Rac2 is Required for Formation of Extracellular Traps in Neutrophils

by

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Faculty of Dentistry
University of Toronto

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Abstract

Recently, it was found that pathogens are trapped and killed by neutrophil extracellular traps (NETs). The role of Rac small GTPases is explored in the formation of NET using neutrophils lacking Rac1, Rac2 or both isoforms. NET formation was observed in both wild-type and Rac1-null neutrophils. In contrast, NET formation was markedly impaired in cells lacking either Rac2 or both Rac2 and Rac1. The defect in NET formation in Rac2-null cells was rescued in the presence of exogenous reactive oxygen species sources, suggesting that Rac2-mediated ROS generation is required. In addition, the role of nitric oxide in NET formation is assessed. Blocking NO production with the nitric oxide synthase inhibitor L-NAME significantly reduced NET formation. Moreover, Rac2-null cells produced significantly less NO than Rac1-null cells or their wild type counterparts. Our data suggest that Rac2 is essential for NET formation via pathways involving both ROS and NO.
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\\textbf{Abbreviations}

**NETs**: Neutrophil Extracellular Traps  
**NO**: Nitric oxide  
**NOS**: Nitric oxide synthase  
**ROS**: Reactive oxygen species  
**NOX**: NADPH oxidase  
**PMA**: Phorbol 12-myristate 13-acetate  
**LPS**: Lipopolysaccharide  
**TEM**: Transmission electron microscopy  
**SEM**: Scanning electron microscopy  
**IF**: Immunofluorescence  
**STBM**: Syncytiotrophoblast microvillus membrane microparticles  
**LDH**: Lactate dehydrogenase  
**DAF-2DA**: 4,5-Diaminofluorescein Diacetate  
**DPI**: Diphenylene iodonium  
**TLR**: Toll-like receptor  
**PKC**: Protein kinase C  
**L-NAME**: L-NG-Nitroarginine methyl ester
**G-CSF**: Granulocyte colony stimulating factor

**MPO**: Myeloperoxidase

**TGN**: Trans-Golgi network

**L-selectin**: Leukocyte selectin

**PAF**: Platelet-activating factor

**CAMs**: Cell adhesion molecules of the Ig-superfamily

**PECAM-1**: Platelet endothelial cell adhesion molecule-1

**JAM**: Junctional adhesion molecule

**ITAMS**: Immunoreceptor tyrosine-based activation motifs

**SOD**: Superoxide dismutase

**HOCl**: Hypohalous acid
1. Neutrophils

1.1 Origin of neutrophils

Neutrophils are the most abundant type of white blood cells in mammals. They originate from a common population of hematopoietic stem cells in the bone marrow. These hematopoietic stem cells are pluripotent and can self-replicate to become uncommitted stem cells, or can differentiate into stem cells with a more limited proliferative potential called progenitor cells. Progenitor cells are far more specific than stem cells because they are destined to differentiate into their “target” cell type through the expression of specific hematopoietic growth factor and cytokine receptors.

1.2 Development of neutrophils

Formation of granulocytes is denoted by the term Granulopoiesis in which the main cytokine, Granulocyte colony stimulating factor (G-CSF) plays a crucial role in proliferation and differentiation of neutrophils in the bone marrow. Neutrophil differentiation is driven primarily by the cytokine granulocyte-colony stimulating factor (G-CSF) which influences the proliferation, survival, maturation and functional activation of neutrophils [1, 2]. A study by Ward et al. [3] clearly showed that the absence of G-CSF causes an increased proportion of neutrophil progenitors undergoing apoptotic cell death, resulting in a significant reduction
in the number of mature neutrophils found in the bone marrow and in the circulation. Hence, G-CSF plays a crucial role in the terminal differentiation of neutrophils.

Development of neutrophils in the bone marrow has classically been divided into five stages (myeloblast, promyelocyte, myelocyte, metamyelocyte, and polymorphonuclear stage) based on cell size, nuclear morphology, and granule content [4].

i. **Myeloblast stage**

In the myeloblast stage of neutrophil development, the myeloblast cells are found to have large, round or oval nuclei with a small amount of cytoplasm. No condensation of chromatin is observed and only 2-5 nucleoli are present. At this stage, granules are absent in the cytoplasm.

ii. **Promyelocyte stage**

In the promyelocyte stage of development, cells are larger than myeloblasts. The nuclei of promyelocytes still retain a round or oval shape, and the nuclear chromatin is diffuse, as in the myeloblast. Primary granules appear at this stage, but secondary granules are not present. The formation of primary granules takes place only at this stage. Consequently, the number of primary granules decreases with each subsequent cell division so that in mature neutrophils, secondary granules are two to three times more numerous than primary granules. Budding of primary granules off the surface of the Golgi complex is also observed.


iii. **Myelocyte stage**

The appearance of secondary granules that are smaller than primary granules is observed at the myelocyte stage of neutrophil development. In this stage, secondary granules stain heavily for glycoproteins which appear as a pinkish ground-glass background. The nuclei of myelocytes are eccentric and round or oval. The nucleoli are smaller and less prominent than in the previous stage of promyelocyte development.

iv. **Metamyelocyte stage**

For the first time, a horseshoe-shaped nucleus without nucleoli is observed in the metamyelocyte stage of neutrophil development. The nuclear chromatin is dense, with considerable clumping along the nuclear membrane. Primary, secondary and tertiary granules occupy the cytoplasm.

v. **Polymorphonuclear stage**

In the final stage of neutrophil development, further condensation of the nuclear chromatin is observed. As the cell develops into the polymorphonuclear stage, two or more lobes connected with filamentous strands are seen as the nucleus. An abundance of specific granules causes the cytoplasm to appear faintly pink due to stain with a reagent.

1.3 **Kinetics and survival of neutrophils**

It is estimated that about 20 times more neutrophils are present in the bone marrow than in the circulation in order to quickly accommodate an increase in demand under stressful conditions [4]. The half-life of mature neutrophils is estimated to range from 6-8 hours in the
circulation to several days when carrying out their protective function against invading microbes [5].

Production of neutrophils takes place in the bone marrow, and an equal number of cells must die in order to maintain homeostasis within the cell [6]. This equilibrium in the number of neutrophils in the system is critical in order to achieve an optimal level of wound healing and to avoid potentially dangerous chronic inflammatory conditions. Thus, tight regulation of neutrophil clearance mechanisms is absolutely crucial.

Programmed cell death, or apoptosis, is responsible for the functional inactivation and elimination of neutrophils [7]. Many neutrophils undergo apoptosis before leaving the bone marrow, and macrophages or dendritic cells remove these apoptotic neutrophils [8]. Cells undergoing apoptosis maintain the integrity of their plasma membranes, thus avoiding damage to other healthy cells by sequestering granules that are otherwise harmful.

Through integrin-mediated signaling, various growth factors and cytokines also generate anti-apoptotic signals. These in turn affect survival of neutrophils during diapedesis and at the site of infection by increasing the life span of neutrophils and accelerating maturation [8]. However, excessive production of neutrophil survival factors such as GM-CSF or G-CSF can lead to accumulation of neutrophils, causing an imbalance in homeostasis within cell [6].

