HEME OXYGENASE-1 REGULATES CCR2/MCP-1 AXIS AND GRANULOMA FORMATION IN A MOUSE MODEL OF NONTUBERCULOUS MYCOBACTERIAL INFECTION.

By

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HEME OXYGENASE-1 REGULATES CCR2/MCP-1 AXIS AND GRANULOMA FORMATION IN A MOUSE MODEL OF NONTUBERCULOUS MYCOBACTERIAL INFECTION

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Non Tuberculous Mycobacterial (NTM) infections are a major cause of disease in animals and humans around the world and have become an increasing source of infection among patients with HIV and AIDS. Recently, there has been an increase in NTM infections in immunocompetent patients, especially in the Southeastern United states. A hallmark of NTM infection is the formation of granulomas which are a conglomeration of the invading pathogen and host inflammatory cells including macrophages, T-cells, B-cells and dendritic cells. Granulomas can contain the pathogen and protect the host from further dissemination of the infection. Heme Oxygenase-1 (HO-1) is a cytoprotective enzyme which breaks down heme. HO-1 is induced by oxidative stress, bacterial infection and many pro-inflammatory cytokines and has been shown to be up-regulated in granulomas and infected tissue. Monocyte Chemotactic Protein-1 (MCP-1) along with its receptor Chemokine Receptor2 (CCR2), are responsible for the recruitment of monocytes from peripheral blood to the site of infection. Our hypothesis is that HO-1 plays a role in the formation of organized (tight) granulomas by regulating the expression of CCR2 and MCP-1 in monocytes and coordinating cellular recruitment form peripheral blood to the site of infection. We have developed a mouse model of
Mycobacterium avium infection in both HO-1 +/+ and HO-1 -/- mice to test whether the lack of HO-1 expression results in the formation of unorganized (loose) granulomas and to test whether HO-1 regulates MCP-1 and CCR2 in this disease model.
CHAPTER 1
INTRODUCTION

Non-Tuberculous Mycobacteria (NTM) are a group of Mycobacteria that are not members of the *Mycobacterium tuberculosis* complex. NTM are a major cause of opportunistic infection in immunocompromised and healthy hosts. These organisms can cause disease in animals and humans and have become an increasing source of infection among patients infected with Human Immunodeficiency Virus (HIV) or Acquired Immune Deficiency Syndrome (AIDS) (1).

*Mycobacterium avium* complex (MAC) is the most common disseminated bacterial infection in untreated patients with AIDS. A hallmark of disseminated MAC infections is the formation of granulomas in multiple organs including the liver, spleen, lymph nodes, bone marrow, and lung (1). Granulomas are lesions in tissue that are formed by the aggregation of activated macrophages, T-cells and fibroblasts and are considered a defense mechanism against invading pathogens. Importantly, bacteria are not always eliminated within the granuloma, but can become dormant, resulting in a latent infection (2). In disseminated mycobacterial disease, the function and structure of granulomas are either absent or compromised. This leads to a failure of the host defenses to contain the bacterium and to further pathology. Our current study aims to elucidate the role of Heme Oxygenase-1 (HO-1), a cytoprotective enzyme, in the formation of organized granulomas. Specifically, we are looking at the role of HO-1 in the regulation of two cytokines, Chemokine Receptor 2 (CCR2) and Monocyte Chemoattractant Protein-1 (MCP-1) both of which are responsible for the recruitment of inflammatory cells to the site of infection. Recruitment of immune cells to the site of infection is a vital step in granuloma formation and if the process is inhibited or altered, the Mycobacterial infection may not be contained.
Hypothesis

Our hypothesis was that HO-1 plays a key role in granuloma formation following mycobacterial infection by regulating the expression of CCR2 and MCP-1 in mouse monocytes, therefore, influencing the recruitment of these inflammatory cells to the site of injury. In HO-1 ++/+ mice, we expected to find high levels of MCP-1 in the lung following infection with \textit{M. avium}, creating a gradient for chemotaxis of immune cells from the periphery to the site of injury. In HO-1 --/-- mice, in which the HO-1 gene was mutated and knocked out, we expected to see high levels of MCP-1 and CCR2 in the periphery as well as in the lung following infection with \textit{M. avium}, therefore, causing the gradient for chemotaxis to be disturbed and inflammation to be disseminated.

Objective

Our objective was to develop a murine model of \textit{M. avium} infection and granuloma formation in order to elucidate the role of HO-1 and its regulation of MCP-1 and CCR2 in mouse monocytes.
CHAPTER 2
BACKGROUND

**Mycobacterium**

Mycobacterium is a genus of Actinobacteria, a gram positive bacterium with a high G-C content. Mycobacteria are aerobic and non-motile. They are classified as gram positive because they lack an outer cell membrane but they characteristically have a thick cell wall comprised of mycolic acid or mycolates (the Latin prefix “myco” pertains to the waxy compounds in the cell wall of these organisms). The Mycobacterial cell wall consists of a hydrophobic mycolate layer and a peptidoglycan (PGN) layer which are held together by arabinogalactan, a polysaccharide (1). This waxy cell wall is an essential virulence factor which contributes to the organism’s survival and gives these organisms their ability to withstand the environment and resist many antibiotics. Mycobacteria can survive long exposure to acids, alkalis, detergents, oxidative burst, and lysis by complement. They can also resist antibiotics that target the cell wall such as penicillin. These organisms are widespread in the environment and typically live in water and food sources (4).

Before the AIDS epidemic, the usual presentation of NTM disease in immunocompetent patients was pulmonary, confined to cervical lymph nodes, limited to skin or in rare cases, disseminated (3). Most patients presenting this disease were older men who had previous underlying pulmonary diseases such as chronic obstructive pulmonary disease (COPD), bronchiectasis, previous tuberculosis and silicosis (5). Other risk factors for NTM disease include: reduced immune competence due to cancer chemotherapy, immunosuppression involved with transplantation, altered chest architecture, alcoholism, and smoking tobacco (4). Recent findings, however, suggest that the occurrence of NTM disease in immunocompetent patients with no underlying lung disease is on the rise. The major pulmonary mycobacterial pathogens
are: *Mycobacterium avium* (*M. avium*), *Mycobacterium kansasii* (*M. kansasii*) and *Mycobacterium intracellulare* (*M. intracellulare*). There is little evidence of person-to-person spread of NTM and it is believed that the environment is the main source or reservoir for these organisms. NTM disease in AIDS is caused primarily by *M. avium* and is second only to AIDS wasting syndrome as the most common cause of death in these patients (6).

**Mycobacterium Avium**

*M. avium* is a non-tuberculous mycobacterium which infects humans and animals alike. *M. avium* is an opportunistic infectious agent that mostly affects patients with underlying conditions that compromise local or systemic immunity (7) and has become a major human pathogen, primarily due to the emergence of the AIDS epidemic. This mycobacterium is found in the environment, particularly in water sources and is in close contact with human populations (7). Human infection is believed to be initiated either through the intestinal tract or by the inhalation of aerosols containing bacteria (7). When inhaled, *M. avium* enters the body via the respiratory route through aerosols and the first host cells to encounter the pathogen are bronchial epithelial cells and alveolar macrophages (7). Mycobacteria use different receptors to gain access into macrophages: mannose receptors, complement receptors (CR3), type A Scavenger receptors and in exposed hosts with mycobacteria-specific antibodies, Fcγ receptors (7, 8).

