REGULATION AND TRAFFICKING OF THE IRON EXPORT PROTEIN, FERROPORTIN1, IN *MYCOBACTERIUM TUBERCULOSIS*-INFECTED MACROPHAGES

DISSERTATION

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By

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ABSTRACT

Infection with *M. tuberculosis* begins when airborne droplets are inhaled. The bacteria travel to the distal regions of the lung and are engulfed by the lung dendritic cells (DCs) and resident alveolar macrophages (AMs). This initial interaction results in the production of inflammatory cytokines, such as IL-12 and TNF-α. The TNF-α produced by infected macrophages acts on other macrophages and results in the production of chemokines, which are sensed by other immune cells expressing chemokine receptors, while the IL-12 is necessary to stimulate cells such as NK and T-cells to produce IFN-γ. At this early point in the infection, *M. tuberculosis* is able to grow and replicate virtually unchecked, moving from cell to cell. Under the control of the chemokines CCR5 and CCR7, the infected dendritic cells migrate to the draining lymph nodes where they activate naïve T-cells. Again, under the control of chemokines, these activated T-cells now travel back to the site of infection and serve as the major source of IFN-γ for naïve monocytes, which are now infiltrating the site. Other cells are also attracted to the site of infection, including monocytes, B-cells, and additional dendritic cells. The end result of the migration of cells into the site of infection is the formation of a granuloma, which is necessary to control the growth of the organism by forcing it into a possibly non-replicative state.

Part of the hosts' immune response is to lower the amount of available iron to the organism via various iron withholding mechanisms, such as the down-regulation of the transferrin receptor and an increase in ferritin expression. However, iron is also utilized by the host as part of the defense against invading pathogens. For instance, our lab has shown that the iron transport protein Nramp1 is present in the mycobacterial-containing phagosome, where it serves to transport iron into the phagosome in order to promote the production of potent reactive oxygen species via the Fenton reaction. Intracellular pathogens, including *M. tuberculosis*, require iron for survival within the macrophage. A recently discovered iron export protein, ferroportin1, has been shown to be the sole iron exporter from mammalian cells. While ferroportin1 is predominantly expressed in the cells of the duodenum where it serves an important role in iron homeostasis, it has also been observed in macrophages.

The regulation of this protein has only recently begun to be examined. However, no studies have been done to examine the expression of ferroportin1 during infection with an intracellular pathogen. Therefore, the goal of this dissertation was to determine the effect of *Mycobacterium tuberculosis* infection on ferroportin1 mRNA and protein expression. To this end, I examined the effect of mycobacterial infection on the expression levels of ferroportin1 in macrophage cell lines and primary mouse macrophages. I studied the intracellular localization of ferroportin1 within mycobacteria-infected macrophages and examined the regulatory mechanisms controlling the

expression of ferroportin1 following infection. I have found that FPN1 mRNA levels in the mouse macrophage cell line RAW264.7 are synergistically increased by treatment with *M. tuberculosis* and IFN-γ. Similar results were obtained using live *M. tuberculosis* and gamma-irradiated, or dead, *M. tuberculosis*. FPN1 mRNA levels were also increased by *M. avium* and IFN-γ in RAW264.7 cells and the mouse alveolar macrophage cell line AMJ2-C8. Treatment of mouse resident peritoneal macrophages with *M. tuberculosis* and IFN-γ resulted in a 6-fold increase in ferroportin1 mRNA expression. In contrast, *M. tuberculosis* and IFN-γ inhibited ferroportin1 expression in bone marrow derived macrophages (BMDMs) and alveolar macrophages. Using confocal microscopy, we observed ferroportin1 rapidly localizing to the mycobacteria-containing phaogosomes, but not to latex bead-containing phagosomes. Examining the regulation of ferroportin1 expression, we observed that the expression is dependent upon TLR2, MyD88, and STAT. Promoter analysis reveals that transcription factor binding sites for PU.1, STAT, and two NFκB binding sites are all important in regulating the transcription of the protein, and iron levels had no effect on the promoter activity, suggesting that iron is acting post-transcriptionally on the expression of ferroportin1.

Based on the observations we have made, we propose that ferroportin1 is the principal iron exporter from the phagosome. In addition, we have observed both ferroportin1 and Nramp1 present on intracellular vesicles. Both Nramp1 and ferroportin1 transport Fe^{2+}. Because iron is oxidized during synthesis of reactive oxygen species, and

ferroportin1 only exports iron in the Fe^{2+} redox state, we would expect that the phagosomal membrane would also contain a ferrireductase that is closely associated with the iron transport proteins. This ferrireductase remains to be identified, however, a possible candidate is the recently characterized ferrireductase Lcytb, which is expressed in lysosomal vesicles and is a member of the cytochrome b_{561} protein family of ascorbate-dependent ferrireductase. While we did not examine anti-mycobacterial activity associated with ferroportin1 in the mycobacteria-containing phagosome, our data shed light on yet another host response to intracellular pathogens. Whether or not this is a mycobacteria-specific phenomenon has also not yet been determined, but another study has indicated that ferroportin1 is able to inhibit the growth of *S. enteritica*. Thus, this could be a general response utilized by the host to deplete iron levels which could be utilized by the pathogen.

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DEDICATION

I wish to dedicate this dissertation to my wife and family for their support and endless encouragement

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Research Publications:

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FIELDS OF STUDY

Major Field: Microbiology

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LIST OF ABBREVIATIONS

Ab – Antibody

AM – Alveolar Macrophage

APC – Antigen Presenting Cell

AG - Arabinogalactin

BMDM – Bone Marrow Derived Macrophage

BSA – Bovine Serum Albumin

Ca – Calcium

CIITA – MHC Class II Transactivator

CFU – Colony Forming Units

DC – Dendritic Cell

DN – Dominant Negative

DNA – Deoxyribonucleic Acid

EEA1 – Early Endosomal Autoantigen 1

FBS – Fetal Bovine Serum

Fe – Iron

FPN1 – Ferroportin1

GM-CSF – Granulocyte Macrophage-Colony Stimulating Factor

HIV – Human Immunodeficiency Virus

IFN – Interferon

IL – Interleukin

IRE – Iron Response Element

IREG1 – Iron Regulated Transporter 1

IRP – Iron Regulatory Protein

JAK – Janus Kinase

KO – Knockout

LAM – Lipoarabinomannan

LAMP1 – Lysosomal-Associated Membrane Protein 1

LPS – Lipopolysaccharide

MDR-TB – Multi Drug Resistant-*Mycobacterium tuberculosis*

MHC – Major Histocompatibility Complex

MOI – Multiplicity of Infection

MR – Mannose Receptor

mRNA – Messenger RNA

MTP1 – Metal Transporter Protein 1

MyD88 – Myeloid Differentiation Factor 88

NK – Natural Killer

NO – Nitric Oxide

Nramp – Natural Resistance Associated Macrophage Protein

PAMP – Pathogen Associated Molecular Pattern

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PG – Peptidoglycan

PI3K – Phosphoinositol 3 Kinase

PI3P – Phosphoinositol 3 Phosphate

PRR – Pattern Recognition Receptor

RES – Reticuloendothelial System

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RT-PCR – Reverse Transcription-Polymerase Chain Reaction

RU – Relative Light Units

SDM – Site Directed Mutagenesis

STAT – Signal Transducer and Activator of Transcription

TLR – Toll-like Receptor

TNF – Tumor Necrosis Factor

U – Units

CHAPTER 1

LITERATURE REVIEW

1.1 Overview

One third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Tuberculosis is spread by inhalation of droplets produced when infected individuals cough and a low dose of *M. tuberculosis* is sufficient to cause disease. Most people who become infected with *M. tuberculosis* do not develop active tuberculosis. In fact, 90% of infected persons develop the latent disease and are at risk for future reactivation. The efforts to control *M. tuberculosis* outbreaks have been hampered by the emergence of a new multi-drug resistant *M. tuberculosis* (MDR-TB) strain and the spread of HIV.

Iron is an essential nutrient, but in excess can result in tissue damage due to increased oxidative stress (Ratledge, 2004; Chung *et al*, 2004), while lack of iron results in anemia. In addition, iron acquisition is a requirement for the intracellular survival of many intracellular pathogens including *Mycobacterium tuberculosis*. Iron homeostasis is maintained by the intestinal uptake of iron from the diet and by recycling of iron from senescent erythrocytes. The reticuloendothelial system (RES) recycles 25 mg of iron each

day from approximately 360 billion senescent erythrocytes (Bratosin *et al*, 1997; Bothwell, 1995). Transport of iron is mediated by transport proteins expressed in the duodenum and reticuloendothelial macrophages. SLC11a2 (Nramp2) is the apical iron transporter of the duodenum and is responsible for the transport of dietary iron into the duodenal enterocyte (Gunshin *et al*, 1997; Crichton *et al*, 2002). Ferroportin1 (also known as IREG1 [iron-regulated transporter 1], MTP1 [metal transporter protein 1], SLC40a1) is the sole iron export protein identified in mammals and is responsible for iron export from both enterocytes of the duodenum and macrophages (McKie *et al*, 2000; Donovan *et al*, 2000; Abboud *et al*, 2000). In the dudonenal enterocytes, ferroportin1 is expressed on the basolaterial membrane and exports iron into blood circulation. In macrophages, ferroportin1 is expressed in intracellular vesicles. Upon phagocytosis of senescent erythrocytes, ferroportin1 localizes to the cell membrane of macrophages where it transports iron out of the macrophages (Chung *et al*, 2004; Delaby *et al*, 2005a, and b; Knutson *et al*, 2003; Knutson *et al*, 2005).

Regulation of ferroportin1 expression in macrophages by infection and inflammatory stimuli has only recently begun to be characterized. Inflammatory stimuli appear to have a negative regulatory effect on ferroportin1 expression. In *in vivo* studies, inflammation induced by LPS in C57BL/6J mice was shown to inhibit the expression of ferroportin1 protein in macrophages of the spleen, liver, and bone marrow (Yang *et al*, 2002). In isolated mouse splenic macrophages and bone marrow derived macrophages,

LPS stimulation also decreased ferroportin1 mRNA expression. Down-regulation of ferroportin1 mRNA expression has also been reported in the human macrophage-like cell lines, THP-1 and U937, stimulated with IFN-γ and LPS. (Ludwiczek *et al*, 2003). Ferroportin1 expression is also positively regulated by macrophage iron levels. In bone marrow derived macrophages (BMDMs) and the mouse J774A.1 macrophage-like cell line, it has been shown that iron loading and phagocytosis of senescent erythrocytes increases the expression of ferroportin1 mRNA and protein. (Delaby *et al*, 2005; Knoutson *et al*, 2003).

The goal of my dissertation reasearch was to determine the effect of *Mycobacterium tuberculosis* infection on ferroportin1 mRNA and protein expression. To this end, I examined the effect of mycobacterial infection on the expression levels of ferroportin1 in macrophage cell lines and primary mouse macrophages. I studied the intracellular localization of ferroportin1 within mycobacteria-infected macrophages and examined the regulatory mechanisms controlling the expression of ferroportin1 following infection.

1.2. Macrophage Biology

Macrophages are a highly versatile cell which constitutes the effector arm of the cellular immune system (Adams *et al*, 1984; Admas *et al*, 1987; Auger *et al*, 1992). Eli Metchnikoff has been credited as the first person to use the term “macrophage” to

describe the large mononuclear phagocytic cells in tissues (Auger *et al*, 1992). He is also credited for his recognition of phagocytosis as a fundamental host defense mechanism in primitive and multicellular organisms (Karnovsky *et al*, 1982; Metchnikoff; Tauber *et al*, 1991). Most resident macrophages in the normal adult originate from pluripotent stem cells in the bone marrow that become distributed amongst the various tissues of the body through the circulation. (Furth, 1989). Once monocytes enter the tissues, they differentiate into the tissue macrophages (Beekhuizen *et al*, 1992), and thus macrophages are named according to the tissues they inhabit, e.g., microglial cells in the central nervous system, alveolar macrophages in the lungs, Kupfer cells in the liver, and mesangial cells in the kidney. The macrophages primarily responsible for the clearance of air borne pathogens, such as *Mycobacterium tuberculosis*, are the alveolar macrophages (Fenton *et al*, 1996, Hirsch *et al*, 1994) and the macrophages primarily responsible for the clearance of blood borne pathogens are the Kupfer cells and the splenic macrophages (Frank *et al*, 1989).

Macrophages are fairly long lasting, surviving in the tissues for 1-3 months. Monocytes migrate into affected sites during local infection or inflammation and this movement is directed by chemotactic factors and other mediators of inflammation such as proinflammatory cytokines (Beekhuizen *et al*, 1992). Both endothelial cells and monocytes contain adhesion molecules which are responsible for the adherence of monocytes to the endothelial cells, and their subsequent migration through the endothelial

layer (Beekhuizen *et al*, 1992). Once at the site of infection, the monocytes differentiate into exudate macrophages which are highly differentiated cells and they exhibit a varying range of morphological, functional, and phenotypic heterogeneity. This is a reflection of their adaptation to a wide range of environmental conditions (Auger *et al*, 1992; Rutherford *et al*, 1993).

Central to macrophage functions in homeostasis, innate and acquired immunity, autoimmunity, and immunopathology is the ability to recognize a wide range of endogenous and exogenous ligands, and to respond appropriately (Karnovsky *et al*, 1982; Metchnikoff; Tauber *et al*, 1991). For this reason, macrophages express a variety of cell surface receptors including mannose receptors, scavenger receptors (CD36, and SR-A), GPI-anchored receptors (CD14), Integrins (CR3), Ig superfamily (FcR), seven transmembrane (CCR2, C5Ar, and EMR2), NK-like C-type lectin-like receptors (Dectin-1), and the Toll-like receptors (TLRs). Janeway was the first to propose the concept of pattern recognition as a means to detect and react to infection and later studies by Medzhitov and Janway shows that pattern recognition receptors play an important role in mediating host defense (Janeway *et al*, 2002). This is based on the recognition of conserved microbial structures known as **p**athogen-**a**ssociated **m**olecular **p**atterns (PAMPs) by a number of germ line-encoded antigen presenting cell (APC) **p**attern **r**ecognition **r**eceptors (PRRs). This is in contrast to the receptors of the T- and B-cells which are generated by somatic recombination and are mainly specific for peptide

antigens. Mannose receptors, Fc receptors, and the complement receptors (CR1, CR3, and CR4) are involved in phagocytosis (Auger *et al*, 1992; Cywes *et al*, 1997; Ernst, 1998; Hirsch *et al*, 1994; Kang *et al*, 1998; Roecklein *et al*, 1992; Schlesinger *et al*, 1996). TLRs in vertebrates are able to sense diverse microbial ligands and are able to transmit signals via adaptor molecules selectively, however, they must depend on other surface molecules such as CD14/MD2 (Akira *et al*, 2001) and C-type lectin-like receptors to aid in recognition (Brown *et al*, 2003). TLR4, in association with CD14, is responsible for the recognition of lipopolysaccharide (LPS) from Gram-negative bacteria bound by LPS binding protein (LBP) and MD2 (Shimazu *et al*, 1999). TLR2, and to a lesser extent, TLR4, have been shown to preferentially bind to the lipomannan (LM) and lipoarabinomannan (LAM) in the cell wall of mycobacteria species. While TLR2 is the most important TLR for recognizing and responding to *M. tuberculosis* infection, it has been shown that TLR4 expression is required to control chronic *M. tuberculosis* infection in mice (Abel *et al*, 2002). In addition to receptors for bacterial ligands, macrophages also contain many receptors for cytokines and chemokines, which are able to modulate the functions of the macrophages following receptor binding. Cytokines such as interleukin-10 (IL-10) and TGF-β are able to down-regulate the production of proinflammatory cytokines by macrophages (Chantry *et al*, 1989; Erspevic *et al*, 1987; Musso *et al*, 1990; Wang *et al*, 1994). IL-2 enhances the tumoricidal and microbicidal activities of macrophages via the induction of tumor necrosis factor alpha (TNF-α)

production and the generation of reactive oxygen intermediates (Erspevic *et al*, 1987). It has been shown that IFN-γ induces, or up-regulates, the expression of MHC class II molecules on the surface of the macrophages (Adams *et al*, 1984; Adams *et al*, 1987; Boehm *et al*, 1997; Gonwa *et al*, 1986). RANTES (Regulated upon Activation, normal T-cell Expressed and Secreted), the chemokines MCP-1, -2, and -3 (macrophage chemotactic protein-1, -2, and -3), and macrophage inflammatory protein-1α and -β (MIP-1α and -β) are responsible for attracting the monocytes to the site of infection (Kitamura, 1997; Miller *et al*, 1992; Rhodes *et al*, 1995; Schall *et al*, 1994; Vaddi *et al*, 1994).

In addition to their phagocytic and immunomodulating properties, following surface and endocytic stimulation, the mature macrophage is able secrete a very large range of high- and low-molecular-weight products. These products include enzymes involved in antimicrobial resistance (lysozyme), neutral proteinases, cytokines such as IL-1 and TNF-α that modulate the activities of other leukocytes and endothelium, and reactive oxygen and nitrogen intermediates implicated in host defense (Auger *et al*, 1992; Shiloh *et al*, 1999). Ligand binding of specific receptors induces various signaling pathways which selectively alter the expression of these genes. Transcription factors such as the $NF_{\kappa}B$ and PU.1 families contribute to macrophage-restricted or activation-dependent changes in gene expression (Clarke *et al*, 1998).