### 1.4 Granules of neutrophils

Antimicrobial molecules within neutrophils are largely responsible for making the neutrophil the first line of defense against biological invaders. These molecules are tightly packed in granules which are released when activated. Initially, only two types of granules were
distinguished in neutrophils based on their affinity for dye: azurophil granules which take up the basic dye azure A and specific granules, which do not [9]. Later on, a clear distinction between the two types of granules was established when identification of myeloperoxidase (MPO) became possible by a peroxidase staining-based method of electron microscopy; MPO was only present in azurophil granules [4]. In addition, high-resolution electron microscopic techniques also revealed the existence of a tertiary granule type, which appears during myeloid maturation, and a fourth category of granules, the secretory vesicles that appear at the final stage of neutrophil maturation [10]. As mentioned above, granules begin to form at the stage of neutrophil maturation marked by transition from myeloblast to promyelocyte [4]. It is believed that formation of granules is achieved by aggregation of immature transport vesicles that bud off from the trans-Golgi network (TGN)[11].

1.5 Neutrophil molecules and functions

1.5.1 Traffic and margination

Neutrophils are distributed in the blood between a circulating pool found in large blood vessels and in the centre stream of small vessels, and a marginating pool [12]. In the marginating pool, neutrophils are briefly retained against the endothelial walls by weak molecular interactions between surface adhesion molecules of neutrophils and endothelial cells which result in “rolling” of neutrophils along the walls of capillaries. The rolling step is mediated by leukocyte selectin (L-selectin) expressed on neutrophils and by E- and P-selectins newly expressed on inflamed endothelial cells [12]. Interestingly, the level of L-selectin expression varies in neutrophils at different stages of maturation; it is highest shortly
after their release from bone marrow but decreases as they undergo normal rolling interactions [13].

1.5.2 Neutrophil priming during rolling

During inflammation, chemoattractants such as platelet-activating factor (PAF), leukotriene B4, and various chemokines are produced by the endothelium of inflamed tissues [12]. Among these chemokines, interleukin 8 (IL-8) is of utmost importance in attracting neutrophils without recruitin monocyte [14, 15]. Neutrophils express several receptors for chemoattractants which belong to the family of receptors associated with intracellular GTP-binding heteroproteins [12]. These G protein-coupled receptors trigger signal transduction cascades leading to firm adhesion of leukocytes and direction-specific movement, chemotaxis [12].

1.5.3 Adhesion

Functional neutrophils must transit rapidly from a circulating, non-adherent state to an adherent state in order to stop rolling and form adhesions with endothelial cells. Firm adhesion appears to occurs through the interaction between neutrophil β2 integrins, heterodimeric transmembrane glycoproteins which are expressed on non-activated neutrophils in a low-affinity state and on their endothelial counterparts [16]. During tissue injury, inflamed tissues release inflammation mediators and new adhesion molecules appear on the endothelium adjacent to the site of inflammation. Activated selectins and inflammatory mediators found on the surface of endothelia stimulate upregulation and
activation of $\beta_2$ integrins expressed on neutrophils to achieve a higher affinity state [17, 18],
leading to firm adhesion between neutrophils and endothelial cells.

1.5.4 Transendothelial Migration

Transmigration of neutrophils takes place at the borders of endothelial cells where tight
junctions between cells are discontinuous. Several adhesion molecules have been shown to
be involved in neutrophil transmigration, including cell adhesion molecules of the Ig-
superfamily (CAMs), the platelet endothelial cell adhesion molecule-1 (PECAM-1), and the
junctional adhesion molecule (JAM) [19, 20]. As mentioned above, rolling and firm adhesion
involves heterophilic interactions between two different classes of molecules. However, in
neutrophil transmigration, interactions between PECAM-1 expressed both on the neutrophil
surface and at the endothelial cell junction effectively pull the neutrophil through the
endothelium into the interstitial fluid [12].

1.5.5 Chemotaxis

Once in the interstitial fluid, neutrophils migrate along a chemotactic gradient towards the
site of injury or infection, a process known as chemotaxis. The term chemotaxis was first
defined in 1884 by Pfeffer, who described it as directional migration of leukocytes along a
chemical gradient [21]. Once a chemoattractant binds to the neutrophil, a series of signaling
pathways are triggered which results in activation of the cytoskeleton of neutrophils [22].
Consequently, neutrophils attain a polarized morphology characterized by an actin-rich
leading lamella, the major cell motility structure permitting rapid cell migration, and a tail-
like uropod at the rear end [22].

8
Under physiologic salt conditions, a 42-kDa actin monomer is assembled into filaments which are used to generate a motile machine capable of efficient and rapid chemotaxis [22]. Directional movement of neutrophils is characterized by actin-dependent cell membrane extension, surface adhesion and cell body contraction. A combination of actin-dependent protrusion of the lamella and contraction of the uropod causes overall movement of neutrophils [23]. Chemotaxis is maintained as long as new or recycled neutrophil receptors at the leading-edge of the cell continuously appear as a result of chemotactic gradient detection [23].

1.6 Microbicidal strategies of neutrophils

The innate immune system is an immediate defense against infectious microorganisms, and is critical for the survival of multicellular organisms [24]. In the innate immune system, neutrophils are essential effector cells that mature in the bone marrow. When they are terminally differentiated, neutrophils are then released into and circulate within the bloodstream, where they have a half-life of only a few hours. In healthy individuals most neutrophils are eliminated and cleared without ever executing their function. However, upon microbial challenge, neutrophils are the first responders deployed to the site of infection/injury and employ several strategies to defeat biological invaders.

1.6.1 Phagocytosis

*Phagocytosis* is an important cellular process of neutrophils which permits engulfment and subsequent elimination of invading pathogens. Two different receptor classes, *FcγReceptors*
(CD32, CD16) and complement receptors (CD35 and CD11b/CD18 integrin) are known to be involved in neutrophil phagocytosis [12]. Among these receptors, CD32 and CR3 are the actual functional receptors, while CR1 and CD16 simply act as co-receptors assisting the function of CD32 and CR3.

Initiation of phagocytosis is marked by activation of Src-tyrosin kinases, which trigger the aggregation of CD32 and CD16 and the phosphorylation of their cytoplasmic ITAMs, immunoreceptor tyrosine-based activation motifs [12]. Phosphorylation of ITAMs establishes a docking site for SH2 domains of Src tyrosine kinase, leading to the activation of PI3-kinase and Rho proteins. Consequently, Rho protein activation causes extension of membrane protrusions over the surface of immunoglobulin G (IgG)-opsonized particles and their engulfment [25-27].

