The Role of Small Rho GTPases Rac1 and Rac2 in Macrophage Polarization

by

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Macrophages are phagocytic cells of hematopoietic origin. They play an important role in both the adaptive and innate immune systems. Macrophages are able to polarize into either M1 or M2 macrophages based on their microenvironment. M1 macrophages are proinflammatory cells that are key regulators of the immune response but are also known to cause tissue damage and destruction. M2 macrophages are anti-inflammatory involved in wound healing. The key functional differences between these two cell types remains to be fully characterized. The Rac members of the Rho family of small GTPases are known to be regulators of macrophage functions however their roles in M1 and M2 macrophages is unknown. We have shown that M1 macrophages produce more ROS and NO compared to M2 cells. We found differences in morphological as well as functional features of M1 and M2 macrophages, which have important implications in their role in the immune response.
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Chapter 1
Statement of Problem

Macrophages are phagocytic cells of hematopoietic origin. They play an important role in both the adaptive and innate immune systems. Macrophages are able to polarize into either M1 or M2 macrophages based on their microenvironment. M1 macrophages are proinflammatory cells that are key regulators of the immune response but are also known to cause tissue damage and destruction. M2 macrophages are anti-inflammatory involved in wound healing. The key functional differences between the two cell types remains to be fully characterized. The Rac members of the Rho family of small GTPases are known to be regulators of macrophage functions however, they roles in M1 and M2 macrophages is unknown. In order to compare the functional differences between M1 and M2 macrophages we studied ROS and NO production, pH of the phagosome, cell shape, and expression of other Rho GTPases. We have shown that M1 macrophages produce more ROS and NO compared to M2 cells. M1 macrophages in Rac1 null cell are unable to acidify their phagosome whereas; M2 cells are able to fully acidify their phagosome. M1 macrophages show a decrease in expression of CDC42, RhoA and Rac1 when compared to M2 macrophages. M1 macrophages appear rounder in shape than M2 macrophages. We are currently running experiments in order to look at chemotaxis, phagocytosis and bacterial killing to characterize these cells in more detail. We are looking at Rac1 and Rac2 null bone marrow derived macrophages in order to look at the differences in the characteristics of M1 and M2 macrophages more closely

1.1 Objectives

1. Characterize the morphological and functional differences between M1 and M2 macrophages.

2. To determine if Rac1 and Rac2 regulate the differentiation of macrophages into a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype?

3. To determine if Rac1 and Rac2 regulate the functional characteristics of an M1 or M2 macrophage (and which functions)?

1.2 Rationales and Hypotheses

Objective 1:
Hypothesis: M1 and M2 macrophages have different morphological as well as functional characteristics.

Rationale: looking at various aspects of M1 and M2 macrophages it is clear that their functions are very different. By their very nature, M1 macrophages are pro-inflammatory and are known to release pro-inflammatory cytokines as well as ROS and Nitrogen species. M1 macrophages are also activated after recognition of pathogen associated molecular patterns and endogenous danger signals. They cause activation of a T-helper1 (Th1) immune response. On the other hand, M2 macrophages are anti-inflammatory cells in which activation of these cells leads to a wound healing phenotype. M2 macrophages activate the T-helper 2 (Th2) associated effector functions and regulate T cell functioning.

Objective 2:

Hypothesis: Rac1 and Rac2 null macrophages show impairments in the differentiation of macrophages into a pro-inflammatory or anti-inflammatory phenotype.

Rationale: In the absence of Rac2, previous data indicates that there was an increase in bone loss during periodontal disease. It was thought that in the Rac2 null mice there was an increase in the polarization of macrophages into an M1 phenotype leading to more ROS being produced as well as more tissue destruction. Rac1 is also implicated in differentiation of many cell types. For example, activation of Rac-1 is shown to potently regulate pre-T cell differentiation and proliferation at the point of T cell antigen receptor (12).

Objective 3:

Hypothesis: Rac1 and Rac2 are involved in various characteristics of M1 and M2 macrophages.

Rationale: The Rho GTPase superfamily controls multiple cellular functions such as cell division, chemotaxis, phagocytosis, NADPH oxidase activation, and superoxide production (3). Rac specifically as been shown to carry out a wide variety of cellular functions including: actin cytoskeletal regulation, cell polarity, vesicle trafficking, and transcription (3). Because M1 and M2 macrophages show very different cell functions, we expect that Rac1 and Rac2 will affect different characteristics of M1 and M2 macrophages.
1.3 Main experimental model

All experiments were carried out using either cultured macrophages or primary mouse cells in culture to study macrophages in their M1 and M2 states. This experimental design has been chosen to study functions and morphology of M1 and M2 macrophages and the role of Rac1 and Rac2 in these cells.
Chapter 2
Introduction

2.1 Inflammation

2.1.1 Chronic inflammation

Chronic inflammation is defined as non resolving acute inflammation which is a protective response involving host cells, blood vessels and proteins (22). Inflammation is meant to be a protective response, however when inflammation is not resolved it can damage affected tissues. Components of inflammation that are capable of destroying microbes can also injure bystanding normal tissue. Chronic inflammation is characterized by an increase in macrophages. Inflammation must be actively terminated when no longer needed, otherwise chronic inflammation ensues (22).

2.1.2 Resolution of inflammation

Macrophages orchestrate initiation and resolution of inflammation. For this reason they are known as the quarterbacks of the immune system. Macrophages are the characteristic cell type involved in chronic inflammation. In order to resolve inflammation it is important for there to be a switch from m1 to m2 macrophages. Defects in the switch from M1 to M2 macrophages may be involved in chronic inflammation. We want to understand what is regulating the shift from M1 to M2 macrophages. There is a balance between M1 and M2 macrophages, either cell type, M1 or M2 can have detrimental effects on the body (18).

2.2 Macrophages

2.2.1 Macrophage progenitors

Monocytes are circulating leukocytic cells that after taking up residence within various tissues, act as progenitors for numerous cell-types including macrophages and dendritic cells (26). Monocytes are derived from myeloid cells, which give rise to these monocytes within the
bone marrow and released from the bone marrow into peripheral blood. Monocytes leave the circulatory system and enter various target tissues in response to chemokinetic signals (1) where they differentiate into various cell-types (26). Approximately 5-10% of circulating leukocytes are monocytes, which have heterogeneous and distinct morphological features including varying size, granularity, and nuclear morphology (26).

2.2.2 Development of Macrophages

Macrophages differentiate from circulating blood monocytes by the expression of F4/80, CD11b, CD18, CD 68 and Fc receptor (7&10). Following migration from the blood into the tissues of the body, monocytes differentiate into macrophages or dendritic cells (10). Monocytes are able to replenish macrophage populations as needed (10). The type of macrophage differentiated is dependant upon the resident tissue (9).

2.2.3 Functional Features of Macrophages

Macrophages are essential as cellular effectors of the innate immune system. Macrophages are phagocytic cells that rid the body of worn out cells and debris, viruses, bacteria, cells that are apoptotic as well as some tumor cells (23). Additionally, macrophages are secretory cells that release a number of mediators or cytokines that regulate various aspects of host defense, inflammation, and homeostasis (23).