1.3. Macrophage Activation

Macrophages have the innate ability to respond to a number of different cell products during the innate and acquired immune response. IFN-γ (originally termed macrophage-activating factor) is among the most important of the macrophage activating factors. IFN-γ activation of macrophages is complex in that activation requires the binding of IFN-γ homodimer to the IFN-γ receptor, which then activates the IFN-γ JAK-STAT signal transduction pathway (Bach *et al*, 1997; Darnell Jr. *et al*, 1994). This initial activation of signaling results in a cascade of early gene expression and these early genes have been shown to be important in the induction of other genes which are expressed following IFN-γ stimulation (Malefyt *et al*, 1991; Pine *et al*, 1994; Reith *et al*, 1995). One specific example of this phenomenon is the major histocompatibility complex (MHC) molecules. Macrophages which have been stimulated with IFN-γ have been shown to express high levels of the MHC II genes. The induction of the MHC II molecules also requires the early induction of yet another IFN-γ-inducible gene, MHC class II transactivator (CIITA). CIITA is an interesting transcription factor in that it can activate transcription without DNA binding (Mach *et al*, 1996; Reith *et al*, 1995). A 1997 study indicated that there is a binding site for IRF-1, also an IFN-γ-inducible gene, in the promoter of CIITA (Muhlethaler-Motte *et al*, 1997). Even though *M. tuberculosis* is able to disrupt the maturation of the phagosome, two independent groups have reported that macrophage activation with IFN-γ can induce the maturation of the mycobacteria-

containing phagosomes (Schaible *et al*, 1998; Via *et al*, 1998). In addition, the ability of gamma-activated macrophages to control the growth of *M. tuberculosis* is enhanced by the induction of nitric oxide synthase (iNOS or NOS2), which is responsible for catalyzing the production of nitric oxide (NO) (Flesch et al, 1991; Chan et al, 1995; McMicking and North, 1997; Flynn and Scanga, 1998).

1.3.1. Gamma interferon

Initial work dealing with viral replication lead to the discovery of IFNs (Isaacs and Lindermann, 1957). While now classified into type I and type II, according to receptor specificity and sequence homology, they were initially classified according to the type of cell secreting them. IFN-γ is the only type II IFN described to date, and structurally it is unrelated to the type 1 IFNs in that IFN-γ binds to a different receptor, and is encoded by a separate chromosomal locus (Schroder *et al*, 2004). While initially believed that $CD4^+$ T helper cells (Th1 lymphocytes), $CD8^+$ cytotoxic T lymphocytes, and NK (natural killer) cells exclusively produced IFN-γ (Bach *et al*, 1997; Young *et al*, 1996), we now know that other cells such as B cells, NKT cells (natural killer T cells) and professional APCs also secrete IFN-γ (Carnaud *et al*, 1999; Gessani *et al*, 1998; Yoshimoto *et al*, 1998; Flaishon *et al*, 2000; Harris *et al*, 2000). The production of IFN-γ by professional APCs, which include monocyte/macrophages and dendritic cells, has been shown to be important in the self-activation of these cells as well as the activation of

nearby cells (Frucht *et al*, 2001; Gessani *et al*, 1998). IFN-γ may also augment microbicidal and tumoricidal activities of macrophages by modulating the expression of cell surface molecules (such as MHC) as well as increasing nitric oxide (NO) production and oxidative burst activity (Adams *et al*, 1987; Boehm *et al*, 1997; Flesch *et al*, 1991; Goldberg *et al*, 1990; Gonwa *et al*, 1986; Green *et al*, 1994; Steeg *et al*, 1982; Takao *et al*, 1996). In addition, the secretion of IFN-γ by NK cells and possibly by professional APCs is likely to be important in the early host defense against infection, whereas T-cells are the major source of IFN-γ in the adaptive immune response (Frucht *et al*, 2001; Sen, 2001).

The production of IFN-γ is primarily controlled by cytokines such as IL-12 and IL-18, which are secreted by APCs (Schroder *et al*, 2004). These cytokines are considered the "bridge" that links infection and IFN-γ production with the innate immune response (Golab *et al*, 2000; Munder *et al*, 2001; Munder *et al*, 1998; Fukao *et al*, 2000; Otani *et al*, 1999; Akira, 2000; Dinarello, 1999). In addition to IFN-γ, macrophage recognition of pathogens is also linked to the secretion of many chemokines; including MIP-1α (Salaza-Mather *et al*, 2000) which serve to attract NK cells to the sit of inflammation, and IL-12 then promotes IFN-γ synthesis in these newly arriving cells (Salaza-Mather *et al*, 2000; Pien *et al*, 2000). There is a synergistic affect of IL-12 and IL-18 on macrophages, NK cells, and T-cells causing increased IFN-γ production (Munder *et al*, 1998; Akira *et al*, 2000; Dinarello *et al*, 1999; Schindler *et al*, 2001; Fukao

et al, 2001). As is the case for most factors involved in mounting an immune response, there are many negative regulators of IFN-γ production, which include the cytokines IL-4, IL-10, TGF-β (transforming growth factor-β) and glucocorticoids (Sen, 2001; Fukao *et al*, 2000; Schindler *et al*, 2001; Fukao *et al*, 2000; Hochrein *et al*, 2001).

IFN-γ primarily signals through the Jak-STAT pathway, which is the same pathway utilized by some 50 cytokines, growth factors, and hormones to affect gene regulation (Subramaniam *et al*, 2001). All STAT proteins contain a SH2 domain that when phosphorylated, mediates homodimerization or heterodimerization with other STAT molecules (Shuai *et al*, 1993; Shuai *et al*, 1994). While STAT1 homodimers are most commonly discussed in the context of macrophage activation, IFN-γ signaling also produces other active complexes such as STAT heterodimers (STAT1:STAT2) and heterotrimers (STAT1:STAT1:IRF-9, STAT1:STAT2:IRF-9) (Darnell *et al*, 1994; Matsumoto *et al*, 1999; Stark *et al*, 1998; Bluyssen *et al*, 1996). It has been shown that phosphorylation of STAT1 at S727 is essential for maximal abilty to activate transcription target genes (Decker and Kovarik, 2000; Zhu *et al*, 1997; Horvath and Darnell, 1996; Goh *et al*, 1999; Wen *et al*, 1995; Zhang *et al*, 1995).

1.4. Mycobacterium Biology

The Mycobacterium genus contains more than 50 species, however, only a few are pathogenic to humans (Barksdale *et al*, 1977; Fenton *et al*, 1996; Grange, 1996).

Initially described by Robert Koch in 1882, *Mycobacterium tuberculosis* is the major cause of tuberculosis around the world. Tuberculosis seems to be a disease of poverty as virtually all tuberculosis-related deaths occur in the developing world; affecting mostly young adults. According to the World Health Organization (WHO), 2 billion people are infected with the TB bacilli that cause tuberculosis, almost 9 million new tuberculosis cases occurred in 2004, and tuberculosis results in the deaths of nearly 2 million people each year. *Mycobacterium africanum* infection is limited to northwestern Africa. *Mycobacterium bovis* can also cause tuberculosis, but the incidence of this occurring has declined due to modern day pasteurization methods and the boiling of consumed milk. *Mycobacterium avium* and *Mycobacterium intracellulare* are usually not human pathogens, but can cause severe pulmonary disease in immunocomprimised hosts. With the arrival of the Acquired Immunodeficiency Syndrome (AIDS) in the early 1980's, *M. avium* infection has become a major human health problem (Bermudez, 1994; Ellner *et al*, 1991; Fu *et al*, 1990). While drug treatment is available for tuberculosis infection, a new form of tuberculosis, or multidrug-resistant tuberculosis (MDR-TB) is on the rise. MDR-TB is a form of tuberculosis that does not respond to the standard drug treatment, and is present in virtually all 109 countries recently surveyed by the WHO (www.who.int/topics/tuberculosis/en/). In fact, 450,000 new MDR-TB cases are estimated to occur every year. The highest rates of MDR-TB are in the countries of the former Soviet Union and China.

Mycobacteria are thin rods with round extremities, 2.5 μm long and 0.2-0.3 μm thick. The cell wall of these organisms is similar to the cell walls of the Gram-positive bacteria. However, the cell walls of Mycobacteria contain higher lipid content and can only be stained with the acid fast stain (Grange, 1996). The ability of *M. tuberculosis* to survive within the host macrophages is thought to be linked to the unusual physiochemical properties of the mycobacterial surface (Brennan, 1989). The bacterium is enclosed within a lipid bilayer cytoplasmic membrane, located beneath the rigid peptidoglycan (PG) layer. A number of proteins, some immunogenic, have been found to associate with PG and between the membrane and PG. (Barnes *et al*, 1989; Brennan, 1989). Continuing outward, PG is covalently linked via phosphodiester bonds to arabinogalactin (AG), which is a polymer of arabinose and galactose (Barksdale *et al*, 1977; Besra *et al*, 1994). Large branched-chain fatty acids (C_{60} to C_{90}) known as mycolic acids are attached to the distal portion of AG (Steck *et al*, 1978). Another important group of cell wall components are the acylated trehalose-2'-sulfates. These may be important for virulence, as the most viulent strains of *M. tuberculosis* elaborate strongly acidic sulfolipids, which may be involved in inactivating the macrophage phagosomes (Besra *et al*, 1994). Another *M. tuberculosis* cell wall component, lipoarabinomannan (LAM), is anchored in the mycobacterial cell membrane and it is thought to extend all the way to the surface (Fenton *et al*, 1996). LAM is found as a heterogenous mixture of arabinose- and mannose-containing phosphorylated high-molecular-weight

lipopolysaccharides (Brennan, 1989; Hunter *et al*, 1986; Venisse *et al*, 1993). LAM is the most important cell wall glycolipid because of its complex immunomodulating properties (Barnes *et al*, 1992; Fenton *et al*, 1996; Sibley *et al*, 1988; Sibley *et al*, 1990). Palmitate and tuberculostearate fatty acids occur in the form of diacylglycerol which is linked to the branched arabinose- mannose-containing polysaccharide via phosphatidyl-*myo*-inositol (Besra *et al*, 1994). Capping of the arabinose termini of LAM from *M. tuberculosis* seems to be important for virulence, as differences in capping significantly affects macrophage responses (Chaterjee *et al*, 1992).

1.4.1. Recognition of Mycobacterium tuberculosis.

The first, and most critical, step for entry of intracellular pathogens into cells is the adhesion of the pathogen to host cell surface. As infection with *M. tuberculosis* is a result of inhalation of the organsism, the primary cell type involved in the initial uptake of *M. tuberculosis* is the alveolar resident macrophage. Following this first encounter, dendritic cells (DCs) and monocyte-derived macrophages also take part in the phagocytic process (Henderson *et al*, 1997; Thurnher *et al*, 1997). Phagocytosis of *M. tubercuolsis* involves many different receptors on the phagocytic cell which either bind to nonopsonized *M. tuberculosis* or recognize opsonins on the surface of the *M. tuberculosis*. Once the mycobacterium has been ingested, the organism will either start to

divide within the phagosome or it will be destroyed by the defense mechanisms of the macrophage.

Nonopsonic mechanisms of mycobacterial phagocytosis involve binding of mycobacteria to a variety of receptors including vitronectin ($\alpha V\beta 3$), fibronectin, and mannose receptors (Abou-Zied *et al*, 1988; Kang *et al*, 1998; Rao *et al*, 1993; Schlesinger *et al*, 1993; Schlesinger *et al*, 1994b; Schlesinger *et al*, 1996). However, the best characterized receptor for nonopsonic-mediated phagocytosis of *M. tuberculosis* is the mannose receptor (MR), which recognizes terminal mannose residues on mycobacteria (Schlesinger *et al*, 1993; Schlesinger *et al*, 1996). The mannose receptor is a monomeric transmembrane protein expressed only on mature macrophages. It has been shown that human monocyte-derived macrophages phagocytose virulent *M. tuberculosis* via mannose receptors through their interaction with the terminal mannosyl units of LAM (Schlesinger *et al*, 1994b; Schlesinger *et al*, 1996). The mannose receptor is also important in mediating the delivery of LAM to the endocytic compartments and thus facilitates mycolic acid and lipoglycan antigen presentation through CD1b to T-cells (Prigozy *et al*, 1997).

In addition to the above mentioned methods of mycoabcterial recognition, macrophages also utilize the Toll-like receptors (TLR) for the recognition of many bacterial and viral components. The mammalian TLRs are a conserved family of membrane associated receptors which posess the ability to signal in either a MyD88-

dependent or -independent manner. Binding of a pathogen associated molecular pattern (PAMP) to the TLR results in the activation of nuclear transcription factors such as NFκB, activating-protein 1 (AP-1), and interferon regulatory factors (IRFs), which results in the production of cytokines, chemokines, and co-stimulatory molecules by the macrophage that can, in turn, activate other cells of the immune system. While TLRs are important in immune receognition and response, it has been shown that TLRs may also be involved in regulating the differentiation of monocytes into dendritic cells and macrophages (Krutzik *et al*, 2005). To date, 11 members of the TLR family have been identified (Uematsu and Akira, 2006). Many studies have shown that mycobacteria and mycobacterial components can act as agonists for TLRs (Pai *et al*, 2004; Quesniaux and Fremond *et al*, 2004; Bafica *et al*, 2005).

1.4.2. Protection against Tuberculosis.

The interaction between infected macrophages and T lymphocytes has been shown to be critical for the successful elimination of *M. tuberculosis*. $CD4^+$ T cells exert their protective effect via the production of cytokines, primarily gamma interferon (IFN-γ) following stimulation with mycobacterial antigens. However, $CD8^+$ T cells have also been implicated in control of tuberculosis infection by secreting cytokines and lysing infected cells (Geluk *et al*, 2000; Stenger *et al*, 1999). The T cell response is, for the most part, antigen specific. For this reason, a lot of attention has been focused on the

identification of immunodominant antigens that could be used for the development of effective vaccines against *M. tuberculosis* (Andersen, 1997). The polymorphisms of the MHC may contribute to differences in disease susceptibility or outcome due to the fact that the acquired T-cell response develops in the context of the MHC (Bothamley *et al*, 1989; Goldfeld *et al*, 1998; Rajalingam *et al*, 1997). The importance of functional diversity of T lymphocytes has also been implicated in the control of tuberculosis infection. It was reported in 1986 that murine helper T (Th) lymphocytes could be divided into two subsets: Th1 and Th2. Th1 clones were characterized by the production of IFN-γ, whereas the Th2 clones were characterized by the production of IL-4 (Mosmann *et al*, 1996). The two subsets develop from naïve T-cells, whose differentiation is influenced by the environment: IL-12, produced by activated macrophages and dendritic cells, has been shown to be the principal Th1-inducing cytokine, while IL-4 promotes the induction of Th2 cells (Abbas *et al*, 1996). However, these cytokines and cellular subsets are not the only ones implicated in the Th1-Th2 concept (Mosmann *et al*, 1987), which are thought to be relevant in many diseases (Lucey *et al*, 1996). It has been shown that IFN-γ gene knockout (KO) mice are highly susceptible to *M. tuberculosis* (Cooper *et al*, 1993), and it has also been shown that individuals lacking receptors for IFN-γ suffer from recurrent, sometimes lethal mycobacterial infections (Flynn *et al*, 1993; Holland *et al*, 1998; Newport *et al*, 1996). In the case of the mycobacterial infection, the Th1-type cytokines seem essential for

protective immunity, wheras the Th2-type cytokines inhibit the *in vitro* production of IFN-γ (Lucey *et al*, 1996; Powrie *et al*, 1993), as well as the activation of macrophages (Appelberg *et al*, 1992), and may therefore weaken host defenses (Malefyt *et al*, 1993). In addition, it has been shown that there is an increase in Th2-type cytokines in tuberculosis patients (Bhattacharyya *et al*, 1999; Dlugovitzky *et al*, 1999; Seah *et al*, 2000; Surcel *et al*, 1994; Crevel *et al*, 2000). However, there are studies that have findings which are not consistent with these (Barnes *et al*, 1993; Hernandez *et al*, 1994; Lai *et al*, 1997; Lin *et al*, 1996).

T-cells are not the only immune cells which have been implicated in the control of tuberculosis infection. Phagocytic cells play a key role in the initiation and direction of adaptive T-cell immunity because the phagocytic cells present mycobacterial antigens and express costimulatory signals and cytokines. Lurie's studies with resistant and susceptible inbred rabbits indicated that innate defense mechanisms of phagocytic cells are important in the control of tuberculosis infection (Lurie *et al*, 1964). In support of this finding, seven days after infection via the inhalation of tubercle bacilli, the lungs of mycobacterium-susceptible rabbits contained 20- to 30-fold more viable mycobacteria than the lungs of mycobacterium-resistant rabbits (Dannenberg *et al*, 1994). Recently it has also been found that acquired T-cell immunity in vaccinated mice protects them from disseminated tuberculosis but does not prevent the initial pulmonary infection, thus indicating that the differences during early infection cannot be attributed to T-cell

immunity (Cooper *et al*, 1997; North *et al*, 1999). The same findings hold true in human disease as well. Genetic studies have shown associations between functional gene polymorphisms and *M. tuberculosis* for various macrophage products (Bellamy *et al*, 1998; Bellamy *et al*, 1999; Wilkinson *et al*, 1999; Wilkinson *et al*, 2000).

