WT and MPO$^{-/-}$ PMNs. Speed is calculated as the total migration distance divided by elapsed time. MPO$^{-/-}$ cells showed no significant difference in speed compared to WT (n=3).

chosen based on the findings of Rosen *et al.* (Rosen *et al.*, 2009). NLE is a synthetic peptide that also binds to the FPR receptor. We set out to confirm that MPO deletion has no impact on the activity of the NLE peptide since this peptide lacks methionine.

To evaluate this, we performed chemotaxis assays with an NLE peptide (100 nM) gradient. As previously stated, cells were added to the lower reservoir of the EZ-TAXIScan chip and the chemoattractant, to the upper reservoir. Image j software was used to track individual cells during migration (Figure 11A). There was no difference in the chemotaxis index for $MPO^{-/-}$ PMN (Figure 11B) when compared to WT and no significant difference in speed (Figure 11C). The results imply that MPO is required for directed migration along a chemotactic gradient in the presence of formyl methionine containing peptides.

Competitive Binding between ^{125}I-fMIFL and unlabeled fMIFL in Isolated PMNs

Neutrophils have been suggested to use spatial cues to navigate in a chemotactic gradient. In polarized cells, receptors that initially sense the gradient are distributed to the leading edge of the cells during migration (Schiffmann 1982). By oxidizing methionine, MPO could play a role in limiting agonist concentration at the FPR and thereby maintaining receptor sensitivity. To test the importance of MPO expression to the binding properties of the FPR, we performed a competitive binding assay using isolated PMNs from WT or $MPO^{-/-}$ mice, in which unlabeled fMIFL was used to displace ^{125}I-labeled fMIFL from the FPR . Results showed that MPO deletion shifted the IC_{50} for labeled fMIFL to lower concentrations (Figure12) by a factor of about 10, i.e., the affinity of agonist for the FPR receptor was increased.



	WT	MPO-/-
IC_{50}	1.824e-007	3.443e-008

Figure 12. Competitive Binding Assay Performed with Isolated PMNs from WT and MPO$^{-/-}$ mice. Competitive binding assays were performed to determine agonist-binding affinity to the FPR receptor. WT and MPO$^{-/-}$ PMNs were isolated from mouse bone marrow and suspended in saline (60 µl) at 10^6 cells per tube. ^{125}I-labeled fMIFL was added to the WT or MPO$^{-/-}$ PMNs together with unlabeled fMIFL at various concentrations from 1 nM to 100 µM for a 30-min period at 4° C. After incubation, cells were washed to remove any free unbound ligand, and then read with a gamma counter. The curve shown represents the specific binding of ^{125}I-labeled fMIFL as a function of unlabeled ligand concentration. Specific binding is the difference between total and nonspecific binding, the latter taken as the cell associated counts remaining in the presence of excess (10^{-4} M) unlabeled fMIFL. IC50 values were determined from the raw data with the aid of Prism 6 software. Table shows the IC50 values obtained for WT (1.824×10^{-7}) and MPO$^{-/-}$ PMNs (3.443×10^{-8}). Hence, there was a ~10-fold decrease in the IC50 of MPO$^{-/-}$ cells compared to WT.

Hence, IC50 values were 3.443 x 10^{-8} M in $MPO^{-/-}$ PMNs and 1.824 x 10^{-7}M in the WT control (Figure 12). Since the assay was performed at 4° C (see Materials and Methods), and internalization of the agonist occupied receptor is known to be temperature sensitive, the results cannot be attributed to differences in receptor internalization.

Effects of Myeloperoxidase Deletion on Calcium Mobilization Induced by Activation of FPRs in PMNs

The activated FPR induces calcium mobilization from intracellular stores in neutrophils. To see if the observed increase in ligand binding affinity in MPO depleted PMNs (see above) affects functional responses to agonists, we evaluated dose-response curves for agonist-induced calcium rise. In the experiments conducted, neutrophils isolated from $MPO^{-/-}$ mice or WT controls were loaded using a Calcium 5 kit (see Materials and Methods) and subsequently challenged with fMIFL. Original recordings of calcium transients obtained from WT and $MPO^{-/-}$ PMNs in response to 10 nM fMIFL are shown in Figure 13A. Transients in MPO deficient cells were increased, as expected. Calcium transients were quantified from the area under the curve (Figure 13B). As can be seen, the transients on average were significantly elevated in the MPO mutant. Additional calcium mobilization assays were done using the non-methionine containing peptide NLE. MPO deletion had no effect on the calcium transient induced by 100 nM NLE (Figure 14A) and mean area values did not differ significantly from WT (Figure 14B). The dose response curve was determined from the peak amplitude of the calcium transient in

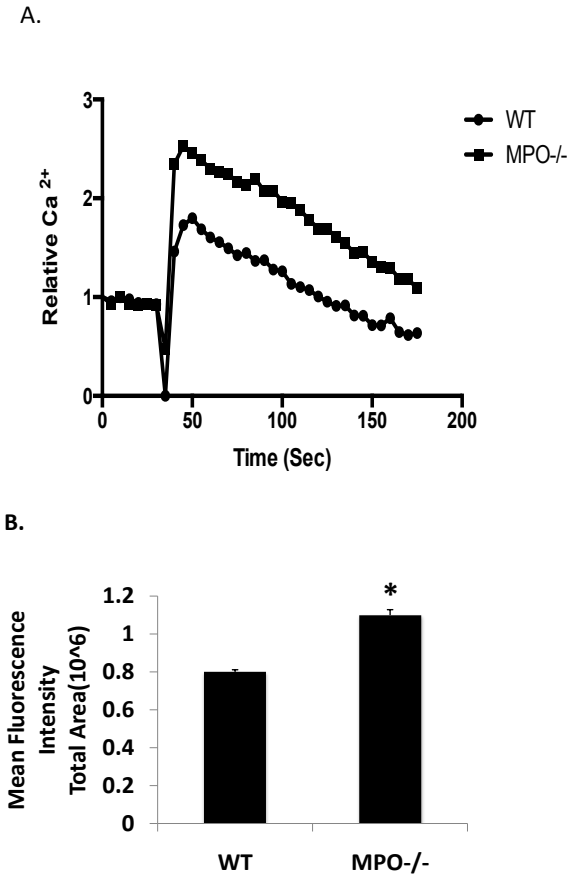


Figure 13. Calcium Mobilization Induced by fMIFL in Isolated PMNs from WT and MPO$^{-/-}$ Mice. 96 well plates were pretreated with 50 µg/ml fibrinogen for 1 hr at room temperature. Bone marrow PMNs were isolated from WT and MPO$^{-/-}$ mice. The PMNs (5 × 10^6 per well) were loaded with the FLIPR calcium sensitive dye for 1 hr at 37° C (see Materials and Methods). FlexStation, a microplate reader with an integrated multichannel pipettor, was programmed to add the chemoattractant fMIFL at a predetermined time, to read the resulting change in cell fluorescence as a function of time (excitation and emission wavelengths of 485 and 525 nm) and to integrate the area under the calcium transient. A: Calcium transients induced by 10 nM fMIFL displayed as plots of relative Ca^{2+} fluorescence (ratio of readings at 525 nm before and after agonist injection) vs. time (sec). Note that the peak calcium transient is of greater amplitude in MPO$^{-/-}$ cells than WT. B: Bar graph of total area under the curve in A. MPO$^{-/-}$ cells showed a significant increase in total area under the curve when compared to WT ($p<0.05$; n=5).