1.6.2 Respiratory burst

When neutrophils recognize bacterial stimuli which induce degranulation, activation of oxidative metabolism is also observed and neutrophils increase their oxygen consumption, a process known as respiratory burst. The respiratory burst is known to be a result of assembly and activation of NADPH oxidase, a plasma and vesicle membrane-bound electron-transferring enzyme complex [9, 28]. The NADPH oxidase complex is made up of six subunits including a transmembrane flavocytochrome b$_{558}$, consisting of subunits gp91$_{phox}$, p22$_{phox}$ and three regulatory cytosolic proteins, p47$_{phox}$, p67$_{phox}$, and Rac small GTPase, all of which are essential for fully functional NADPH oxidase activity [29].
The major product of NADPH oxidase is a free radical, superoxide (O$_2^-$), known to kill ingested microbes either by direct superoxide-mediated damage or by activation of other enzymes such as proteases and peroxidases [30]. Superoxide can also undergo superoxide dismutase (SOD)-mediated catalytic conversion to hydrogen peroxide (H$_2$O$_2$) which serves as a co-substrate for myeloperoxidase (MPO) in the production of hypohalous acid (HOCl), a very effective antimicrobial agent.

1.7 A new strategy: Neutrophil Extracellular Traps (NETs)

More recently, evidence has unveiled a new phagocytosis-independent defense strategy of neutrophils, Neutrophil Extracellular Traps (NETs) [31]. The formation of NETs was found to be quite different from the other two strategies in that neutrophils release web-like structures consisting primarily of chromatin and antimicrobial proteins released by neutrophils to trap and kill pathogens in the extracellular space [31, 32].

1.7.1 The structure of NETs

High-resolution scanning electron microscopy (SEM) showed that NETs consist of smooth fibers with a diameter of 15 to 17nm, which are comprised of a sequence of nucleosomes from unfolded chromatin, and globular domains of around 25nm [31]. Analysis by immunofluorescence (IF) also revealed the major constituent of these fibers to be DNA containing proteins such as neutrophil elastase, cathepsin G, and myeloperoxidase [31]. In addition, NETs also contain histones H1, H2A, H2B, H3, and H4 [31]. The strong association of several DNA intercalating dyes with NETs and disintegration of NETs following treatment with DNase indicated that DNA is the main structural component and
scaffold of NETs [31]. Cytoplasmic proteins such as actin and tubulin are excluded from NETs [31].

1.7.2 The genesis of NETs

The exact mechanism that triggers the formation and release of NETs still remains elusive. However, several stimuli are known to activate neutrophils to initiate a programme that leads to the formation of NETs: phorboal myristate acetate (PMA), lipopolysaccharide (LPS), interleukin-8 (IL-8) or pathogens such as *Salmonella typhimurium* and the fungus *Candida albicans* [32]. Induction of NET release is most efficient in response to microorganisms compared to stimulation with isolated bacterial constituents such as LPS [31, 32], suggesting that simultaneous stimulation of multiple signaling pathways through several receptors and their physical recruitment is required for the most effective NET formation by neutrophils.

Two models have been proposed to explain the release of NETs: 1) a novel cell death mechanism; and 2) DNA release from intact cells. Fuchs et al. [32] have determined that the NET generation is indeed a novel form of cell death by monitoring individual cells via live video microscopy. Experiments demonstrated that the cell membrane of neutrophils ruptures and NETs are released by dying cells. However, NET formation is clearly different from necrosis and apoptosis. In contrast to apoptosis and necrosis, cells forming NETs clearly show disintegration of the nuclear envelope and mixing of nuclear and cytoplasmic material, as well as loss of internal membranes and disappearance of cytoplasmic organelles [32]. In addition, the cytoplasmic marker lactate dehydrogenase (LDH) is not detected during NET death, suggesting that this process is not the result of cell leakage seen during necrotic cell death [33]. The distinctive necrotic morphology was also not seen in PMA-activated
neutrophils, even after long periods of incubation [32]. The process of NET formation also occurs as early as 10 min after activation, too rapidly to be caused by apoptotic cell death [31, 32]. In addition, DNA fragmentation and exposure of phosphatidylserine typically seen in apoptotic cell death are absent in NET-forming neutrophils [34]. Together, these data indicate that neither apoptosis nor necrosis leads to NET formation, and that formation of NETs seems to be the consequence of a novel cell death mechanism.

During NET formation, the normal shape of nuclei is lost and homogenization of eu- and hetero chromatin takes place. As the nuclear envelope and the granule membranes disintegrate, components of NETs mix and come into contact with granules. Finally, the cell membrane breaks and NETs are released [32].

1.7.3 Molecular basis of NET formation

i. ROS requirement for NET formation

Stimulation of receptors such as Toll-like receptors 4 (TLR4s), as well as cytokine and Fc receptors [31, 35] that are suspected to be involved in NET formation activate protein kinase C (PKC). Subsequently, activated PKC initiates a signal transduction cascade leading to the assembly and activation of the NADPH oxidase complex. Several lines of evidence have already demonstrated that NET formation depends on the generation of reactive oxygen species (ROS) by NADPH oxidase. First, inhibition of respiratory burst in neutrophils by diphenylene iodonium (DPI) effectively impairs the formation of NETs in human neutrophils [32]. Second, the exogenous catalase which degrades H$_2$O$_2$ to molecular oxygen and water impedes the formation of NETs, whereas an inhibitor of this catalase increases NET formation. Third, neutrophils from patients with chronic granulomatous disease with mutated
NADPH oxidase do not produce NETs in response to PMA but produce NETs in response to hydrogen peroxide, indicating that ROS are a requirement for NET formation [32]. Although it is undeniable that ROS are required for proper NET formation, how ROS promotes the formation of NETs remains largely unknown. It is speculated that ROS act as second messengers to promote the downstream signaling events that culminate in NET formation.

ii. Histone deimination

Inside the nucleus, histones package and order DNA into highly condensed chromatin. Thus, histones are also suspected to be involved in NET formation. Recent studies by Wang et al. [36] and Neeli et al. [37] showed the deimination of histones in neutrophil-derived NETs, reinforcing the idea of histone involvement in NET formation. However, no experiments have been done to demonstrate that blocking histone deimination prevents NET formation.

1.7.4 The antimicrobial effect of NETs

Phagocytosis, respiratory burst and NETs are all actively employed to provide protection against invading pathogens. NETs particularly appear to be effective against a wide range of both Gram-positive and Gram-negative bacteria, parasites, and fungi [31, 38-40]. Although the mechanism by which NETs entrap microorganisms is still unknown, it has been proposed that electrostatic attraction between positively charged components of NETs and the negatively charged surface of microorganisms mediates entrapment. Electrostatically entrapped pathogens are then likely to be killed by highly concentrated antimicrobial proteins found in NETs such as neutrophil elastase or histones [31]. Although NETs appear to be an efficient way to contain and kill most biological invaders, some bacteria have evolved
strategies to evade this defense strategy by producing DNases. DNases can degrade the DNA backbone of NETs which allows bacteria to evade entrapment and killing [39, 40].