The interaction of *M. tuberculosis* with TLR2 has been shown to result in the production of many inflammatory cytokines such as TNF-α and IL-1 (Underhill *et al*, 1999). In addition, the interaction of *M. tuberculosis* with TLR2 results in an inhibition of MHC class II antigen presentation of mycobacterial components (Noss *et al*, 2001). Because TLR2-deficient mice have impaired defense against virulent *M. avium*, TLR2 is thought to serve a pivitol role in the protection against mycobacterial infection (Feng *et al*, 2003). In support if this, it has been shown that $TLR2^{-/-}$ macrophages display impaired production of TNF-α (Tjarnlund *et al*, 2006) which has been shown to be a critical cytokine for controlling the mycobacterial infections (Roach *et al*, 2002).

1.5. Mycobacterial resistance mechanisms.

The ability of *M. tuberculosis* to be a successful pathogen is directly dependent on the fact that it is a highly adapted organism. One such adaptation has enabled *M. tuberculosis* to interfere with intracellular membrane trafficking while inside the host macrophage. David Russell (Vergne *et al*, 2004a) and his lab have shown that *M. tuberculosis* actively blocks the biogenesis of the phagolysosome, thus evading

elimination within the phagocytic cell. The fact that *M. tuberculosis* is able to enter the macrophage and reside within a pathogen-friendly phagosome that does not mature into the phagolysosome (Armstrong *et al*, 1971) is critical for tuberculosis infection, disease activation, spread, latency, and suppression of detection by the host (Flynn *et al*, 2003; Hanekom *et al*, 2003; Kincaid *et al*, 2003; Mustafa *et al*, 1999; Hmama *et al*, 1998; Russell *et al*, 2002; Stenger *et al*, 1998; Ting *et al*, 1999; Pancholi *et al*, 1993).

Because the *M. tuberculosis* phagosome does not mature into a phagolysosome, phagolysosome maturation in infected macrophages has been the subject of numerous studies. Several groups (Oh *et al*, 1996; Crowle *et al*, 1991; Sturgill-Koszycki *et al*, 1994) have shown that there is incomplete acidification of the luminal region of the phagosome. Further, mature lysosomal hydrolases appear to be absent from the phagosome (Malik *et al*, 2000; Malik *et al*, 2001; Ullrich *et al*, 1999). Recent studies suggest that Ca^{2+} manipulation by the pathogen may be responsible for the previous two findings (Malik *et al*, 2000; Malik *et al*, 2001; Malik *et al*, 2003). In addition, the trafficking of MHC molecules has also been shown to be interrupted in the mycobacteria-containing phagosomes (Clemens *et al*, 1995). It has been shown that the mycobacteria have access to transferrin-bound iron (Sturgill-Koszycki *et al*, 1996), there is an accumulation of transferrin receptor on the phagosomes (Clemens *et al*, 1996), and the mycobacteria have access to glycosphingolipids (Russell *et al*, 1996).

Studies dealing with the mechanistic processes which lead to the block of phagosomal maturation began with the analysis of endocytic Rabs. A 1997 study (Via *et al*, 1997) showed that the block of maturation occurs between the maturation stages controlled by the small GTP-binding proteins Rab5, which are early endocytic markers, and Rab7, late endosomal markers. These proteins are responsible for directing intracellular trafficking and maintaining the identity of the endocytic vesicles. While Rab5 accumulates in the mycobacterial phagosomes, Rab7 was not present at the usual times expected for its recruitment (Via *et al*, 1997). Due to initial observations dealing with Rabs, many labs have taken on the issue of determining the exact roles of both Rab5 and Rab7 in mycobacterial phagosome maturation (Clemmens *et al*, 2000 a, and b; Kelley *et al*, 2003). While Rabs appear to be important in the maturation of the mycobacterial phagosome, it has been shown recently that Rab5 may also play a role in facilitating the acquisition of iron by the intracellular mycobacteria (Kelley *et al*, 2003). It has been shown that the majority of Rab5-effectors are recruited to mycobacterial phagosomes as well as control (latex bead) phagosomes with the one exception being EEA1 or early endosomal autoantigen 1. This was an interesting finding as it is known that EEA1 serves as an organelle tethering molecule by bridging membranes which are destined for fussion with other membranes (Christofordis *et al*, 1999; Simonsen *et al*, 1998). It has also been shown that EEA1 not only interacts with Rab5 (Simonsen *et al*, 1998), but that its recruitment and subsequent association with the endosomal membranes

is strengthened via the binding of its FYVE domain to PI3P (Simonsen *et al*, 1998). Thus, the reduced recruitment of EEA1 to the mycobacterial phagosomes (Fratti *et al*, 2001) has implicated PI3P and PI3K in the mycobacterial phagosome maturation block (Fratti *et al*, 2003). More recently, two proteins of the mycobacterial cell wall have been shown to be important in the inhibition of phagosomal maturation. Phosphatidylinositol mannoside (PIM) and LAM are both phosphatidylinositol analoges and a recent study (Vergne *et al*, 2004b) has indicated that PIM stimulates fusion of early endosomes with other endosomes, which most likely allows the mycobacterial phagosome to gain access to endocytosed nutrients, including iron bound to transferrin.

While the fusion of phagosomes with lysosomes in neutrophils is Ca^{2+} dependent, the rise in Ca^{2+} elicited during phagocytosis of serum-opsonized particles in macrophages was initially seen as unnecessary for phagosome-lysosome fusion. This was done by monitoring the phagosomal acquisition of LAMP-1 (lysosomal-associated membrane protein 1) (Zimmerli *et al*, 1996). While early studies showed that LAMPs are delivered to the phagosomes via a PI3P-independent route (Karlsson *et al*, 1998), it is now known that additional, PI3P-dependent trafficking routes contribute to phagosomal biogenesis by delivering markers other than LAMPs (Malik *et al*, 2001; Malik *et al*, 2003; Fratti *et al*, 2003). It was previously shown that EEA1 plays an essential role in the maturation of phagosomes as it is recruited to membranes by Rab5 and by PI3P, the product of hVPS34 (Simonsen *et al*, 1998), and it has now been demonstrated that Ca^{2+} and calmodulin also

influence the recruitment of the PI3KhVPS34 complex to phagosomes in macrophages (Vergne *et al*, 2003).

Thus, it is now possible to link the mycobacterium-dependent inhibition of Ca^{2+} rise (Malik *et al*, 2000) with the recruitment of PI3P-dependent tethering molecules such as EEA1 to the mycobacterial phagosomes as indicated by Fratti et al (Fratti *et al*, 2001). From this information, it has been determined that the mycobacterial interference with the Ca^{2+}/CaM/CaMKII cascade brings about a block in hVPS34 recruitment (Vergne *et al*, 2003). This implicates a drop in the generation of PI3P on the mycobacterial phagosome, thus preventing the delivery of critical hydrolases and H^+ATPase (Vergne *et al*, 2004b).

1.6. Iron transport proteins and innate immunity

Iron is an essential nutrient for most all living cells and is required for the functioning of a large number of enzymes. In humans, 30-40 mg of iron is required daily for internal use, which includes the synthesis of hemoglobin. In contrast, humans only absorb 1-2 mg of iron per day from our diet (Testa, 2002). Thus, the supply of iron needed comes from the recycling of about 20 mg of iron from senescent erythrocytes. Iron may be attached as part of a heme nucleus or to various amino acids near the active sites of proteins. Iron is a key inorganic element used in electron transport because it can undergo oxidation and reduction reations (i.e. redox reactions) in which the iron oscillates

between its oxidized form, ferric iron (Fe^{3+}), and its reduced form, ferrous (Fe^{2+}). For a pathogenic microorganism growing within the host, iron must be acquired from the host for survival, thus creating a competition between the host and the pathogen for iron.

1.6.1. Iron and Mycobacterium tuberculosis

With respect to *M. tuberculosis*, Kochan (Kochan, 1973) showed that its growth in human serum was inhibited by the iron-withholding actions of transferrin, thus, *M. tuberculosis* requires iron from the host in order to survive and persist. Just how the organism obtains the iron has been the subject of numerous papers form varying groups (Bullen *et al*, 1999; Ratledge *et al*, 2000; Sigel *et al*, 1999; Winkelmann *et al*, 1994; Winkelmann *et al*, 1997; Woodridge *et al*, 1993). Some studies have indicated that *M. tuberculosis* obtains iron from extracellular transferrin and lactoferrin and from the intracellular iron (Fe^{2+}) pool (De Voss *et al*, 1999; Ratledge, 2004). These same studies also indicate that the primary source of iron for intraphagosomal *M. tuberculosis* is the Fe^{2+} intracellular iron pool. *M. tuberculosis* also expresses siderophores (mycobactin and carboxymycobactin) capable of binding Fe^{3+}, which are also thought to be important in the acquisition of iron within the phagosome (De Voss *et al*, 1999; Ratledge, 2004). Although there are three main categories of siderophores, the mycobacteria siderophores fall into the hydroxamte and mixed ligand types (Ratledge, 2004). Recently, two mycobacterial genes (irtA and irtB) have been identified that code for an ABC transporter

that is thought to be important important in the transport of iron bound to carboxymycobactin into the mycobacteria (Rodriguex *et al*, 2006). *M. tuberculosis* also expresses a divalent cation transporter, Mramp, which belongs to the Nramp family (Agranoff *et al*, 1999; Boechat *et al*, 2002). While Mramp transports Fe^{2+}, studies have shown that deletion of this gene does not alter the growth of *M. tuberculosis* in iron deficient medium or in mouse macrophages (Boechat *et al*, 2002), suggesting that Mramp is not essential for the uptake of iron by *M. tuberculosis*.

1.6.2. Nramp1

Nramp1, or SLC11A1, is a transporter of Fe^{2+} as well as many other divalent metal cations, and is expressed by cells of the myeloid lineage (Vidal *et al*, 1993; Govoni *et al*, 1997). Nramp1 is a large (90-100kDa), highly glycosylated, protein composed of 12 transmembrane domains (Vidal *et al*, 1993; Gruenheid *et al*, 1997; Searle *et al*, 1998). Studies have shown that Nramp1 is expressed in late endosomal and lysosomal vesicles, and following the phagocytosis of a pathogen or latex beads, localizes to the phagosomal membrane (Gruenheid *et al*, 1997; Searle *et al*, 1998). Originally, this gene was identified as one that determines innate resistance to a range of pathogens (Vidal *et al*, 1993; Skamene *et al*, 1982; Blackwell *et al*, 1991; Vidal *et al*, 1995b; Vidal *et al*, 1995a). However, the gene has two alleles in mice, a wild-type resistant allele and a naturally occurring mutant allele with a susceptible phenotype. The mutant Nramp1 differs from

the wild-type by only a single amino substitution of a glycine with a aspartic acid at position 169 (Vidal *et al*, 1993) that causes misfolding of the protein and its subsequent retention with the ER (Vidal *et al*, 1996; White *et al*, 2004). While mice with the susceptible allele are more susceptible to infection with *Leishmania donavani*, *Salmonella typhimurium*, and *Mycobacterium bovis*, Nramp1 also confers susceptibility to several other mycobacterial species including *M. avium*, *M. intracellulare*, and *M. lepraemurium*. Whether or not Nramp1 dtermines susceptibility to *M. tuberculosis* is still in doubt, since mice with a deletion of the Nramp1 gene are equally resistant to infection with *M. tuberculosis* as compared to normal, wild-type, mice (North *et al*). In addition, another study has shown that *M. tuberculosis* growth in mice expressing the mutant allele is greater than in mice with the wild type allele (Arias *et al*, 1997). In humans, the G169D mutation is not present. However, other Nramp1 polymorphisms have ben associated with risk of microscopy-positive tuberculosis and severe forms of tuberculosis, but not susceptibility for tuberculosis (Sebøbrg *et al*, 2002; Zhang *et al*, 2005). Taken together, these data suggest that the variant human Nramp1 alleles affect mycobacterial growth rather than susceptibility to infection.

The directional transport of iron by Nramp1 is still being debated. Our laboratory was the first to show that Nramp1 transports iron in macrophages (Kuhn *et al*, 1999), and our studies led us to propose that Nramp1 transports iron into phagosomes and mediates resistance to mycobacteria by catalyzing the production of hydroxyl radicals by the

Fenton reaction. In support of this, we have shown that phagocytosis of *M. avium* induced the production of hydroxyl radicals in RAW264.7 macrophages expressing wild-type Nramp1. However, this is not the case in macrophages expressing the mutant Nramp1 allele (Kuhn *et al*, 1999). Our laboratory has also shown that the addition of a small amount of iron to cultures of resident peritoneal macrophages from Nramp1 wild-type mice stimulated antimicrobial activity against *M. avium* (Zwilling *et al*, 1999). Inhibition of hydroxyl radical generation prevented the increased antimicrobial activity.

However, Philipe Gros and his colleagues (Jabado *et al*, 2000) examined the effects of Nramp1 on iron transport when the phagosomes were first loaded with iron. They reasoned that Nramp1 transports iron and other divalent cations out of the phagosome, thus depriving the bacteria of iron needed for growth. Their experiments utilized phagosomes that were formed in macrophages containing zymosan with a bound fluorescent probe and Mn^{2+}, which quenched the fluorescence. They then showed that the return of fluorescence was greater in Nramp1 wild-type macrophages, indicating that Nramp1 was transporting the Mn^{2+} out of the phagosome. In yet another study, Mulero *et al* investigated the release of iron following phagocytosis of radiolabeled iron bound to immune complexes. They have concluded that Nramp1 regulated the release of iron from the phagosomes following phagocytic uptake of iron.

In yet another study, Goswami *et al* (2001) have shown that Nramp1-mediated iron transport is bidirectional, thus providing a possible explanation for the apparent

differences between the previous studies. They showed that frog oocytes which express mouse Nramp1 on the cell surface transports Fe^{2+}, Zn^{2+}, and Mn^{2+} in either direction and this directional transport was found to be dependent on the presence of a pH gradient, with the direction opposite to that of the H^+ gradient. While these studies have yielded different results, there are some consistencies in all of them. The first is that a pH gradient is required. The second is that iron transported by Nramp1 is iron concentration-dependent.

1.6.3 Ferroportin1

Ferroportin1 (SLC40a1), also known as MTP1 and IREG1, was first discovered independently by three groups (Donavan *et al*, 2000; Abboud *et al*, 2000; McKie *et al*, 2000). It is a 62kDa integral membrane protein with 9 or 12 membrane spanning domains and functions as an Fe^{2+} export protein. To date, it is the only iron export protein discovered in mammalian cells. Mutations of ferroportin1 in humans has been shown to result in haemochromatosis type 4, an iron overload disease with accumulation of iron in macrophages due to impaired iron export (Pietrangelo, 2004; McGregor *et al*, 2005; Liu *et al*, 2005a; De Domenico *et al*, 2005). Ferroportin1 is expressed on duodenal enterocytes, liver hepatocytes, reticuloendothelial macrophages, and placental synctiotrophoblasts (Donavan *et al*, 2000; Abboud *et al*, 2000; McKie *et al*, 2000). Ferroportin1 functions in placental meterno-fetal iron transfer, intestinal iron absorption,

and recycling of iron by the reticuloendothelial macrophages and hepatocytes. In the intestine, ferroportin1 is expressed on the basolateral membrane and exports iron from the enterocyte into the blood circulation. In macrophages, it is expressed in intracellular vesicles. Upon phagocytosis of senescent erythrocytes, or iron treatment of the macropage, ferroportin1 was found to be highly expressed at the plasma membrane of mouse bone marrow-derived macrophages (Delaby *et al*, 2005a; Knutson *et al*, 2005). Confirmation of iron export by ferroportin1 has been demonstrated in experiments that showed that ferroportin1 expression in the plasma membrane of frog oocytes stimulated radiolabeled iron efflux (McKie *et al*, 2000; McGregor *et al*, 2005).

The expression of ferroportin1 is regulated by both transcriptional and post-transcriptional pathways. Ferroportin1 mRNA is constitutively expressed by macrophages, but the level is higher in mouse bone marrow-derived macrophages (BMDMs) as compared to the mouse macrophage cell lines RAW264.7 and J774A.1 (Canonne-Hergaux *et al*, 2006). *In vivo*, the expression of ferroportin1 mRNA and protein has been shown to be down-regulated in the spleen, liver, and bone marrow by inflammation induced by LPS (Yang *et al*, 2002). In addition, LPS has been shown to down-regulate the expression of ferroportin1 mRNA in isolated splenic macrophages and bone marrow-derived macrophages (Liu *et al*, 2005b). However, the phagocytosis of erythrocytes opsonized with Ig, as well as aged erythrocytes transiently increase the expression of ferroportin1 mRNA (Liu *et al*, 2005b; Delaby *et al*, 2005b) in

macrophages. It has also been shown that overloading J774A.1 macrophages with iron also increased ferroportin1 mRNA expression, while iron chelation decreased mRNA expression (Delaby *et al*, 2005b).