*M. avium* can spread from the initial site of infection to the rest of the body through the lymphatics or through the blood. At the main site of infection, the bacilli are phagocytosed by alveolar macrophages (4). This is the first event in which the host develops a response to the invading pathogen and is vital to the outcome of infection. Of the many virulence factors of Mycobacteria, their ability to reside and multiply within alveolar macrophages is seen as one of the most important. Other virulence factors of NTM are: prevention of acidification of phagocytic vesicles, prevention of phagosomes-lysosome fusion, delay in tumor necrosis factor
secretion by infected host cells, uptake by epithelial cells and production of receptors for macrophage binding (9). Phagocytosed pathogens are normally transported from the phagosomes to lysosomes within the macrophages and are effectively destroyed after phago-lysosomal fusion. The most well-characterized antimicrobial mechanisms of the macrophage are the ones that involve oxidative damage: the respiratory burst and nitric oxide (NO) production (9). These two systems work synergistically in killing many microbes (7, 10). Macrophages that have phagocytosed the bacilli normally die and form a central area of necrosis or a caseum; the pathogen is then contained within these caseous granulomas (11).

**Mycobacterium Avium Complex (MAC)**

*Mycobacterium avium* complex (MAC) is generally referred to as an opportunistic pathogen since it usually infects patients with established lung damage (11). Mycobacteria included in the MAC family are classified as acid-fast, slowly growing bacilli that may produce a yellow pigment in the absence of light (1). Other species of Mycobacteria which are included in MAC include: *M. avium paratuberculosis, M. avium silvaticum, M. avium hominissuis* and *M. avium colombiense* (3). MAC in AIDS patients most often causes a disseminated illness (bacteria is spread through the blood stream) and can cause many symptoms throughout the body. The risk of MAC is inversely related to the patient’s CD4+ T-cell count, and increases significantly when the CD4+ T-cell count decreases below 50 cells/mm3 of blood (3). 98% of MAC infections in AIDS patients are due to *M. avium* (1). Isolates from MAC infected patients are commonly referred to as *Mycobacterium avium hominissuis*, since this strain is found mostly in humans. Like most NTM, MAC bacteria are found in air, water, soil, foods, some tobacco products, and in many animals. Covert et al showed that person-to-person transmission of MAC bacteria is unlikely, suggesting that humans contract this disease from the environment (12). Recently, there has been a growing concern about human exposure to MAC through drinking
water due to its high resistance to most chemical disinfectants in water treatment processes and its high prevalence in biofilms in water distribution systems (13, 14, 15). Because most Mycobacteria are intracellular pathogens, the host cells, alveolar macrophages (AM) in this case, can actually serve as a barrier to the delivery of drugs to the intracellular environment (16). MAC organisms have shown to have resistance to many antimycobacterial drugs, which adds to the challenges of treating this disease.

**Host Response To Infection**

After mycobacterial infection, the innate and adaptive immune systems respond with the recruitment of immune cells to the site of injury. The bacilli are recognized by host immune cells via Toll-Like Receptor (TLR) 2 ligation (17). TLRs are pattern recognition receptors (PRRs) which recognize and bind to mycobacterial cell wall components and other pathogenic materials. TLRs are believed to represent key receptors for the recognition of mycobacterial antigens and activation of macrophages and Dendritic cells (DC), as well as other cells of innate immunity, thereby likely modulating the adaptive immune response (15,17,19). A variety of microbial products recognize and activate mammalian TLRs, facilitating the transcription of genes involved in regulating the adaptive immune response, including chemokines, cytokines and co-stimulatory molecules (15,20,21,22). Mammalian TLRs represent a structurally conserved family of membrane receptors, which have homology to the Drosophila Toll system (17, 20). TLR signaling leads to the nuclear translocation of the transcription factor nuclear factor-κB (NFκB) via a MyD88 dependent or independent manner (9) (Figure 2-1). NFκB is involved in the expression of many immune response genes, such as those encoding cytokines. TLR signaling also triggers differentiation of monocytes into macrophages and dendritic cells allowing for a more potent innate and adaptive immune response (9, 24). Cytokines are likely to be important mediators during the pulmonary inflammatory response (25). It is also believed
that the ordered influx of leukocytes to the site of inflammation is controlled by different regulatory Chemokines (26, 27). Chemokines are a large family of structurally related secreted proteins that are important for leukocyte trafficking. These small (8-12kDa) inducible secreted cytokine proteins act primarily as chemoattractants and activators of leukocytes (28). Chemokines can be divided into four subfamilies (C-C, C-X-C, C, and C-X-X-X-C) depending on spacing of highly characteristic cysteine residues within their amino terminal regions (29). Chemokines are produced by a variety of cells, including leukocytes, epithelial cells, endothelial cells, fibroblasts and numerous other cell types following stimulation by cytokines or microbial products (25 30, 31). Chemokines exert their effect via a distinct group of seven-transmembrane-domain protein receptors that signal through heterodimeric GTP-binding proteins (29, 32).

Two important molecules involved in the recruitment of monocyte-macrophages and T-cells to the site of infection are Monocyte chemoattractant protein-1 (MCP-1) and Chemokine receptor 2 (CCR2). MCP-1, acting through its receptor CCR2, is a potent chemoattractant for mononuclear cells (25).

**Formation Of Granuloma**

Granulomas are formed as a consequence of chronic antigen persistence and are thus a hallmark of all mycobacterial infections (32). In general, the production of pro-inflammatory cytokines and chemokines is essential for the recruitment of inflammatory cells to the site of infection and for the formation and maintenance of granuloma. This will not only localize and sequester the bacteria, but will also localize the immuno-pathologic responses to the bacteria (34). Granuloma formation involves the interaction of bacteria, fibroblasts, immune cells, including macrophages, and T cells, as well as immune effectors such as chemokines and cytokines (Figure 2-2). This process involves a cytokine network which drives the T-helper cell
immune response. Formation of granulomas is dependent on sequential release of specific cytokines and chemokines, which activate and chemoattract T lymphocytes and monocytes, the cellular components of granulomas. In vivo granuloma formation is induced and maintained by various cytokines (35). It is believed that small, solid (organized) granulomas can contain the infection and prevent further pathology, whereas, large, necrotic (loose) granulomas may lead to the dissemination of the bacteria and cause greater infection. For this reason, the integrity of a developing granuloma is crucial in terms of disease progression and survival. Ehlers et al., (32) have shown that mice lacking a Tumor Necrosis Factor (TNF) receptor have granulomas that disintegrate and cause widespread inflammatory apoptosis of both granulomas and surrounding tissue, leading to higher mortality. Granulomas can provide antibacterial protection in host tissue, but can also induce tissue damage and inflammation concomitantly. Thus, the outcome of mycobacterial disease is linked to granuloma formation and maintenance of granuloma integrity (16, 36, 37, 38). We believe that MCP-1 forms a gradient for immune cells expressing CCR2 on their surfaces following mycobacterial infection. It is this gradient which allows immune cells such as monocyte/macrophages, T-cells, and Dendritic cells to be recruited to the site of infection from the peripheral blood. If this gradient is somehow lost, then the immune response will not be effective in clearing the infection since the proper inflammatory cells will not be able to migrate towards the injury and will cause widespread inflammation and further pathology of the disease.