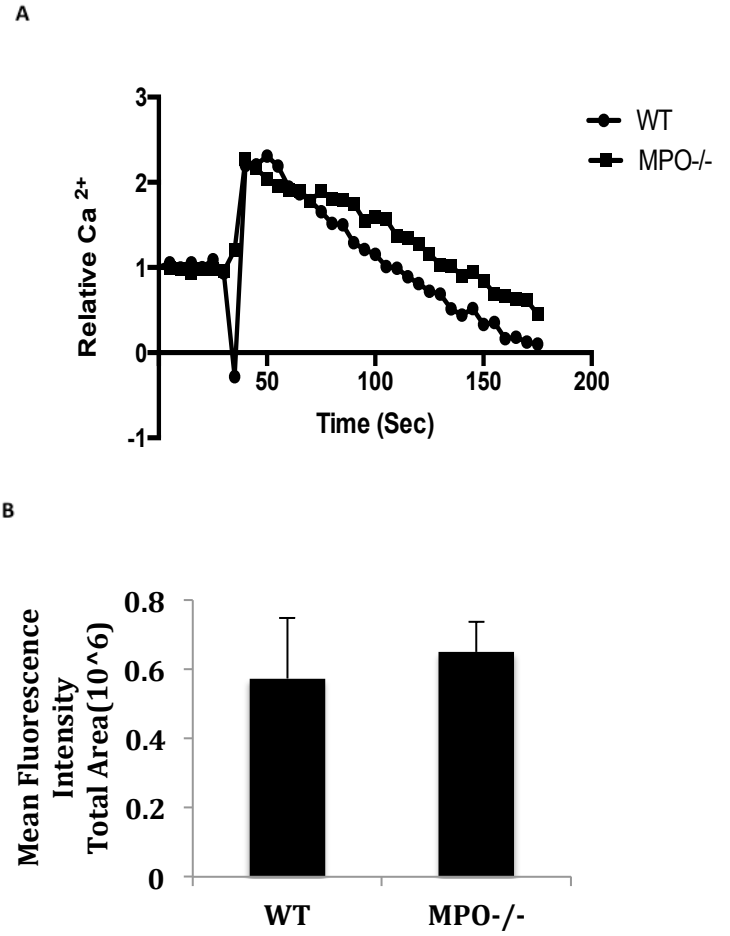
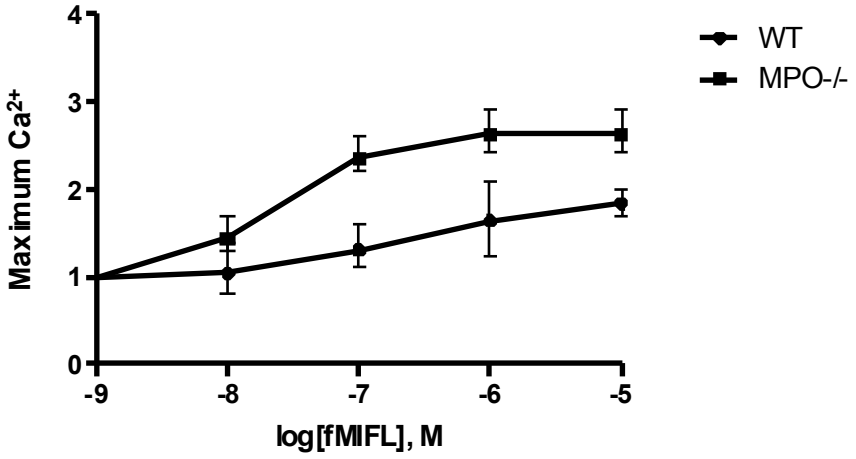
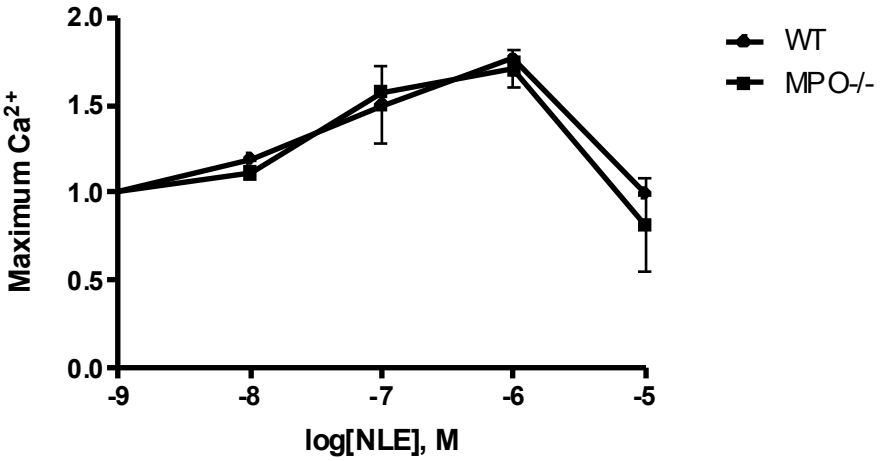


Figure 14. Calcium Mobilization Induced by NLE in Isolated PMNs from WT and MPO$^{-/-}$ Mice. 96 well plates were pretreated with 50 µg/ml fibrinogen for 1 hr at room temperature. Bone marrow PMNs were isolated from WT and MPO$^{-/-}$ mice. The PMNs (5 × 10^6 per well) were loaded with the FLIPR calcium sensitive dye for 1 hr at 37° C (see Materials and Methods). FlexStation, a microplate reader with an integrated multichannel pipettor, was programmed to add the chemoattractant NLE at a predetermined time, to read the resulting change in cell fluorescence as a function of time (excitation and emission wavelengths, 485 and 525 nm) and to integrate the area under the calcium transient. A: Calcium transients induced by 100 nM NLE are displayed in plots of relative Ca^{2+} fluorescence (ratio of readings at 525 nM before and after agonist injection) vs. time (sec). B: Bar graph of total area under the curve in A. MPO$^{-/-}$ cells showed no significant difference in total area under the curve when compared to WT (n=5).



	WT	MPO$^{-/-}$
EC_{50}	2.307e-007	2.877e-008

Figure 15. Dose Dependent Activation of Calcium Mobilization in Isolated PMNs at Various Concentrations of fMIFL. 96 well plates were pretreated with 50 µg/ml fibrinogen for 1 hr at room temperature. Bone marrow PMNs were isolated from WT and MPO$^{-/-}$ mice. The PMNs (5×10^6 per well) were loaded with FLIPR calcium sensitive dye for 1 hr at 37° C (see Materials and Methods). FlexStation, a microplate reader with an integrated multichannel pipettor, was programmed to add the chemoattractant fMIFL at a predetermined time, to read the resulting change in cell fluorescence as a function of time (excitation and emission wavelengths of 485 and 525 nm) and to integrate the area under the calcium transient. Graph: Dose-response curve for the effect of fMIFL on amplitude of calcium transients. Table: The EC50 values calculated from the dose-response curve are 2.307×10^{-7} for WT and 2.877×10^{-8} for MPO$^{-/-}$. Note the 10-fold decrease in the EC50 value in MPO$^{-/-}$ cells when compared to WT (n=5).



	WT	$MPO^{-/-}$
EC_{50}	1.627e-007	1.089e-007

Figure 16. Dose Dependent Activation of Calcium Mobilization in Isolated PMNs at Various Concentrations of NLE. 96 well plates were pretreated with 50 µg/ml fibrinogen for 1 hr at room temperature. Bone marrow PMNs were isolated from WT and $MPO^{-/-}$ mice. The PMNs (5×10^6 per well) were loaded with FLIPR calcium sensitive dye for 1 hr at 37° C (see Materials and Methods). FlexStation, a microplate reader with an integrated multichannel pipettor, was programmed to add the chemoattractant NLE at a predetermined time, to read the resulting change in cell fluorescence as a function of time (excitation and emission wavelengths of 485 and 525 nm) and to integrate the area under the calcium transient. Graph: Dose-response curve for the effect of NLE on amplitude of calcium transients. Table: The EC_{50} values calculated from the dose-response curve are 1.627×10^{-7} for WT and 1.089×10^{-7} $MPO^{-/-}$. Note lack of difference between EC50 values in $MPO^{-/-}$ cells and WT (n=5).

response to increasing concentrations of fMIFL and NLE. Analysis of the curves showed that fMIFL activated the FPR with a 10-fold higher potency. The EC_{50} values obtained were 2.3O7 x 10^{-7} M (WT) and 2.877 x 10^{-8} M ($MPO^{-/-}$)(Figure 15), consistent with the ligand binding study (above). In contrast to fMIFL, NLE gave closely similar EC_{50} values of 1.627 x 10^{-7} M (WT) and 1.089 x 10^{-7} fM ($MPO^{-/-}$) (Figure 16), indicating that MPO deletion has no effect on responses to NLE. This result is expected since NLE lacks methionine and is not subject to oxidation.