Ferroportin1 mRNA contains a 5' iron response element (IRE) in its mRNA (Abboud *et al*, 2000). Under low iron levels in macrophages, iron regulatory proteins (IRPs) bind to the 5' IRE, blocking translation of ferroportin1 mRNA (Krause *et al*, 2000; Park *et al*, 2001). When the iron levels are high, IRP activity is inhibited and translation of mRNA is increased. *In vivo*, the expression of ferroportin1 in the cell membrane of enterocytes and macrophages is also regulated by hepcidin (Krause *et al*, 2000; Park *et al*, 2001).

1.6.4. Hepcidin

Recent studies have shown the hepcidin acts as a negative regulator of iron absorption by the duodenum (Laftah *et al*, 2004; Yamaji *et al*, 2004) and inhibits the release of iron by macrophages (Knutson *et al*, 2005). Hepcidin acts by binding to ferroportin1, triggering the internalization and degradation of ferroportin1. This results in decreased iron export by macrophages and enterocytes (Nemeth *et al*, 2004). Hepcidin is a peptide which was originally isolated from human urine (Park *et al*, 2001) as well as plasma (Krause *et al*, 2000). While the liver is the main source of hepcidin (Park *et al*, 2001; Krause *et al*, 2000; Pigeon *et al*, 2001), recent studies have indicated that hepcidin

is also synthesized in bacteria-infected neutrophils (Peyssonaux *et al*, 2006) and macrophages (Peyssonaux *et al*, 2006; Sow and Florence, 2007).

Hepcidin has been shown to possess antimicrobial activity against gram-negative and gram positive bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis*, as well as antifungal activity against *Candida albicans*, *Aspergillus niger* and *Aspergillus fumigatus* (Park *et al*, 2001; Krause *et al*, 2000).

CHAPTER 2

THE IRON EXPORT PROTEIN FERROPORTIN1 IS DIFFERENTIALLY EXPRESSED IN MOUSE MACROPHAGE POPULATIONS AND IS PRESENT IN THE MYCOBACTERIA-CONTAINING PHAGOSOME

2.1. Introduction

Iron is an essential nutrient, but in excess can result in tissue damage due to increased oxidative stress (Ratledge, 2004; Chung *et al*, 2004), while lack of iron results in anemia. Iron homeostasis is maintained by the intestinal uptake of iron from the diet and by recycling of iron from senescent erythrocytes. The reticuloendothelial system (RES) recycles 25 mg of iron each day from approximately 360 billion senescent erythrocytes (Bratosin *et al*, 1997; Bothwell,1995). Transport of iron is mediated by transport proteins expressed in the duodenum and reticuloendothelial macrophages. Ferroportin1 (also known as IREG1 [iron-regulated transporter 1], MTP1 [metal transporter protein 1], SLC40a1) is the sole iron export protein identified in mammals and is responsible for iron export from both enterocytes of the duodenum and macrophages (McKie *et al*, 2000; Donovan *et al*, 2000; Abboud *et al*, 2000).
In the dudonenal enterocytes, ferroportin1 is expressed on the basal membrane and exports iron into the portal blood circulation. In macrophages, ferroportin1 is expressed in intracellular vesicles. Upon phagocytosis of senescent erythrocytes, ferroportin1

localizes to the cell membrane of macrophages where it transports iron out of the macrophages (Chung *et al*, 2004; Delaby *et al*, 2005; Knutson *et al*, 2003; Knutson *et al*, 2005).

During infection, a competition for iron occurs between the macrophage of the infected host and the pathogen. Invading pathogens secrete siderophores or utilize host-derived iron storage/transport proteins in order to capture iron from the host (Weinberg, 1998). The host macrophage attempts to suppress pathogen proliferation by complex iron-withholding mechanisms. One such mechanism involves changes in the levels of iron transport proteins. Our laboratory has shown that infection of macrophages with the intracellular pathogen *M. avium* results in an increase in expression of the mRNA of the iron transport proteins Nramp1 and Nramp2, and a decrease in the expression of transferrin receptor mRNA (Zhong *et al*, 2001).

Regulation of ferroportin1 expression in macrophages by infection and inflammatory stimuli has only recently begun to be characterized. Inflammatory stimuli appear to have a negative regulatory effect on ferroportin1 expression. *In vivo*, inflammation induced by LPS in C57BL/6J mice was shown to inhibit expression of ferroportin1 protein in macrophages of the spleen, liver, and bone marrow (Yang *et al*, 2002).

In isolated mouse splenic macrophages and bone marrow derived macrophages, LPS stimulation also decreased ferroportin1 mRNA (Liu *et al*, 2005). Down-regulation of ferroportin1 mRNA expression has also been reported in the human macrophage-like cell lines THP-1 and U937 stimulated with IFN-γ and LPS. (Ludwiczek *et al*, 2003). Ferroportin1 expression is also positively regulated by macrophage iron levels. In bone marrow derived macrophages (BMDM) and the mouse J774A.1 macrophage-like cell line, it has been shown that iron loading and phagocytosis of senescent erythrocytes increases the expression of ferroportin1 mRNA and protein. (Delaby *et al*, 2005; Knoutson *et al*, 2003).

The purpose of the present study was to determine the effect of an intracellular bacterium on ferroportin1 mRNA and protein expression. We show that ferroportin1 mRNA expression is both positively and negatively regulated in different macrophage populations following infection with *M. avium* and *M. tuberculosis* and stimulation with IFN-γ. Further, we localized ferroportin1 to the phagosome in *M. tuberculosis* infected RAW264.7 macrophages. These studies indicate that ferroportin1 expression is regulated by pathogenic mycobacteria and the key cytokine IFN-γ and likely plays a role in regulating iron availability in the mycobacterial phagosome.

2.2. Materials and Methods

2.2.1. Mycobacteria

Mycobacterium avium strain Mac 101 (ATCC 70998) was obtained from the American Type Culture Collection (Fredrick, MD). Mycobacteria were cultured in Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (ODACC) (Difco, Detroit, MI) at 37°C until mid-log phase. Bacteria were aliquoted in 1 ml amounts at 2 X 10^6 CFU/ml and stored frozen in 10% glycerol at -80°C until used. The number of bacteria was confirmed by plate counting on 7H11 agar plates. Gamma-irradiated *Mycobacterium tuberculosis*, H37Rv (Colorado State University; National Institutes of Health Contract NIAID-N01-AI-40091) was re-suspended in PBS, briefly sonicated, and centrifuged at 800 rpm for 10 minutes to eliminate bacterial clumping. The protein concentration of the supernatant was determined via the Bradford protein assay (Biorad, Hercules, CA). Live *M. tuberculosis* H37Rv (ATCC 27294) was grown for 9–11 days on Middlebrook 7H11 agar. Immediately prior to the infection of macrophages with *Mycobacterium tuberculosis*, bacteria were scraped from the plates into a 2.0 ml polypropylene tube containing two 3 mm glass beads and 1 ml of DMEM medium. In order to reduce clumping, the samples were pulse-vortexed six times (~1 s per pulse) and the resulting suspensions were allowed to settle for 30 min as described previously (Schlesinger *et al*, 1990).

This bacterial suspension contained $1x10^8$ bacteria /ml with minimal clumping (<10%). The number of bacteria present in the suspension was confirmed by counting in a Petroff–Hauser chamber.

2.2.2. Mice

Male C57/BL6 mice 4-6 weeks of age were purchased from Charles Rive Laboratories (Wilmington, MA). They were allowed free access to water and fed a standard diet. The mice were treated according to ILACUC guidelines regarding the use of animals for research at The Ohio State University.

2.2.3. Cell Culture

The RAW 264.7 mouse macrophage cell line (ATCC TIB-71) and the AMJ2-C8 mouse alveolar cell line (ATCC CRL-2455) were plated at $5x10^6$ cells per well in six-well culture plates containing DMEM media supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and penicillin-streptomycin. The macrophages were allowed to adhere for 4 hours at 37°C in 5% CO_2 incubator. The nonadherent cells were then washed away with DMEM media without antibiotics and the macrophages monolayers were treated with *Mycobacterium tuberculosis* as indicated in each experiment. At the same time, the macrophage monolayers were treated with either 200 U/ml mouse IFN-γ or *M. tuberculosis* and 200 U/ml mouse IFN-γ.

2.2.4. Bone Marrow Macrophage Isolation

Bone marrow cells were isolated from the marrow of the femurs and tibias of C57/BL6 mice. The cells were plated in complete DMEM supplemented with 10 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ). On the third day of culture, 50% of the medium was removed and replaced with fresh media supplemented with GM-CSF. On the fifth day of culture, 75% of the media was removed and replaced with fresh media supplemented with GM-CSF. Mature adherent bone marrow derived macrophages were obtained after 7 days of culture. The macrophages were then plated in 6-well culture plates at a concentration of 4×10^6 macrophages per well. After adhering overnight, the macrophages were stimulated for 8 hours with 100 μg/ml of gamma-irradiated *M. tuberculosis*, 200 Units/ml of IFN-γ, or a combination of IFN-γ and gamma-irradiated *M. tuberculosis*.

2.2.5. Lung Macrophage Isolation

Mice were anesthetized with CO_2 and the lungs were perfused via the heart with 0.02% EDTA in PBS. The lungs were removed and sliced into 1-2 mm pieces and then incubated in RPMI with 10% FBS, penicillin-streptomycin, 10mM HEPES, collagenase (0.70 mg/ml), and DNAse (50 U/ml) for 1.5 hours at 37°C. A single cell suspension was obtained by pipetting vigorously and washed twice with HBBS supplemented with 2% FBS. The cells were then resuspended in RPMI with 10% FBS,

glutamine, penicillin-streptomycin, 1mM pyruvate and 10mM HEPES. The macrophages were isolated by adherence to plastic by culturing the cells for 1.5 hours at 37° in 100 mm petri dishes at a concentration of 20 million cells/ petri dish. The non-adherent cells were removed by washing 3 times in warm HBBS containing 2% FBS. The adherent cells were then removed by gently scrapping. The cells were then counted and plated at 4 x 10^6 macrophages per well in 6 well culture plates. Following a 24 hour incubation period, the media was replaced and the cells stimulated as indicated.

2.2.6. RNA Isolation

RAW264.7 monolayers were lysed using Qiagen lysis buffer containing 2-mercaptoethanol and homogenized by passing the cell lysates through QiaShredders (Qiagen,Valencia, CA). The RNA was then isolated using the RNeasy Mini Kit (Qiagen). RNA was isolated from bone marrow derived macrophages and lung macrophages using High Pure RNA Isolation Kit (Roche, Indianapolis, IN). In both procedures, residual DNA was removed during RNA purification by on-column DNAse digestion using RNase-Free DNase.

2.2.7. Quantitative RT-PCR

One μg total RNA was reversed transcribed using 100 μM dNTPs, 15 units AMV Reverse Transcriptase and 0.5 μg Oligo $(dT)_{15}$ primer in reverse transcription buffer for 1

hr at 42°C (Promega, Madison, WI). The expression of mouse GAPDH, and ferroportin1 mRNA were analyzed by real-time RT-PCR using SYBR green polymerase chain reaction master mix (Roche). Reactions were run on the Roche LightCycler 2. The following sense and antisense sequences were employed: mouse GAPDH, sense (5'-3'), GTGTGAACGGA-TTTGGCCGTATTGGGCG, antisense (5'-3') TCGCTCCTGGAAGATGGTGATGGGC; mouse ferroportin1, sense (5'-3') TGGATGGGTCCTTACTGTCTGCTAC, antisense (5'-3') TGCTAATCTGCTCCTGTTTTCTCC. Primers used for GAPDH and Ferroportin1 were designed using the MacVector primer software (Accelrys, San Diego, CA). The amplification conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 5 seconds and 72°C for 20 seconds. The relative expression of each sample was calculated using mouse GAPDH as a reference and the delta Ct method as previously described (Livak *et al*, 2001).

2.2.8. Immunofluorescence

RAW264.7 cells were plated on coverslips in 24 well plates at a concentration of 2.5 x 10^5 cells/well. The cells were then treated with live *M. tuberculosis* and stimulated with IFN-γ for 8 hours. Cells were fixed with 4% paraformaldeyde for 20 minutes and permeabilized with 0.10% Triton X-100 for 10 minutes at room temperature. The cells were washed twice PBS and blocked for three hours at room temperature using a

blocking solution containing 1% BSA and 10% heat inactivated goat serum in PBS. Rabbit anti-mouse ferroportin1 antibody (Alpha Diagnostic) was absorbed twice with gamma-irradiated *M. tuberculosis* overnight at 4°C to remove *M. tuberculosis* reactive antibodies. Removal of *M. tuberculosis* antibodies was confirmed by Western blot of SDS lysates of *M. tuberculosis* and by immuofluoresence with *M. tuberculosis* adhered to coverslips. The absorbed antibody was then added to each well at a final concentration of 1:500 overnight. After washing, the secondary antibody, Alexa 488-coupled $(Fab')_2$ goat anti rabbit IgG antibody (Invitrogen, Carlsbad, CA), was added at room temperature for 1 hour. In order to detect *M. tuberculosis* in the infected cells, coverslips were stained with auramine-rhodamine (Difco Laboratories, Detroit, MI) and counterstained with 5% potassium permanganate as previously described (Feruson *et al*, 2002). Expression of ferroportin1 was then detected as described above. Coverslips were removed from the 24 well plates and mounted on slides with ProLong® Gold antifade reagent (Invitrogen). Fluorescence was visualized with a Zeiss Meta 510 confocal microscope. Green fluorescence intensity was analyzed from images using Sigma ScanPro image analysis software (SPSS Science, Chicago, IL). For the latex bead experiments, green fluorescent beads, 2.0 μm Fluoresbrite® YG Microspheres (PolySciences, Warrington, PA) were first diluted 1:100 in PBS and washed twice in complete DMEM media. RAW264.7 cells plated on coverslips in 24 well culture plates were treated with latex beads at 100:1 beads/macrophage for the indicated amounts of time. Coverslips were fixed with

paraformaldeyde, premeablized with Triton X-100, and blocked as described above. The presence of ferroportin 1 was determined using rabbit anti-FPN1 antibody (Alpha Diagnostic) and Alexa 594-coupled $(Fab')_2$ goat anti rabbit IgG secondary antibody (Invitrogen). The location of LAMP1 was determined using a rat monclonal anti-LAMP1 antibody (Santa Cruz biotechnology, Santa Cruz, CA) followed by a Alexa 594-coupled $(Fab')_2$ goat anti rat IgG secondary antibody (Invitrogen).

The coverslips were mounted on glass slides and fluorescence visualized by confocal microscopy as described above.

2.2.9. Statistics

Results were analyzed by one-way analysis of variance (ANOVA) with Turkey's test using SigmaSTAT (SPSS Science, Chicago, IL).

2.3. Results

2.3.1. Infection of RAW264.7 cells with mycobacteria and stimulation with IFN-γ synergistically induces the expression of ferroportin1 mRNA.

We first examined the expression of ferroportin1 mRNA in the mouse RAW264.7 macrophage cell line by real-time RT-PCR. The basal expression of ferroportin1 was low in these cells. The results in Fig.1A show that ferroportin1 mRNA expression is induced

in RAW264.7 cells infected with *M. avium* and stimulated with IFN-γ. IFN-γ was chosen based on its importance in activating macrophages. Interferon-gamma treatment of macrophages has been shown to increase the expression of MHCII as well as enhance the ability of macrophages to control the growth of *M. tuberculosis* via the induction of nitric oxide synthase (iNOS or NOS2), which is responsible for the production of nitroc oxide (NO) (Flesch *et al*, 1991; Chan *et al*, 1995; McMicking *et al,* 1997; Flynn *et al*, 1998). A 12-fold increase in the expression of ferroportin1 mRNA was observed after 12 hours of stimulation. Treatment with *M. avium* alone resulted in a 4-fold increase in expression and stimulation with IFN-γ alone had no effect on ferroportin1 mRNA expression. *M. avium* infection and stimulation with IFN-γ also increased ferroportin1 mRNA expression in AMJ2-C8, a mouse alveolar macrophage cell line (Fig. 1B).

Stimulation of RAW264.7 cells with gamma-irradiated *M. tuberculosis* and IFN-γ also synergistically increased ferroportin1 mRNA expression (Fig. 1C). Expression peaked at 8 hours of stimulation with a 30-fold increase and returned to baseline by 24 hours. Treatment with gamma-irradiated *M. tuberculosis* alone resulted in only a 5-fold increase in ferroportin1 mRNA levels. Similarly, infection with live *M. tuberculosis* and stimulation with IFN-γ resulted in a 15-fold induction following 8 hours of stimulation, and only a 5-fold increase when treated with live *M. tuberculosis* alone (Fig. 1D). Thus, these results show that both live and gamma-irradiated *M. tuberculosis* induces a similar level of ferroportin1 mRNA. To determine if ferroportin1 mRNA is induced in a dose-

dependent manner, RAW264.7 cells were treated with varying doses of gamma-irradiated and live *M. tuberculosis* and stimulated with IFN-γ. Results in figure 2A show that ferroportin1 mRNA expression increased with increasing doses of irradiated *M. tuberculosis*. Maximal induction of ferroportin1 mRNA expression was obtained at a concentration of 100 μg protein/ml of gamma-irradiated *M. tuberculosis*. Ferroportin1 mRNA expression also increased with increasing doses of live *M. tuberculosis* (Fig. 2B). Peak levels of ferroportin1 mRNA were obtained with a multiplicity of infection (MOI) of 20 bacteria/macrophage. Results in figure 2C show that the effect of IFN-γ on ferroportin1 expression in *M. tuberculosis*-infected macrophages is also dose-dependent.