Macrophages function as the first line of defense against non-self-molecules and other materials and debris (2). In addition to the adaptive immune system, macrophages play an important role in the innate immune system, and tissue homeostasis (4). They contribute to the recognition, uptake and killing of microorganisms and multicellular parasites, antigen presentation to T and B lymphocytes (32). Finally, macrophages must develop and respond to rapid changes in the environment (6).

Macrophages are known as professional phagocytes. Phagocytosis occurs by an actin-dependent mechanism and is, generally defined as the uptake of particles of 1µ or greater in diameter. Macrophages use phagocytosis in order to uptake and degrade infectious agents and senescent cells. Phagocytosis by macrophages is essential in development, tissue remodeling, immune response, and inflammation (31). Particle internalization is initiated by an interaction with specific receptors on the surface of the phagocyte with the surface of the particle (31).
Macrophages have an abundance of receptors used for receptor-mediated uptake, with specific adaptations to internalize larger particles rapidly. This mechanism optimizes their ability to degrade internalized materials. Macrophages use receptor-mediated contact with pathogens in order to internalize and degrade these harmful particles.

Following tissue injury where there is a necessity for increased numbers of macrophages, it is essential for monocytes to exit the circulatory system and then to enter the affected tissue. Specific interactions must occur between the macrophages and the endothelial cells that line the blood vessels to allow for monocytic ingress and subsequent differentiation into macrophage-like cells into the affected site. This is a crucial part of the inflammatory process. Activation of the cytoskeleton and adhesive integrins allow these leukocytes to migrate in an extracellular matrix where they are sensitive to variations in the concentrations of chemoattractants; a process known as chemotaxis (25). Bacterial products in addition to cytokines and chemokines released by cells at the site of inflammation are chemoattractants that influence the migration of leukocytes to the affected site (25).

2.2.4 Classification of Macrophages

Macrophages are heterogeneous cells and are able to polarize based on their microenvironment (2). There are two main classifications of macrophages, based on their functional activity (7). Classically activated macrophages, M1, mediate host defense and are generally pro-inflammatory. On the other hand there are M2, or alternatively activated macrophages, which are anti-inflammatory and are, involved in wound healing (7). Macrophages have the ability to induce and resolve immune responses (7).

2.2.5 M1- classically activated macrophages

M1 macrophages are bacterial products that are induced by Induced by Interferon gamma (IFN-γ), and lipopolysaccharides (LPS). M1 macrophages induce the production of pro-inflammatory cytokines, as well as production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (6). M1 macrophages are known to release the interleukin-12 (IL-12) and interleukin-23 (IL-23) cytokines, which promote strong pro-inflammatory Th1 responses (21). As well, anti-proliferative and cytotoxic activities are promoted by M1 macrophages, which result in ROS and RNS production (23). These macrophages are produced during cell-mediated
immune responses and enhance microbicidal and tumoricidal capacity (8). M1 macrophages are also activated after recognition of pathogen associated molecular patterns and endogenous danger signals (23). Produced by innate or adaptive immune cells; natural killer cells produce IFN-γ, which in response to stress and infections can prime macrophages to produce pro-inflammatory cytokines and increased amounts of ROS and nitrogen radicals (8). When natural killer cells produce IFN-γ, the response is usually transient and cannot terminally differentiate a population of macrophages to the M1 phenotype (8). Th1 cells, part of the adaptive immune system produce IFN-γ and maintain classically activated macrophages that can kill indiscriminately (8).

Pro-inflammatory cytokines produced by M1 macrophages are important in host defense but can alternatively cause major tissue damage to the host, thus their activation must be tightly controlled (8). Demonstrated by many acute infections where Escherichia coli (E. coli), is implicated, excessive M1 activation is postulated to be deleterious to the host (2). Induction of M1 macrophages by E. coli though the recognition of LPS by toll like receptor 4 (TLR4) causes the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and LPS induced tumor necrosis factor alpfa (TNF-α) up-regulation (2). Patients with E. coli induced sepsis have increased circulating concentrations of M1 cytokines which is highly correlated with mortality (2). Conversely, patients who demonstrated a mixed M1/M2 macrophage phenotype were more likely to survive the infection (2). Some bacteria such as the Salmonella species are able to neutralize M1 related effectors, and specifically S. typhimurium inhibits phagosome relocalization of Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase causing inhibition of the activity of oxidative microbes (2). Other mediators that are released by M1 macrophages are proteases and bioactive lipids (23).

2.2.6 M2- alternatively activated macrophages

IL-4, IL-13, and IL-10 anti-inflammatory cytokines are produced by Th2-type responses and released in response to tissue destruction by M2 macrophages (9,23). Activation of M2 macrophages leads to a wound healing phenotype, which produces extracellular matrix components (8). M2 macrophages activate the Th2-associated effector functions and regulate T cell functioning (23). Resolving inflammation, phagocytizing apoptotic neutrophils and producing mediators that are important in tissue remodeling and angiogenesis including
Transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) are hallmarks of M2 activity (23). In addition to wound healing, M2 macrophages are involved in humoral immunity and allergic and anti-parasite responses (9). M2 macrophages express higher levels of scavenger receptors and pro-angiogenic factors than M1 macrophages (21). M2 macrophages antagonize toxic M1 responses as well as diminish inflammation (9). They produce growth factors that stimulate epithelial cells and fibroblasts, contributing to wound repair via myofibroblasts and the inhibition of degradation of the extracellular matrix by tissue inhibitors of metalloproteinase (TIMPs) (10). High expression of arginase-1 by M2 macrophages lowers the level of NO secretion by M1’s, which causes tissue destruction (4). CD206 is upregulated in M2 macrophages and is a marker for M1 macrophages and M2’s. CD206, also known as a mannose receptor, which helps bind high mannose structures on pathogens allowing them to be easily phagocytosed by M2 macrophages (8).

Additionally, M2 macrophages play an important role in fibrosis (10). Reprogramming of macrophages toward an M2 phenotype is implicated in the chronic evolution of infectious disease (2). For example, chronic brucellosis is associated with IL-10 mediated M2 polarization (2). This polarization suppresses the host’s ability to fight the infection due to increased amount of M2 macrophages over M1 (2). M2 promoting cytokines, interleukin -4 (IL-4) and interleukin 10 (IL-10) can be neutralized thus increasing the destruction of Brucella abortus by IFN-γ production and subsequent restoration of M1 macrophages (2).

M2 macrophages have been subdivided into three categories; M2a, M2b, and M2c (23). M2a macrophages are activated by IL-14 and IL-13,. M2b macrophages on the other hand are activated by immune complexes and IL-1beta or LPS,. Lastly, M2c macrophages are activated by IL-10, TGF-β, or glucocorticoids (23). In this study we are using M2a macrophages, stimulating them with IL-4.