**Heme Oxygenase-1 (HO-1)**

Heme Oxygenase (HO) is a ubiquitously expressed protein which catalyzes the degradation of heme to produce three catabolic byproducts: carbon monoxide (CO), Iron (Fe2+) which is sequestered by ferritin and Biliverdin which is converted to Bilirubin by Biliverdin reductase (39) (Figure 2-3). There are three isoforms of HO including: one inducible form, HO-
1, and two constitutive forms, HO-2 and HO-3 (40). Also known as Heat Shock protein 32 (HSP32), HO-1 is the rate limiting enzyme involved in the degradation of heme, a potent oxidant. In addition to its role as a key enzyme in heme degradation, HO-1 has been shown to be anti-inflammatory and anti-oxidative through its breakdown of heme (41). HO-1 is a 32 kDa microsomal stress response protein that is induced by oxidative stress, inflammation and bacterial infection and is secreted by many cell types (41). Mitzuno et al. (41) have shown that among peripheral blood leukocytes, monocytes are the principal source of HO-1 when stimulated in vitro and that his production plays a role in systemic inflammation. HO-1 has been shown to be induced by glutathione depletion, lipopolysaccharide (LPS) and both hyperoxia and hypoxia in vivo (42). In addition, HO-1 is induced by various cytokines and growth factors (43). The 5'-flanking region of the HO-1 gene contains binding sites for the transcription factors that regulate inflammation, including nuclear factor-kappa B and activator protein 1 (74). However, these do not appear to mediate lipopolysaccharide-induced HO-1 gene expression. This transcription factor may also be important in the regulation of HO-1 by pro-inflammatory stimuli (44). HO-1 plays a central role in the defense against oxidative and inflammatory insults in the lung and has been shown to be upregulated in many pulmonary diseases (45). HO-1 deficiency, however, is associated with chronic inflammation and increased leukocyte recruitment in mice and in humans (41). Zampetaki et al (46) have shown that HO-1 deficiency in humans and mice results in chronic inflammation and increased leukocyte recruitment. The byproducts of HO-1 enzymatic activity are cyto-protective, anti-inflammatory and may act as anti-oxidants at the right concentrations (44). HO-1 has been implicated in the protection of lung tissue during oxidative stress, but the expression and cellular distribution of HO-1 is poorly characterized. In healthy lung tissue, HO-1 has been shown to be expressed in alveolar macrophages and in
bronchial epithelial cells (46). HO-1 is recognized to alter both chemokine production as well as chemokine receptor expression (74). Recruitment of monocytes to the site of infection is a complex phenomenon dependant on the expression of chemokines and expression of receptors for these chemokines on peripheral blood monocytes. HO-1 has been shown to act in cell mediated immunity in the pleura and infections with *M. avium* have been shown to increase the expression of HO-1 in mononuclear cells and pleural mesothelial cells (47). HO-1 has been shown to downregulate the inflammatory response in the lungs by attenuating the expression of adhesion molecules and inhibiting the recruitment of leukocytes or by repressing the induction of cytokines and chemokines such as MCP-1 (47). It is not known how HO-1 regulates and suppresses cytokine expression. It is possible that HO-1 inhibits the actions of NFκB and therefore abrogates an inflammatory response (45). HO-1 has been shown to directly regulate MCP-1 mRNA expression trough the degradation of heme (48). Furthermore, HO-1 induction results in suppression of monocyte chemotactic response via inhibition of CCR2 expression (49).

**Monocyte Chemoattractant Protein-1 (MCP-1)**

MCP-1 (Also known as monocyte chemotactic and activating factor (MCAF)) is a 76 amino acid peptide that is selectively chemotactic for peripheral blood monocytes (50). MCP-1 is an 8.7-kDa protein that has specific chemoattractant and activating activity for monocytes in acute inflammatory conditions (25). The regulated interactions of chemokines with their respective receptors are thought to mediate the controlled recruitment of specific leukocyte subpopulations required during host defense and inflammation. MCP-1, a member of the CC motif chemokine family, is secreted by pleural mesothelial cells, endothelial cells, fibroblasts, monocytes, macrophages and leukocytes in response to inflammatory signals such as IL-1 and TNF-alpha and bacterial infection (51). CC chemokines have two conserved, adjacent cysteines near their amino terminus and are predominantly chemotactic for mononuclear cells (65). MCP-
1 is a primary and specific mononuclear cell chemoattractant from a class of cytokines that are referred to as the platelet factor 4 (PF4) super family and are related by predicted primary structural similarities and by conservation of a 4 cysteine motif (51). MCP-1 was first cloned from mouse fibroblasts as a PDGF-inducible gene termed JE (24,52) and is homologous to human MCP-1 (24, 53, 54, 55, 56,57). It has been shown that increased levels of MCP-1 following bacterial infection correlate with the influx of CD4+ T cells and macrophages in to the lungs (25). However, other results suggest that MCP-1 is a mediator of inflammation and can specifically stimulate the directional migration of T cells as well as monocytes and may play an important role in immune cell recruitment into sites of antigenic challenge (47).

Previous results show that MCP-1, along with several other chemokines, is controlled by the Nuclear Factor Kappa Beta (NFκB) signaling pathway (58, 59, 60). MCP-1 creates a gradient for chemotaxis of monocytes-macrophages, T-cells and natural killer (NK) cells towards the site of infection and is the ligand for CCR2 which is expressed on cell surfaces of leukocytes (28). This receptor-chemokine interaction has been shown to induce haptotaxis (the migration of cells along the gradient of substrate-bound attractant molecules) in mesothelial cells (28). MCP-1 is characterized by its ability to evoke chemotaxis mainly in monocytes (50). MCP-1 has also been shown to aid in the recruitment of other mononuclear phagocytes such as: CD45ARO+ T lymphocytes, B cells, and NK cells (61). In vitro, MCP-1 is chemotactic for mononuclear cells, but not Neutrophils (62). However, Neutrophils in an inflammatory milieu, can express CCR2, potentially enabling their chemotaxis to MCP-1 (63). The association between augmented levels of MCP-1 and the recruitment of mononuclear cells expressing CCR2 plays a crucial role during chronic inflammatory disease (62). MCP-1 has been shown to be upregulated in HO-1 knockout mice (64). This suggests a possible inhibitory effect by HO-1 on MCP-1. Alternatively,
Kanakiriya et al (65) have shown that inhibition of HO-1 with Zinc protoporphyrin (ZnPP), a specific HO-1 inhibitor, prevented the hemin-induced expression of MCP-1 mRNA and that the active form of Iron can also induce the expression of both HO-1 and MCP-1 (45). During infection and inflammatory disease, the increased release of MCP-1 attracts T-lymphocytes and macrophages to the site of infection and results in granuloma formation (54). ZnPP prevented the expression of MCP-1 by hemin at 6 hours; examination at later time points demonstrate an increasing level of expression of MCP-1 mRNA despite the continued presence of this inhibitor of HO activity; indeed, at 18 hours, the level of expression of MCP-1 was increased almost threefold in the presence of ZnPP (47). Thus, MCP-1 can also be induced by HO-1 independent pathways, but this leads to a delayed expression of the chemokine (47). It is believed that MCP-1 plays a crucial role in the development of disseminated MAC disease. Some studies have shown that MCP-1 can act as a negative regulator for the CCR-2 cell surface receptor (66).