2.3.2. Ferroportin1 mRNA is increased in resident peritoneal macrophages by M. tuberculosis and IFN-γ, but not in bone marrow derived macrophages or lung macrophages.

We examined the mRNA levels of ferroportin1 in three different macrophage sub-populations from C57BL/6J mice. Constitutive expression of ferroportin1 mRNA in unstimulated resident peritoneal macrophages was low. Treatment with gamma-irradiated *M. tuberculosis* and stimulation with IFN-γ resulted in a 5-fold increase in ferroportin1 mRNA compared to a 2-fold increase with gamma-irradiated *M. tuberculosis* alone (Fig. 3A). In contrast, expression of ferroportin1 mRNA was constitutively high in bone marrow derived macrophages and lung macrophages. Expression of ferroportin1 mRNA

was inhibited following treatment with gamma-irradiated *M. tuberculosis* or IFN-γ (Fig 3B). Expression was also inhibited by the TLR2 ligand PAM_3CSK_4 and the TLR4 ligand LPS consistent with previous reports in the literature (Yang *et al*, 2002; Liu *et al*, 2005). When the cells were treated with the combination of IFN-γ and *M. tuberculosis* or the TLR ligands, IFN-γ did not change the down-regulation of ferroportin1 expression (Fig 3B). Stimulation of lung macrophages with gamma-irradiated *M. tuberculosis* or LPS also resulted in a down-regulation of ferroportin1 mRNA expression (Figure 3C). Again, the addition of IFN-γ did not alter the down-regulation by *M. tuberculosis* or LPS.

2.3.3. Ferroportin1 localizes to the mycobacteria-containing phagosomes.

Previous studies (Delaby *et al*, 2005; Knutson *et al*, 2005) have shown that ferroportin1 in macrophages is present in intracellular vesicles and upon phagocytosis of senescent erythrocytes traffics to the plasma membrane. Since trafficking of ferroportin1 during phagocytosis of a pathogen has not been studied, we examined ferroportin1 expression by confocal microscopy in RAW264.7 cells infected with live *M. tuberculosis* and stimulated with IFN-γ. We observed a punctuate pattern of fluorescence in unstimulated RAW264.7 cells, consistent with localization of ferroportin1 within intracellular vesicles (Fig 4A). There was little localization to the plasma membrane. When the RAW264.7 cells were treated with IFN-γ, the intensity of ferroportin1 immunfluoresence was comparable to control unstimulated RAW264.7 cells (Fig. 4B).

Pretreatment of the rabbit anti-ferroportin1 antibody with a blocking peptide abolished activity (no fluorescence), indicating that the immunofluoresence was specific (Fig. A and B). Also, controls without primary antibody showed no fluorescence (not shown).

In RAW264.7 cells infected with *M. tuberculosis,* ferroportin1 was highly localized to the *M. tuberculosis* phagosome. There was no expression of ferroportin1 in the plasma membrane (Fig 4C). Ferroportin1 also localized to the *M. tuberculosis* phagosome in RAW264.7 cells infected with *M. tuberculosis* and stimulated with IFN-γ (Fig 4D). Again, pretreatment of the rabbit anti-ferroportin1 antibody with a blocking peptide abolished activity (no fluorescence), indicating that the immunofluoresence was specific (Fig. 4E). Quantitative analysis showed that the fluorescence intensity of ferroportin1 localized to the *M. tuberculosis* phagosome was significantly greater in RAW264.7 cells that were infected with *M. tuberculosis* and stimulated with IFN-γ compared to cells that were only infected with *M. tuberculosis*. The fluorescence intensity of phagosomes stimulated with *M. tuberculosis* and IFN-γ was $1.23 \pm 0.13 \times 10^5$ green fluorescence intensity units/phagosome compared to $0.742 \pm 0.059 \times 10^5$ for phagosomes in macrophages infected with *M. tuberculosis* alone ($p<0.005$). However, the magnitude of the increase (1.7X) was much less than we observed in ferroportin1 mRNA expression. These results suggest that ferroportin1 localizes to the phagosome following phagocytosis of *M. tuberculosis*.

In order to examine the trafficking of ferroportin1 to the mycobacteria-containing phagosomes, we next examined ferroportin1 expression in a time course experiment following infection of RAW264.7 cells with *M. tuberculosis* (Fig. 5). Ferroportin1 appears to rapidly localize to the *M. tuberculosis*-containing phagosomes with partial co-localization by 30 minutes of infection and stimulation with IFN-γ (Fig. 5A). Ferroportin1 is almost completely associated with the *M. tuberculosis*-containing phagosomes by 1 hour (Fig. 5B) and completely associated following two hours of infection (Fig. 5C).

We next determined if ferroportin1 also localizes with latex beads within phagolysosomes. RAW264.7 cells were incubated with green fluorescence latex beads and the localization of ferroportin1 and the late endosomal lysosomal marker LAMP1 was determined by confocal microscopy (Fig. 6A and B). There was no localization of ferroportin1 to the latex bead phagosome, whereas, LAMP1 localized to the phagosome as expected (Fig. 6C).

2.4. Discussion

Previous studies have shown that ferroportin1 expression in mouse macrophages is negatively regulated by LPS and the gram-negative bacteria *Pseudomonas aerginosa* (Peyssonnaux *et al*, 2006). In this report, we demonstrate that ferroportin1 mRNA expression is differentially expressed in macrophage subpopulations and macrophage cell

lines following infection with mycobacteria and stimulation with IFN-γ. Two patterns of expression were observed. In bone marrow derived macrophages and lung macrophages, ferroportin1 mRNA expression was down-regulated in response to infection. In resident peritoneal macrophages and the macrophage cell lines, RAW264.7 and AMJ2-C8, infection with mycobacteria and stimulation with IFN-γ synergistically increased ferroportin1 mRNA expression. Thus, in these cells ferroportin1 expression is increased by the classical two signal pattern of macrophage activation, one signal being initiated by the interaction of the mycobacteria with the macrophage and the second signal being initiated by IFN-γ. One possible explanation for the different results is that the different macrophage populations are engaging different Toll-like receptors. This does not appear likely, since both a TLR2 ligand (Pam_3CSK_4) and a TLR4 ligand (LPS) also inhibited ferroportin1 expression in the bone marrow derived macrophages. This differential expression may relate to the basal level of ferroportin1 mRNA in unstimulated macrophages. Resident peritoneal macrophages, RAW264.7 cells, and AMJ2-C8 cells have low levels of constitutive mRNA expression of ferroportin1. Bone marrow derived macrophages and lung macrophages have high constitutive mRNA expression. Another possible explanation for differential expression of ferroportin1 mRNA in macrophage populations results from differences in transcriptional activation of the ferroportin1 gene in both unstimulated and activated macrophages. Although the 5' upstream region of the mouse ferroportin1 gene has been shown to have promoter activity (Liu *et al*, 2002), the

signaling pathways and transcription factors involved in regulating expression are unknown.

In this study, we also examined ferroportin1 protein expression in RAW264.7 cells by confocal microscopy. In unstimulated cells a constitutive level of ferroportin1 expression was observed that localized to intracellular vesicles. In cells infected with *M. tuberculosis* and stimulated with IFN-γ, a small (1.7X) increase in ferroportin1 immunofluorescence in the phagosome was observed when compared to cells that were only infected with *M. tuberculosis.* This increase in protein is substantially lower than the increase in mRNA expression. A possible explanation for this difference is that ferroportin1 is also post-transcriptionally regulated in these cells. Previous studies have shown that ferroportin1 protein expression is regulated post-transcriptionally by intracellular iron levels. The mRNA of ferroportin1 contains an iron responsive element (IRE) in the 5' untranslated region (UTR) that has been shown to confer translational regulation by iron through the iron regulatory proteins (IRPs) in a manner similar to ferritin and other 5'UTR-IRE-regulated genes (McKie *et al*, 2000; Abboud *et al*, 2000; Liu *et al*, 2002). These studies have shown that the ferroportin1 IRE is functional. IFN-γ has been shown to decrease macrophage iron levels by decreasing expression of the transferrin receptor and ferritin synthesis, resulting in a decrease in total macrophage iron levels (Taetle and Honeysett, 1998; Byrd and Horwitz, 1993; Mulero and Block, 1999; Zhong *et al*, 2001). Thus, the difference in ferroportin1 mRNA and protein expression

in the RAW264.7 cells following infection and stimulation with IFN-γ is most likely due to the activity of the IRE in the 5'UTR of the ferroportin1 mRNA, which under low iron represses translation of the protein.

Previous studies have shown that upon phagocytosis of senescent erythrocytes, ferroportin1 localizes to the cell membrane (Chung *et al*, 2004; Delaby *et al*, 2005; Knutson *et al*, 2003; Knutson *et al*, 2005). Here, we show that ferroportin1 localizes to the phagosome after phagocytosis of *M. tuberculosis* and is not expressed on the cell membrane. The localization of ferroportin1 to the phagosome was rapid. The lack of trafficking of ferroportin1 to the cell membrane is possibly due to the low levels of iron in these cells. Previous studies (Delaby *et al*, 2005; Knutson *et al*, 2003) have shown that iron loading of macrophages induces trafficking of ferroportin1 to the plasma membrane, which suggests that the trafficking of ferroportin1 to the plasma membrane is regulated by a signal pathway that is activated by intracellular iron levels. Thus, we speculate that since following phagocytosis of *M. tuberculosis* and stimulation with IFN-γ intracellular iron levels decrease, there is no trafficking of ferroportin1 to the plasma membrane. Trafficking of ferroportin1 to the *M. tuberculosis* phagosome is also most likely regulated by signaling pathways induced by the interaction of *M. tuberculosis* with the macrophage, since ferroportin1 localized to the phagosome following phagocytosis of *M. tuberculosis* but not to the phagosome following phagocytosis of latex beads.

The iron transporter, Nramp1 (SLC11a1), which is also expressed in macrophages, is present in late endosomal vesicles (Gruenheid *et al*, 1997; Searle *et al*, 1998) that fuse with the phagosome. The Nramp1 gene in mice has two alleles that determine resistance to a range of intracellular pathogens, a wild-type resistant Nramp1^{Gly169} allele, and a mutant susceptible Nramp1^{Asp169} allele (Vidal *et al*, 1993, Vidal *et al*, 1995). Previous studies (Searle *et al*, 1998, Kuhn *et al*, 2001) have shown Nramp1 to localize to phagosomes containing *M. avium* in macrophages that express the wild-type Nramp1^{Gly169} allele, whereas Nramp1 is retained within the endoplasmic reticulum in macrophages that express the mutant Nramp1^{Asp169} allele (Vidal *et al*, 1996, White *et al*, 2004). In addition, we have found that Nramp1 localizes to the *M. tuberculosis* phagosome within 2 hours after infection of RAW264.7 cells stably transfected with wild-type Nramp1 (unpublished observations). In the current study, we found that ferroportin1 also localizes to the *M. tuberculosis* phagosome within two hours after infection. The presence of both iron transport proteins in the mycobacteria containing phagosome raises the question of what their functions are as iron transporters during infection. Our lab was the first to show that Nramp1 transports iron into phagosomes and mediates resistance to pathogens by catalyzing the production of hydroxyl radicals by the Fenton reaction (Kuhn *et al*, 1999; Kuhn *et al*, 2001; Zwilling *et al*, 1999). However, others (Jabado *et al*, 2000) have suggested that Nramp1 transports iron out of the phagosome and mediates resistance to pathogens by removing iron required for growth. Expression of Nramp1 in frog oocytes suggests that iron transport by

Nramp1 is actually bi-directional with the direction determined by iron and proton gradients (Goswami *et al*, 2001). Thus, Nramp1 would be expected to transport iron out of phagosomes with high iron content, such as phagosomes containing senescent erythrocytes and transport iron into phagosomes with low iron content. Expression of ferroportin1 in oocytes results in iron export out of the oocyte, confirming that ferroportin1 is an iron export protein (McKie *et al*, 2000). Thus, the presence of ferroportin1 in the phagosomal membrane suggests that ferroportin1 is responsible for the export of iron from the phagosome into the cytosol.

We favor a model (Fig. 7) in which Nramp1 and ferroportin1 act together to regulate phagosomal iron levels. In this model, Fe^{+2} is transported into the mycobacteria-containing phagosome, where the iron catalyzes the production of highly toxic hydroxyl radicals. As result of the Fenton reaction the iron is oxidized to Fe^{+3}. *M. tuberculosis* express mycobactin, exochelins, and a siderophore that captures Fe^{+3} (DeVoss *et al*, 1999; Gobin *et al,* 1995). Thus it is critical that the oxidized iron be removed from the phagosome to prevent growth of surviving mycobacteria. We propose that ferroportin1 is the principal iron exporter from the phagosome. If the iron concentrations are high enough, Nramp1 may also be involved in iron export. Both Nramp1 and ferroportin1 transport Fe^{+2}. Thus, we would expect that the phagosomal membrane also contains a ferrireductase that is closely associated with the iron transport proteins. This ferrireductase remains to be identified, but a possible candidate is the recently

characterized ferrireductase Lcytb, which is expressed in lysosomal vesicles and is a member of the cytochrome b_{561} protein family of ascorbate-dependent ferrireductases (Su and Asard, 2006; Zhang *et al,* 2006).

In conclusion, we demonstrate that ferroportin1 is differentially expressed in macrophage populations and expression is regulated at multiple levels, including mRNA expression, protein expression, and trafficking. We also show that ferroportin1 localizes to the *M. tuberculosis* phagosome, where it may have anti-mycobacterial activity by limiting iron available to the mycobacteria. Although the anti-mycobacterial activity of ferroportin1 needs to be determined experimentally, ferroportin1 has recently been shown to have anti-bacterial activity against the intracellular growth of *Salmonella enterica* (Chlosta *et al*, 2006). Further studies to determine if ferroportin1 is expressed in macrophages during tuberculosis may lead to the development of a strategy to up-regulate the expression of ferroportin1, which may contribute to the treatment of the disease caused by *M. tuberculosis*.

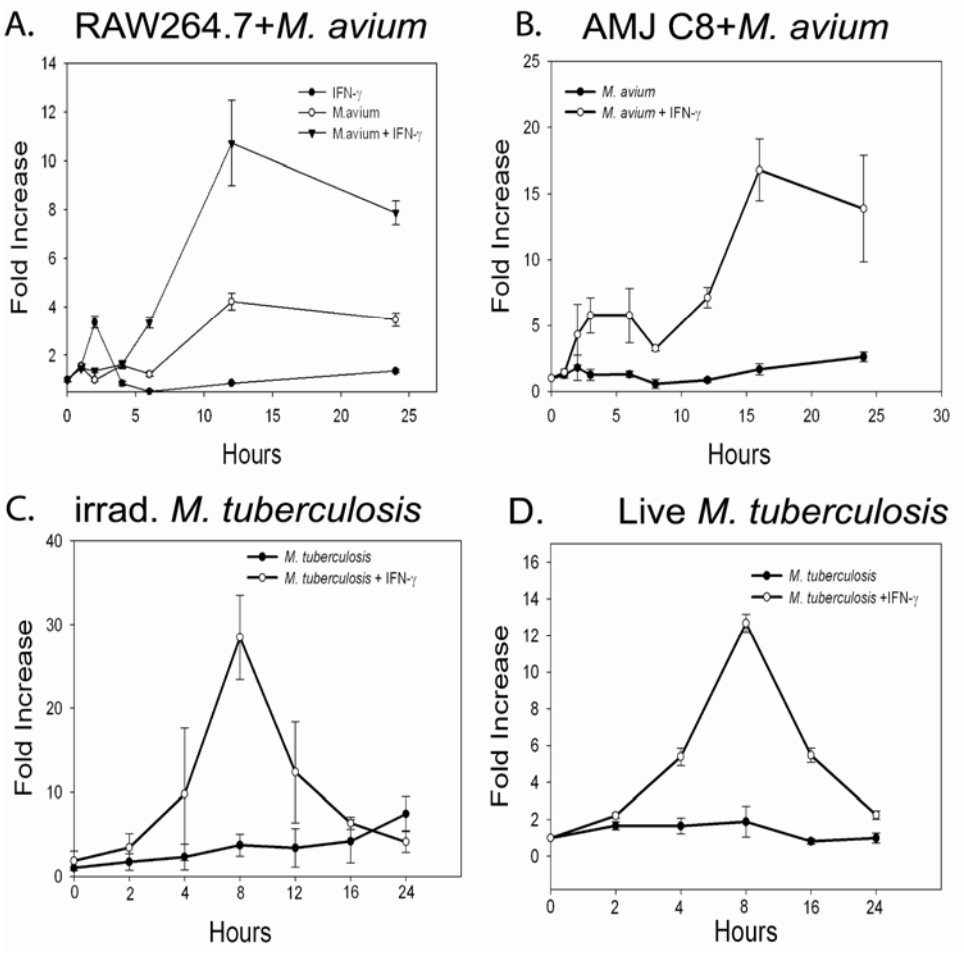

Figure 1: Effect of mycobacteria infection on ferroportin1 mRNA expression in RAW264.7 and AMJ2-C8 cells. A. RAW264.7 cells were infected with 20:1 *M. avium* and stimulated with 200 Units/ml IFN-γ. B. AMJ2-C8 cells were treated with 20:1 *M. avium* and stimulated with 200 Units/ml IFN-γ. C. RAW264.7 cells were treated with gamma-irradiated *M. tuberculosis* (100 μg protein/ml) and stimulated with IFN-γ (200 Units/ml). D. RAW264.7 cells were infected with 10:1 live *M. tuberculosis* H37Rv and stimulated with 200 Units/ml IFN-γ. RNA was isolated at each time point and mRNA expression of ferroportin1 determined by real-time RT-PCR. The results were normalized to GAPDH and expressed as the fold-increase in ferroportin1 expression compared to control unstimulated cells. The results are the mean +/- S.D. of three independent experiments.