2.2.7 Macrophages and disease

1. Metabolic disorders: M2 macrophages found in adipose tissue, are known regulators of metabolic functions such as: maintenance of adipocyte function, insulin sensitivity, and glucose tolerance; essential elements in prevention of the development of obesity and type 2 diabetes (10). IL-4 has been implicated in maintaining M2 macrophages in order to maintain non-obese mice.. When obesity
does develop, M2 macrophages switch to the M1 phenotype and create a pro-inflammatory environment (10).

2. Tumorigenesis: M1 macrophages provide a protective role in tumorigenesis by activating tumor-killing mechanisms and suppress activities that promote tumor growth. In addition they provide a positive feedback loop in the anti-tumor response via amplification of t helper 1 (Th1) responses (10). Conversely, tumor-associated macrophages, which are similar in phenotype to M2 macrophages, contribute to tumor progression. This is consistent with the hypothesis that IL-4 and interleukin 13 (IL-13) have tumor promoting tendencies (10).

3. Autoimmunity: M1 produced cytokines have been implicated in a number of chronic inflammatory and auto-immune diseases such as Crohn's disease, rheumatoid arthritis, and periodontal disease (10). These cytokines released by M1 macrophage result in inflammation in the associated tissues as well as tissue destruction (10). In addition contrasting roles exist in these diseases with respect to the different subsets of macrophages (10). For example in rheumatoid arthritis, M1 macrophages produce inflammatory cytokines which lead to chronic polyarthritis, but they also produce ROS which activates T-cells, leading to protection from arthritis (10).

4. Atherosclerosis: the formation of obstructive atherosclerotic plaques is in part caused by macrophages becoming lodged in the intima and subintima of the arteries (10). IFN-γ produced by Th1 cells, stimulates the differentiation of highly active macrophages that promote unstable lesions and increased uptake of low-density lipoprotein (10). Cytokines that are produced by Th2 cells, IL-10, block the formation of pathogenic M1-like macrophages in these plaques (10).

5. Pathogenesis of fibrosis: macrophages are regulators of many progressive fibrotic diseases by producing mediators that activate fibroblasts (10). Macrophages that are pro-fibrotic also secrete their own matrix metalloproteins (MMP's) and TIMP’s as well as secreting pro-fibrotic cytokines (10).

A number of detrimental occurrences can happen when dysregulation of the functioning of macrophages occurs (23). If there is an increased response of M1 macrophages, tissue damage and cytotoxicity can occur (23). On the other hand, an increase in the response of M2
macrophages can lead to fibrosis, exacerbate cytotoxic and allergic responses, and cancer (23).

2.3 Cytokines

2.3.1 Macrophage Colony Stimulating factor (M-CSF) and Granulocyte-macrophage colony stimulating factor (GM-CSF)

Colony stimulating factors are a group of glycoprotein molecules needed for macrophage survival, proliferation, and activation (14). In order for macrophages to grow and differentiate from monocytes in the bloodstream, M-CSF or GM-CSF is required (9). Addition of M-CSF and IL-4 to a culture of bone marrow derived monocytes will induce a classically activated, M1, macrophage to become differentiated (13). Alternatively, addition of GM-CSF and IFN-γ + LPS will induce an alternatively activated macrophage, M2 (9). Immune or inflammatory stimuli can activate macrophages or T cells to produce GM-CSF (15).

2.3.2 Lipopolysaccharide (LPS)

As a principal component of the outer membrane of gram-negative bacteria, LPS is recognized by monocytes and macrophages of the innate immune system (17). Monocytes are able to respond to LPS by expressing a number of inflammatory cytokines such as TNF-α, interleukin-1 (IL-1), interleukin-6 (IL-6), granulocyte colony stimulating factor (G-CSF), GMCSF, and M-CSF (17). LPS binds to the LPS binding protein, which brings it to CD14 on the cell surface, where it interacts with TLR4, a signaling receptor (17). MAPK pathways including ERK, JNK, and p38 are activated by LPS (17). Of particular importance, Rac1 is an upstream signaling molecule in the P38 pathway (17). LPS and Interferon gamma together have the ability to polarize macrophages into the M1 phenotype, which have cytotoxic and anti-tumoral properties (18).

2.3.3 Interferon-gamma

Upon appropriate stimulation, Natural killer (NK) cells and T-helper 1 (TH1) cells will produce IFN-γ (19). Gene expression induced by IFN-γ is accomplished by the activation of the JAK-STAT pathway (19). Along with LPS, IFN-γ is able to polarize macrophages from monocytes into classically activated M1 macrophages with tumoricidal and microbicidal
Properties (19). IFN-γ stimulates macrophages to produce TNF-α, creating a resistance to intracellular bacteria (19). When IFN-γ is overexpressed, there is an increase in expression of MHC class I and II localized inflammation and tissue destruction (19). In a recent study, where mice had a defect in producing IFN-γ, it was shown that they were unable to resist infection caused by some bacteria (19).

2.3.4 IL-4

IL-4, a cytokine known for its ability to polarize macrophages into the M2 phenotype causes the upregulation of the mannose receptor and MHC class II molecules by macrophages, which stimulates endocytosis and antigen presentation (9). In addition, IL-4 induces the expression of arginase, which is important for cell recruitment and granuloma formation (9). T helper 2 (TH2) cells are the main source of IL-4, however mast cells and basophils are also known to produce the cytokine (9). IL-4 also has the ability to cause T cells to differentiate into TH2 cells even in the absence of other important cytokines (9). IL-4 binds to two receptor complexes, interleukin-4 R alpha (IL-4R alpha), a high affinity heterodimer and the interleukin R2 (IL-R2), which is expressed in various levels by T and B cells as well as mast cells and macrophages (9). IL-4Ralpha transduces the signal when bound to IL-4 to activate the STAT6 pathway (9). The actions of IFN-γ, a known activator of M1 macrophages is decreased in the presence of IL-4 (9).

2.4 Rac GTPases

Rac GTPases are enzymes belonging to the superfamily of Rho GTPases, which transduce signals from surface receptors to intracellular signaling pathways (7). The Rho GTPase superfamily controls multiple cellular functions such as cell division, chemotaxis, phagocytosis, NADPH oxidase activation, and superoxide production (6). Active Rho GTPases have GTP bound and can switch to an inactive GDP bound conformation (6). Guanine nucleotide exchange factors are responsible for activation of Rho GTPases, which cause the release of GDP (6). On the other hand, GTPase activating proteins catalyze GTP hydrolysis leading to the formation of GDP, causing the GTPase to be inactive (6). Rac specifically has been shown to perform a wide variety of cellular functions including: actin cytoskeletal regulation, cell polarity, vesicle trafficking, and transcription (book). There are three Rac isoforms consisting of Rac1, Rac2, and Rac3 (11) Rac 1 is a hemopoietic cell specific isoform and has 92% identical amino acid
sequences to Rac 2, which is expressed in many tissues (11). Although Rac 1 and Rac 2 contain identical GEF binding effector domains at amino acids 26-45, association-dissociation affinities have been shown to be 6x greater in Rac 2 (Yamauchi, Marchal et al. 2005). The difference is due to the C-terminal domains of Rac1 and Rac 2, where the greatest divergence in amino acid sequence is observed (Glogauer, Marchal et al. 2003; Yomauchi, Marchal et al 2005). Phagocytosis is an important aspect of macrophage biology and Rac is known for regulating many phagocytic functions (11). NADPH oxidase requires Rac for its activation, which is able to catalyze the production of the superoxide anion (11). Studies have shown that when one inhibits Rac GTPase there is a blockage of actin surface extensions (Hall 1998). Rac is required for normal remodeling of the actin cytoskeleton.