Quantification of Methionine Oxidation in Isolated Bone Marrow PMNs

Methionine oxidation has been shown to be a key contributor to the bactericidal activity of PMNs. Methionine is an amino acid that contains sulfur and is oxidized during the respiratory burst (Ling *et al.*, 1978). In our studies, we used mass spectrometry to prove that MPO causes methionine oxidation in normal PMNs. Oxidation is easily assessed by mass spectrometry, because the addition of oxygen to methionine would increase its molecular weight by 16. In the experiments conducted we assessed the ability of a standard number of PMNs (WT or $MPO^{-/-}$) to oxidize fMIFL. We expressed extent of oxidation as the measured ratio between a non-oxidized fragment of fMIFL containing methionine and oxidized fMIFL (see Materials and Methods). The results showed that MPO deletion causes a 10-fold decrease in methionine oxidation compared to WT, suggesting that MPO is a major contributor to oxidation of methionine in PMNs (Figure 17). Since the oxidation product was recovered from the external medium after spinning out the cells, PMNs must have released oxidants into the extracellular space.

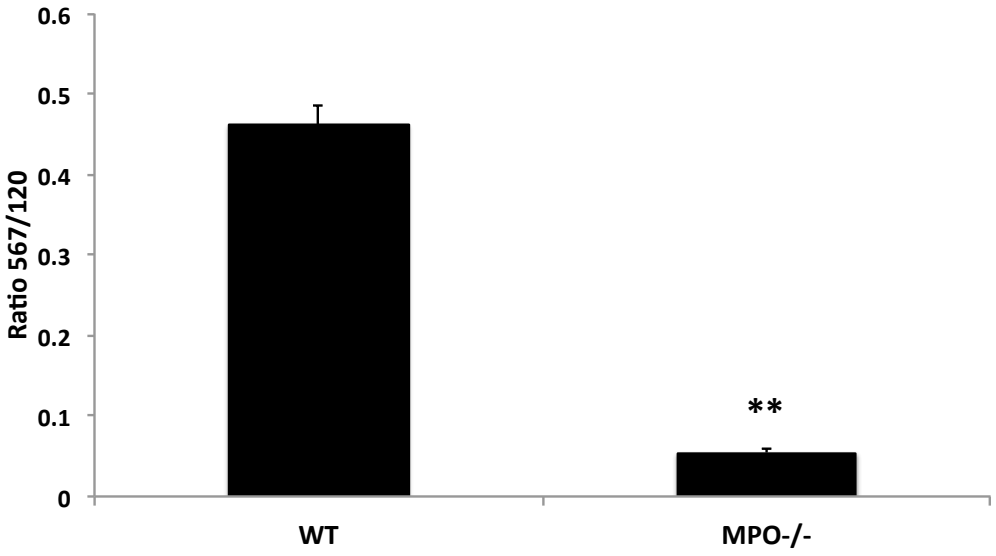


Figure 17. Oxidation of the Methionine Residue in fMIFL. Bone marrow PMNs isolated from WT and $MPO^{-/-}$ mice were suspended at 10^6 cells per tube. Subsequently, cells of each genotype were challenged with 10 nM fMIFL for 30 min at room temperature. Cells were then pelleted and the supernatant collected for analysis by mass spectroscopy (see Materials and Methods). Graph quantifies the ratio of an unmodified peptide fragment (FW, 120) to the oxidized methionine-containing peptide (FW, 567). $MPO^{-/-}$ PMNs show significantly less oxidation of the methionine residue than that of WT. (**, $p<0.005$; n=3).

DISCUSSION

Neutrophils are required for protection of the host from pathogens during an inflammatory response, and function as the initial defense against invading microorganisms. They sense chemical cues in the bloodstream and have the ability to follow a chemical gradient to its source (chemotaxis)(Harris, 1954). Neutrophils go through three different steps to activate chemotaxis. The first step is ligand-receptor interaction, resulting in adhesion to substrate and cell polarization; the second step involves signaling to motility elements (actin polymerization and myosin contractility); and the terminal step is activation of the motility elements that produce cell migration (Downey, 1994; Cicchetti *et al.*, 2002). Our understanding of cell migration has significantly improved in recent years. However, our understanding of how neutrophils achieve spatial sensing at the molecular level and in particular the role of cell surface receptors remains limited. It has been reported that, in the neutrophil, FPRs accumulate at the leading edge of the migrating cell and are required for directional sensing (Zigmond *et al.*, 1981; Cassimeris & Zigmond, 1990). Saturation and desensitization of the receptor hinder the neutrophil, which may become nonresponsive to the chemoattractant, implying the importance of unoccupied receptors in sensing the chemotactic gradient. It is also suggested that receptor recycling is required for adequate cell migration. Most MPO-related research thus far has focused on the enzyme's physiological function in the phagosome, where it produces deleterious agents to aid in destruction of invading microorganisms (Klebanoff, 2005). One of those agents is the superoxide

anion, O_2^- (Babior *et al.*, 2002). It was demonstrated that inhibition of NADPH oxidase interferes with PMN chemotaxis (Hattori *et al.*, 2010). Interestingly, superoxide is almost immediately dismutated to H_2O_2, which in turn serves as an MPO substrate along with chloride ions (Graham *et al.*, 2007). These considerations gave rise to the possibility that limitation of MPO substrates may lead to impairment of chemotaxis. Hence, the question arose whether MPO also plays a role in chemotaxis. In this study, we identify a novel mechanism for MPO in mediating proper directional sensing.

The initial observation in support of such a mechanism was the loss of neutrophil chemotaxis toward fMLP upon inhibition of MPO. For screening purposes and to provide proof of concept, these early studies made use of differentiation of HL60 cells into neutrophils as an inexpensive *in vitro* model of PMN chemotactic behavior. In our studies, we inhibited myeloperoxidase activity in these primary neutrophils with 4-ABH (50 μM), a blocker of MPO (Kettle *et al.*, 1997), and the results demonstrated loss of chemotaxis in treated HL-60 cells. To show the physiological significance of these observations, we tested the chemotactic behavior of isolated PMNs. PMNs isolated from MPO $^{-/-}$ mice exhibited a striking loss of chemotaxis in an fMIFL gradient compared to WT counterparts, thus demonstrating for the first time the importance of MPO in PMN chemotaxis. The agonist fMIFL was used because it is the most potent chemoattractant in the case of murine PMNs, whereas fMLP is optimal in the human case (i.e., HL60 cells) (Southgate *et al.*, 2008). Although depletion of MPO impaired neutrophil chemotaxis, the cells still displayed responsiveness to the chemoattractant. In this

study, we noted the presence of agonist-induced chemokinesis (nonvectorial locomotion in response to a chemoattractant) in both WT and MPO impaired PMNs, suggesting that MPO deficiency did not ablate the FPR. Our binding data (see below) support such an inference.

Having provided *in vitro* and *ex vivo* data supporting the role of MPO expression in neutrophil chemotaxis, we obtained evidence for an *in vivo* role of MPO in two different models of neutrophil infiltration. The *in vivo* setting is rather different from the *in vitro* or *ex vivo* situations in that there is a barrier (i.e., the vessel wall) interposed between the vascular neutrophil and the chemotactic source (Harris, 1954; Wong *et al.*, 2010). However, there is abundant evidence in the literature that chemoattractants, including formyl peptides, not only attract PMNs but also activate them, thus promoting adhesion to and migration across the vessel wall (Harris, 1954; Le *et al.*, 2002; Wong *et al.*, 2010). As expected, we showed that i.p. fMIFL attracted substantially fewer migratory neutrophils to the peritoneal cavity in MPO deficient mice than in the WT counterpart. It was not clear whether this result could be attributed to differences in the size of circulating PMN populations in WT and MPO deficient mice, since those data are not available for the mouse to our knowledge. However, in a second experimental series, we circumvented this problem by injecting known numbers of labeled PMNs into the circulation via the jugular. All of the injected PMNs must initially pass through the pulmonary vascular bed before reaching the systemic circulation. We determined neutrophil migration by recovering labeled neutrophils from BALF after baiting them with i.t. fMIFL. As significantly fewer migratory neutrophils with MPO

deficiency were recovered relative to the control, the results clearly confirmed that MPO deficiency impairs PMN migration *in vivo*.

It is well known that neutrophil adhesion to substratum is necessary for normal cell motility. Migrating neutrophils cyclically attach to substrate at the leading edge and detach at the trailing edge to pull themselves toward a chemoattractant (Cassimeris & Zigmond, 1990; Munevar *et al.*, 2001). Therefore, we conducted *in vitro* experiments to see if MPO genetic deletion alters the adhesiveness of PMNs to the endothelial monolayer. These experiments showed that MPO deletion did not affect adhesion of mouse neutrophils to the endothelial monolayer. The experimental system used had the virtue of permitting direct observation of PMN adhesion, but did not address the *in vivo* relevance of the observations. Therefore, additional experiments were conducted to see if MPO deficits affect binding of autologous PMNs to mouse lung microvessels. In the studies conducted, we introduced labeled PMNs via the jugular vein and subsequently collected the lung tissue after purging unbound neutrophils from the pulmonary circulation. This protocol assured that only firmly bound PMNs were counted. We found that binding of labeled PMNs to lung microvessels was statistically indistinguishable between WT and $MPO^{-/-}$ mice, thus confirming that MPO deficiency does not impair binding of PMNs *in vivo*. Hence, this mechanism is ruled out as a potential explanation for the strong impact of MPO impairment on PMN chemotaxis.