**Chemokine (C-C Motif) Receptor 2 (CCR2)**

CCR2 is a seven-transmembrane-spanning G-protein coupled receptor expressed on the cell surface of leukocytes (60) (Figure 2-4). This receptor binds MCP-1 with high affinity and is activated upon ligand binding (60). MCP-1 interacts with CCR2 which has two isoforms, CCR2A and CCR2B (29, 68). Activation of CCR2 leads to downstream activation of Phospholipase C (PLC) which cleaves the molecule Phosphotidyl inositol (4, 5) bisphosphate (PIP2) (68). This cleavage forms two second messenger molecules called Inositol trisphospahete (IP3) and Diacylglycerol (DAG) which induce intracellular signaling events and trigger inflammation (68). Specifically, DAG has been shown to activate Protein Kinase C (PKC) and IP3 has been shown to trigger the release of calcium from internal cellular stores (60). These events promote the activation of the Mitogen Activated Protein Kinase (MAPK) pathway and induce differentiation and chemotaxis of leukocytes (60) (Figure 2-5). CCR2 is expressed on
many different cell type surfaces and provides a mechanism for specific interaction with certain chemokines. Belperio et al. (60) have shown that CCR2-/- mice have a reduced infiltration of mononuclear phagocytes and neutrophils in a mouse model using tracheal allografts. However, the levels of lymphocyte infiltration were not changed and the absence of CCR2 expression did not change the protein levels of MCP-1 (60). Additionally, it has been shown that CCR2-/- mice showed a decreased level of recruitment of mononuclear phagocytes to the site of granulomas (69). Other experiments have shown that the CCR2 receptor can regulate MCP-1 levels by binding and internalizing MCP-1, creating a self-limiting negative feedback loop and abrogating the influx of inflammatory cells to the site of injury (70). Lu et al., (71) reported that CCR2 is probably the only receptor for MCP-1 in vivo. We believe that expression of CCR2 on monocytes plays an integral role in granuloma formation. This receptor allows immune cells to chemotax along an MCP-1 gradient towards the site of infection.
Figure 2-1. Recognition of an appropriate ligand by TLR-2 (for example, lipopolysaccharide) triggers the Toll-like receptor to recruit MyD88. MyD88 interacts with the Toll-like receptor through its own Toll/IL-1 receptor domain and in turn engages the serine-threonine kinase IRAK though a death domain. Signal transduction factors such as TRAF6 carry the signal through a series of phosphorylations until NFκB is ultimately released to the nucleus where it can activate the transcription of appropriate immune response genes. Allan Genome Biology 2000 1:reports0079 doi:10.1186/gb-2000-1-6-reports0079
Figure 2-2. Formation of granuloma involving pathogen, macrophages, T-cells, and B cells.
Figure 2-3. Degradation of heme by HO-1. HO-1 catalyzes heme degradation to biliverdin-IX, CO, and iron. These metabolites mediate the antiapoptotic, anti-inflammatory, vasodilatory, anticoagulant, antioxidant, and antiproliferative properties of HO-1 (Fredenburgh et. al).

Figure 2-4. CCR2 structure. Regions implicated in ligand binding, coupling of effector molecules and suggested targets for GRK and JAK are highlighted. (apresslp.gvpi.net)
Figure 2-5. Schematic representation of the signaling pathways activated through the CCR2. Ligand-binding to CCR2 induces conformational changes in the receptor, leading to its dimerization; this in turns disables JAK2 association and activation. JAK activation initiates the JAK/STAT pathway and enables efficient G protein coupling to CCR2. G proteins mediate signaling cascades through GRK and arrestins, leading to receptor desensitization and internalization. Other signaling molecules triggered through CCR2 include PI-3 kinase, PLC, and MAPK (apresslp.gypi.net)
CHAPTER 3
MATERIALS AND METHODS

HO-1 Knockout Mouse Model

C57BL/6 mice were used for this model since it has previously been shown that this strain develops focused lesions that grow into large granulomas following \textit{M. avium} infection; these granulomas exhibit central necrosis (caseous necrosis) similar to human granulomas described in tuberculosis lesions (7). We used the HO-1 knockout model developed by Kenneth D. Poss and Susumu Tonegawa as described (72). Briefly, the construct was designed to remove a 3.7kb XhoI-HindIII fragment of murine DNA with intron sequence and coding sequence for the final 226 amino acids (Figure 3-1). Chimeric mice were generated as described (71, 73) and HO-1 -/- (Hmox1-/-) were obtained by mating male chimeras with C57BL/6 females; these Hmox1+/+ animals were intercrossed to produce Hmox1-/- mice. HO-1 wild type (+/+), heterozygous (+/-), and knockout (-/-) mice were maintained in a breeding colony in a specific pathogen free (SPF) animal housing facility under Animal Care Services at the University of Florida. HO-1 -/- male mice of 8 weeks of age were paired with heterozygous (+/-) females of the same age and subsequent litters were genotyped for the HO-1 gene. Genotyping was done upon weaning mouse pups 21 days after birth. Mouse tissue was obtained by cutting a small piece (1cm) off the tip of the tail. This tissue was digested and genomic DNA was isolated using Extract-N-Amp™ Tissue PCR Kit (Sigma, St. Louis, MO). PCR was performed using specific primers for wild type and knockout (mutant) HO-1 genes. HO-1 Wild type primer sequence (forward, 5’-GTA CAC TGA CTG TGG TRG GGG GAG-3’, reverse, 5’- AGG GCC GAG TAG ATA TGG TAC-3’) and HO-1 mutant primer sequence (forward, 5’- GCT TGG GTG GAG AGG CTA TTC-3’, reverse, 5’- CAA GGT GAG ARG ACA GGA GAT C-3’). The PCR product was run on a 2% agarose gel
supplemented with Ethidium bromide for UV imaging of DNA. The molecular weight of the wild type and mutant gene products are 180 base pairs and 290 base pairs respectively.

**M. Avium culture**

*M. avium* subspecies avium Chester (ATCC# 15769) was maintained in ATCC medium 90 Lowenstein Jenson medium and grown in Lowenstein-Jensen Medium Slants (BD Biosciences, San Jose, CA) according to manufacturer’s instructions. Alternatively, colonies were grown on Middlebrook and Cohn 7H10 agar plates (BD Biosciences, San Jose, CA) and incubated for 14 days at 37 degrees in 95% oxygen and 5% carbon dioxide. A stock solution was prepared and the concentration was measured by reading the optical density (OD) using a spectrophotometer at 600nm (BioRad, Hercules, CA). Bacteria were frozen in 1ml aliquots in 10% glycerol at -80° C at a concentration of $1.3 \times 10^9$ Colony forming units (cfu)/ml. Frozen aliquots were thawed and briefly sonicated before each use.

**Collection Of Serum**

The mice were euthanized in a desiccator with an overdose of Isoflurane, an analgesic (Webster veterinary supply, Sterling, MA). Then, whole blood was collected through the abdominal aorta using a 25 gauge needle. The blood was left at room temperature for 30 minutes to clot and then centrifuged at 12,000 rpm to separate the serum from the red blood cells (RBCs). The serum was then aliquoted and stored in a -80° C freezer for further analysis.