1.7.5 The role of NETs in host defense

NETs have been implicated in diseases in vivo. NETs are abundant at sites of infection and in samples from experimental shigellosis in rabbits as well as in spontaneous appendicitis in humans [31]. NETs have also been observed in the alveolar lungs in mice infected with S. pneumoniae [39], as well as in bovine mastitis [41]. The antimicrobial properties of NETs that kill invading pathogens are an undeniably important function of NETs [31, 39, 41].

Surprisingly, NETs have the capacity to provide protection even in the absence of microbicidal activity. Microbes such as Streptococcus pneumonia, Group A Streptococci (GAS) and Mycobacterium are trapped but not killed by NETs, suggesting a significant role for NET-mediated microbial trapping alone in immune defense [39, 40, 42]. NETs also function as a physical barrier to confine microbes to a local site of infection, thus preventing further spread of pathogens [39, 40, 42]. In addition, NETs also immobilize and confine components of neutrophil granules structure to keep potentially noxious proteins such as proteases from diffusing away and inducing damage in healthy tissue adjacent to the site of inflammation and infection.

1.7.6 The contribution of NETs to pathological conditions

Production of NETs might cause deleterious effects in the host. For example, release of large amounts of DNA and histones has the potential to contribute to development of autoimmune diseases such as lupus erythematosus which is induced by autoimmune reactions to the host’s
own DNA [43]. NETs might also play a role in the pathogenesis of non-infectious diseases like pre-eclampsia, a serious disorder of late pregnancy causing fetal and maternal mortality worldwide [44]. Pre-eclampsia is a multisystem disorder that can be characterized by two generalized processes of vasospasm and endothelial dysfunction [45]; these disturbances are believed to result from reduced placental perfusion due to abnormal trophoblast differentiation as well as elevated release of inflammatory microparticles and cytokines that play a role in the overt activation of the maternal innate immune system observed in pre-eclampsia [44, 46, 47]. Analysis by Gupta et al. [45] revealed that placentally derived syncytiotrophoblast microvillous membrane microparticles (STBM) and soluble factors efficiently activated neutrophils to generate NETs, and that the formation of NETs in the intervillous space reduces $O_2$ transport through the placenta, causing hypoxia in the fetus and fetal loss [45].

Interestingly, NETs also seem to be involved in sepsis. Clark et al. [35] reported that in cases of severe sepsis, platelet Toll-like receptor 4 (TLR4) detected TLR4 ligands in blood and induced platelet binding to adherent neutrophils. This led to the activation of neutrophils and led to the production of NETs. NETs retained their integrity under flow conditions and ensnared bacteria within the vasculature.

1.7.7 NET production by innate immune cells other than neutrophils

The formation of extracellular traps has also been observed in other granulocyte types such as mast cells and eosinophils. Mast cells are tissue cells preferentially located at the host-environment interface. Upon activation, they release various cytokines and mediators, particularly histamine [48]. Aside from being able to release histamine, mast cells can act as
mediators to eliminate bacteria at sites of infection through the release of TNF-α and recruitment of neutrophils [49, 50]. Interestingly, it was reported by Von Kockritz-Blickwede M et al. [51] that mast cells can form extracellular traps to kill bacteria.

Similar to NETs, ROS-dependent pathways also seems to be implicated in mast cell extracellular trap formation. As shown for neutrophils, the formation of extracellular traps in mast cells can be induced by PMA, H₂O₂, or bacterial pathogens [51]. The ability of mast cells to release extracellular traps supports the notion that these granulocytes are also capable of directly responding to invading microbes [51].

Another intriguing NET-like mechanism has also been observed in eosinophils. Studies with eosinophils indicate that cells that are primed with interleukin-5 or interferon-γ release fibers suggestive of extracellular traps containing eosinophil granular proteins into the extracellular space upon stimulation with LPS [52]. Eosinophilic extracellular traps have also been observed in colon samples of patients with Crohn’s disease [52].

Whether or not other cells of the innate immune system (e.g. macrophages) also have the ability to release these fibrous structures into the extracellular space requires further investigation.
Chapter 2

Introduction

Neutrophils play a critical role as first line of defense by the host innate immune system against invading pathogens. During inflammation or infection, neutrophils are recruited to the site of injury and provide protection by eliminating potential threats via phagocytosis or release of bactericidal substances [53]. Recently, a novel defense mechanism of neutrophils was described in which web-like structures (neutrophil extracellular traps: NETs) consisting of chromatin and proteins are produced to trap and kill pathogens [31, 32]. However, our knowledge of the underlying mechanisms regulating NET formation is still limited. Studies using neutrophils from patients with chronic granulomatous disease (CGD) have provided evidence that ROS are essential for the formation of NETs [32, 54]. Neutrophils from CGD patients lack significant ROS formation due to a genetic defect in components of the NADPH oxidase, resulting in an inability to produce NETs which may partly account for increased susceptibility to microbial infections. This finding led us to investigate the specific roles of Rac1 and Rac2, members of the Rho family of small GTPases in the formation of NETs, as Racs are a key regulator of ROS generation via the NADPH oxidase system [55]. Despite the fact that Rac1 and Rac2 share 92% homology in amino acid sequence, recent studies using Rac2-deficient neutrophils suggest that each isoform has unique roles in neutrophil function [55-57]. To determine the involvement of Rac1 and Rac2 in the formation of NETs, mice in which Rac1 or Rac2 or both genes are selectively disrupted in cells of the
granulocyte/monocyte lineage were used. It is demonstrated here for the first time that Rac2-null neutrophils have significant defects in formation of NETs in vitro. Moreover it is demonstrated that NET formation in Rac2-null cells can be partially rescued by stimulation with exogenous ROS, suggesting that activation of NADPH oxidase by Rac2 plays a pivotal role. In addition, data describing the role of nitric oxide (NO) in the formation of NETs is presented here. Several studies including Lee et al.[58] and Pieper et al. [59] have identified NO as a crucial ROS modulator in neutrophils. Importantly, NO has been shown to play an important role in NET formation in human neutrophils [60]. Here, it is confirmed that NO production is also required for formation of NETs in murine neutrophils and that Rac2 is required for efficient NO production. Our data suggest that Rac2 is an essential regulator of NET formation through dual regulation of ROS and NO generation in a Rac isoform-specific manner.
Chapter 3