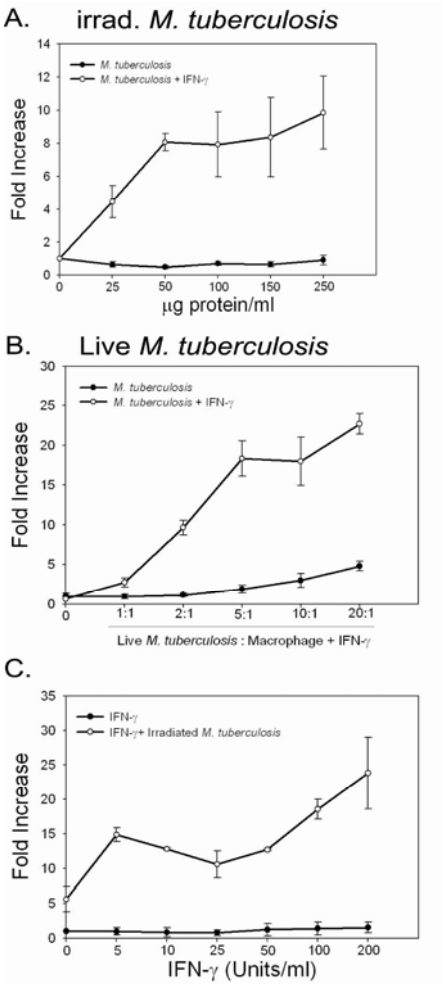

Figure 2: Effect of varying doses of IFN-γ and mycobacteria on ferroportin1 mRNA expression. A. RAW264.7 cells were treated with varying does of gamma-irradiated *M. tuberculosis* and stimulated with 200 Units/ml IFN-γ. B. RAW264.7 cells were infected with varying doses of live *M. tuberculosis* and stimulated with 200 Units/ml IFN-γ. C. RAW264.7 cells were stimulated with varying doses of IFN-γ and treated with 100 μg protein/ml gamma-irradiated *M. tuberculosis*. RNA was isolated from each treatment at 8 hours and expression of ferroportin1 mRNA determined by real-time RT-PCR. The results were normalized to GAPDH and expressed as fold-increase in ferroportin1 expression compared to control unstimulated cells. The results are the mean +/- S.D. of three independent experiments.

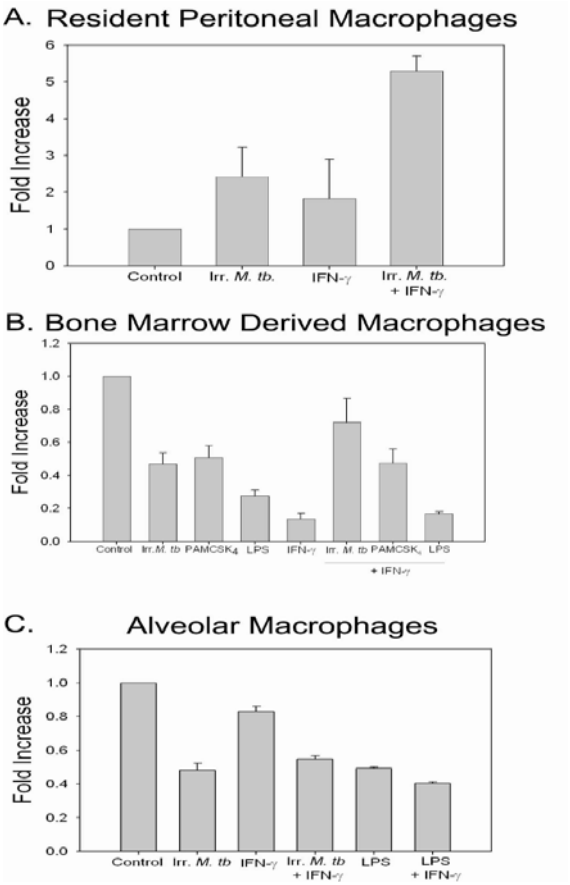

Figure 3: Expression of ferroportin1 mRNA in primary mouse macrophages. A. Resident peritoneal macrophages from C57/BL6 mice were treated with gamma-irradiated *Mycobacterium tuberculosis* (100 μg/ml) and stimulated with 200 units/ml IFN-γ. B. Bone marrow derived macrophages from C57/BL6 mice were treated with gamma-irradiated *Mycobacterium tuberculosis* (100 μg/ml), Pam_3CSK4 (500 ng/ml), LPS (100 ng/ml), and stimulated with 200 units/ml IFN-γ. C. Alveolar macrophages from C57/BL6 mice were treated with gamma-irradiated *Mycobacterium tuberculosis* (100 μg/ml), LPS (100 ng/ml) and stimulated with 200 units/ml IFN-γ. RNA was isolated from each treatment and expression of ferroportin1 mRNA determined by real-time RT-PCR. The results were normalized to GAPDH and expressed as fold-increase in ferroportin1 mRNA expression compared to control unstimulated cells. The results are the mean +/- S.D. of three independent experiments.

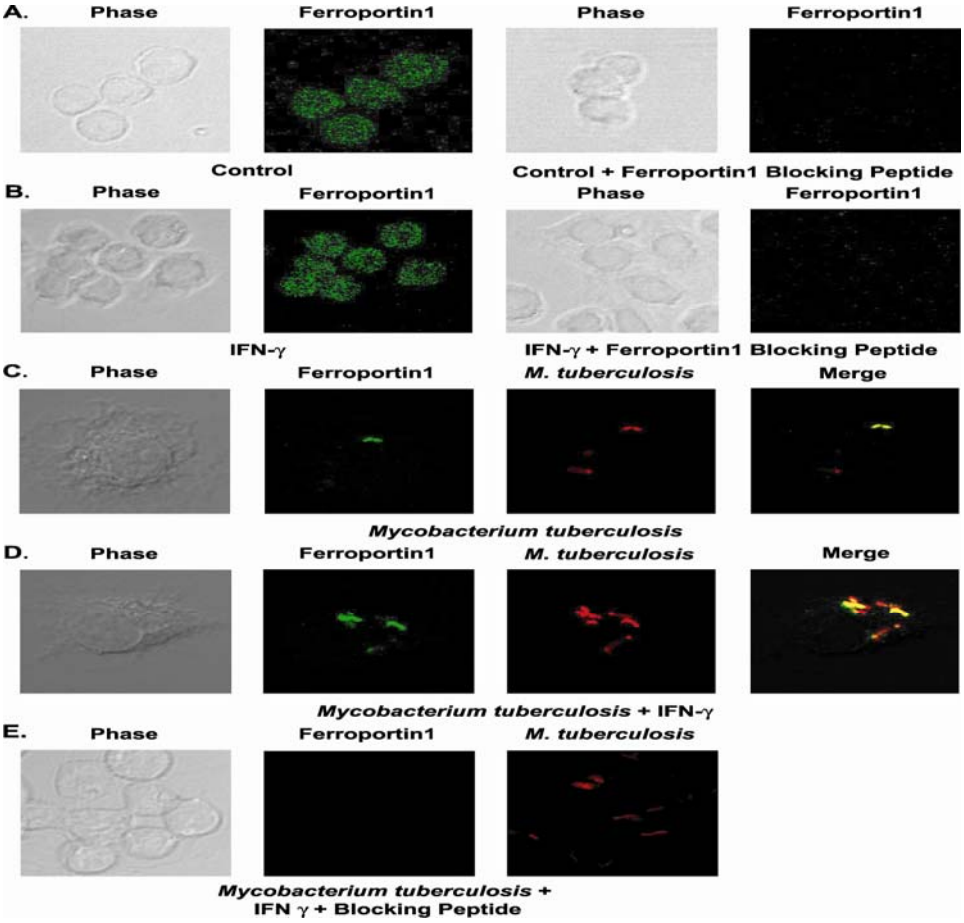

Figure 4: Localization of ferroportin1 to the mycobacteria-containing phagosomes. RAW264.7 cells were stimulated with IFN-γ (200 U/ml), live *M. tuberculosis* (5:1), and IFN-γ + live *M. tuberculosis* for 8 hours. *M. tuberculosis* was detected by staining with auramine-rhodamine. Ferroportin1 was detected by cross-sectional confocal microscopy using rabbit anti-mouse FPN1 antibody. The secondary antibody was Alexa488-coupled $(Fab')_2$ goat anti-rabbit IgG. A. Representative confocal images of unstimulated control RAW264.7 cells (A), RAW264.7 cells were stimulated with IFN-γ (B), RAW264.7 cells infected with live *M. tuberculosis* (C) and RAW264.7 cells infected with live *M. tuberculosis* and stimulated with IFN-γ (D). A and B shows RAW264.7 cells with ferroportin1 contained in small intracellular vesicles. A and B also show a control experiment in which the anti-ferroportin1 antibody was preabsorbed with the ferroportin1 control peptide. C and D shows ferroportin1 localized to the *M. tuberculosis* containing phagosomes.

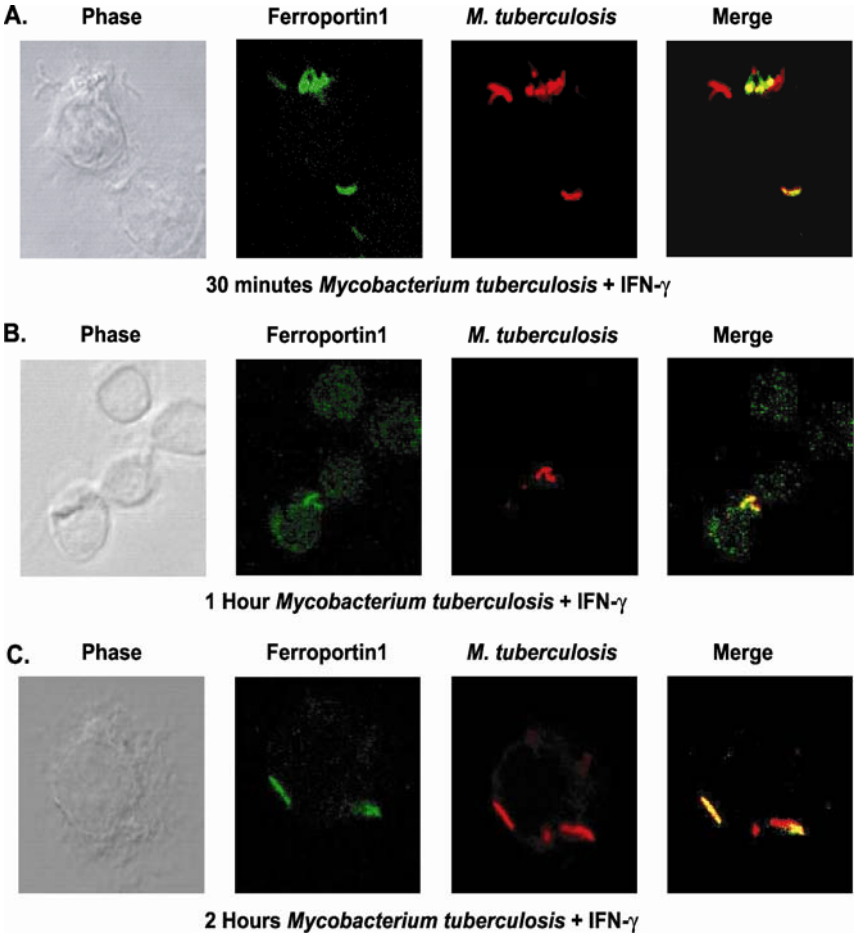

Figure 5: Trafficking of FPN1 to the mycobacteria-containing phagosomes. RAW264.7 cells were infected with live *M. tuberculosis* for 30 minutes, 1 hour, and 2 hours. Detection of *M. tuberculosis* was carried out with auramine-rhodamine and counterstained with 5% potassium permanganate. FPN1 was detected by immunofluorescence using rabbit anti-mouse FPN1 primary antibody. The secondary antibody was Alexa488-coupled $(Fab')_2$ goat anti-rabbit antibody.

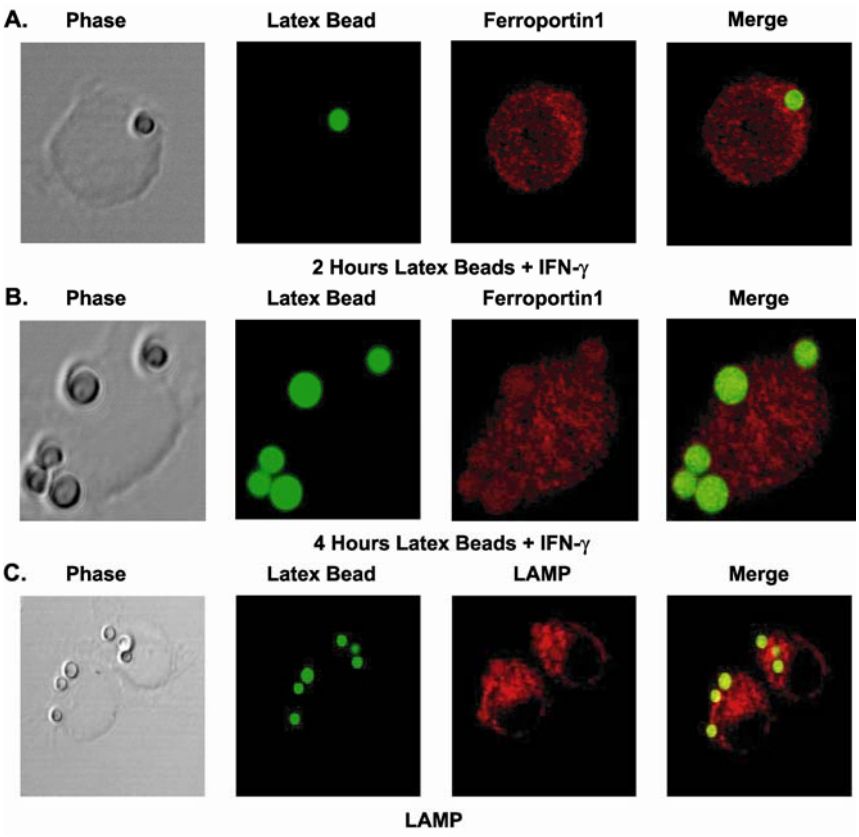

Figure 6: Effect of latex bead stimulation on the trafficking of FPN1. RAW264.7 cells were treated with latex beads (100:1) for 2, and 4 hours. The presence of FPN1 was determined using rabbit anti-FPN1 antibody and detected using 594-coupled $(Fab')_2$ goat anti-rabbit secondary antibody. Localization of LAMP was determined using rat anti-LAMP1 antibody and detected using 594-coupled $(Fab')_2$ goat anti-rat secondary antibody. This figure shows that FPN1 does not localize to the latex bead-containing phagosomes while LAMP1 does.

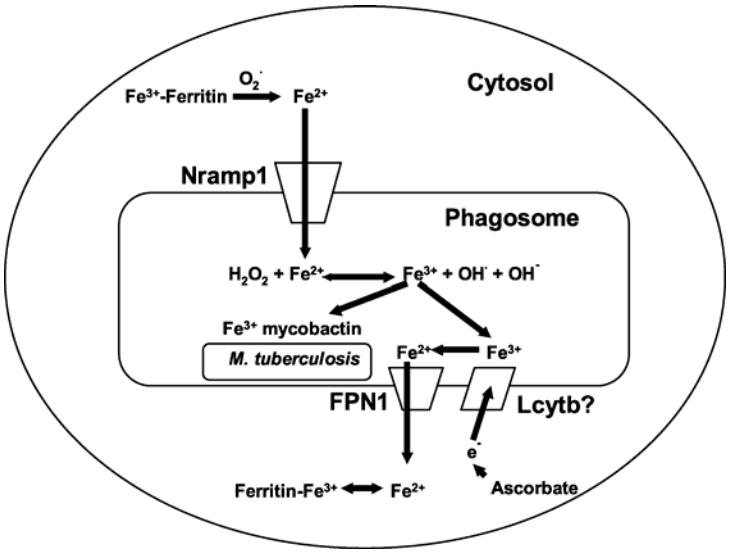

Figure 7: Proposed model for action of ferroportin1 and Nramp1 in the mycobacteria-containing phagosome. Iron (Fe^{+2}) is transported from the intracellular iron pool into the phagsome by Nramp1, possibly facilitated by the release of Fe^{+2} from ferritin through the action of reactive oxygen species. In the phagosome, iron catalyzes the production of hydroxyl radicals via the Fenton reaction, resulting in the oxidation of Fe^{+2} to Fe^{+3}. Since Fe^{+3} can be acquired by *M. tuberculosis* via the mycobacterial mycobactins, Fe^{+3} needs to be quickly recycled out of the phagosme. In this model, Fe^{+3} is reduced by a phaogsomal membrane associated ferrireductase, possibly Lcytb, and exported out of the phagosme by ferroportin1 into the cytoplasm, where it can re-associate with ferritin.