Another possible explanation that was not excluded related to the fact that the chemoattractant fMIFL contains a formyl-methionine group that is susceptible

to oxidation (Rosen *et al.*, 2009). We therefore tested if MPO deletion affects the response to a selective peptide agonist of FPR lacking methionine. In the experiments conducted, we compared the chemoattractant properties of NLE toward WT and MPO deficient mouse PMNs. Interestingly, MPO deletion did not affect the migration of PMNs in an NLE gradient, thus indicating that MPO activity affects responses only to formyl-methionine containing agonists. These findings also imply that MPO most likely acted by modifying the agonist through oxidation rather than by impairing the FPR receptor.

We next investigated if MPO deletion modifies receptor binding properties, by conducting binding assay on isolated mouse PMNs using iodinated fMIFL. These studies demonstrated that FPRs in MPO deficient cells have 10-fold higher agonist affinity than the WT control. The binding maxima and slope of the steepest region of the curves were statistically indistinguishable in WT and MPO depleted cells, suggesting that the effect of MPO deletion on receptor function was limited to affinity increase. We can also ruled out differences in receptor internalization, which is well known to occur at physiologic temperature (Hofman *et al.*, 2010), since the binding studies were conducted at a temperature of 4°C. The observation of increased agonist affinity in the MPO $^{-/-}$ cells has an important functional implication. In a chemotactic gradient, a higher affinity receptor will saturate sooner and thereby impair chemotaxis. This inference is borne out by the dose-response curve of calcium mobilization, which was also shifted to the left by a factor of 10 in the MPO$^{-/-}$ cells and was thus observed to saturate sooner (see Fig. 15). As expected, MPO deletion failed to alter dose-response curves to the FPR agonist NLE

in the calcium mobilization assay. This observation confirms the importance of the methionine residue in agonists such as fMIFL in the effects of MPO deletion.

Lastly, to establish the importance of MPO in the oxidation of methionine residues, we performed mass spectroscopy to quantify oxidation of fMIFL by WT or $MPO^{-/-}$ PMNs. In these studies, we incubated identical numbers of WT or $MPO^{-/-}$ PMNs with fMIFL. By using this technique, we were able to verify that WT PMNs indeed oxidize fMIFL and that MPO deficiency substantially curtails oxidation by a factor of about 10. We infer from these direct observations that hypochlorous acid (HOCl), the chief product of the MPO system, is required for efficient formyl-methionine oxidation to formyl-methionine sulfoxide in PMNs. These results are physiologically important because it is well established in the literature that, once agonist is oxidized, it loses activity at the FPR (Clark *et al.*, 1980; Rosen *et al.*, 2009). In principle, the oxidized agonist, which was reported in the above studies to function as an extremely weak partial agonist, might be expected to contribute to a rightward shift in the dose-response curve for formyl peptide agonists. Such an explanation dovetails with the results of our calcium mobilization assay, in which the WT curve was shifted to the right.

In conclusion, we examined the molecular properties of MPO as they affect neutrophil chemotaxis, calcium mobilization, formyl-methionine oxidation, and FPR binding affinity both *in vitro* and *in vivo*. MPO is necessary for and mediates sustained neutrophil chemotaxis by helping to maintain FPR availability. MPO proves to be important in chemotaxis only when challenged with a methionine containing N-formyl peptide. The present study demonstrated that HOCl is the

likely agent of methionine oxidation that inactivates FPR agonists. The probable function of oxidation of methionine containing agonists may be to prevent premature saturation of FPRs in a chemotactic gradient, thereby facilitating the bacterial clearance function of PMNs.

REFERENCES

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA & Weisman GA. (2006). International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacological reviews* **58,** 281-341.

Adler J. (1969). Chemoreceptors in bacteria. *Science* **166,** 1588-1597.

Adler J, Hazelbauer GL & Dahl MM. (1973). Chemotaxis toward sugars in Escherichia coli. *Journal of bacteriology* **115,** 824-847.

Agner K. (1947). Detoxicating effect of verdoperoxidase on toxins. *Nature* **159,** 271.

Agner K. (1958). Crystalline myeloperoxidase. . *Acta Chem Scand A* **12,** 89-94.

Arcaro A & Wymann MP. (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *The Biochemical journal* **296 (Pt 2),** 297-301.

Aswad D & Koshland DE, Jr. (1975). Isolation, characterization and complementation of Salmonella typhimurium chemotaxis mutants. *Journal of molecular biology* **97,** 225-235.

Aswanikumar S, Corcoran B, Schiffmann E, Day AR, Freer RJ, Showell HJ & Becker EL. (1977). Demonstration of a receptor on rabbit neutrophils for chemotactic peptides. *Biochemical and biophysical research communications* **74,** 810-817.

Babior BM. (2004). NADPH oxidase. *Current opinion in immunology* **16,** 42-47.

Babior BM, Lambeth JD & Nauseef W. (2002). The neutrophil NADPH oxidase. *Archives of biochemistry and biophysics* **397,** 342-344.

Baggiolini M, Horisberger U, Gennaro R & Dewald B. (1985). Identification of three types of granules in neutrophils of ruminants. Ultrastructure of circulating and maturing cells. *Laboratory investigation; a journal of technical methods and pathology* **52,** 151-158.

Benard V, Bohl BP & Bokoch GM. (1999). Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *The Journal of biological chemistry* **274,** 13198-13204.

Bentwood BJ & Henson PM. (1980). The sequential release of granule constitutents from human neutrophils. *Journal of immunology* **124,** 855-862.

Blocker A, Severin FF, Burkhardt JK, Bingham JB, Yu H, Olivo JC, Schroer TA, Hyman AA & Griffiths G. (1997). Molecular requirements for bi-directional movement of phagosomes along microtubules. *The Journal of cell biology* **137,** 113-129.

Blumenreich MS. (1990). The White Blood Cell and Differential Count. In *Clinical Methods: The History, Physical, and Laboratory Examinations*, 3rd edn, ed. Walker HK, Hall WD & Hurst JW. Boston.

Bokoch GM. (1995). Chemoattractant signaling and leukocyte activation. *Blood* **86,** 1649-1660.

Bokoch GM. (2005). Regulation of innate immunity by Rho GTPases. *Trends in cell biology* **15,** 163-171.

Bokoch GM, Bohl BP & Chuang TH. (1994). Guanine nucleotide exchange regulates membrane translocation of Rac/Rho GTP-binding proteins. *The Journal of biological chemistry* **269,** 31674-31679.

Bokoch GM & Diebold BA. (2002). Current molecular models for NADPH oxidase regulation by Rac GTPase. *Blood* **100,** 2692-2696.

Borregaard N. (2010). Neutrophils, from marrow to microbes. *Immunity* **33,** 657-670.

Borregaard N & Cowland JB. (1997). Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89,** 3503-3521.

Borregaard N, Miller LJ & Springer TA. (1987). Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. *Science* **237,** 1204-1206.

Borregaard N, Sorensen OE & Theilgaard-Monch K. (2007). Neutrophil granules: a library of innate immunity proteins. *Trends in immunology* **28,** 340-345.

Boxio R, Bossenmeyer-Pourie C, Steinckwich N, Dournon C & Nusse O. (2004). Mouse bone marrow contains large numbers of functionally competent neutrophils. *Journal of leukocyte biology* **75,** 604-611.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y & Zychlinsky A. (2004). Neutrophil extracellular traps kill bacteria. *Science* **303,** 1532-1535.

Burner U, Obinger C, Paumann M, Furtmuller PG & Kettle AJ. (1999). Transient and steady-state kinetics of the oxidation of substituted benzoic acid hydrazides by myeloperoxidase. *The Journal of biological chemistry* **274,** 9494-9502.

Carp H. (1982). Mitochondrial N-formylmethionyl proteins as chemoattractants for neutrophils. *The Journal of experimental medicine* **155,** 264-275.