**Bronchial Alveolar Lavage Fluids (BALF)**

BALF was collected by ligating the trachea using thread and placing a catheter into the airway through the trachea. Fluids were collected by injecting 800-1000 μL of Hanks’ Balanced Salt Solution (HBSS) into the mouse lung via canulation of the trachea. The fluid was then re-drawn and the process was repeated twice for a total volume of 1600-2000 μL BALF. The fluid was placed on ice and then centrifuged at 1500g to pellet the cells and separate the supernatant.
The supernatant was aliquoted and stored in a -80° C freezer until further use. The BAL cell pellet was stored in Cell Freezing Medium (Sigma, St. Louis, MO) and stored in a -80° C freezer.

**Tissue Harvest**

We collected the lungs, liver, spleen and kidneys from each mouse following euthanasia. Some tissue was frozen in Optimal Cutting Temperature (OCT) compound (Sakura, Torrance, CA) and stored in -80° C. The rest was fixed in 4% Paraformaldehyde and stored in room temperature for 24 hours. This tissue was then transferred to 70% ethanol and stored at room temperature until used.

**Immunohistochemistry**

Lung tissue from sacrificed mice was fixed in 4% Paraformaldehyde for 24 hours then transferred to 70% Ethanol. Tissue was sent for paraffin embedding and Hematoxylin and Eosin (H&E) staining to Histology Tech Services, Gainesville Florida. Paraffin-embedded slides were stained for CCR2 using Goat anti mouse primary antibody (Capralogics, Hardwick, MA) and ABC staining kit (Vector laboratories, Burlingame, CA) according to manufacturer recommendations. Alternatively, slides were stained with primary antibody for HO-1 (Calbiochem, San Diego, CA). Briefly, the slides were de-paraffinzed using Xylenes (Fisher Scientific, Fairlawn, NJ). Slides were placed in Xylene solution for 5 minutes twice. The slides were then washed with 1X Phosphate Buffered Saline (PBS) for 2 minutes. Next, the slides were permeated by incubating with triton X 100 (Sigma Aldrich, St. Louis, MO) for 10 minutes and incubated with Hydrogen Peroxide for (H₂O₂) for 30 minutes. Normal goat serum (ABC kit) was then added for 30 minutes to block tissue from background or non-specific binding. Primary antibody for CCR2 was added to slides for 1 hour followed by washing and addition of secondary antibody (ABC kit) for 30 minutes. The slides were then incubated for 5 minutes in
DAB peroxidase substrate solution (Vector laboratories, Burlingame, CA) for color development. All slides were counter-stained with Hematoxylin (fisher Scientific, Fair Lawn, NJ) for 30 seconds. Sections were visualized using a Nikon DIAPHOT inverted microscope. The ocular is magnified to 10 times (10x) and the objectives used were magnified to 10x, 20x or40x. Pictures were taken using the SPOT Imaging Solutions software (Sterling Heights, MI).

TIB-71 (RAW 264.7) Cells

RAW 264 (7TIB-71) cells were obtained from American Type Culture Collection (Manassas, VA). RAW 264 cells are macrophages derived from BALB/C mice and are known to produce lysozyme. The base medium for this cell line is ATCC-formulated Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), Fungizone (MP Biomedicals United States, Solon, OH) and Penicillin-Streptomycin solution (MP Biomedicals United States, Solon, OH). Cells were grown to confluence in 6-well plates and the culture medium was replaced with serum-free medium (SFM) to perform the experiments. Camhi et al. have shown that HO-1 is induced in RAW 264.7 cells with treatment of LPS (74).

Co-Culture of RAW 264.7 Cells With M. Avium

TIB-71 cell cultures were plated on 60mm culture plates (Corning, Lowell, MA) at a concentration of 1x10^6 cells per plate and treated with M. avium in SFM at a concentration of 50x10^6 bacteria per plate or a ratio of 50:1 bacteria per cell. After reaching confluence, the plates were incubated at 37° C for different time points (4, 8, and 24 hours) and the cells and supernates were harvested. All treatments were done in triplicate. Cell culture supernates were stored in a -80° C freezer. Cell pellets were stored in either Buffer RLT (Qiagen, Valencia, CA) or RIPA cell lysis buffer (Sigma Aldrich, St. Louis, MO) for RNA isolation and Western Blot protein analysis respectively. The resulting samples were stored in a -80° C freezer until needed.
**HO-1 Inhibition By Zinc Protoporphyrin-IX In Cell Cultures**

Zinc protoporphyrin-IX (ZnPP) is a normal metabolite that is formed in trace amounts during heme biosynthesis (75). ZnPP may regulate heme catabolism through competitive inhibition of HO-1 enzyme activity. ZnPP is found in erythrocytes and is deposited in the spleen along with heme where both bind HO-1. This process slows down heme degradation by HO-1. ZnPP inhibits the enzyme activity of HO-1 but does not alter the gene and protein expression of HO-1. Cells in culture were pretreated with 10μM ZnPP-IX (Frontier Scientific, Logan, UT) for thirty minutes in SFM. The media is then changed to fresh SFM and the cells are exposed to different treatments as described above and incubated for different time points. Cells are collected, spun down and stored in RLT Lysis Buffer (10 parts RLT Lysis buffer, Qiagen sciences, MD, USA and 1 part 2-Mercaptoethanol , Bio-Rad Laboratories Inc., Hercules, CA) for RNA isolation. Cell Supernates were also collected and stored in a -80° C freezer for cytokine studies.

**Mouse Model For M. Avium Infection**

We have developed a murine model for *M. avium* infection and chronic colonization which leads to granuloma formation in the lungs and other tissue. C57BL/6 wild type and HO-1 knockout mice were inoculated intranasal with 7x10⁶ *M. avium* in PBS once per week for three weeks. Alternatively, wild type control mice were inoculated with equal volume of PBS. Throat swabs were done once a month and plated on M. avium-specific plates to monitor colonization. The swabs were plated on the aforementioned 7H10 plates (BD) and incubated for 14 days in a 37° C incubator with 95% O2 and 5% CO2. Colonies are visualized and counted on each plate to determine the level of colonization in each mouse. The mice were sacrificed at different time
points (2, 4 and 6 months) and the lungs, spleen and liver were harvested. BALF, BAL cells and serum were collected.

**Cytokine Studies**

MCP-1 levels in cell culture supernatants, mouse sera and BALF were measured using Enzyme-Linked ImmunoSorbent Assay (ELISA). We used a Quantikine Mouse CCL2/JE/MCP-1 immunoassay kit according to manufacturer recommendation (R&D systems Inc., Minneapolis, MN). The cells and media are collected and centrifuged at 2000 RPM for 5 minutes. The culture supernates were collected and stored in a -80 °C freezer until needed. 50 μL of each sample (supernates, BALF, or serum) was used in the assay to measure MCP-1 levels. The OD was measured at 450 nm in a Biotek Synergy 2 microplate reader using Gene5 software (Biotek Industries, Winooski, VT).