CHAPTER 3

THE REGULATION OF FERROPORTIN1 EXPRESSION IN MYCOBACTERIA-INFECTED MACROPHAGES.

3.1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, is a major cause of mortality (Dye *et al*, 1999). Tuberculosis is primarily spread by the inhalation of air-borne droplets of *Mycoabcterium tuberculosis* (Zahrt, 2003). In the host, alveolar macrophages are the first cells to encounter the organism, thus these cells are an important component of the innate immune system and its response to the invading pathogen. The ability of the host to respond to, and eradicate, infection largely depends upon the macrophages, and a macrophages ability to kill pathogens is greatly enhanced by the macrophage activating cytokine IFN-γ, which is produced by activated T cells and by Natural Killer (NK) cells. IFN-γ acts by inducing gene expression via the Jak-STAT pathway (Darnell *et al*, 1994; Ihle, 1996), leading to the translocation of STAT1 to the nucleus, where it induces the expression of IFN-γ-responsive genes (Boehm *et al*, 1997). Macrophages respond to IFN-γ stimulation by increasing the antimicrobial activity which includes the production of reactive oxygen species (ROS) and nitric oxide (NO) (Chan *et al*, 1992; McMicking *et al*, 1997a).

In addition to activation by IFN-γ, macrophages also utilize the Toll-like receptors (TLRs) for the recognition of many bacterial and viral components. The mammalian TLRs are a conserved family of membrane associated receptors which recognize highly conserved structures, which have been referred to as pathogen associated molecular patterns (PAMPs). Binding of a PAMP to the TLR results in the activation of nuclear transcription factors such as NFκB, activating-protein 1 (AP-1), and interferon regulatory factors (IRFs), which leads to the production of cytokines, chemokines, and co-stimulatory molecules by the macrophage that can, in turn, activate other cells of the immune system. Several studies have shown that mycobacteria and mycobacterial components can act as agonists for TLRs (Pai *et al*, 2004; Quesniaux and Fremond *et al*, 2004; Bafica *et al*, 2005). Specifically, TLR2, and to a lesser extent, TLR4, have been shown to preferentially bind to the lipomannan (LM) and lipoarabinomannan (LAM) in the cell wall of mycobacteria species.

During infection, the host macrophage attempts to limit the availability of iron to *Mycobacterium tuberculosis*. In turn, the bacteria attempts to survive by capturing iron from the macrophage through the secretion of siderophores or utilization of host derived proteins (Weinberg, 1998). IFN-γ has been shown to reduce the ability of macrophages to acquire iron from transferrin by down-regulating the expression of the transferrin receptors (Mulero and Brock, 1999). In addition, iron withholding mechanisms have been shown to play an important role in host defense (Weiss, 1995). Several reports have

shown that the acquisition of iron from the host is essential for the growth and survival of mycobacteria *in vivo* (de Voss *et al*, 2000; Gold *et al*, 2001; Hobson *et al*, 2002). It has also been shown that the ability of *M. tuberculosis* to acquire iron worsens the outcome of infection (Lounis *et al*, 2001). Recently, an iron-regulated iron export protein, ferroportin1 (SLC40a1) has been discovered (Donavan *et al*, 2000; Abboud *et al*, 2000; McKie *et al*, 2000). This 62kDa integral membrane protein consists of 9 or 12 membrane spanning domains and is the only Fe^{2+} export protein which has been discovered in mammals to date. Mutations in ferroportin1 in humans has been shown to result in haemochromatosis type 4, an iron overload disease with accumulation of iron in macrophages due to impaired iron export (Pietrangelo, 2004; McGregor *et al*, 2005; Liu *et al*, 2005a; De Domenico *et al*, 2005). Ferroportin1 mRNA is constitutively expressed by macrophages, but the level is higher in mouse bone marrow-derived macrophages (BMDMs) as compared to the mouse macrophage cell lines RAW264.7 and J774A.1 (Canonne-Hergaux *et al*, 2006). *In vivo*, the expression of ferroportin1 mRNA and protein has been shown to be down-regulated in the spleen, liver, and bone marrow by inflammation induced by LPS (Yang *et al*, 2002). In addition, LPS has been shown to down-regulate the expression of ferroportin1 mRNA in isolated splenic macrophages and bone marrow-derived macrophages (Liu *et al*, 2005b). However, the phagocytosis of erythrocytes opsonized with Ig, as well as aged erythrocytes transiently increase the expression of ferroportin1 mRNA (Liu *et al*, 2005b; Delaby *et al*, 2005b) in macrophages. It has also been shown that overloading J774A.1 macrophages with iron

also increased ferroportin1 mRNA expression, while iron chelation decreased mRNA expression (Delaby *et al*, 2005b).

We have shown in previous studies that ferroportin1 mRNA is synergistically induced in macrophages by IFN-γ and *M. tuberculosis* (Van Zandt, K.E. *et al*, 2007). Furthermore, we have shown that the ferroportin1 protein localizes to *Mycobacterium tuberculosis*-containing phagosomes (Van Zandt, K.E., *et al*, 2007). In the present study, we examined the regulation of ferroportin1 mRNA in macrophages infected with *M. tuberculosis* and stimulated with IFN-γ. We show that the induction of ferroportin1 in activated macrophages is dependent upon iron levels, TLR2, MyD88, and STAT1. Further, we show that the synergistic effect of *M. tuberculosis* and IFN-γ occurs at the transcriptional level, and that the transcription factors PU.1, STAT1, and NFκB play an important role in the transcription of the ferroportin1 gene.

3.2. Materials and Methods

3.2.1. Mycobacteria

Gamma-irradiated *Mycobacterium tuberculosis*, H37RV (Colorado State University; National Institutes of Health Contract NIAID-N01-AI-40091) was re-suspended in PBS, briefly sonicated, and centrifuged at 800 rpm for 10 minutes to

eliminate bacterial clumping. The protein concentration of the supernatant was determined via the Bradford protein assay (Biorad, Hercules, CA).

3.2.2. Reagents

FBS was purchased from Atlanta Biologicals (Lawrenceville, GA). Mouse IFN-γ was obtained from Roche (Indianapolis, IN). DNA polymerase (Klenow fragment), Dulbecco's MEM (DMEM), sodium pyruvate, penicillin-streptomycin, and hygromycin B were obtained from Invitrogen (Carlsbad, CA). [^{32}P]dCTP (300 Ci/mmol) was obtained from Amersham (Piscataway, NJ).

3.2.3. RAW264.7 Cell Lines

The RAW264.7 mouse macrophage cell line (ATCC TIB-71) was cultured in DMEM supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 U/ml Penicillin, 100 μg/ml streptomycin. The RAW264.7 cell line expressing Flag-tagged dominant negative (DN) TLR2, which lacks 14 amino acids in the cytoplasmic domain (Curry, Alvarez *et al*, 2004), and the RAW264.7 cell line expressing Flag-tagged DN MyD88, which lacks the death domain (Lafuse, Alvarez *et al*, 2006), were maintained in DMEM medium supplemented with hygromycin B. A RAW264.7 cell line expressing STAT1 siRNA was created by transfecting RAW264.7 cells with STAT1 siRNA pSilencer 4.1-CMV hygro plasmid (Ambion, Austin, TX) and selecting

stable clones with hygromycin B and limiting dilution cloning. A siRNA target sequence (aagcaagcgtaatctccaggga, 225-275) was identified in mouse STAT1 (NM-009283) using the on-line siRNA Database (Protein Lounge.com). STAT1 hairpin siRNA encoding oligonucleotides were annealed and ligated into the pSilencer 4.1-CMV hygro plasmid vector. Sequence of the plasmid was confirmed by DNA sequencing. The STAT1 siRNA RAW264.7 clones were screened for decreased STAT1 expression by Western blotting and two clones lacking STAT1 expression were used in this study.

3.2.4. RNA Isolation

RAW264.7 cell lines were plated at 5 x 10^6 cells per well for RNA isolation experiments. All cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and penicillin-streptomycin, and allowed to adhere overnight. The next morning, the medium was removed and fresh DMEM medium supplemented with 10% fetal bovine serum and penicillin-streptomycin was added to each well. The cells were then treated with gamma-irradiated *M. tuberculosis* (100 μg/ml), IFN-γ (200 Units/ml), or a combination of both for 8 hours. Macrophages were lysed using Qiagen lysis buffer containing 2-mercaptoethanol and homogenized by passing the cell lysates through QiaShredders (Qiagen, Valencia, CA). The RNA was then isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). Residual DNA was removed during the RNA purification by on-column DNase digestion.

3.2.5. Quantitative RT-PCR

One μg total RNA was reversed transcribed using 100 μM dNTPs, 15 units AMV Reverse Transcriptase and 0.5 μg Oligo $(dT)_{15}$ primer in reverse transcription buffer for 1 hr at 42°C (Promega, Madison, WI). The expression of mouse GAPDH, and ferroportin1 mRNA were analyzed by real-time RT-PCR using SYBR green polymerase chain reaction master mix (Roche). Reactions were run on the Roche LightCycler 2. The following sense and antisense sequences were employed: mouse GAPDH, sense (5'-3'), GTGTGAACGGA-TTTGGCCGTATTGGGCG, antisense (5'-3') TCGCTCCTGGAAGATGGTGATG-GGC; mouse ferroportin1, sense (5'-3') TGGATGGGTCCTTACTGTCTGCTAC, antisense (5'-3') TGCTAATCTGCTCCTGTTTTCTCC. Primers used for GAPDH and Ferroportin1 were designed using the MacVector primer software (Accelrys, San Diego, CA). The amplification conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 5 seconds and 72°C for 20 seconds. The relative expression of each sample was calculated using mouse GAPDH as a reference and the delta Ct method as previously described (Livak *et al.*, 2001).

3.2.6. Cloning of mouse ferroportin1 promoter and generation of deletion constructs

A luciferase reporter containing mouse ferroportin1 promoter was constructed by cloning the -1175 to +96 sequence of the mouse ferroportin1 gene into the pGL3 basic

luciferase vector (Promega, Madison, WI). The DNA fragment containing the -1175 to +96 sequence was synthesized by PCR using the bacterial artificial chromosome (BAC) clone (RPCI-23 183P22) obtained from Invitrogen containing the mouse ferroportin1 gene and primers based on the published ferroportin1 genomic sequence. The PCR product was digested with HindIII and XhoI, and the digested fragment was ligated into the pGL3 basic luciferase vector (Promega, Madison, WI). The construct was designated FPN-1175/+96. Constructs containing 5' –deletions of the mouse ferroportin1 promoter (FPN-769/+96, -580/+96, -509/+96, -432/+96, and -387/+96) were also generated by PCR using the FPN-1175/+96 plasmid as a template. The following forward primers were designed: -796/+96: 5'-GCGCGCTCGAGCCTGGTTACTTCCCTTCAGCACTTGGAGCC-3'; -580/+96: 5'-GCGCGCTCGAGGAGCTTTGATTCCGCA-3'; -509/+96: 5'-GCGCGCTCGAGAGGCAGGCCGGACATGACTTGTATTTACTT-3';-432/+96: 5'-GCGCGCTCGAGTTCCGCGCCAGCTCCCGCTGAGCTTT-3'; -387/+96: 5'-GCGCGCTCGAGTCTGGTGGCCAGAGAGAAGAGCTTCCTT-3'. The reverse primer used was 5'-GCGCGAAGCTTTGAAGTTGGAAAGCCAAAGCCTTATGGG-3'. After digestion with the HindIII and Xho1 restriction enzymes, the PCR fragments for the deletion constructs were also cloned into the pGL3-basic vector. All constructs were verified by DNA sequencing.

3.2.7. Site directed mutagenesis

Using FPN-1175/+96-pGL3 plasmid as a template, mutations were made to the following consensus binding sequences: PU.1, STAT, NFκB site #1 (N1), and NFκB site #2 (N2). Mutations were carried out using the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen). The primers used were as follows (mutated nucleotides are in bold): PU.1 forward 5'-GCAGGTATGCCCTGGGGAGGA**G**ACGGAGGTCAT-3'; PU.1 reverse 5'-TCCTCCCCAGGGCATACCTGCTTCTTGTGTCA-3'; STAT forward 5'-GCCCTCTCTATAGTTCGGAG**GC**GGAAAACATTC-3'; STAT reverse 5'-CTCCGAACTATAGAGAGGGCTCTCTCTCAAA-3'; N1 forward 5'-TTCCGAAGGGTAGCTAGGGT**AT**GAGGCTCCTTT-3'; N1 reverse 5'-ACCCTAGCTACCCTTCGGAATGCAGGTAGCT-3'; N2 forward 5'-CTCCCGCTGAGCTTTGCCTA**AT**GACTGTCCGGCA-3'; N2 reverse 5'-TAGGCAAAGCTCAGCGGGAGCTGGCGCGGAA-3'. All constructs were verified by DNA sequencing.

3.2.8. Transient transfection

Plasmid DNA was isolated using the Endofree plasmid maxi prep kit (Qiagen, Valencia, CA). RAW264.7 cells were cultured overnight at 2.5 x 10^5 cells/well in 24-well culture plates. RAW264.7 cells were then transfected with 0.5 μg of the ferroportin1-pGL3 plasmids/well using Lipofectamine Plus (Invitrogen), according to the

manufacturer's instructions. Following overnight incubation, triplicate wells were stimulated with 200 Units/ml IFN-γ, 100 μg/ml gamma-irradiated *M. tuberculosis*, or a combination of both. After 8 hours, cellular lysates were prepared, and luciferase activity was measured using the Promega luciferase assay system (Promega). The protein concentration was determined using the Bio-Rad protein assay. The results are expressed as relative light units (RLU)/ μg protein.

3.2.10. Statistics

Results were analyazed by one-way analysis of varience (ANOVA) with Turkey's test using SigmaSTAT (SPSS Science, Chicago, IL).

3.3 Results

3.3.1. Expression of ferroportin1 mRNA in activated macrophages requires Toll-like receptor stimulation.

To determine whether the induction of ferroportin1 mRNA expression by *M. tuberculosis* and IFN-γ is dependent upon Toll-like receptor signaling, we examined ferroportin1 mRNA in RAW264.7 cell lines expressing dominant negative (DN) TLR2 and MyD88 (Curry, Alvarez, *et al*, 2004; Lafuse, Alvarez *et al*, 2006). Figure 8A shows the results of infection of the RAW-TLR2 DN and the RAW-MyD88 DN cell lines. The

expression of ferroportin1 mRNA was nearly completely inhibited in both the RAW-TLR2 DN and RAW-MyD88 DN cells treated with *M. tuberculosis* and stimulated with IFN-γ (Fig.8A). Ferroportin1 mRNA is also synergistically induced in RAW264.7 cells by IFN-γ and the tri-acylated lipopeptide Pam_3CSK_4, a TLR2 ligand (Fig. 8B). However, treatment of RAW-TLR2 DN, and RAW-MyD88 DN cell lines with Pam_3CSK_4 and IFN-γ results in very little induction of ferroportin1 mRNA expression (Fig. 8B). Thus, these results suggest that ferroportin1 expression is dependent, at least in part, on TLR2 and the TLR signaling protein MyD88.

3.3.2 Expression of ferroportin1 mRNA in requires STAT1 activation.

We determined if induction of ferroportin1 mRNA by *M. tuberculosis* and IFN-γ is dependent on activation of STAT1. In these experiments, we used two RAW264.7 cell lines that stably express STAT1 siRNA. These cells lack expression of STAT1 and fail to express CIITA (class II transactivator) mRNA, a STAT1 dependent gene, in response to IFN-γ (not shown). Fig. 9 shows the results of ferroportin1 mRNA in the STAT1 siRNA expressing cell lines compared to control RAW264.7 cells. In the STAT1 siRNA expressing RAW264.7 cells, induction of ferroportin1 mRNA by *M.tuberculosis* and IFN-γ is reduced nearly 100%, indicating that induction of ferroportin1 mRNA expression is STAT1 dependent.

3.3.3. Macrophage Expression of ferroportin1 mRNA in activated macrophages is dependent upon iron levels.

The ferroportin1 gene contains a 5' iron response element (IRE) in its mRNA (Abboud *et al*, 2000) and studies have shown that ferroportin1 protein expression is regulated by iron levels in the macrophage. Under low iron levels in macrophages, iron regulatory proteins (IRPs) bind to the 5' IRE, blocking translation of ferroportin1 mRNA (Krause *et al*, 2000; Park *et al*, 2001). We sought to determine whether or not iron is also involved in the regulation of ferroportin1 mRNA expression in the mouse macrophage-like cell line RAW264.7. In a previous study (Van Zandt *et al*, 2007) we showed that *M. tuberculosis* and IFN-γ synergistically induced ferroportin1. Gamma-irradiated *M. tubeculosis* was equally effective as live *M. tuberculosis* in inducing expression. Figure 10A shows the effect of chelating iron on the induction of ferroportin1 mRNA by gamma-irradiated *M. tuberculosis* and IFN-γ. Increasing doses of desferrioxamine, an iron chelating agent, results in decreased expression of ferroportin1 mRNA. We also found that incubating cells with iron, 1 hour before infection, results in increased expression of ferroportin1 mRNA (Fig. 10B). Thus, the results show that iron not only regulates the translation of ferroportin1, but the expression of ferroportin1 mRNA as well.