Cassimeris L & Zigmond SH. (1990). Chemoattractant stimulation of polymorphonuclear leucocyte locomotion. *Seminars in cell biology* **1,** 125-134.

Chen Y, Shukla A, Namiki S, Insel PA & Junger WG. (2004). A putative osmoreceptor system that controls neutrophil function through the release of ATP, its conversion to adenosine, and activation of A2 adenosine and P2 receptors. *Journal of leukocyte biology* **76,** 245-253.

Chertov O, Yang D, Howard OM & Oppenheim JJ. (2000). Leukocyte granule proteins mobilize innate host defenses and adaptive immune responses. *Immunological reviews* **177,** 68-78.

Cicchetti G, Allen PG & Glogauer M. (2002). Chemotactic signaling pathways in neutrophils: from receptor to actin assembly. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists* **13,** 220-228.

Clark RA, Szot S, Venkatasubramanian K & Schiffmann E. (1980). Chemotactic factor inactivation by myeloperoxidase-mediated oxidation of methionine. *Journal of immunology* **124,** 2020-2026.

Claus V, Jahraus A, Tjelle T, Berg T, Kirschke H, Faulstich H & Griffiths G. (1998). Lysosomal enzyme trafficking between phagosomes, endosomes, and lysosomes in J774 macrophages. Enrichment of cathepsin H in early endosomes. *The Journal of biological chemistry* **273,** 9842-9851.

Coffer PJ, Geijsen N, M'Rabet L, Schweizer RC, Maikoe T, Raaijmakers JA, Lammers JW & Koenderman L. (1998). Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. *The Biochemical journal* **329 (Pt 1),** 121-130.

Dale DC, Boxer L & Liles WC. (2008). The phagocytes: neutrophils and monocytes. *Blood* **112,** 935-945.

Davies MJ, Hawkins CL, Pattison DI & Rees MD. (2008). Mammalian heme peroxidases: from molecular mechanisms to health implications. *Antioxidants & redox signaling* **10,** 1199-1234.

DeLeo FR, Goedken M, McCormick SJ & Nauseef WM. (1998). A novel form of hereditary myeloperoxidase deficiency linked to endoplasmic reticulum/proteasome degradation. *The Journal of clinical investigation* **101,** 2900-2909.

Desjardins M & Descoteaux A. (1997). Inhibition of phagolysosomal biogenesis by the Leishmania lipophosphoglycan. *The Journal of experimental medicine* **185,** 2061-2068.

Dharmawardhane S, Brownson D, Lennartz M & Bokoch GM. (1999). Localization of p21-activated kinase 1 (PAK1) to pseudopodia, membrane ruffles, and phagocytic cups in activated human neutrophils. *Journal of leukocyte biology* **66,** 521-527.

Dinauer MC, Pierce EA, Bruns GA, Curnutte JT & Orkin SH. (1990). Human neutrophil cytochrome b light chain (p22-phox). Gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. *The Journal of clinical investigation* **86,** 1729-1737.

Doerschuk CM. (2000). Leukocyte trafficking in alveoli and airway passages. *Respiratory research* **1,** 136-140.

Downey GP. (1994). Mechanisms of leukocyte motility and chemotaxis. *Current opinion in immunology* **6,** 113-124.

Dusenbery DB. (1998). Spatial sensing of stimulus gradients can be superior to temporal sensing for free-swimming bacteria. *Biophysical journal* **74,** 2272-2277.

Dusi S, Donini M & Rossi F. (1996). Mechanisms of NADPH oxidase activation: translocation of p40phox, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47phox or p67phox. *The Biochemical journal* **314 (Pt 2),** 409-412.

Edwards SW, Nurcombe HL & Hart CA. (1987). Oxidative inactivation of myeloperoxidase released from human neutrophils. *The Biochemical journal* **245,** 925-928.

Ehrengruber MU, Coates TD & Deranleau DA. (1995). Shape oscillations: a fundamental response of human neutrophils stimulated by chemotactic peptides? *FEBS letters* **359,** 229-232.

el-Hag A & Clark RA. (1987). Immunosuppression by activated human neutrophils. Dependence on the myeloperoxidase system. *Journal of immunology* **139,** 2406-2413.

el-Hag A, Lipsky PE, Bennett M & Clark RA. (1986). Immunomodulation by neutrophil myeloperoxidase and hydrogen peroxide: differential susceptibility of human lymphocyte functions. *Journal of immunology* **136,** 3420-3426.

Faurschou M & Borregaard N. (2003). Neutrophil granules and secretory vesicles in inflammation. *Microbes and infection / Institut Pasteur* **5,** 1317-1327.

Floris R & Wever R. (1992). Reaction of myeloperoxidase with its product HOCl. *European journal of biochemistry / FEBS* **207,** 697-702.

Frohlich D, Spertini O & Moser R. (1998). The Fcgamma receptor-mediated respiratory burst of rolling neutrophils to cytokine-activated, immune complex-bearing endothelial cells depends on L-selectin but not on E-selectin. *Blood* **91,** 2558-2564.

Furtmuller PG, Obinger C, Hsuanyu Y & Dunford HB. (2000). Mechanism of reaction of myeloperoxidase with hydrogen peroxide and chloride ion. *European journal of biochemistry / FEBS* **267,** 5858-5864.

Furtmuller PG, Zederbauer M, Jantschko W, Helm J, Bogner M, Jakopitsch C & Obinger C. (2006). Active site structure and catalytic mechanisms of human peroxidases. *Archives of biochemistry and biophysics* **445,** 199-213.

Gao JL, Chen H, Filie JD, Kozak CA & Murphy PM. (1998). Differential expansion of the N-formylpeptide receptor gene cluster in human and mouse. *Genomics* **51,** 270-276.

Gao JL, Lee EJ & Murphy PM. (1999). Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. *The Journal of experimental medicine* **189,** 657-662.

Garifulin O & Boyartchuk V. (2005). Listeria monocytogenes as a probe of immune function. *Briefings in functional genomics & proteomics* **4,** 258-269.

Graham DB, Robertson CM, Bautista J, Mascarenhas F, Diacovo MJ, Montgrain V, Lam SK, Cremasco V, Dunne WM, Faccio R, Coopersmith CM & Swat W. (2007). Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-PLCgamma2 signaling axis in mice. *The Journal of clinical investigation* **117,** 3445-3452.

Grandvaux N, Soucy-Faulkner A & Fink K. (2007). Innate host defense: Nox and Duox on phox's tail. *Biochimie* **89,** 1113-1122.

Grinstein S, Nanda A, Lukacs G & Rotstein O. (1992). V-ATPases in phagocytic cells. *The Journal of experimental biology* **172,** 179-192.

Gulden PH, Fischer P, 3rd, Sherman NE, Wang W, Engelhard VH, Shabanowitz J, Hunt DF & Pamer EG. (1996). A Listeria monocytogenes pentapeptide is presented to cytolytic T lymphocytes by the H2-M3 MHC class Ib molecule. *Immunity* **5,** 73-79.

Hampton MB, Kettle AJ & Winterbourn CC. (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92,** 3007-3017.

Harris H. (1954). Role of chemotaxis in inflammation. *Physiological reviews* **34,** 529-562.

Haslam RJ, Koide HB & Hemmings BA. (1993). Pleckstrin domain homology. *Nature* **363,** 309-310.

Hattori H, Subramanian KK, Sakai J, Jia Y, Li Y, Porter TF, Loison F, Sarraj B, Kasorn A, Jo H, Blanchard C, Zirkle D, McDonald D, Pai SY, Serhan CN & Luo HR. (2010). Small-molecule screen identifies reactive oxygen species as key regulators of neutrophil chemotaxis. *Proceedings of the National Academy of Sciences of the United States of America* **107,** 3546-3551.

He R, Tan L, Browning DD, Wang JM & Ye RD. (2000). The synthetic peptide Trp-Lys-Tyr-Met-Val-D-Met is a potent chemotactic agonist for mouse formyl peptide receptor. *Journal of immunology* **165,** 4598-4605.

Heyworth PG, Bohl BP, Bokoch GM & Curnutte JT. (1994). Rac translocates independently of the neutrophil NADPH oxidase components p47phox and p67phox. Evidence for its interaction with flavocytochrome b558. *The Journal of biological chemistry* **269,** 30749-30752.

Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F & Wymann MP. (2000). Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* **287,** 1049-1053.