Materials and Methods
Chapter 3

Materials and Methods

i. Chemicals and Reagents

**Bone Marrow Neutrophil Isolation:** MEM alpha cell culture media (Invitrogen, Carlsbad, CA); Percoll (P4937, Sigma); Dulbecco’s Phosphate Buffered Saline (D1408, Sigma); Hanks’ Balanced Salt Solution (14175, 14025, Invitrogen, CA). **Neutrophil Extracellular Traps Assay:** Phorbol 12-myristate 13-acetate (P1585, Sigma); Lipopolysaccharides from *Salmonella enteric* serotype enteritidis Bioxtra (L7770, Sigma); 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97% (238813, Sigma); N-Acetyl-L-cysteine (A7250, Sigma); GR ACS Hydrogen Peroxide solution 30% W/W (HX0635, EMD, Gibbs-town, NJ); SYTOX Green Nucleic Acid Stain (S7020, Invitrogen, CA); Triton X-100 (22686, USB, Ohio); DNase I, RNase-free (Fermentas, Ontario, Canada). **Nitric Oxide Quantification Assay:** DAF-2 diacetate (85165, Cayman Chemical, Michigan). **Microscopy:** Poly-L-Lysine (P8920, Sigma); Paraformaldehyde (158127, Sigma); Histone H3 (D1H2) Rabbit mAb (1:150) (Cell Signaling, Danver, MA); Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, CA); Glycine (56-40-6, Bio Basic inc, Markham); Albumin from Bovine Serum (A7906, Sigma); Karnovsky’s fixative (2.5% glutaraldehyde in a 0.1 M cacodylate buffer with 5mM CaCl₂ and Reynold’s lead citrate were kind gifts from the Microscopy Imaging Laboratory at Faculty of Medicine at the University of Toronto.

ii. Mice and neutrophil isolation

Rac1-deficient mice were generated according to protocols described in Glogauer et al [55]. In brief, selective disruption of Rac1 expression in granulocytes/neutrophils was achieved by
using conditional Rac1<sup>cre</sup>LysM<sup>cre</sup> mice in which the Cre recombinase is expressed under the control of the murine lysozyme M gene regulatory region. Rac2-null mice were previously generated according to protocols described in Roberts et al [61]. Mice were euthanized by either cervical dislocation or via carbon dioxide asphyxiation, and the tibia and femora were aseptically removed and dissected free of adherent soft tissue. Bone ends were cut, and the marrow space was flushed out using a sterile 26-gauge needle with cell culture medium (alpha MEM). The flushed marrow was passed through a 20-gauge needle several times to create a homogenous, single cell suspension. Cells were then layered onto discontinuous Percoll gradients of 80%/65%/55% [62] and centrifuged for 30 min at 1300g at 4°C. Mature neutrophils found at the 80% and 65% interfaces were collected. Neutrophils obtained were counted by using a Z1 Coulter Particle Counter (Coulter Electronics, Hialeah, FL). Routinely, more than 85% of isolated cells were neutrophils, confirmed by Wright-Giemsa staining. All experiments were performed with mice older than 4 weeks. All mice were used in accordance with the Guide for the Human Use and Care of Laboratory Animals and the approval of the University of Toronto animal care committee.

iii. Neutrophil Extracellular Traps (NETs) assay

To quantify NETs, neutrophils in 1x HBSS containing CaCl<sub>2</sub>/MgCl<sub>2</sub> were seeded in a black 96-well plate (Cayman Chemical, Michigan). The cells were treated with 100nM PMA or 100ng/ml LPS or left untreated. Cells were also lysed with 0.1% triton X-100 as a control for the total DNA in different wells for each time point. They were then incubated at 37°C for 3 hours. The cell-impermeable DNA-binding dye, Sytox green was added at a concentration of 5µM at each time point to bind extracellular DNA, and fluorescence was quantified using a
fluorescence spectrophotometer; excitation/emission: 485/520nm (Fisher Scientific, Ontario, Canada). To ensure that the NET assay only detected extracellular NET-DNA in control samples, neutrophils were incubated with 5 units of RNase-free DNase I for 1 hour at 37°C prior to extracellular DNA measurement (Fig. S1).

iv. **Nitric oxide (NO) quantification**

To measure the amount of nitric oxide in live cells, neutrophils were incubated with 1 µM DAF-2DA for 30 min at 37°C. Culture media were then replaced to remove excess dye, and the neutrophils were either incubated in the presence or absence of 100nM PMA. NO was measured every 15 min for a period of 3 hours using a fluorescence spectrophotometer (Fisher Scientific, Ontario, Canada).

v. **Confocal Microscopy**

Prior to microscopy, 13-mm cover slips were pre-coated with two drops of 0.001% poly-l-lysine in 1x HBSS with CaCl₂/MgCl₂. The slips were incubated at 37°C for 30 min, taken out to be washed with 1x HBSS and let dry. To visualize NETs using immunofluorescence, neutrophils were seeded on 13-mm pre-coated cover slips and left in the incubator at 37°C for 15, 90 and 180min. Cells were then washed and fixed with 4% paraformaldehyde for at least 8 hours. 0.1% Triton X-100 was added for 5 min to permeabilize the cells. The cells were then blocked with 100nM glycine and 1% BSA for 1 hour each at room temperature. The cells were then incubated with primary antibody, Histone H3 antibody (1:150) and secondary antibody, Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:200) for 1 hour each to stain for histones. Five µM Sytox green was used to stain for DNA. Prepared samples were analyzed using confocal microscopy. Scale bar represents 10µm.
vi. **Transmission Electron Microscopy**

Sample preparation for TEM was done by Microscopy Imaging Lab at University of Toronto. In brief, the cell mono-layers are fixed for one hour with Karnovsky’s fixative at room temperature. The fixative is removed and replaced with fresh fixative, and the sample is stored at 4°C until the next day. Dehydration was then performed using a graded series of ethanol: 30%, 50%, 70%, 95% twice each for 5 minutes followed by three changes of 100% ethanol for ten minutes each, all at room temperature. The cells are then infiltrated with a mixture of Epon-Araldite and ethanol; 1:1 for ½ hour, 3:1 for 1 hour, and 100% Epon-Araldite for 2 hours all at room temperature with rotation. After the subsequent polymerization step for 48 hours in a 60°C oven, samples were sectioned and stained using saturated uranyl acetate followed by Reynold’s lead citrate. The sections were then examined and photographed in a Hitachi H7000 transmission electron microscope at the magnification of 5000 and 9000.

vii. **Statistical Analysis**

For experiments in which there were multiple observations per sample, numerical results were expressed as mean ± SEM. Each experiment had a sample size of n≥3, unless otherwise stated. Statistical analysis was performed using a Student’s two-tailed t-test unless otherwise specified and a p value of less than 0.05 or 0.01 was considered statistically significant.
Chapter 4

Results
Chapter 4

Results

Neutrophil Extracellular Trap (NET) release is induced by phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS).

To measure NET formation, we adapted an assay developed by Brinkmann et al. [31] which is based on fluorescent quantification of extracellular DNA, the major structural component of NETs. To validate the NETs assay, 5 units of DNase were added alone or with PMA treatment (methods). DNase-mediated degradation of extracellular DNA almost completely abolished the fluorescent signal from Sytox Green, demonstrating the specificity of the assay for extracellular DNA (Supplemental Figures; Fig. S1). NET formation in murine neutrophils was induced with PMA (100 nM) or LPS (100 ng/ml), and extracellular DNA was quantified after 90 and 180 min of stimulation (methods). Unstimulated neutrophils were used as a baseline for normalization, and NET formation was expressed as the ratio of extracellular DNA divided by total DNA. To measure total DNA, replicates were pre-treated with Triton X-100 prior to staining with Sytox green. NETs formation was undetectable 15 min after stimulation (data not shown). However, we found that approximately 4% and 15% of the total DNA is released as NETs after 90 and 180 min of PMA stimulation, respectively (Fig. 1). In contrast, LPS stimulated neutrophils released only about 1% and 5% of total DNA after 90 and 180 min stimulation, respectively (Fig. 1).