**Real-Time Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA from cultured RAW 264 cells was purified and diluted with RNase-free water to 100 ng/L; then, 10 μL of each sample were reverse transcribed into combinational DNA (cDNA). After the reverse transcription reaction was finished, 80 μL of RNase-free water were added to each sample. 10μL of diluted cDNA product were mixed with 25 μL of SYBR Green JumpStart Taq ReadyMix, 0.5 μL of internal reference dye, H2O and 2 μL of specific oligonucleotide primers (80 nM final concentrations) to a total volume of 50 μL for quantification of the real-time polymerase chain reaction (PCR). The quantification of real-time PCR was performed by using the SYBR Green method on the Applied Biosystems 7500 Real Time PCR System with the following profile: 1 cycle at 94°C for 2 minutes; 40 cycles at 94°C for 15 seconds, at 60°C for 1 minute, and at 72°C for 1 minute; and the at acquired fluorescent signal from the elongation step. Beta-actin or 18s rRNA was used as a house-keeping internal control. The real-time PCR products were confirmed by electrophoresis on 2% agarose gels.
cDNA was amplified using mRNA–specific primers for mouse 18S rRNA (forward: 5’-TGTCTCAAAGATTAAGCCATGCAT-3’, reverse: 5’-AACCATAACTGATTTAATGAGCCATTC-3’), mouse MCP-1 (forward: 5’-GGCTCAGCCAGATGCAGTTAA-3’, reverse: 5’-CCTACTCATTTGGGATCATCTTTGCT-3’), mouse CCR2 (forward: 5’-CAACTCCTTCACTCAGGCACAR-3’, reverse: 5’-GGAAAGAGGCAGTTTGCAAAG-3’), mouse HO-1 (forward: 5’-CTTTTCAGAAGGGYCACGGTGWCC-3’, reverse: 5’-GTGGAGMCYCTTYACRTAGYG-3’).

Data analysis was carried out by using the ABI sequence-detection software using relative quantification. The threshold cycle (Ct), which is defined as the cycle at which PCR amplification reaches a significant value, is expressed as the mean value. The relative expression of messenger RNA (mRNA) was calculated by using the Ct method (where Ct is the value obtained by subtracting the Ct value of the housekeeping gene -actin mRNA from the Ct value of the target mRNA). The amount of the target relative to ß-actin or 18s rRNA mRNA was expressed as $2^{-\Delta Ct}$.

**Statistical Analysis**

The data obtained from independent experiments are presented as the mean +/- standard deviation; they were analyzed by the unpaired student t-test using Statview software version 5.0, SAS Inst. INC. (Cary, NC). All experiments were done in triplicate. Differences were considered significant at P < 0.05 level.
Figure 3-1. Targeted disruption of the Hmox1 gene. (a) Hmox1 genomic locus and targeting vector. A 3.7-kb region including exons 3, 4, and a portion of 5 (e3-e5) was replaced with a pkg-neo cassette. The 5’ and 3’ probes used for screening ES cell clones and genotyping mice are shown. The 5’ probe hybridizes to an 11-kb KpnI fragment of the native Hmox1 gene and a 6.5-kb fragment from the disrupted gene. D, HindIII site; K, KpnI; X, XhoI; Xb, Xbal. (Poss, Tonegawa, 1997).
CHAPTER 4
RESULTS

In Vitro Results

HO-1 is induced by *M. avium* in RAW 264.7 cells

We stained RAW 264.7 cells for HO-1 to test whether the expression levels of HO-1 are increased after insult with *M. avium*. Our results showed that HO-1 was induced by *M. avium* in RAW 264.7 cells after 4 hours (Figure 4-2) as opposed to controls (Figure 4-1). HO-1 was still expressed at 8, 24 and 48 hours after exposure to *M. avium* as well (results not shown).

We next looked at mRNA levels of HO-1 in RAW 264.7 cells in the presence and absence of *M. avium* in order to see whether *M. avium* induces gene expression of HO-1. Our results showed that HO-1 expression was induced in a time dependent manner in this mouse monocyte cell line after *M. avium* treatment (Figure 4-3).

MCP-1 relative gene expression in RAW 264.7 cells

Following the same protocol as explained above, we analyzed the mRNA levels of MCP-1 in RAW 264.7 cells using real time PCR. After treatment with *M. avium*, MCP-1 mRNA levels increased in a time dependent manner as compared to controls, but these levels were abrogated when we treated the cells with ZnPP, a competitive inhibitor of HO-1 enzymatic activity, prior to *M. avium* exposure (Figure 4-4).

CCR2 relative gene expression in RAW 264.7 cells

In order to test whether *M. avium* infection and HO-1 inhibition affect gene expression of CCR2, mRNA levels of CCR-2 were measured in TIB-71 (RAW 264.7) cells. Our results indicate that when these cells are treated with ZnPP, CCR2 expression is significantly increased on the surface of mouse monocytes and this increase is time dependent (Figure 4-5).
MCP-1 levels in Cell Culture Supernates

To support our previous results obtained with RNA, we measured the levels of the same cytokine in RAW 264.7 cell culture supernates using ELISA. MCP-1 levels were significantly increased in a time dependent manner in cell culture supernates of cells that were treated with *M. avium* for 4, 8 and 24 hours, but these levels were abrogated after treatment with ZnPP-IX even with concurrent infection with *M. avium* (Figure 4-6).

In Vivo Results

Mouse tissue

After euthanasia, we checked the lungs, liver and spleen of HO-1 +/+ and HO-1 -/- mice to ensure granuloma formation and therefore, infection has occurred. We observed that the lungs of HO-1 +/+ and HO-1 -/- mice showed detectable differences in granuloma formation. Figure 4-8 shows that HO-1 +/+ mice infected with *M. avium* formed granuloma in the lungs as compared to saline controls (figure 4-7). We then examined the lungs of HO-1 -/- mice to see whether they form similar lesions following infection with *M. avium*. Figure 4-10 shows that HO-1 -/- mice inoculated with *M. avium* do not form organized granulomas and have disseminated infection in the lung as compared to saline controls (figure 4-9).

Immunohistochemistry

To confirm the mouse model of *M. avium* infection, we examined the formation of granuloma in lung tissue of infected mice. From analyzing our H&E stained tissue sections, it was evident in HO-1+/+ mice developed granulomas after 6 months exposure to *M. avium* via intra-nasal inoculation as compared to saline controls (Figure 4-11). These mice exhibited organized granuloma formation and were able to contain the pathogen to the site of infection. HO-1 -/- mice displayed loose, unorganized lesions in the lungs after exposure to *M. avium* as compared to their control counterparts (Figure 4-12).
HO-1 protein expression in mouse lung tissue

We stained the lungs tissue of HO-1 +/+ control and infected mice to study whether *M. avium* infection induces HO-1 expression. HO-1 levels were up-regulated in granulomatous tissue in HO-1 +/+ mouse lungs infected with *M. avium* as compared to Control mice. Figure 4-13 shows a 100x magnification of a control lung from an HO-1 +/+ mouse which was inoculated with saline (PBS) which was stained for HO-1. This tissue exhibited basal levels of HO-1. Figures 4-14 and 4-15 show a 200x magnification of an HO-1 +/+ mouse lung stained for HO-1. This mouse was inoculated with *M. avium* for 6 months and shows upregulation of HO-1 in granulomatous tissue.