Hofman EG, Bader AN, Voortman J, van den Heuvel DJ, Sigismund S, Verkleij AJ, Gerritsen HC & van Bergen en Henegouwen PM. (2010). Ligand-induced EGF receptor oligomerization is kinase-dependent and enhances internalization. *The Journal of biological chemistry* **285,** 39481-39489.

Hurley JV & Spector WG. (1965). A Topographical Study of Increased Vascular Permeability in Acute Turpentine-Induced Pleurisy. *The Journal of pathology and bacteriology* **89,** 245-254.

Jahraus A, Tjelle TE, Berg T, Habermann A, Storrie B, Ullrich O & Griffiths G. (1998). In vitro fusion of phagosomes with different endocytic organelles from J774 macrophages. *The Journal of biological chemistry* **273,** 30379-30390.

Jamuar MP & Cronkite EP. (1980). The fate of blood granulocytes. *Experimental hematology* **8,** 884-894.

Janetopoulos C & Firtel RA. (2008). Directional sensing during chemotaxis. *FEBS letters* **582,** 2075-2085.

Jannat RA, Robbins GP, Ricart BG, Dembo M & Hammer DA. (2010). Neutrophil adhesion and chemotaxis depend on substrate mechanics. *Journal of physics Condensed matter : an Institute of Physics journal* **22,** 194117.

Jesaitis AJ, Buescher ES, Harrison D, Quinn MT, Parkos CA, Livesey S & Linner J. (1990). Ultrastructural localization of cytochrome b in the membranes of resting and phagocytosing human granulocytes. *The Journal of clinical investigation* **85,** 821-835.

Kaibuchi K, Kuroda S & Amano M. (1999). Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annual review of biochemistry* **68,** 459-486.

Karlsson J, Fu H, Boulay F, Bylund J & Dahlgren C. (2006). The peptide Trp-Lys-Tyr-Met-Val-D-Met activates neutrophils through the formyl peptide receptor only when signaling through the formylpeptide receptor like 1 is blocked. A receptor switch with implications for signal transduction studies with inhibitors and receptor antagonists. *Biochemical pharmacology* **71,** 1488-1496.

Kawaguchi A, Ohmori M, Harada K, Tsuruoka S, Sugimoto K & Fujimura A. (2000). The effect of a Rho kinase inhibitor Y-27632 on superoxide production, aggregation and adhesion in human polymorphonuclear leukocytes. *European journal of pharmacology* **403,** 203-208.

Kent JD, Sergeant S, Burns DJ & McPhail LC. (1996). Identification and regulation of protein kinase C-delta in human neutrophils. *Journal of immunology* **157,** 4641-4647.

Kettle AJ, Gedye CA, Hampton MB & Winterbourn CC. (1995). Inhibition of myeloperoxidase by benzoic acid hydrazides. *The Biochemical journal* **308 (Pt 2),** 559-563.

Kettle AJ, Gedye CA & Winterbourn CC. (1997). Mechanism of inactivation of myeloperoxidase by 4-aminobenzoic acid hydrazide. *The Biochemical journal* **321 (Pt 2),** 503-508.

King CC, Jefferson MM & Thomas EL. (1997). Secretion and inactivation of myeloperoxidase by isolated neutrophils. *Journal of leukocyte biology* **61,** 293-302.

Kinkade JM, Jr., Pember SO, Barnes KC, Shapira R, Spitznagel JK & Martin LE. (1983). Differential distribution of distinct forms of myeloperoxidase in different azurophilic granule subpopulations from human neutrophils. *Biochemical and biophysical research communications* **114,** 296-303.

Kjeldsen L, Bjerrum OW, Askaa J & Borregaard N. (1992). Subcellular localization and release of human neutrophil gelatinase, confirming the existence of separate gelatinase-containing granules. *The Biochemical journal* **287 (Pt 2),** 603-610.

Klebanoff SJ. (1968). Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *Journal of bacteriology* **95,** 2131-2138.

Klebanoff SJ. (1970). Myeloperoxidase: contribution to the microbicidal activity of intact leukocytes. *Science* **169,** 1095-1097.

Klebanoff SJ. (2005). Myeloperoxidase: friend and foe. *Journal of leukocyte biology* **77,** 598-625.

Knall C, Worthen GS & Johnson GL. (1997). Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 3052-3057.

Knall C, Young S, Nick JA, Buhl AM, Worthen GS & Johnson GL. (1996). Interleukin-8 regulation of the Ras/Raf/mitogen-activated protein kinase pathway in human neutrophils. *The Journal of biological chemistry* **271,** 2832-2838.

Kobayashi SD, Voyich JM & DeLeo FR. (2003). Regulation of the neutrophil-mediated inflammatory response to infection. *Microbes and infection / Institut Pasteur* **5,** 1337-1344.

Laudanna C, Mochly-Rosen D, Liron T, Constantin G & Butcher EC. (1998). Evidence of zeta protein kinase C involvement in polymorphonuclear neutrophil integrin-dependent adhesion and chemotaxis. *The Journal of biological chemistry* **273,** 30306-30315.

Le Y, Gong W, Li B, Dunlop NM, Shen W, Su SB, Ye RD & Wang JM. (1999). Utilization of two seven-transmembrane, G protein-coupled receptors, formyl peptide receptor-like 1 and formyl peptide receptor, by the synthetic hexapeptide WKYMVm for human phagocyte activation. *Journal of immunology* **163,** 6777-6784.

Le Y, Murphy PM & Wang JM. (2002). Formyl-peptide receptors revisited. *Trends in immunology* **23,** 541-548.

Lefkowitz DL, Gelderman MP, Fuhrmann SR, Graham S, Starnes JD, 3rd, Lefkowitz SS, Bollen A & Moguilevsky N. (1999). Neutrophilic myeloperoxidase-macrophage interactions perpetuate chronic inflammation associated with experimental arthritis. *Clinical immunology* **91,** 145-155.

Lefkowitz DL, Mills K, Morgan D & Lefkowitz SS. (1992). Macrophage activation and immunomodulation by myeloperoxidase. *Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine* **199,** 204-210.

Lefkowitz DL, Mills KC, Moguilevsky N, Bollen A, Vaz A & Lefkowitz SS. (1993). Regulation of macrophage function by human recombinant myeloperoxidase. *Immunology letters* **36,** 43-49.

Lefkowitz SS, Gelderman MP, Lefkowitz DL, Moguilevsky N & Bollen A. (1996). Phagocytosis and intracellular killing of Candida albicans by macrophages exposed to myeloperoxidase. *The Journal of infectious diseases* **173,** 1202-1207.

Li Z, Hannigan M, Mo Z, Liu B, Lu W, Wu Y, Smrcka AV, Wu G, Li L, Liu M, Huang CK & Wu D. (2003). Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. *Cell* **114,** 215-227.

Li Z, Jiang H, Xie W, Zhang Z, Smrcka AV & Wu D. (2000). Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science* **287,** 1046-1049.

Liberles SD, Horowitz LF, Kuang D, Contos JJ, Wilson KL, Siltberg-Liberles J, Liberles DA & Buck LB. (2009). Formyl peptide receptors are candidate chemosensory receptors in the vomeronasal organ. *Proceedings of the National Academy of Sciences of the United States of America* **106,** 9842-9847.

Lincoln JA, Lefkowitz DL, Cain T, Castro A, Mills KC, Lefkowitz SS, Moguilevsky N & Bollen A. (1995). Exogenous myeloperoxidase enhances bacterial phagocytosis and intracellular killing by macrophages. *Infection and immunity* **63,** 3042-3047.

Ling N, Minick S & Guillemin R. (1978). Amino-terminal extension analogs of methionine-enkephalin. *Biochemical and biophysical research communications* **83,** 565-570.

Loitto VM, Rasmusson B & Magnusson KE. (2001). Assessment of neutrophil N-formyl peptide receptors by using antibodies and fluorescent peptides. *Journal of leukocyte biology* **69,** 762-771.

Machesky LM & Gould KL. (1999). The Arp2/3 complex: a multifunctional actin organizer. *Current opinion in cell biology* **11,** 117-121.

Machesky LM & Insall RH. (1998). Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Current biology : CB* **8,** 1347-1356.

Marasco WA, Phan SH, Krutzsch H, Showell HJ, Feltner DE, Nairn R, Becker EL & Ward PA. (1984). Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by Escherichia coli. *The Journal of biological chemistry* **259,** 5430-5439.

McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CC, Beck PL, Muruve DA & Kubes P. (2010). Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science* **330,** 362-366.

Mellman I. (1992). The importance of being acid: the role of acidification in intracellular membrane traffic. *The Journal of experimental biology* **172,** 39-45.

Mollinedo F, Nakajima M, Llorens A, Barbosa E, Callejo S, Gajate C & Fabra A. (1997). Major co-localization of the extracellular-matrix degradative enzymes heparanase and gelatinase in tertiary granules of human neutrophils. *The Biochemical journal* **327 (Pt 3),** 917-923.

Mukherjee S, Ghosh RN & Maxfield FR. (1997). Endocytosis. *Physiological reviews* **77,** 759-803.

Munevar S, Wang YL & Dembo M. (2001). Distinct roles of frontal and rear cell-substrate adhesions in fibroblast migration. *Molecular biology of the cell* **12,** 3947-3954.

Nathan C. (2002). Points of control in inflammation. *Nature* **420,** 846-852.

Nathan C. (2006). Neutrophils and immunity: challenges and opportunities. *Nature reviews Immunology* **6,** 173-182.

Neptune ER, Iiri T & Bourne HR. (1999). Galphai is not required for chemotaxis mediated by Gi-coupled receptors. *The Journal of biological chemistry* **274,** 2824-2828.

Nicolaides AN. (2005). Chronic venous disease and the leukocyte-endothelium interaction: from symptoms to ulceration. *Angiology* **56 Suppl 1,** S11-19.

Niedergang F & Chavrier P. (2004). Signaling and membrane dynamics during phagocytosis: many roads lead to the phagos(R)ome. *Current opinion in cell biology* **16,** 422-428.

Niggli V. (1999). Rho-kinase in human neutrophils: a role in signalling for myosin light chain phosphorylation and cell migration. *FEBS letters* **445,** 69-72.

Niggli V & Keller H. (1997). The phosphatidylinositol 3-kinase inhibitor wortmannin markedly reduces chemotactic peptide-induced locomotion and increases in cytoskeletal actin in human neutrophils. *European journal of pharmacology* **335,** 43-52.

Nijhuis E, Lammers JW, Koenderman L & Coffer PJ. (2002). Src kinases regulate PKB activation and modulate cytokine and chemoattractant-controlled neutrophil functioning. *Journal of leukocyte biology* **71,** 115-124.

O'Brien PJ. (2000). Peroxidases. *Chemico-biological interactions* **129,** 113-139.

Pamer EG, Wang CR, Flaherty L, Lindahl KF & Bevan MJ. (1992). H-2M3 presents a Listeria monocytogenes peptide to cytotoxic T lymphocytes. *Cell* **70,** 215-223.

Parent CA & Devreotes PN. (1999). A cell's sense of direction. *Science* **284,** 765-770.

Perez HD, Elfman F, Lobo E, Sklar L, Chenoweth D & Hooper C. (1986). A derivative of wheat germ agglutinin specifically inhibits formyl-peptide-induced polymorphonuclear leukocyte chemotaxis by blocking re-expression (or recycling) of receptors. *Journal of immunology* **136,** 1803-1812.

Philips MR, Feoktistov A, Pillinger MH & Abramson SB. (1995). Translocation of p21rac2 from cytosol to plasma membrane is neither necessary nor sufficient for neutrophil NADPH oxidase activity. *The Journal of biological chemistry* **270,** 11514-11521.

Pryzwansky KB & Breton-Gorius J. (1985). Identification of a subpopulation of primary granules in human neutrophils based upon maturation and distribution. Study by transmission electron microscopy cytochemistry and high voltage electron microscopy of whole cell preparations. *Laboratory investigation; a journal of technical methods and pathology* **53,** 664-671.

Quinn MT, Evans T, Loetterle LR, Jesaitis AJ & Bokoch GM. (1993). Translocation of Rac correlates with NADPH oxidase activation. Evidence for equimolar translocation of oxidase components. *The Journal of biological chemistry* **268,** 20983-20987.

Rabinowitz S, Horstmann H, Gordon S & Griffiths G. (1992). Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages. *The Journal of cell biology* **116,** 95-112.

Rice WG, Ganz T, Kinkade JM, Jr., Selsted ME, Lehrer RI & Parmley RT. (1987). Defensin-rich dense granules of human neutrophils. *Blood* **70,** 757-765.

Rickert M, Boulanger MJ, Goriatcheva N & Garcia KC. (2004). Compensatory energetic mechanisms mediating the assembly of signaling complexes between interleukin-2 and its alpha, beta, and gamma(c) receptors. *Journal of molecular biology* **339,** 1115-1128.

Riviere S, Challet L, Fluegge D, Spehr M & Rodriguez I. (2009). Formyl peptide receptor-like proteins are a novel family of vomeronasal chemosensors. *Nature* **459,** 574-577.

Roberts AW, Kim C, Zhen L, Lowe JB, Kapur R, Petryniak B, Spaetti A, Pollock JD, Borneo JB, Bradford GB, Atkinson SJ, Dinauer MC & Williams DA. (1999). Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity* **10,** 183-196.

Romani L, Mencacci A, Cenci E, Puccetti P & Bistoni F. (1996). Neutrophils and the adaptive immune response to Candida albicans. *Research in immunology* **147,** 512-518.

Rosen H, Klebanoff SJ, Wang Y, Brot N, Heinecke JW & Fu X. (2009). Methionine oxidation contributes to bacterial killing by the myeloperoxidase system of neutrophils. *Proceedings of the National Academy of Sciences of the United States of America* **106,** 18686-18691.

Rot A, Henderson LE, Copeland TD & Leonard EJ. (1987). A series of six ligands for the human formyl peptide receptor: tetrapeptides with high chemotactic potency and efficacy. *Proceedings of the National Academy of Sciences of the United States of America* **84,** 7967-7971.

Rot A, Henderson LE & Leonard EJ. (1986). Staphylococcus aureus-derived chemoattractant activity for human monocytes. *Journal of leukocyte biology* **40,** 43-53.

Rot A, Henderson LE, Sowder R & Leonard EJ. (1989). Staphylococcus aureus tetrapeptide with high chemotactic potency and efficacy for human leukocytes. *Journal of leukocyte biology* **45,** 114-120.

Sanders LC, Matsumura F, Bokoch GM & de Lanerolle P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. *Science* **283,** 2083-2085.

Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-dos-Santos AJ, Stanford WL, Bolon B, Wakeham A, Itie A, Bouchard D, Kozieradzki I, Joza N, Mak TW, Ohashi PS, Suzuki A & Penninger JM. (2000). Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* **287,** 1040-1046.

Schieven GL, de Fex H & Stephenson L. (2002). Hypochlorous acid activates tyrosine phosphorylation signal pathways leading to calcium signaling and TNFalpha production. *Antioxidants & redox signaling* **4,** 501-507.

Schiffmann E, Corcoran BA & Wahl SM. (1975a). N-formylmethionyl peptides as chemoattractants for leucocytes. *Proceedings of the National Academy of Sciences of the United States of America* **72,** 1059-1062.

Schiffmann E, Showell HV, Corcoran BA, Ward PA, Smith E & Becker EL. (1975b). The isolation and partial characterization of neutrophil chemotactic factors from Escherichia coli. *Journal of immunology* **114,** 1831-1837.

Schoonbroodt S, Legrand-Poels S, Best-Belpomme M & Piette J. (1997). Activation of the NF-kappaB transcription factor in a T-lymphocytic cell line by hypochlorous acid. *The Biochemical journal* **321 (Pt 3),** 777-785.

Schultz J. (1958). Myeloperoxidase. *Annals of the New York Academy of Sciences* **75,** 22-30.

Schultz J, Corlin R, Oddi F, Kaminker K & Jones W. (1965). Myeloperoxidase of the leucocyte of normal human blood. 3. Isolation of the peroxidase granule. *Archives of biochemistry and biophysics* **111,** 73-79.

Schultz J & Kaminker K. (1962). Myeloperoxidase of the leucocyte of normal human blood. I. Content and localization. *Archives of biochemistry and biophysics* **96,** 465-467.

Schultz J & Shmukler HW. (1964). Myeloperoxidase of the Leucocyte of Normal Human Blood. Ii. Isolation, Spectrophotometry, and Amino Acid Analysis. *Biochemistry* **3,** 1234-1238.