Microscopic analysis confirms the presence of neutrophil extracellular traps in murine neutrophils in response to PMA

To confirm the results obtained with the NETs assay, we used confocal laser microscopy to visualize NET formation at various time points. Although microscopic visualization of NET
is useful for qualitative assessment, it may not be very suitable for quantitative purposes since NETs are very fragile [63]. To detect the appearance of NETs and investigate the morphological changes leading to NET formation by confocal microscopy, we used Sytox green to stain DNA and an antibody against histone H3, one of major structural component of NETs [64]. In unstimulated cells NET formation was absent and nuclei maintained their normal donut-like shape. In addition, histone H3 was seen to co-localize with nuclei at all time points (Fig. 2A). Fifteen minutes after stimulation, the nuclei of neutrophils still exhibited their normal donut-like shape comparable to unstimulated neutrophils. In contrast, we observed apparent changes in nuclei and overall shape of neutrophils at later time points after stimulation with PMA or LPS. Ninety minutes after stimulation, extracellular DNA release by a small number of neutrophils was observed. The nuclei of some neutrophils no longer maintained their initial shape, but started to expand and fill the cytoplasmic space. The overall cell shape also changed from circular to elongated (Fig. 2A). Three hours after stimulation, the nuclei of activated neutrophils expanded and filled most of the cytoplasmic space. At this time point, we also found that histone H3 co-localized with DNA in NETs (Fig. 2A). The most abundant amount of extracellular DNA between neutrophils was observed at this time point (Fig. 2A).

Transmission electron microscopic (TEM) images further confirmed the change in nuclear morphology during the process of NET formation (Fig. 2B). After 15 min of PMA stimulation, no apparent morphological changes were detected. However, after 90 min the plasma membrane of some neutrophils started to rupture, possibly marking the initial process of NET release. Extracellular structures suggestive of NETs were also observed at this time point (Fig. 2B). At 180 min after stimulation, more abundant NET release was observed than
at the 90 min time point and many of the neutrophils seemed to have lost their initial smooth plasma membrane structure. At this time point, we also observed that the nuclei of many of neutrophils filled most of the cytoplasmic space (Fig. 2B).

**Reactive oxygen species (ROS) are required for the formation of NETs**

To confirm the requirement for ROS [32] during NET formation in our assay, we stimulated murine neutrophils with PMA (Fig. 3A) or LPS (Fig. 3B) in the presence of the ROS scavenger, Trolox. In response to PMA, Trolox significantly reduced the formation of NETs to about 0 and 3% at 90 and 180 min after stimulation, respectively. As expected, Trolox also significantly reduced the amount of NETs formation to less than 0.5% at both 90 and 180 min after the stimulation by LPS, demonstrating ROS as an important requirement for efficient NET formation (Fig. 3A, B). N-Acetyl cysteine (NAC) was used as an alternative ROS scavenger and also resulted in significant reduction of NET formation (data not shown).

In addition, the NADPH oxidase inhibitor, DPI also showed significant reduction in NET formation in response to PMA at 180 min after stimulation (Fig. S2).

**Rac2 is required for neutrophil extracellular trap formation**

Since the Rac small GTPases are key regulators of ROS generation through activation of NADPH oxidase in neutrophils, we hypothesized that Rac is also important for NET formation. Murine neutrophils express two Rac isoforms, Rac1 and Rac2 [55]. In order to identify the isoform-specific contribution of Rac, NET formation was induced in neutrophils deficient in Rac1, Rac2 or both isoforms. Approximately, 2% and 16% of total DNA was released as NETs in Rac1-null neutrophils in response to PMA after 90 and 180 min stimulation, respectively (Fig. 4A). In Rac1-null neutrophils, the amount of NETs released
was slightly lower at 90 min and higher at 180 min compared to wild-type neutrophils but the
difference was statistically insignificant. Similar amounts of NET formation were also
observed in WT and Rac1-deficient cells after stimulation with LPS (Fig. 4B). Rac1-null
neutrophils released about 2% and 4% of total DNA as extracellular traps, similar to the
amounts observed in wild-type neutrophils at 90 and 180 min after stimulation (Fig. 4B). In
contrast, NET formation in Rac2-deficient neutrophils was dramatically reduced in both
PMA and LPS stimulated cells. In response to both stimuli, less than 1% and 2% of the DNA
from Rac2-null neutrophils was released as NETs at 90 and 180 min after stimulation,
respectively. NET formation was virtually undetectable in cells that lacked both Rac
isoforms (Rac1/2null) (Fig. 4A, B).

**Hydrogen peroxide induces NET formation in wild-type neutrophils and rescues NET
formation in Rac2-null neutrophils**

LPS or PMA are known activators of NADPH oxidase and induce the production of
ROS [65]. Since Rac2 is the major isoform involved in NADPH oxidase complex activation
[66] and ROS production is severely impaired in Rac2-null neutrophils [55], we tested
whether reduced NET formation in Rac2-null cells is caused by defective ROS generation.
Since it was reported that ROS alone can trigger NET formation [32], we induced NETs
formation in wild-type neutrophils using hydrogen peroxide and glucose oxidase (GO), a
hydrogen peroxide producing enzyme. Both hydrogen peroxide and GO induced NET
formation in wild-type neutrophils (Fig. 5A). Stimulation with hydrogen peroxide (50 µM)
triggered roughly 6% of total DNA to be released as NETs after 180 min stimulation (Fig.
5A). GO (10 mU/ml) induced NET formation slightly more effectively (10% DNA release)
(Fig. 5A). NET formation induced by hydrogen peroxide was confirmed by
immunofluorescence microscopy (data not shown). In Rac2-null cells, H2O2 and GO induced NET formation as much as that in Wild-type cells, unlike PMA-stimulated Rac2-null neutrophils which were impaired to produce NETs. Analysis of NET release by confocal microscopy also confirmed these results, demonstrating that impaired NET formation in Rac2-null cells is – at least partially – caused by reduced NADPH oxidase mediated ROS generation (Fig. 5B).