2.4.1 Actin cytoskeleton and the Rho family of small GTPases

Remodeling and formation of the cytoskeleton is of utmost importance to cellular structure and shape (14). The cytoskeleton, which is composed of an array of filamentous actin, (F-actin) mediates cell functions such as providing structural framework, motility, and cell division (14).

It has been shown that the expression of Rac 1 in Rac 2 null macrophages was not affected by the deletion of the Rac 2 gene (11). Furthermore, in the mouse, Rac 1 can compensate for the deletion of the Rac 2 gene (16). However it is still remains relatively unclear how the Rac isoforms function independently and whether they are both required for normal cell function (16).

Consequently, knowing the roles of Rac 1 and Rac 2 in polarization of cells, cell morphology and the actin cytoskeleton, encourages further studies to investigate the role of these proteins in the polarization of M1 and M2 macrophages.

2.4.2 Rho GTPases and disease

Due to their role in cell polarization, cell adhesion, and cytoskeletal regulation, it is essential to study Rho GTPases and their involvement in migration, invasion, and metastasis of human diseases and cancers (15). More specifically, overexpression of Rac 1 has been implicated in causing colorectal tumors (14).
Alternatively, Rac 2 knockout mice demonstrate a phenotype that is closely related to the human leukocyte adhesion deficiency and chronic granulomatous disease as well as exhibiting leukocytosis (16). This has also been noted in a study that was being used to screen for severe combined immunodeficiency (16). Newborns who had a noted infection around their umbilical stumps, were characterized with defects in neutrophil adhesion and migration in addition to neutrophilia and leukocytosis (16). As well, these newborns were shown to have selective defects in NADPH oxidase activity, a feature of chronic granulomatous disease (16).

2.4.3 Rac 1 and Rac 2

Ubiquitously expressed, Rac 1 is the predominant isoform of the 3 Rac isoforms and is responsible mainly for the rearrangement of the actin cytoskeleton (Hall 1998). In murine macrophages, it has been seen that Rac 1 is present in approximately 4-fold excess of Rac 2(11). Rac 1 regulates the morphology of macrophages, and affects macrophage cell migration (12). There is a defect in Rac 1 null macrophages associated with cell spreading as well as a defect in their ability to ruffle their membranes (12).

Rac 2 is expressed primarily in haematopoietic cells, and is essential for normal regulation of superoxide production, phagocytosis, and the accumulation of macrophages to sites of inflammation (11+12). In a previous study Rac 2 null mice show defects in phagocytosis and superoxide production (16). Rac 2, along with Rac 1 is required for normal morphology of macrophages (12). There is a third isoform, Rac 3 which is found in all cell types, however it will not be studied here.

2.5 Mouse model

The use of transgenic animal models has enabled the study of a variety of diseases through selective gene mutations. Advances in genetic technology have allowed the generation of mouse knockout models to manipulate individual, or multiple genes in order to facilitate their study (3). Mice share 60-70% of their genetic homology with humans and are therefore serve as a useful model in the study of genetic diseases affecting humans (12). With respect to Rac GTPases, the amino acid sequence of murine Rac1 and human Rac1 are reported to be identical. In addition, murine Rac2 differs from human Rac 2 by only two amino acids, aspartate/glutamate substitution at position 148 and proline/alanine at position 188 (5).
Despite similarities between human and murine models, there is a limit to the extent the animal models can translate into human application. Due to the genetic differences between animals and humans, these models will never translate 100% to the human response. However, despite limitations, the use of knock-out mice models allows the advancement of studies beyond in vitro cell cultures to determine the overall effect in vivo.

Chapter 3
Materials and Methods

3.1 Chemicals and Reagents

Cell culture medium: MEM alpha and D-MEM cell culture media (Invitrogen, Carlsbad, CA). Western blotting: RIPA buffer (9806, cell signaling technologies, Danvers, MA); 50x Protease Inhibitor Cocktail (51-21426Z, BD Bioscience Pharmingen, Franklin Lakes, NJ); BCA Protein Assay Kit (23225, Pierce, Rockford, IL); Nitrocellulose Membrane (8549062, GE Healthcare, Piscataway, NJ);
3.2 Isolation of Murine Bone Marrow Derived macrophage Progenitors and differentiation into M1 and M2 macrophages

All procedures described were performed in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the University of Toronto Animal Care Committee. Bone marrow monocytes (BMMs) were taken from 4-12 week old WT, Rac1 null, and Rac2 null mice. Mice were sacrificed via cervical dislocation and under aseptic conditions; the tibia and femora were removed and stripped of adherent soft tissue inside a laminar air flow bio-safety cabinet. Epiphyses of the bones were cut and the bone marrow was flushed with a 25-gauge needle using α-MEM culture media. The flushed bone marrow was then passed through a 20-gauge needle in order to break down the bone marrow into a homogeneous, single cell suspension. Cells were then spun down and resuspended in 12ml of α-MEM containing 10% FBS and 10% antibiotics and cultured overnight in a t-75 flask. Non-adherent BMMs are taken from the t-75 flask and counted using a Z1 Coulter Particle Counter (Coulter Electronics, Hialeah, FL, USA). M1 and M2 macrophages were created by seeding 1x10^6 cells in a 6- well tissue culture plastic plate or by seeding 0.3x10^6 cells in a 12-well tissue culture plastic plate, depending on the experiment being run. Cells to become M1 macrophages are stimulated with 10ng/ml of GM-CSF whereas the M2 macrophages are stimulated with 20ng/ml of M-CSF for 48 hours. 48 hours later another round of 10ng/ml GM-CSF is placed on the M1 macrophages and 20ng/ml of M-CSF is placed on the M2 macrophages. After another 24 hours, cells are treated with new α-MEM containing 10% FBS and 10% antibiotics and M1 macrophages are again given 10ng/ml of GM-CSF as well as 1ng/ml of IFN- γ and 10ng/ml of LPS e.coli. M2 macrophages were given 20ng/ml of M-CSF as well as 10ng/ml of IL-4 for 48 hours. After 48 hours various experiments were run with the M1 and M2 macrophages.