Schwiebert EM & Zsembery A. (2003). Extracellular ATP as a signaling molecule for epithelial cells. *Biochimica et biophysica acta* **1615,** 7-32.

Sengelov H, Kjeldsen L & Borregaard N. (1993). Control of exocytosis in early neutrophil activation. *Journal of immunology* **150,** 1535-1543.

Serrador JM, Nieto M, Alonso-Lebrero JL, del Pozo MA, Calvo J, Furthmayr H, Schwartz-Albiez R, Lozano F, Gonzalez-Amaro R, Sanchez-Mateos P & Sanchez-Madrid F. (1998). CD43 interacts with moesin and ezrin and regulates its redistribution to the uropods of T lymphocytes at the cell-cell contacts. *Blood* **91,** 4632-4644.

Shepherd VL & Hoidal JR. (1990). Clearance of neutrophil-derived myeloperoxidase by the macrophage mannose receptor. *American journal of respiratory cell and molecular biology* **2,** 335-340.

Sheppard FR, Kelher MR, Moore EE, McLaughlin NJ, Banerjee A & Silliman CC. (2005). Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. *Journal of leukocyte biology* **78,** 1025-1042.

Simmons A, Leaverton P & Elbert G. (1974). Normal laboratory values for differential white cell counts established by manual and automated cytochemical methods (Hemalog D-TM). *Journal of clinical pathology* **27,** 55-58.

Simon SI, Burns AR, Taylor AD, Gopalan PK, Lynam EB, Sklar LA & Smith CW. (1995). L-selectin (CD62L) cross-linking signals neutrophil adhesive functions via the Mac-1 (CD11b/CD18) beta 2-integrin. *Journal of immunology* **155,** 1502-1514.

Sinha S, Watorek W, Karr S, Giles J, Bode W & Travis J. (1987). Primary structure of human neutrophil elastase. *Proceedings of the National Academy of Sciences of the United States of America* **84,** 2228-2232.

Southgate EL, He RL, Gao JL, Murphy PM, Nanamori M & Ye RD. (2008). Identification of formyl peptides from Listeria monocytogenes and Staphylococcus aureus as potent chemoattractants for mouse neutrophils. *Journal of immunology* **181,** 1429-1437.

Spudich JL & Koshland DE, Jr. (1975). Quantitation of the sensory response in bacterial chemotaxis. *Proceedings of the National Academy of Sciences of the United States of America* **72,** 710-713.

Stasia MJ, Jouan A, Bourmeyster N, Boquet P & Vignais PV. (1991). ADP-ribosylation of a small size GTP-binding protein in bovine neutrophils by the C3 exoenzyme of Clostridium botulinum and effect on the cell motility. *Biochemical and biophysical research communications* **180,** 615-622.

Steinman RM, Mellman IS, Muller WA & Cohn ZA. (1983). Endocytosis and the recycling of plasma membrane. *The Journal of cell biology* **96,** 1-27.

Stephens L, Milne L & Hawkins P. (2008). Moving towards a better understanding of chemotaxis. *Current biology : CB* **18,** R485-494.

Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweis PC & Hawkins PT. (1994). A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. *Cell* **77,** 83-93.

Stephens LR, Eguinoa A, Erdjument-Bromage H, Lui M, Cooke F, Coadwell J, Smrcka AS, Thelen M, Cadwallader K, Tempst P & Hawkins PT. (1997). The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell* **89,** 105-114.

Stossel TP. (1994). The machinery of cell crawling. *Scientific American* **271,** 54-55, 58-63.

Suire S, Coadwell J, Ferguson GJ, Davidson K, Hawkins P & Stephens L. (2005). p84, a new Gbetagamma-activated regulatory subunit of the type IB phosphoinositide 3-kinase p110gamma. *Current biology : CB* **15,** 566-570.

Takano T, Fiore S, Maddox JF, Brady HR, Petasis NA & Serhan CN. (1997). Aspirin-triggered 15-epi-lipoxin A4 (LXA4) and LXA4 stable analogues are potent inhibitors of acute inflammation: evidence for anti-inflammatory receptors. *The Journal of experimental medicine* **185,** 1693-1704.

Tapper H, Karlsson A, Morgelin M, Flodgaard H & Herwald H. (2002). Secretion of heparin-binding protein from human neutrophils is determined by its localization in azurophilic granules and secretory vesicles. *Blood* **99,** 1785-1793.

Tiffany HL, Gao JL, Roffe E, Sechler JM & Murphy PM. (2011). Characterization of Fpr-rs8, an atypical member of the mouse formyl peptide receptor gene family. *Journal of innate immunity* **3,** 519-529.

Underhill DM & Ozinsky A. (2002). Phagocytosis of microbes: complexity in action. *Annual review of immunology* **20,** 825-852.

Van Epps DE, Simpson S, Bender JG & Chenoweth DE. (1990). Regulation of C5a and formyl peptide receptor expression on human polymorphonuclear leukocytes. *Journal of immunology* **144,** 1062-1068.

Vieira OV, Botelho RJ & Grinstein S. (2002). Phagosome maturation: aging gracefully. *The Biochemical journal* **366,** 689-704.

Wagner JG & Roth RA. (2000). Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharmacological reviews* **52,** 349-374.

Ward PA & Lentsch AB. (1999). The acute inflammatory response and its regulation. *Archives of surgery* **134,** 666-669.

Witko-Sarsat V & Descamps-Latscha B. (1994). Neutrophil-derived Oxidants and Proteinases as Immunomodulatory Mediators in Inflammation. *Mediators of inflammation* **3,** 257-273.

Wong CH, Heit B & Kubes P. (2010). Molecular regulators of leucocyte chemotaxis during inflammation. *Cardiovascular research* **86,** 183-191.

Worthen GS, Avdi N, Buhl AM, Suzuki N & Johnson GL. (1994). FMLP activates Ras and Raf in human neutrophils. Potential role in activation of MAP kinase. *The Journal of clinical investigation* **94,** 815-823.

Xu J, Wang F, Van Keymeulen A, Herzmark P, Straight A, Kelly K, Takuwa Y, Sugimoto N, Mitchison T & Bourne HR. (2003). Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. *Cell* **114,** 201-214.

Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, Serhan CN & Murphy PM. (2009). International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacological reviews* **61,** 119-161.

Ye RD, Cavanagh SL, Quehenberger O, Prossnitz ER & Cochrane CG. (1992). Isolation of a cDNA that encodes a novel granulocyte N-formyl peptide receptor. *Biochemical and biophysical research communications* **184,** 582-589.

Yoshinaga-Ohara N, Takahashi A, Uchiyama T & Sasada M. (2002). Spatiotemporal regulation of moesin phosphorylation and rear release by Rho and serine/threonine phosphatase during neutrophil migration. *Experimental cell research* **278,** 112-122.

Zaas AK & Schwartz DA. (2005). Innate immunity and the lung: defense at the interface between host and environment. *Trends in cardiovascular medicine* **15,** 195-202.

Zarbock A & Ley K. (2008). Mechanisms and consequences of neutrophil interaction with the endothelium. *The American journal of pathology* **172,** 1-7.

Zhang P, Summer WR, Bagby GJ & Nelson S. (2000). Innate immunity and pulmonary host defense. *Immunological reviews* **173,** 39-51.

Zheng L, Eckerdal J, Dimitrijevic I & Andersson T. (1997). Chemotactic peptide-induced activation of Ras in human neutrophils is associated with inhibition of p120-GAP activity. *The Journal of biological chemistry* **272,** 23448-23454.

Zigmond SH. (1977). Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *The Journal of cell biology* **75,** 606-616.

Zigmond SH. (1980). Polymorphonuclear leucocyte chemotaxis: detection of the gradient and development of cell polarity. *Ciba Foundation symposium* **71,** 299-311.

Zigmond SH, Joyce M, Borleis J, Bokoch GM & Devreotes PN. (1997). Regulation of actin polymerization in cell-free systems by GTPgammaS and Cdc42. *The Journal of cell biology* **138,** 363-374.

Zigmond SH, Levitsky HI & Kreel BJ. (1981). Cell polarity: an examination of its behavioral expression and its consequences for polymorphonuclear leukocyte chemotaxis. *The Journal of cell biology* **89,** 585-592.

Zu YL, Qi J, Gilchrist A, Fernandez GA, Vazquez-Abad D, Kreutzer DL, Huang CK & Sha'afi RI. (1998). p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-alpha or FMLP stimulation. *Journal of immunology* **160,** 1982-1989.

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