CCR2 expression in mouse lung tissue

To test and confirm previous results that showed that HO-1 regulates the expression of CCR2, we looked at CCR2 expression in the lung tissue of HO-1 +/+ and HO-1 -/- infected and control mice. Figure 4-16 shows that HO-1 -/- mice have higher basal levels of CCR2 in lung tissue. CCR2 levels were up-regulated in lung tissue of infected HO-1 +/+ mice as compared to uninfected controls (Figure 4-17). Additionally, we observed higher levels of CCR2 in infected lungs of HO-1 -/- mice as compared to infected HO-1 +/+ mice (Figure 4-18). In HO-1 +/+ mouse lung tissue, CCR2 co-localized to granulomas (figure 4-19). Our results showed that CCR2 is normally regulated by HO-1 in the lungs of HO-1 +/+ mice following infection. Figure 24 shows that CCR2 is co-localized to the granuloma and is not expressed in other areas of the lung. In HO-1 -/- mice we see that this regulation is lost and CCR2 is expressed in a much larger area of the lung and is disseminated after infection as compared to CCR2 expression in HO-1 +/+ tissue from infected mice (Figure 4-20).
MCP-1 levels in BALF and serum

When we analyzed the levels of MCP-1 in both BALF and serum, we observed a significant increase of MCP-1 in the BALF of infected wild type C57BL/6 mice as compared to uninfected controls and increased levels in infected HO-1 -/- mice as compared to knockout controls as measured with ELISA (Figure 4-21). Additionally, MCP-1 levels were higher in HO-1 -/- control mice as compared with wild type controls. MCP-1 levels were also increased in the sera of mice infected with *M. avium* as compared to control mice and the levels of MCP-1 in sera of HO-1 -/- mice infected with *M. avium* were significantly higher than wild type controls and HO-1 -/- controls (Figure 4-22).
Figure 4-1. 400x magnification stain of RAW 264.7 cells after 4 hours of incubation in SFM showing basal levels of HO-1. Blue color shows counter-stain with Hematoxylin.

Figure 4-2. 400x stain of RAW 264.7 cells after 4 hours treatment with *M. avium* showing increased levels of HO-1 expression (brown). Blue color shows counter-stain with Hematoxylin.
Figure 4-3. HO-1 relative apparent gene expression significantly increases in a time dependent manner in mouse monocytes after treatment with M. avium. HO-1 levels increase significantly (p < .001) in cells treated with M. avium (*). RAW 264.7 cells were treated for 4, 8, 24 and 48 hours in serum free medium (C), or with M. avium.
Figure 4-4. MCP-1 Relative Gene Expression significantly increased (p < .001) in monocytes after treatment with *M. avium* for 8 and 24 hours as compared to controls (*) and were significantly lowered when cells were pretreated with ZnPP for 8 and 24 hours (**) as compared to *M. avium* alone for the same time points. RAW 264.7 cells were incubated for 4, 8, and 24 hours in serum free media (Control) or treated with *M. avium*, or both ZnPP and *M. avium*.
Figure 4-5. CCR-2 Relative Gene Expression was significantly increased (p<.05) in *M. avium* treated monocytes as compared with controls at 4 hours (*), 8 hours (**), and 24 hours (***) and in cells treated with ZnPP and *M. avium* as compared to controls. RAW 264.7 cells were incubated for 4, 8, and 24 hours in serum free media (Control), treated with *M. avium* or treated with both ZnPP and M. avium
Figure 4-6. MCP-1 levels significantly increase (p < .05) in cell supernates in a time dependent manner after treatment with *M. avium* as compared to controls (*) and are significantly decreased (p < .05) after inhibition of HO-1 with ZnPP as compared with *M. avium* treatment alone (**).
Figure 4-7. HO-1+/+ 6 month control lung. Control mice were inoculated with intranasal PBS

Figure 4-8. HO-1 +/+ lung showing granuloma formation after 6 months exposure to M. avium via intranasal inoculation
Figure 4-9. HO-1 -/- 6 month control lung. Control mice were inoculated with intranasal PBS.

Figure 4-10. HO-1 -/- lungs showed disseminated infection and loose granuloma formation following 6 month exposure to M. avium.
Figure 4-11. A) 100x magnification H&E stain of an HO-1 +/+ control mouse lung. B) 100x magnification H&E stain of an HO-1 +/+ mouse infected with *M. avium* for 6 months showing granuloma formation.

Figure 4-12. A) 100x magnification H&E stain of HO-1 -/- saline control lung. B) 100x magnification H&E stain of HO-1 -/- showing loose, unorganized granulomas and disseminated infection in lung tissue following 6 month exposure to *M. avium*.
Figure 4-13. 100x magnification of control lung from HO-1 +/+ mouse showing basal levels of HO-1 (brown).

Figure 4-14. 200x magnification of *M. avium* infected HO-1 +/+ mouse lung tissue showing increased expression of HO-1 (brown) in granuloma. Blue color shows counter-stain with Hematoxylin.
Figure 4-15. 200x magnification of granuloma in *M. avium* infected HO-1 +/- mouse lung showing increased expression of HO-1 in granuloma. Blue color shows counter-stain with Hematoxylin

Figure 4-16. A) 100x magnification of control HO-1 +/- control lung showing basal levels of CCR2 (brown). B) 100x magnification of HO-1 -/- control mouse lung showing basal levels of CCR2 (brown). Blue color shows counter-stain with Hematoxylin
Figure 4-17. A) 100x magnification of an HO-1+/+ control mouse lung tissue showing basal levels of CCR2 (brown). B) 100x magnification of an HO-1 +/+ mouse lung inoculated with *M. avium* for 6 months showing increased expression of CCR2 (brown). Blue color shows counter-stain with Hematoxylin.

Figure 4-18. A) 100x magnification of an HO-1 +/- mouse lung after 6 months *M. avium* infection showing CCR2 expression (brown). B) An increase in CCR2 expression in HO-1 -/- mouse lung after 6 months of *M. avium* infection. Blue color shows counter-stain with Hematoxylin.
Figure 4-19. A and B. 100x magnification of an HO-1 +/+ mouse lung after 6 month infection with *M. avium* showing CCR2 (brown) co-localizes with granuloma. Blue color shows counter-stain with Hematoxylin

Figure 4-20. A and B. 100x magnification of an HO-1 -/- mouse lung following 6 month infection with *M. avium* showing disseminated expression of CCR2 (brown). Blue color shows counter-stain with Hematoxylin
Figure 4-21. MCP-1 levels are significantly increased (p < .001) in BALF of infected HO-1 +/+ and HO-1 -/- mice as compared with controls (*). Basal levels of MCP-1 are significantly higher (p < .001) in HO-1 -/- control mice as compared to HO-1 +/+ control mice (**).
Figure 4-22. MCP-1 levels are increased in mouse serum after 6 month *M. avium* infection and are significantly higher (p < .0001) in infected HO-1 +/- and HO-1 -/- mice (*) as compared with saline controls. Significantly higher basal levels of MCP-1 are seen in HO-1 -/- sera then HO-1 +/- sera (**).
CHAPTER 5
DISCUSSION