3.3 Western blot analysis

Macrophages in culture were washed three times with PBS and lysed in RIPA buffer supplemented with 1 mM PMSF and 1x protease inhibitor cocktail and collected by cell scraping. The cells are centrifuged at 16000xg for 5 minutes at 4oC. Total protein concentration was determined using a BCA protein assay kit. 5x Laemmli sample buffer was added to 10ug of
total cell lysates and heated at 100°C for 5min. Samples were resolved via SDS-PAGE on 12% polyacrylamide gels. Following electrophoresis, samples were transferred onto a nitrocellulose membrane. Non-specific sites were blocked with 5% milk powder in TBST for 1 hour at room temperature. Membranes were incubated with primary antibodies (see below) overnight at 4°C on a shaker, washed three times with TBST, incubated with the corresponding secondary antibody for 1 hour and washed three times with TBST. Immuno-reactive proteins were detected using chemiluminescence with Amersham ECL Plus Western Blotting Detection System, upon exposure to Bioflex MSI film. Films were developed using the Kodak M35A X-OMAT Processor, scanned digitally using the Epson Perfection 1250 scanner and band intensities were quantified by densitometry using Image J 1.41 software. Band intensities were normalized against total β-actin. N=3 for all western blots.

3.4 NBT assay

Zymosin was thawed in at 37°C water bath for 2 minutes. After stimulating the Bone Marrow Derived Macrophages with cytokines for 48 hours 50 microliters of NBT and 25 microliters of the thawed zymosin were added to each well of a 12-well plate. The plate was spun at 500xg for 1minute and then incubated at 37°C for 5 minutes. The cells were then washed 3x with α-MEM containing 10% FBS and 10% antibiotics. 50 microliters of NBT is then added again and incubated at 37°C for 20-30 minutes. Cells were washed 3x with PBS and then fixed with 4% paraformaldehyde for 15 minutes. Cells were washed again 3x with PBS. Five to ten pictures were taken for each well and the amount of blue staining is measured (precipitation of ROS). N=3 for all NBT assays performed.

3.5 Cell Migration Assay

DMEM media containing 10% FBS and 10% antibiotics (700 microliters) is added to the wells of a 24-well cell culture plate, followed by incubation of the transwells in the wells at 37°C for 1-2 hours to equilibrate the membrane. Cells that were stimulated 48 hours prior to the experiment are scraped from the bottom of the wells and counted. 2 x 10⁶ cells/ml are spun down at 300g for 5 minutes and resuspended in 1ml of complete media. Media from the equilibrated chambers are aspirated and 0.1ml of the cell suspension is added to each transwell with 0.5ml of
medium in the lower well followed by an incubation for 2 hours at 37°C to allow the cells to attach to the membrane. After the 2 hours, 0.5ml of fresh media containing 20ng/ml of M-CSF (the chemoattractant) is added to spare wells and the transwells are added to these wells. On top of each transwell insert, 0.1ml of complete media without the chemotactrant was added. These wells were incubated for 1-2 hours to allow the cells to migrate. At the end of the incubation cells were washed with PBS 3x and fixed with 4% Paraformaldehyde for 30 minutes at room temperature. Again the cells were washed with PBS 3x and stained with DAPI for 10 minutes at room temperature in a dark place. Cotton swabs were used to remove cells on the upper side of the membrane and the membrane was removed with a razor blade. Membrane was then washed with PBS and mounted with anti-fade mounting medium on a glass slide with the cells facing up. The migrated cells that migrated through the membrane were counted. N=3 for the cell migration assay.

3.6 Cell shape and morphology

After stimulating the Bone Marrow Derived Macrophages with cytokines for 48 hours, cells were washed 3x with PBS and then fixed with 4% paraformaldehyde for 20 minutes at room temperature. Cells were again washed 3x with PBS and glycine was added for 10 minutes and washed 3x with PBS. 0.1% Triton in PBS was added to the cells and left on for 5 minutes and then removed from the plate. 1% BSA +0.1% Triton in PBS was added to the cells for 30 minutes at room temperature. 200 microliters of a 1:40 dilution of 488 phalloidin in 1% BSA +0.1% Triton in PBS was added to the cells and incubated in the dark for 20 minutes. Cells were washed 3x with PBS and coverslips were mounted on a glass slide. Pictures were taken of cells and their lengths and widths were measured to determine the ratio of these parameters. N=3

3.7 Griess assay

Protocol taken from Promega Griess Reagent System. For each assay a nitrite standard curve was prepared for quantitation of NO\textsubscript{2}\textsuperscript{-} levels. Reference curves were prepared in α MEM with 10% antibiotics and 10% FBS. A 1ml of a 100 µM nitrite solution was prepared by diluting the provided 0.1M nitrite standard 1:1000 in α MEM with 10% antibiotics and 10% FBS. 24 wells in a 96-well plate were designated for the nitrite standard reference curve. 50 µl of α MEM with 10% antibiotics and 10% FBS was added into the wells in rows B-H. 100 µl of the 100 µM
nitrite solution was added to the remaining 3 wells in row A. 6 serial twofold dilutions (50µl/well) were performed in triplicates down the plate to generate the nitrite standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56µM) and 50µl from the 1.56µM set of wells was discarded. No nitrite solution was added to the last set of wells.

![Figure 1. Suggested plate format for the Nitrite Standard reference curve.](image)

The Sulfanilamide solution and NED solutions were equilibrated to room temperature for 15-30 minutes. 50 µl of each experimental sample was added to wells in duplicates or triplicates. 50 µl of the sulfanilamide solution was added to all experimental samples and wells containing the dilution series for the nitrite standard reference curve. The plate was then incubated for 5-10 minutes at room temperature in a dark place. 50 µl of the NED solution was then added to all the wells. The plate was incubated again at room temperature for 5-10 minutes in a dark place. Within 30 minutes the absorbance was measured with a filter between 520nm and 550nm.
3.8 Phagosome pH measurement

In order to measure the pH of the phagosome a probe must be used. A suitable dye should be available to target the phagosome and must have a pKa near the expected pH of the compartment of interest. FITC is used as a pH indicator and it is loaded into the phagosome by covalent conjugation to sheep red blood cells (sRBCs). $10^6$ cells/mL macrophages are plated on 25-mm diameter glass coverslips and differentiated according to the protocol above. After the 7 days of differentiation, cells are washed twice with incubation buffer to remove non-adherent macrophages. 1mL of PBS is added to the covers slips and it is placed in a chamber holder maintained at 37°C above the 100x objective to clearly resolve the internalized particles. A field
was found with several adherent macrophages. 500 µL of a suspension of FITC- labeled and opsonized sRBCs is added to the cells. The cells are monitored using bright field video microscopy until one or more of the sRBCs are seen to bind to a macrophage. fluorescent images are acquired by alternatively illuminating the field with 490nm and 440nm wavelength light as the particle is internalized for the duration of the assay. An in situ calibration is performed after the desired maturation time course. The cells were washed once with the initial calibration solution and then 1mL of the same calibration solution containing nigericin is added. The cells of interest always remain in the field of view and in focus after all solution changes. After 3-5 minutes of equilibration of the phagosomal pH with the calibration solution a series of images is acquired. The images were used to calculate the 490nm/440nm fluorescent ratio value corresponding to the pH of the first calibration solution. With the use of image analysis software a region of interest is defined covering the internalized particle and the average fluorescent intensity of that region of interest is recorded in both the 490nm and 440nm channels. Background readings at both wavelengths from a comparable size region of innerst in the region outside the cells were obtained and was subtracted from the phagosomal readings. From the background corrected values, the 490nm/440nm ratio was computed. Proceeding through the time course and calibration it was ensured that the region of interest was aligned over the phagosome in case a movement had occurred. A calibration curve was constructed by plotting the background-corrected 490nm/440nm ratio as a function of pH. The pH of the phagosome was determined throughout the time course by interpolating the pH from the calibration curve using the measured ratios (10). The same experiment was conducted by adding 10 micromolar of Diphenyleneiodonium (DPI) at the time when FITC is added. DPI is an inhibitor of inducible isoform of Nitric oxide synthase (iNOS) N=5