**Nitric oxide generation is involved in NET formation and is decreased in Rac2-null cells**

Recently, nitric oxide (NO) was shown to be important for NET formation in human neutrophils [60]. To determine if NO has a comparable role in NET formation in murine neutrophils, we induced NETs in wild-type neutrophils while blocking the activity of nitric oxide synthase (NOS). Using the pharmacological inhibitor L-NAME to block NO generation, we observed a significant reduction in NET formation at both 90 min and 180 min after stimulation with PMA (Fig. 6A), demonstrating a similar requirement for NO in NET generation in mouse neutrophils. Interestingly, Rac is able to interact and regulate the activity of constitutive NO synthases [67]. Therefore, we investigated the possible impact of Rac deficiency on NO production in Rac1-null and Rac2-null neutrophils. After stimulation with PMA, the amount of NO was monitored every 15 min over a period of 3 hours by measuring the fluorescent emission of the NO-probe, DAF2-DA. NO production in PMA stimulated WT and Rac1-null neutrophils constantly increased over a period of 3 hours (Fig. 6B). NO production in Rac2-null neutrophils was markedly lower and after 180 min the amount of NO generated in Rac2-null neutrophils was about half of compared to wild type neutrophils (Fig. 6B).
Chapter 5

Discussion
Chapter 5

Discussion

For the survival of multicellular organisms, the innate immune system, which provides an effective defense strategy against invading pathogens, is absolutely essential. Neutrophils are key in the innate immune response which have strategies such as phagocytosis and respiratory burst to deal with pathogenic invaders. Recently, a third strategy was unveiled i.e. the formation of Neutrophil Extracellular Traps (NETs). How NET formation is exactly regulated at the molecular level remains largely unknown. Therefore, in an effort to further elucidate the mechanisms involved in NET formation, we investigated the role of Rac GTPase, a key regulator of neutrophil functions, in the formation of NETs. In murine neutrophils, two isoforms of Rac small GTPase, Rac1 and Rac2 are expressed in similar amounts [56]. Although Rac1 and Rac2 have 92% homology in amino acid sequence [68], the two proteins seem to play non-overlapping roles in neutrophil extracellular trap formation. In this report, we investigate the specific roles of Rac1 and Rac2 in NET formation using neutrophils derived from Rac-deficient mice.

**Rac2 is a key component for proper NET formation in vitro**

Our data show that the formation of NETs is normal in Rac1-null neutrophils whereas in Rac2-deficient neutrophils NET formation is greatly reduced (Fig.4). Two possible explanations may provide explanation for this observation. First, Rac1 and Rac2 may not be functionally redundant despite their homology in amino acid sequence. Murine neutrophils express similar amounts of Rac1 and Rac2, unlike human neutrophils which express Rac2
predominantly [56]. Our observation that Rac1 cannot fully compensate for deletion of Rac2 suggests functional independence of the two isoforms, at least in the process of NET formation. A study by Li et al. [56] supports this notion by demonstrating differential activation of Rac1 and Rac2 downstream of chemoattractant receptor activation by targets; FMLP or PMA stimulation of wild-type murine neutrophils results in activation of both Rac1 and Rac2, but finds that substantially more Rac2-GTP was detected than Rac1-GTP. This indicates differential activation of Rac isoforms by agonist-induced signals, suggesting a mechanism which could contribute differentiation of the roles of Rac1 and Rac2. Another possibility is that proper NET formation mainly relies on NADPH oxidase-dependant ROS generation, which is preferentially regulated by Rac2 in neutrophils [55]. This explains why absence of Rac2 expression results in abnormal formation of NETs compared with absence of Rac1. A study by Dorseuil et al. [69] demonstrated that non-prenylated Rac2 has a 6-fold higher affinity for p67phox, a key component of the NADPH oxidase system than non-prenylated Rac1, suggesting that Rac2 is a preferential regulator of NADPH oxidase. Glogauer et al. [55] further confirmed that Rac1 is not required for production of superoxide generation in neutrophils, yet Rac2 is specifically required. Our findings show that NADPH-inhibited wild type neutrophils exhibit a significant reduction in NET formation 180min after stimulation. In addition, a downstream product of NADPH oxidase, hydrogen peroxide, is able to induce the formation of NETs in Rac2-null neutrophils as much as in wild type neutrophils. These findings indicate that the ROS generation required for proper NET formation may occur primarily through the NADPH oxidase system, which is regulated preferentially by Rac2 [55].
**Possible role of NO in NET formation**

Recently, the free radical NO has gained attention as a possible player in NET formation, since it is an important signaling molecule that can act in concert with ROS to regulate cell functions [60]. This led us to investigate a possible role of NO in NET formation. Indeed, our data showed that reduction in NO production by inhibition of NOS significantly reduced the NET formation, indicating the involvement of NO in the process of NET formation through functional NOS. A recent study by Satyananda et al. [60] also shows the role of NO in NET formation by demonstrating that NO induces extracellular traps in human neutrophils by facilitating the NADPH oxidase-mediated generation of free radicals. In addition, the ability of NO to promote the generation of free radicals has also been extensively reported and accepted [58, 59, 70]. Therefore it is likely that NO production in murine neutrophils also potentiates NADPH oxidase-mediated ROS generation leading to effective formation of NETs.

**Rac2 is a preferential isoform involved in regulating NO production**

A very interesting study by Selvakumar et al. [67] recently demonstrated that Rac small GTPase directly interacts with and regulates constitutively-expressed nitric oxide synthases in endothelial cells. This led us to suspect an additional role of Rac2 as a NOS activator in addition to its role as a preferential regulator of NADPH oxidase-mediated ROS generation. To prove this, we measured NO production in Rac deficient neutrophils. Our data (Fig.6) showed that Rac2 but not Rac1 is the Rac small GTPase isoform in murine neutrophils that is preferentially involved in production of NO in response to PMA; decreased production of nitric oxide in neutrophils was only seen in Rac2-null neutrophils whereas NO production
was unaffected in Rac1-null neutrophils. This supports the notion that Rac2 may also regulate NO production directly through NOS in murine neutrophils, as seen in an earlier study [17].

**Rac2 is an essential regulator of NET formation through dual regulation of ROS and NO generation**

According to Satyananda et al. [60], NO potentiates ROS production by NADPH oxidase, which of course requires functional Rac2 to be activated. Our preliminary data (not shown) also indicated that NO alone in Rac2-null cells has no effect on NET formation since there is no Rac2-activated NADPH oxidase activity to enhance.

Since Rac2 is found to have an important role in the formation of NETs through both NADPH-mediated ROS generation and NOS-mediated ROS generation, Rac2 is proposed to have a dual role of activating NADPH oxidase and enhancing its activity by increasing levels of NO through interaction with NOS.
Figure legends

Figure 1. Neutrophil Extracellular Trap (NET) release is induced by phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS). Neutrophils were isolated from wild-type mice and were treated with 100 nM PMA, 100 ng/ml LPS or mock-treated. Staining of extracellular DNA with Sytox Green was used to quantify NET formation (see methods). The percentage of extracellular DNA was calculated by subtracting the fluorescence of unstimulated neutrophils from the fluorescence of stimulated neutrophils at each time point, divided by the total DNA. Data are derived from 3 or more independent experiments, each of which was performed in triplicate (n=3, * indicates p<0.05).