Since the advent of HIV and the AIDS epidemic, NTM infections have become a major source of disease in the United States and around the world (1). Although NTM typically do not cause disease in humans, MAC infections have been on the rise in both immune competent patients as well as the immunocompromised (2). The formation of granuloma is a vital part of the body’s immune defense against invading pathogens. After infection with M. avium, failure to develop organized granulomas results in the dissemination of the infection and greater injury to the host. The regulated recruitment of immune cells including monocyte-macrophages, T-cells, dendritic cells and B-cells to the site of infection and injury is essential for the host to contain and eliminate invading pathogens (33). In this study, we tested the interaction and regulation of three proteins (HO-1, MCP-1 and CCR2) in the process of granuloma formation. HO-1, an inducible, cyto-protective protein has been implicated in many inflammatory disease as well as mycobacterial infections (45). We hypothesized that HO-1 plays a role in granuloma formation by regulating MCP-1 and CCR2 expression in mouse monocytes and therefore regulating the cellular recruitment of these immune cells from the periphery to the site of infection and injury. MCP-1 is a chemokine responsible for creating a gradient for the chemotaxis of immune cells from the periphery to the site of infection and CCR2 is the cell surface receptor which is bound by MCP-1 (25). MCP-1 and CCR2 are both highly involved in the process of cellular recruitment and in this study we demonstrate that HO-1 is a key regulator of these cytokines and therefore the immune recruitment process and granuloma formation. From our in vitro experiment using RAW 264.7 mouse monocytes, we observed that HO-1 is induced by M. avium as early as 4 hours post treatment and stays highly expressed for up to 48 hours (Figure 9). Once activated by M. avium, these monocytes began to express higher levels of both MCP-1 (Figure
10) and CCR2 (Figure11). However, when we treated the cells with ZnPP, a competitive inhibitor for HO-1 enzyme activity, we saw a decreased expression of MCP-1 and an increase expression of CCR2, suggesting that HO-1 levels are directly correlated with MCP-1 expression and inversely correlated with CCR2 expression. Previous findings have given contradicting evidence in terms of the correlation between HO-1, MCP-1 and CCR2. In many studies, the level of both MCP-1 mRNA and protein were reduced with the inhibition of HO-1 (34), but in others, the opposite finding was observed (57). Zampetaki et al (45) have shown that despite high mRNA levels, significantly lower levels of cytokine protein were found in BALF of mice over-expressing HO-1. These findings suggest that HO-1 over-expression can suppress the induction of cytokine mRNA levels and that this regulation might act through the NFκB signaling pathway. Our in vitro and in vivo results, however, show that inhibiting HO-1 enzyme activity led to a decrease in MCP-1 protein levels in cell supernates (Figure 12), BALF (Figure 27) and mouse serum (Figure 28), suggesting that HO-1 is directly correlated to MCP-1 expression in mouse monocytes. Since we observed an induction of HO-1 and increased levels of MCP-1 in the lung tissue, BALF and serum of infected HO-1 +/+ mice, it is possible that an MCP-1 gradient was established in order to recruit monocytes from the peripheral blood to the site of infection. This corresponds to a normal immune response in which cells expressing CCR2 are recruited from the periphery and chemotax along an MCP-1 gradient towards the site of insult in order to from granulomas (28). When testing MCP-1 protein levels in infected mice, we observed higher basal levels of MCP-1 in sera and BALF in control HO-1 -/- mice as compared to wild type controls. These results support previous findings (46) and suggest that HO-1 -/- mice are in a chronic state of inflammation. This chronic inflammatory state could lead
to the destruction of the MCP-1 chemotactic gradient which would then disrupt cellular recruitment to the site of infection.

Previous findings along with our results suggest that different cell types in different organ systems may react differently to altered levels of HO-1. This could be the result of direct regulation by HO-1 on MCP-1 gene expression via NFκB or the result of over expression of the CCR2 receptor on monocytes and macrophages in the vicinity of the infection which creates a negative feedback loop between CCR2 and MCP-1. It has been reported that when HO-1 enzymatic activity is inhibited by ZnPP, the levels of CCR2 increase (48, 49) suggesting that HO-1 somehow regulates the expression of CCR2 on the surface of monocytes, macrophages and other immune cells. Our results show that HO-1 -/- mice indeed had increased levels of CCR2 on monocyte/macrophage cell surfaces as seen by relative gene expression (Figure 11) and immunohistochemistry (Figure 26). Our findings also suggest that HO-1 normally regulates the expression of the CCR2 during infection and allows these cells to chemotax along an MCP-1 gradient towards infection. When we analyzed the results from our in vivo mouse model, we observed that the loss of HO-1 expression led to loose or unorganized granuloma formation in mice following *M. avium* infection. The inability of these mice to form solid granulomas led to a disseminated infection with more inflammation and more lesions covering a greater area of the lungs, liver and spleen. As Figure 18 shows, HO-1 -/- mice had loose, unorganized granulomas in the lungs. It is possible that these mice did not contain the mycobacterium as compared to HO-1 +/+ mice and therefore had disseminated infections. These findings suggest that HO-1 plays a critical role in the regulation of cellular recruitment to the site of infection and in organized granuloma formation. When analyzing our HO-1 -/- mouse tissue and immunohistochemistry, we observed that the lack of the HO-1 gene in mice infected with *M. avium* led to severe
disseminated infection, loose, unorganized granulomas and greater pathology as compared with their HO-1 +/- counterparts which formed organized granulomas after infection. This is a significant finding because we can now focus our efforts on the direct role of HO-1 in the process of granuloma formation and cellular recruitment to the site of infection in the live animal. Our HO-1 -/- mouse model has given us a useful tool in the study of granuloma formation following *M. avium* infection. Our studies have helped shed light on the role of HO-1 in the regulation of MCP-1 and CCR2 and in the process of cellular recruitment during and following mycobacterial infection.
CHAPTER 6
FUTURE CONSIDERATIONS

Having established an HO-1 -/- animal model of *M. avium* infection and granuloma formation, we can now focus on the direct role of HO-1 in the innate and adaptive immune responses, specifically focusing on granuloma formation. We plan to further elucidate the role of HO-1 in regulating cytokines such as MCP-1 and CCR2 and how this regulation affects cellular recruitment from the periphery to sites of infection and inflammation. As stated previously, HO-1 expression may exert different physiological changes in different organ systems and cell types. We can now study the effects of HO-1 or lack thereof, in several organs, cell types and in different states of health. We can also concentrate on the different aspects of heme catabolism by HO-1 which may affect this immune process. We plan on testing the affects of free iron (Fe+), Billirubin, and CO2 on granuloma formation and the immune response to *M. avium* infection. Another challenge will be to test whether it is possible to rescue the cyto-protective role of HO-1 in HO-1 -/- mice by treating them with one or more of the catabolic byproducts of heme degradation. Another possibility is the use of HO-1 therapy to treat NTM infected patients with HIV or AIDS in the hopes of rescuing their immune function. With these future studies, we hope to contribute not only to the scientific field of microbiology, but also to the clinical and therapeutic areas of medicine. Hopefully our findings will help us understand the mechanisms of NTM infection and help prevent such diseases in the future. Alternatively, we hope that our study will help elucidate the role of HO-1 in NTM disease and will lead to a better understanding of how to approach and treat other disease caused by both NTM and Mycobacterium tuberculosis.
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BIOGRAPHICAL SKETCH

Doron Regev was born in Tel-Hashomer, Israel. He grew up in Kfar Saba, Israel and moved to the United States at the age of 8. Doron spent most of his childhood in Rochester, NY and graduated from Brighton High School in 1995. He earned his bachelor’s degree in Biology from the University at Albany (NY) in 1999. Doron also excelled as a collegiate football player at Albany, earning a first team All-American award as a place kicker his senior year. Following college, Doron pursued a career as a football player and in 2001, played for the Rochester Brigade, a professional arena football team in Rochester, NY. He later decided to embark on a scientific career and began his research at the University of Rochester, where he worked in a cardiovascular research lab for two years. He then moved to Gainesville, Florida where he worked in a pulmonary research lab under Dr. Veena Antony and entered the MS program at the University of Florida. Upon completion of his master’s degree, Doron will continue his studies in the IPD program at the University of Florida in the hopes of attaining a Ph.D. in medical sciences.