3.9 Flow cytometry

BM-derived macrophages were recovered by scraping of the plate. Surface marker expression was analyzed by flow cytometry after 24 and 48 hours post stimulation with M1 and M2 cytokines (BD FACS Aria Flow Cytometer, Erembodegem, Belgium). Cells were harvested, washed in 1XPBS twice and incubated for 30 minutes with antibodies against F4/80 (clone BM8, Biolegend), CD36 (clone 72.1, eBioscience), CD80 (clone 16-10A1, Biolegend), CD206 (clone C068C2, Biolegend) at room temperature, in the dark. Fixed in 2% PFA for 15 minutes on ice,
washed twice again and re-suspended in flow buffer. The surface expression levels for each marker were measured on day 2 of polarization, after adding fresh DMEM with 10% FBS and polarizing cytokines. The concentration of the cytokines was modulated in dose–response experiments. Data was analyzed with Flow Jo Flow Cytometry Analysis software (Tree Star, Ashland, OR) after gating on the live cells population in the Fixable Viability Dye eFluor 506/FSC-A window. Values were expressed as the ratio of the median fluorescence intensity (MFI). N=5

3.10 Phagocytosis assay

Fluorescent labeled zymosan was spun down for 5 minutes at 2000rpm and then resuspended in 0.5ml of mouse serum and incubated at 37°C for 30 minutes. The zymosan was then re-suspended in 0.5ml of α-MEM+/+. 10 ul of zymosan was added to each well of bone marrow derived marphages taken from WT, Rac1 null, and Rac2 null mice and differentiated into M1 and M2 macrophages for 48 hours. The plate was then centrifuged for 1 minute at 1500 rpm and then incubated for 30 minutes at 37°C. The wells were then washed 3x with α-MEM+/+. The plate was then placed on ice and media was replaced with α-MEM+/+ containing secondary mouse anti-body with FITC attached. The plate was incubated on ice for 30 minutes and then wells were washed with α-MEM+/+ 3x. The cells were then fixed with 4% PFA for 20 minutes and mounted onto slides using fluorescent mounting media. Confocal microscopy was used to view the slides. N=3

3.11 Bacterial killing assay

A colony from a master plate of DH5α bacteria was picked and put into 10ml of LB broth. The bacteria was incubated shakingly overnight at 37°C. The bacteria was spun down at 400rpm for 10 minutes and then resuspended in 2ml of PBS+/+. The amount of bacteria was counted by taking 50ul and mixing it with 2ml of PBS+/+ in a cuvette. The OD600 reading was taken and the concentration was calculated giving 10⁹ cells/ml. The stock of bacteria was then diluted to 1x10⁹ cells/ml and a serial dilution was performed from 1x10⁹ to 1x10² cells/ml. 100ul of 1x10⁴, 1x10³, and 1x10² cells/ ml were plated on agar plates. The tube of 1x10⁹ cells/ml was
opsonized for 30 minutes in heat-inactivated bovine serum. The bacteria was then washed and resuspended in 1ml of PBS+/. A serial dilution was performed from 1x10^9 cells/ml to 1x10^2 cells/ml. 100ul of 1x10^4, 1x10^3, and 1x10^2 cells/ml were plated on agar plates. The suspension of bacteria in the 1x10^8 cells/ml was diluted to a concentration of 6x10^7 cells/ml. WT M1 and WT M2 cells were each diluted to a concentration of 10x10^6 cells/ml and added to separate eppendorf tubes containing 6x10^7 cells/ml of bacteria. These tubes were incubated shakingly at 2500rpm at 37°C for 1 hour. The mixture was then washed and re-suspended in 200ul of 1% saporin to lyse the macrophages. Serial dilutions were made for both M1 and M2 samples to make 10^3, 10^2, and 10^1 cells/ml and each concentration was plated on agar plates. All the plated were incubated overnight and the number of colonies were counted. N=3

Chapter 4

Results

4.1 Small Rho GTPase expression

Rac1, CDC42, and RhoA have increased expression in WT M2 macrophages vs. WT M1 macrophages. Western blot analysis has indicated that Rac1, CDC42, RhoA expression is increased in WT M2 macrophages vs. WT M1 macrophages derived from primary monocytes taken from WT mouse bone marrow. BMMs were induced to undergo differentiation into M1 and M2 macrophages, as well as stimulation with only M-CSF to get a partial M2 macrophage and stimulation with only GM-CSF to get a partial M1 phenotype, protein was isolated and a western blot was run for Rac1, CDC42, and RhoA.
4.2 Macrophage shape and morphology

WT M1 macrophages display a rounder shape than WT M2 macrophages, which display a long thinner shape. The lengths and widths of 100 macrophages were measured and the ratio of length: width was measured. The smaller the length: width ratio the rounder the cell shape was whereas the larger the ratio, the thinner and longer in shape the cells were. On average, the M1 macrophages gave a ratio of 1.46 whereas the M2 macrophages have a ratio of 7.85, which is significantly different (p<0.05) from 1.46. M1 and M2 macrophages were also stained with phalloidin in order to visualize the difference in the shapes of the cells.
4.3 Production of ROS

WT M1 macrophages produce more ROS than M2 macrophages. An NBT assay was performed in order to measure the amount of ROS produced by M1 and M2 WT macrophages. NBT acts as an oxidant therefore when in contact with ROS it will precipitate into a blue dye. The amount of blue dye was measured on 15 fields for each M1 and M2 macrophages. There was a significant difference (p<0.05) in the % of blue dye in the field between M1 and M2 macrophages with the average percent for each cell type being 11.81% for M1 macrophages and 2.72% for M2 macrophages indicating that M1 macrophages produce more ROS than M2 macrophages.
4.4 NO production

WT M1 macrophages produce more NO than WT M2 macrophages. A Griess assay was performed in order to measure the amount of NO produced by M1 and M2 macrophages. On average, M1 macrophages produced significantly more (p<0.05) NO than M2 macrophages. The amount of NO produced is measured by the absorbance of the coloured azo compound. The average absorbance of the WT M1 macrophages was 989 whereas the M2 macrophages had an average absorbance of 380.

4.5 Acidification of phagosomes

M1 macrophages acidify their phagosomes faster than M2 macrophages. WT M1 and M2 show a slight alkination within the first 5 minutes and then begin to acidify after with the M2s slightly lagging. Small bouts of alkination and acidification take place once acidified. Both M1 and M2 WT macrophages are able to acidify their phagosomes.