Figure 2. Microscopic analysis of neutrophil extracellular trap formation in murine neutrophils. Isolated neutrophils were seeded on poly-L-lysine coated glass slides, stimulated with 100 nM PMA for the indicated time and fixed before processing for confocal laser microscopy or transmission electron microscopy. (A) DNA was stained with Sytox Green and histone 3 was detected by a specific antibody against histone 3, followed by an Alexa-568 conjugated secondary antibody (red). Confocal laser scanning microscopy demonstrated initial NET formation after 90 min with co-localization of extracellular DNA and histone 3 in NETs. (B) Transmission electron microscope (TEM) images show morphological changes in the nucleus and the formation of NETs at 90 min and 180 min after stimulation. Scale bars = 10 µm.
Figure 3. **Reactive oxygen species (ROS) are required for NET formation.** Neutrophils from wild-type mice were stimulated with PMA (100 nM) or LPS (100 ng/ml) in the presence of 100 µM Trolox or vehicle. NET formation was measured at 90 minutes and 180 minutes post stimulation. Data are derived from 3 or more independent experiments, each of which was performed in triplicate (n=3, p<0.05).

Figure 4. **Rac2 is required for neutrophil extracellular trap formation.** Neutrophils were isolated from WT, Rac1-null, Rac2-null and Rac1/2-null mice, and NET formation was induced with (A) 100 nM PMA or (B) 100 ng/ml LPS. Formation of NETs was quantified at 90 minute and 180 minute post stimulation. Data are derived from 3 or more independent experiments, each of which was performed in triplicate (n=3, * indicates p<0.05).

Figure 5. **Hydrogen peroxide induces NET formation in wild-type and rescues NET formation in Rac2-null neutrophils.** (A) Wild-type neutrophils and Rac2null neutrophils were stimulated with 100 nM PMA, 50 µM hydrogen peroxide or 10 mU/ml glucose oxidase (GO) to induce NET formation and extracellular NETs were quantified at the indicated time points. Data are derived from 3 or more independent experiments, each of which was performed in triplicate (n=3, * indicates p<0.01). (B) Partial rescue of NET formation in Rac2-null neutrophil by hydrogen peroxide was confirmed by confocal microscopy (DNA stained by Sytox Green, Histone 3 by Alexa 568 in red).
Figure 6. Nitric oxide generation is involved in NET formation and is decreased in Rac2-null cells. (A) PMA-induced NET formation was significantly reduced by inhibiting nitric oxide generation with the nitric oxide synthase inhibitor, L-NAME (5mM). (B) Nitric oxide formation was measured every 15 minutes in wild-type, Rac1-null and Rac2-null neutrophils after stimulation with PMA. Data are derived from 3 or more independent experiments, each of which was performed in triplicate (n=3, * indicates p<0.05).
Supplemental figure legends

**Figure S1. Neutrophil Extracellular Trap quantification assay specifically measures the extracellular DNA**

Neutrophils from the wild-type mice were stimulated with PMA alone (100 nM), DNase alone (5 units/ml), PMA and DNase together or left untreated (control). Staining of extracellular DNA with Sytox Green was used to quantify NET formation (see methods). The amount of extracellular DNA was measured at 90 minutes and 180 minutes post stimulation. The percentage of extracellular DNA was calculated by subtracting the fluorescence of unstimulated neutrophils from the stimulated neutrophils at each time point and divided by the total DNA signal. Data are derived from 3 or more independent experiments, each of which was performed in triplicate (n=3, p<0.05).

**Figure S2. Diphenylene iodonium (DPI), an NADPH oxidase inhibitor, significantly reduces neutrophil extracellular trap formation**

Neutrophils from wild-type mice were stimulated with PMA (100nM) or PMA and DPI (10μM) together. As expected, NET formation was observed in PMA-stimulated neutrophils. In neutrophils stimulated with both PMA and DPI, no significant reduction in NET formation was observed at 90 minutes after stimulation. However, NET formation was significantly reduced at 180 minutes after stimulation. Data are derived from 3 or more independent experiments, each of which was performed in triplicate (n=3, p<0.05).
Figures

Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure S1.

![Graph showing extracellular DNA as a percentage of total DNA for different treatments at 90min and 180min.]

Figure S2.

![Graph showing extracellular DNA as a percentage of total DNA for PMA and PMA+DPI treatments at 90min and 180min.]

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Chapter 6

Future Directions
Chapter 6

Future Directions

In this project, major strides have been taken towards determining the role of Rac small GTPases in neutrophil extracellular trap formation. However, there are a few questions that still remain unanswered.

First, the conclusion that Rac2-mediated NET formation is dependent on superoxide generation from NADPH oxidase was based on the initial application of two ROS scavengers, Trolox and NAC. Although these data clearly implicated ROS in NET formation, this needed to be strengthened to completely implicate NADPH oxidase in NET formation. Thus, we conducted an experiment with NADPH oxidase inhibitor DPI to specifically examine the role of NADPH oxidase in NET formation. As expected, DPI treatment significantly reduced the formation of NETs 180min after stimulation. However, even a wide range of concentrations of DPI did not completely inhibit the formation of NETs. One possible explanation for this observation is that Rac2-mediated NADPH oxidase-dependent ROS production may be the major but not the only pathway involved in the NET formation. It is also possible that DPI may be less effective in treatment of bone marrow-derived murine neutrophils. In order to completely clarify this, similar experiments should be performed using neutrophils from mutant mice lacking expression of NADPH oxidase in cells of the hematopoietic lineage.

Secondly, this report proposes that NET formation is mainly dependent on Rac2-mediated ROS production and yet involves the free radical NO. This raises a question: which one is the
main contributor to Rac2-mediated NET formation? According to Patel et al. [60], NO potentiates ROS production by NADPH oxidase, which requires functional Rac2 to be activated. Our preliminary data (not shown) also indicated treatment of Rac2-null cells with NO alone has no effect on NET formation since there is no Rac2-activated NADPH oxidase activity to enhance. Thus we believe that in wild type neutrophils, Rac2 plays a dual role in activating NADPH oxidase and enhancing its activity by increasing NO production.

In our study, a mouse model was used to gain insight into possible pathways involved in the formation of NETs. Neutrophils isolated from mouse bone marrow are representative of human neutrophils. However, blood neutrophils might be more mature than bone marrow neutrophils and therefore, it is also important to take this factor into account when applying this notion to humans or to mice.
Chapter 7

Conclusion
Chapter 7

Conclusions

This was the first report that examined the role of Rac small GTPases in the formation of neutrophil extracellular traps. It is shown here that Rac2 is required for the formation of NETs in an isoform-specific manner. Indeed, NET formation in Rac1-null cells was not affected. Because NET formation is tightly linked to generation of ROS, it is likely that the defect in NET formation is caused by lack of ROS formation in Rac2-null cells [56]. This is supported by the finding that exogenous addition of ROS induces NET formation in Rac2-null cells as seen in wild type cells. In addition, we identified an isoform-specific function for Rac2 in NO generation in neutrophils. Rac2-null cells produce roughly half the amount of NO produced by wild type cells. Since NO plays an important role in stimulating ROS production required for NET formation [71], we propose a dual role for Rac2 in NET formation that besides being the preferential Rac isoform for NAPDH oxidase, Rac2 also augments ROS production through NO-mediated stimulation of free radical generation by NADPH-oxidase.
References