4.6 Flow cytometry

Macrophages stimulated with GMCSF+IFN+LPS display increased M1 markers whereas macrophages stimulated with MCSF+IL4 display increased M2 markers. Using flow cytometry 48 hours post stimulation, it has been shown that the M1 markers, CD80 and CD36, are upregulated in cells that are stimulated with GMCSF+IFN+LPS when compared to cells that are stimulated with MCSF+IL-4 in all cases, WT, Rac1 null, and Rac2 null bone marrow derived macrophages (there is a significant difference, p<0.05). We see no difference in these M1 markers between WT, Rac1 null, and Rac2 null bone marrow derived macrophages, however there is a significant increase in the amount of these markers in double knockout bone marrow derived macrophages stimulated with GMCSF+IFN+LPS. Using the M2 marker, CD206 there is a significant increase in the amount of CD206 in cells that are stimulated with MCSF+IL-4 when compared to cells stimulated with GMCSF+IFN+LPS. There is also a significant increase in the amount of CD206 in the double knockout bone marrow derived macrophages as well as the Rac1 null bone marrow derived macrophages stimulated with MCSF+IL-4 when compared to macrophages from WT and Rac2 null bone marrow derived macrophages stimulated with MCSF+IL-4. It has been shown that 24 hours post stimulation; there is a significant decrease in
the amount M1 surface markers CD36 and CD80 in cells stimulated with M1 cytokines when compared to cells treated with M1 cytokines for 48 hours.

Figure 5. Macrophage differentiation based on surface marker expression occurs at 48h. Flow cytometry to assess surface marker expression was used to determine the ideal timing of macrophages polarization towards M1 and M2 phenotypes. Comparison of the expression of CD36, CD80 and CD206 in macrophages polarized towards M1 and M2 phenotypes for 24h and 48h was assessed. (A) Representative histograms (n=3). B. Average median fluorescence intensity ± SEM for surface markers, CD36, CD80, and CD206 (n=3). (MFI) Median fluorescence intensity.
4.7 M1 macrophages have decreased amounts of various small Rho GTPases

Using a Western blot it has been shown that Rac1 has increased expression in M2 macrophages when compared to M1 macrophages. Rac1 has a greater increase in Rac2 null M2 macrophages when compared to WT M2 macrophages suggesting a compensatory effect. As well, other small Rho GTPases, CDC24 and RhoA show higher expression in M2 macrophages when compared to M1.

4.8 Macrophage shape

Rac1 and Rac2 null macrophages M1 macrophages are rounder than their M2 counterparts. It has been shown that the WT, Rac1 null, and Rac 2 null M1 macrophages all have a rounder cell shape when compared to their M2 counterparts. As well it has been shown that
Rac1 null and Rac2 null M2 macrophages are rounder than the WT M2 macrophages. Rac1 null M1 macrophages have a length:width ratio of 1.97 whereas M2s length:width ratio is 5.01 (p<0.05). Rac2 null M1 macrophages have a length width ratio of 2.0 and M2s is 5.61.

4.9 M1 macrophages produce more ROS and NO than their M2 counterparts

We used an NBT assay to measure the amount of superoxide produced by bone marrow derived macrophages from WT M1, WT M2, Rac1 null M1, Rac1 null M2, Rac2 null M1, and Rac2 null M2. It had been shown that in all the cases, M1 macrophages produce significantly more superoxide than their M2 counterparts. Rac1 and Rac2 null M1 macrophages produce slightly less superoxide than WT M1 macrophages however the difference is not significant. The percent area covered by the blue staining that is produced by the ROS from RAC1 null M1, Rac1 null M2, Rac2 null M1 and Rac2 null M2 is 6.53, 1.58, 8.65, and 0.38 respectively. Using a Griess assay to measure NO production from these same groups including DKO M1 and DKO M2, it has been shown that in all cases M1 macrophages produce significantly more NO than M2 macrophages with no significant differences between WT, Rac1 null, Rac2 null, and DKO.
4.10 M1 macrophages display heterogeneity in their abilities to acidify their phagosomes.

Phagosomal pH was measured in WT, Rac1 null, and Rac2 null M1 and M2 macrophages. In all cell types, WT and Rac1 null, and Rac2 null macrophages, there was heterogeneity in the M1 populations. There were some M1 macrophages where the pH of the phagosome was acidified within 5-20 minutes. On the other hand, there were populations of M1 macrophages that were unable to acidify their phagosomes, pH oscillates between pH6 and pH6.8. There is a significant difference between the final pH of the M1 macrophage that fail to acidify when compared to the M1 macrophages that are able to acidify. In all cell types, M2 macrophages display a homogeneous population where all cells are able to acidify their
phagosomes. When DPI, an NADPH oxidase inhibitor, was added, the population of M1 macrophages becomes homogeneous. All M1 macrophages are able to acidify their phagosomes.

Figure 8. M1 macrophage populations display heterogeneity whereas M2 populations are homogeneous. A. M1 macrophages are a heterogeneous population. Some cells produce more ROS than others, as well some are able to acidify their phagosomes while some are not able. B. M2 macrophages are a homogenous population and they are all able to acidify their phagosomes at 9.8 minutes. There is a significant difference in the M1 macrophages that do not acidify their phagosomes when compared to the ones that are able to acidify their phagosomes. There is no significant difference between M1 macrophages that are able to acidify their phagosomes when compared to M2 macrophages. n=3.
4.11 Macrophage migration

M2 macrophages migrate more than M1 macrophages. Using a transwell assay, a chemoattractant is used in order to stimulate macrophage migration. Macrophages tend to move towards the chemoattractant M-CSF and it has been shown that M2 macrophages are better able to migrate than M1 macrophages. There is a significant difference between M1 and M2 macrophages in all genotypes (WT, Rac1 null and Rac2 null) with Rac1 showing a significant increase in M1 migration compared to WT M1 and Rac1 null M2 show a significant increase in migration when compared to WT M2. Cells migrate through a matrix and the matrix on the side that the cells migrate from is wiped clean so that only cells that have migrated on the opposite side of the matrix are stained with DAPI and visualized in the microscope. Cells that are stained with DAPI are counted to determine the number of cells that have migrated. WT M1

Figure 9. DPI homogenizes the population of M1 macrophages and causes acidification in all M1 macrophages. All M1 macrophages with the addition of DPI are able to acidify their phagosomes. DPI is a NADPH oxidase inhibitor. A. both M1 and M2 macrophages are able to acidify their phagosomes. There is now a homogeneous population of M1 macrophages. B. Rac1 null macrophages. C. Rac2 null macrophages n=3.
macrophages display on average 1.6 cells per field whereas M2 macrophages display on average 16.6 cells per field. Rac1 null macrophages show increased migration with Rac1 null M1 macrophages migrating more than M2 but both migrate more than their WT counterparts. Rac1 null M1 and M2 macrophages display an average of 11.2 and 35.4 cells per field respectively. Rac2 null M1 macrophages tend not to migrate at all whereas M2s show an average of 16.8 cells per field.

Figure 10. Rac1 null M2 macrophages more chemotactic than WT and Rac2 null macrophages. Cells cultured in 24-well plates and stimulated 48 hours prior to the experiment, scraped, counted, seeded (2 x 10^6 cells/mL) and incubated to allow the cells to attach to the membrane. Membrane was mounted with anti-fade mounting medium on a glass slide and the migrated cells were counted. An increase in M2 macrophages were found on the membranes when compared to M1 macrophages. n=3.
4.12 Bacterial Killing assay

M1 macrophages are more efficient at killing bacteria than M2 macrophages. There is a significant difference between the amount of bacteria that survived with the M1 bacteria, 3.65±0.2% and the M2 bacteria, which has a survival rate of 10.87±0.29%. M1 and M2 macrophages are left with the bacteria for 1 hour n=3.

Figure 11. **Rac1 M2 macrophages migrate more than WT and Rac2 M2 macrophages.** Cells cultured in 24-well plates and stimulated 48 hours prior to the experiment, scraped, counted, seeded (2 x 10^6 cells/mL) and incubated to allow the cells to attach to the membrane. Membrane was mounted with anti-fade mounting medium on a glass slide and the migrated cells were counted. A significant increase in Rac1 M2 macrophages were found on the membranes when compared to WT and Rac2 M2 macrophages (p<0.05). n=3.
Chapter 5

Discussion

Playing an important role in host defense, macrophages also play a conspicuous role in innate and acquired immunity (30). Macrophages are an important component of the mononuclear phagocyte system that is comprised of cells of bone marrow origin, including monocytes and tissue macrophages (30). Although one of the primary roles of macrophages is tissue repair and remodeling, it is also appreciated that, during inflammation, macrophages can contribute to tissue damage via production of inflammatory mediators, such as ROS (30). Three major functions of macrophages during inflammation include; phagocytosis, antigen presentation, and immune-modulation through production of various cytokines and growth

Figure 12. M1 macrophages are more efficient at killing bacteria than M2 macrophages. There is a significant difference between the amount of bacteria that survived with the M1 bacteria, $3.65\pm0.2\%$ and the M2 bacteria, which has a survival rate of $10.87\pm0.29\%$. M1 and M2 macrophages are left with the bacteria for 1 hour $n=3$. 
factors (30). Macrophages also display many other functions such as ROS and NO release, chemotaxis, bacterial killing, phagosome acidification as well as many other functions. These functions differ between M1 and M2 macrophages almost being able to categorize them as different cell types. Rac1 and Rac2 are members of the Rho family of small GTPases, which regulate a wide number of cellular functions (26). It has been reported that knockouts of Rac1 and Rac2 have a variety of deleterious affects on cell function and shape, which have been demonstrated to have different effects in M1 and M2 macrophages. In order to study the functional characteristics of M1 and M2 macrophages bone marrow derived macrophages were stimulated with IFN, LPS and GM-CSF and M-CSF and IL-4 respectively.

According to our flow cytometry results we can see that Rac1 and Rac2 do not affect the ability of the macrophages to polarize into M1 and M2 macrophages, however there are aspects of the functions of M1 and M2 macrophages that are changed when Rac1 or Rac2 are knocked out. First of all, it has been shown that ROS production is affected when Rac1 and Rac2 are knocked out. M1 macrophages have a greater ability to produce ROS than M2 macrophages, as previously described (6). Here we have shown that when Rac1 is knocked out we get a reduction in the amount of ROS produced when compared to the WT, as well an even greater reduction in ROS production when Rac2 is knocked out. Rac is important for NADPH oxidase (Nox) function and is therefore most likely responsible for the decreased ROS production in the knockout macrophages. Nox deliberately produces ROS and participates in other biological processes including innate immunity. Nox requires the small GTPase Rac in addition to other proteins to produce superoxide. It has been previously shown in neutrophils with Rac2 knocked out a significant decrease in ROS production (27).

We have also shown that M2 macrophages are more chemotactic than M1 macrophages. This is most likely due to the homeostatic function of the M2 macrophages. They are required by the immune system to migrate more so than the M1 macrophages, they must have the ability promote tissue repair as well as tissue remodeling. We see in M2 macrophages there are increased levels of small Rho GTPases such as CDC42, RhoA, and Rac1. Because Rho GTPases alter the arrangement of the actin filaments and the actin cytoskeleton is central to cell migration, it is likely that increased levels of these GTPases would cause increase levels of migration (26). It is known that Rac1 drives lamellipodium extension at the front of migrating cells, Rho
stimulates actomyosin contractility at the back, and CDC42 is required for determining the direction of migration, which is important in chemotaxis (26).

Chapter 6

Future directions

In this project we have taken some steps towards showing some functional differences between M1 and M2 macrophages. As well we have shown some defects in M1 and M2 macrophages when Rac is knocked out. However, there are a number of important questions that still remain unanswered. This section is devoted to describing necessary future experiments needed to resolve these remaining issues.

Going further it would be interesting to look into the reasons why not all M1 macrophages are able to acidify their phagosomes. The production of ROS within a macrophage during an immune response is crucial for antimicrobial activity as well as causing inflammation. Because excess oxidants could cause tissue damage and oxidative stress, phagocytes must control the timing and location of the NADPH oxidase activity. It is very important to know acidification activity in M1 and M2 macrophages and their differences. It could be a number of factors such as phagosome sealing, ATPase pump, and NADPH oxidase malfunctions. It would be important to know why when DPI, an NADPH oxidase inhibitor is added to a population of M1 macrophages, they are all able to acidify their phagosomes. It is known that Rac1 and Rac2 play an important role in NADPH oxidase activity, therefore looking further into their roles in this process is very important.

In this project we looked at macrophage chemotaxis towards the chemoattractant M-CSF. We were able to show that M2 macrophages are more chemotactic than their M1 counterparts. As well, we showed that Rac1 null macrophages were able to chemotax more so than Rac2 null macrophages. It would be important to look at various chemoattractants and discover whether M1 and M2 macrophages are able to chemotax at different rates depending on the chemoattractant present. Sites of infection or injury secrete an many inflammatory cytokines that act as chemoattractants which recruit macrophages (22). Macrophages have the ability to sense
and migrate along a gradient of these factors. Macrophages use cytoskeletal remodeling to move through tissues of various physical properties. Because Rac1 and Rac2 both play important roles in cytoskeletal rearrangement it is important to look at their role in chemotaxis further.

In this project there were difficulties looking at phagocytosis in M1 and M2 macrophages. Phagocytosis is an important mechanism for homeostasis as well as for removal of harmful pathogens. Interestingly, phagocytosis requires the cell to change shape in order to be able to engulf particles. It is known that the actin cytoskeleton is a major force driving phagocytosis (22). There are many receptors and ligands that can trigger phagocytosis to occur. Different Rho GTPases are activates at different times and locations during phagocytosis, Rac1 and Rac2 are localized near the base of the phagocytic cup, which is the base of the phagosome (22). It would be important to look at differences in M1 and M2 macrophage phagocytosis. As well it is important to look at Rac1 and Rac2 null macrophages in phagocytosis, as they play an important role in this process.
References