3.3.4. IFN-γ and Mycobacterium tuberculosis synergistically induce ferroportin1 promoter activity.

In order to determine if IFN-γ and *M. tuberculosis* activate transcription of the ferroportin1 gene, we cloned the promoter region of the mouse ferroportin1 gene into the pGL3-basic luciferase vector. This luciferase reporter plasmid contained 1175 bp of sequence upstream of the transcription start site and 96 bp downstream of the transcription start sequence. The construct was designed not to contain the IRE element in the untranslated region of the mRNA, so that differences in luciferase activity are solely due to promoter activity. The ferroportin1-pGL3 plasmid was transiently transfected into RAW264.7 cells and the effect of stimulation on promoter activity was determined. As shown in figure 11, IFN-γ and gamma-irradiated *M. tuberculosis* synergistically induced ferroportin1 promoter activity. Since we have shown that the addition of iron increased ferroportin1 mRNA expression induced by IFN-γ and *M. tuberculosis*, while the chelation of iron with desferroxamine decreased expression, we sought to determine whether ferroportin1 promoter activity induced by *M. tuberculosis* and IFN-γ was also increased by the addition of iron and decreased by the chelation if iron. The addition of iron or the chelation of iron had no effect on the ferroportin1 promoter activity (not shown), suggesting that the iron regulation of ferroportin1 mRNA expression is not due to iron regulation of promoter activity.

3.3.5. Analysis of ferroportin1 promoter activity following stimulation of macrophages with IFN-γ and M. tuberculosis.

To investigate the DNA sequence required for promoter activity, deletion constructs were cloned into the luciferase reporter vector pGL3-Basic and transiently transfected into RAW264.7 cells (Fig. 12A). However, the results in figure 12B show that the highest level of luciferase activity was obtained with the FPN-1175 construct. Deletion of the PU.1 site resulted in complete loss of promoter activity in the FPN-769, FPN-580, FPN-509, FPN-432, FPN-387 constructs. This suggests that the PU.1 site is essential for promoter activity. To determine the contribution of the STAT1 site and the two NFκβ sites to promoter activity, we created constructs of the FPN-1175 with mutations of these sites. The results in figure 13 show the activity of the ferroportin1 promoter following the mutation of the STAT and NFκB sites. The results show that a mutation of either of the NFκB sites, or the mutation of the STAT recognition site eliminates the activity of the promoter. These results suggest that the induction of ferroportin1 promoter acitivity by *M. tuberculosis* and IFN-γ is dependent upon both STAT1 and NFκB and either alone is not sufficient to induce promoter activity. Mutation of the PU.1 site also eliminated promoter activity, confirming that this site is also essential for promoter activity.

3.4 Discussion

Regulation of ferroportin1 expression in macrophages by infection and inflammatory stimuli has only recently begun to be characterized. Previous studies (Yang *et al*, 2002; Liu *et al*, 2005; Ludwiczek *et al*, 2003) have suggested that inflammatory stimuli, such as LPS, have a negative regulatory effect on ferroportin1 expression. However, our previous studies (Van Zandt *et al*, 2007) have shown that ferroportin1 mRNA is differentially expressed in macrophage populations infected with mycobacteria. We showed that in resident peritoneal macrophages and the macrophage cell lines, RAW264.7 and AMJ2-C8, IFN-γ and *M. tuberculosis* infection synergistically induced expression of ferroportin1 mRNA. However in bone marrow derived macrophages and lung macrophages infection with mycobacteria down-regulated ferroportin1 expression, consistent with the previous studies. In the current study, we sought to determine the regulatory mechanisms behind the production of ferroportin1 mRNA in RAW264.7 macrophages stimulated with IFN-γ and the intracellular pathogen *Mycobacterium tuberculosis*.

We determined if TLR2 is involved in the induction of ferroportin1 mRNA. Several studies have shown that mycobacteria and mycobacterial components can act as agonists for TLR2 (Pai *et al*, 2004; Quesniaux and Fremond *et al*, 2004; Bafica *et al*, 2005), although a role for TLR4 in the recognition of *M. tuberculosis* has been also been suggested (Abel *et al,* 2002). Interaction of *M. tuberculosis* with TLR2 has been shown to

result in the production of many inflammatory cytokines such as TNF-α and IL-1 (Underhill *et al*, 1999). Mice deficient in TLR2 have impaired defense against virulent *M. avium*, suggesting TLR2 plays a pivotal role in the protection against mycobacterial infection (Feng *et al*, 2003). In support of this, it has been shown that $TLR2^{-/-}$ macrophages also display impaired production of TNF-α (Tjarnlund *et al*, 2006), which has been shown to be a critical cytokine for controlling mycobacterial infections (Roach *et al*, 2002). When RAW264.7 macrophages expressing the dominant negative form of TLR2 and the Toll-like receptor signaling molecule MyD88 were infected with *Mycobacterium tuberculosis* and stimulated with IFN-γ, there was markedly reduced levels of ferroportin1 mRNA expression, suggesting that Toll-like receptor signaling, specifically TLR2, is required for the induction of ferroportin1 mRNA. Similarly, there was no induction of ferroportin1 in the TLR2 and MyD88 dominant negative macrophages by the the TLR2 agonist Pam_3CSK_4 and IFN-γ. These studies indicate that the syngeristic induction of ferroportin1 mRNA by *M. tuberculosis* and IFN-γ requires activation of TLR2.

Activation of macrophages with IFN-γ results in the activation of the Jak-STAT signaling pathway, and the subsequent nuclear translocation of the transcription factor STAT1 (Darnell *et al*, 1994; Ihle *et al*, 1996). Since IFN-γ is required for the expression of ferroportin1 mRNA, we investigated whether STAT1 was required for ferroportin1 production. Using macrophages which have been transfected with a STAT1 siRNA, we

showed that ferroportin1 expression is completely lost following infection with *M. tuberculosis* and stimulation with IFN-γ. Thus, the syngeristic induction of ferroportin1 is dependent on both TLR2 signaling and STAT1 activation by IFN-γ.

Previous studies (Canonne-Hergaux *et al*, 2006; Yang *et al*, 2002; Liu *et al*, 2005; Delaby *et al,* 2005) have shown that expression of ferroportin1 is regulated by both transcriptional and post-transcriptional pathways. In the current study, we determined if the induction of ferroportin1 mRNA by IFN-γ and *M. tuberculosis* is at the level of transcription by studying ferroportin1 promoter activity in activated macrophages. We found that, similar to mRNA expression, promoter activity is induced by IFN-γ and *M. tuberculosis* together, but not independently of one another.

In this study, we cloned and sequenced the 1175-bp DNA fragment containing the mouse ferroportin1 promoter region. This segment contained an up-stream PU.1 site, two NFκB binding sites and one STAT binding site. Deletion analysis and site-directed mutagenesis was used to examine the role of these sites in promoter activity. Deletion analysis showed that all promoter activity induced by IFN-γ and *M. tuberculosis* was lost when the nuclear transcription factor-binding site for PU.1 was removed. Mutation of the PU.1 site also resulted in loss of promoter activity. This is expected as it is known that PU.1 is needed for maximal induction of inflammatory proteins in macrophages. Site directed mutagenesis of the two NFκB binding sites and the one STAT binding site also resulted in loss of promoter activity induced by IFN-γ and *M. tuberculosis.* Both NFκβ

sites were required for promoter activity, as deletion of either site caused loss of promoter activity. Thus, our promoter studies provide further evidence for the requirement of both IFN-γ/STAT signaling and NFκB/Toll-like receptor signaling for the synergistic induction of ferroportin1 mRNA by IFN-γ and *M. tuberculosis.*

In this study, we also examined whether iron levels within the macrophage affect the induction of ferroportin1 following infection with *M. tuberculosis* and stimulation with IFN-γ. Previous studies have shown that both mRNA and protein expression of ferroportin1 is regulated by iron. Ferroportin1 mRNA contains a 5' iron response element (IRE) in its mRNA (Abboud *et al*, 2000). Under low iron levels in macrophages, iron regulatory proteins (IRPs) bind to the 5' IRE, blocking translation of ferroportin1 mRNA (Krause *et al*, 2000; Park *et al*, 2001). When the iron levels were high, IRP activity was inhibited and translation of mRNA is increased. Ferroportin1 mRNA expression was found to be increased in J774A.1 mouse macrophages overloaded with iron and decreased by iron chelation (Delaby *et al*, 2005b). In the current study, we also show that ferroportin1 mRNA expression induced by IFN-γ and *M. tuberculosis* in RAW264.7 cells is increased by the addition of iron and inhibited by iron chelation. However, when we examined the effect of iron on ferroportin1 promotor activity, iron had no effect on promoter activity induced by IFN-γ and *M. tuberculosis.* This suggests that the effect of iron on ferroportin1 mRNA expression is not due to transcriptional regulation by iron levels. One possible explanation is that iron is acting post-transcriptionally by influencing

ferroportin1 mRNA stability, such that under low iron conditions ferroportin1 mRNA is less stable. This could occur through binding of IRPs to the ferroportin1 mRNA not only blocking translation but also increasing degradation of the mRNA.

In conclusion, our studies have demonstrated that the production of ferroportin1 by IFN-γ and *M. tuberculosis* activated macrophages is dependent on both the TLR and Jak-STAT pathways, and that iron levels within the macrophage play a role in the level of ferroportin1 mRNA expression in RAW264.7 cells. While further studies are needed to examine the anti-mycobacterial properties of ferroportin1, knowing that both the IFN-γ and Toll-like receptor signaling pathways are required for the induction of ferroportin1 may provide researchers with a basis to develop new ways of fighting *M. tuberculosis* infections world wide.

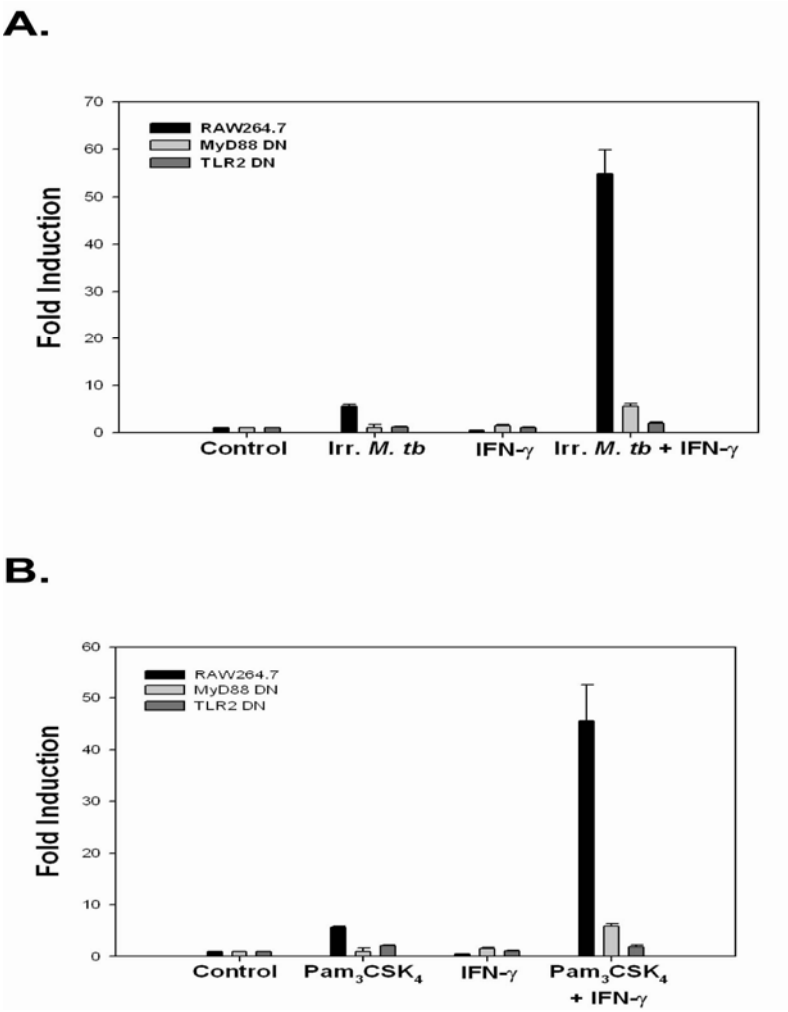

Figure 8: Contribution of Toll-like receptors to the expression of ferroportin1. RAW264.7 cells transfected with either a dominant negative form of TLR2 or MyD88 were treated with 100 μg/ml gamma-irradiated *M. tuberculosis* (A), or 500 ng/ml Pam_3CSK_4 (B) and stimulated with 200 Units/ml IFN-γ for 8 hours. RNA was isolated and mRNA expression of ferroportin1 determined by real-time RT-PCR. The results were normalized to GAPDH and expressed as the fold-increase in ferroportin1 expression compared to control unstimulated cells. The results are the mean +/- S.D. of three independent experiments.

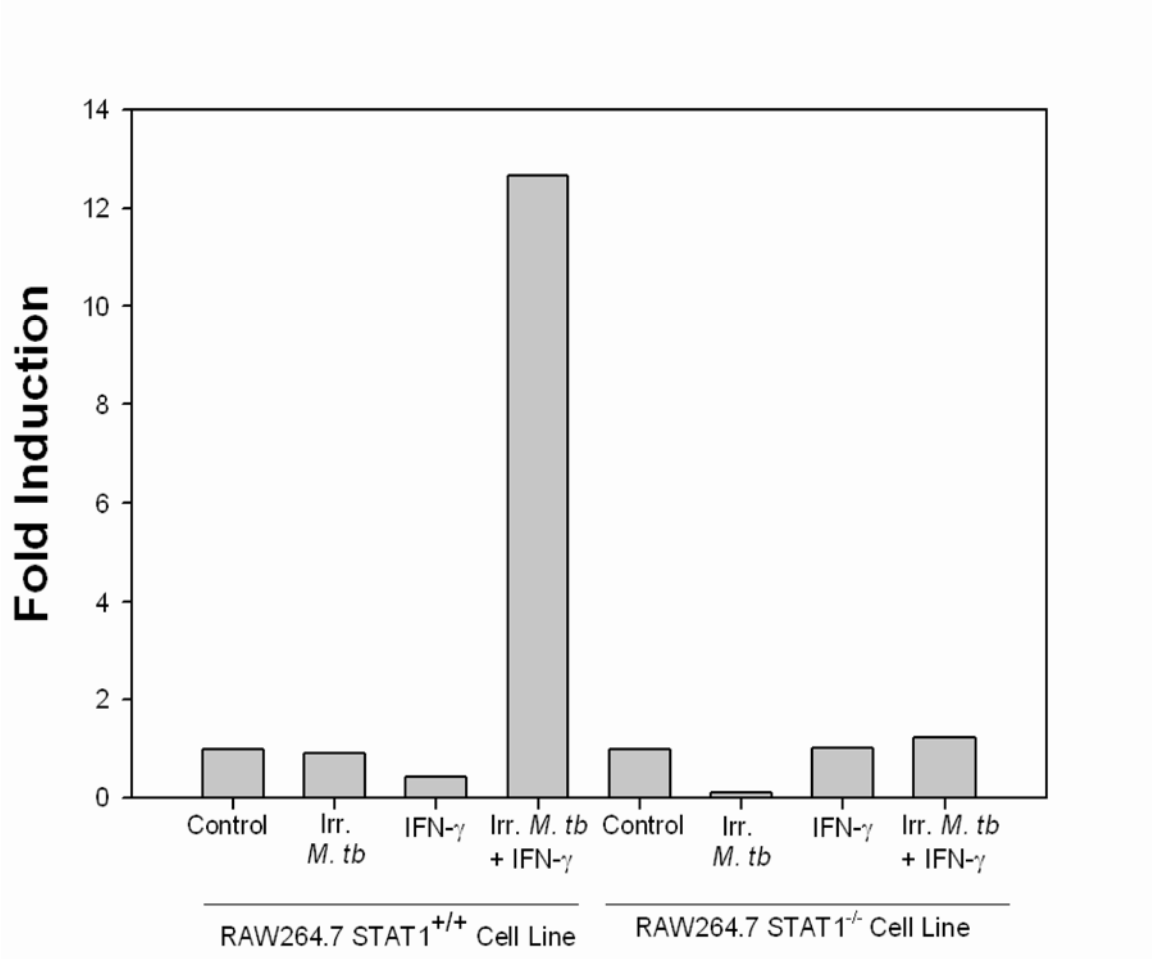

Figure 9: STAT1 mediated ferroportin1 expression. RAW264.7 cells transfected with STAT1 siRNA (STAT1-/-) and RAW264.7 cells with normal STAT1 expression (STAT1+/+) were treated with 100 μg/ml gamma-irradiated *M. tuberculosis* and stimulated with 200 Units/ml IFN-γ. RNA was isolated and mRNA expression of ferroportin1 determined by real-time RT-PCR. The results were normalized to GAPDH and expressed as the fold-increase in ferroportin1 expression compared to control unstimulated cells. The results are the mean +/- S.D. of three independent experiments.

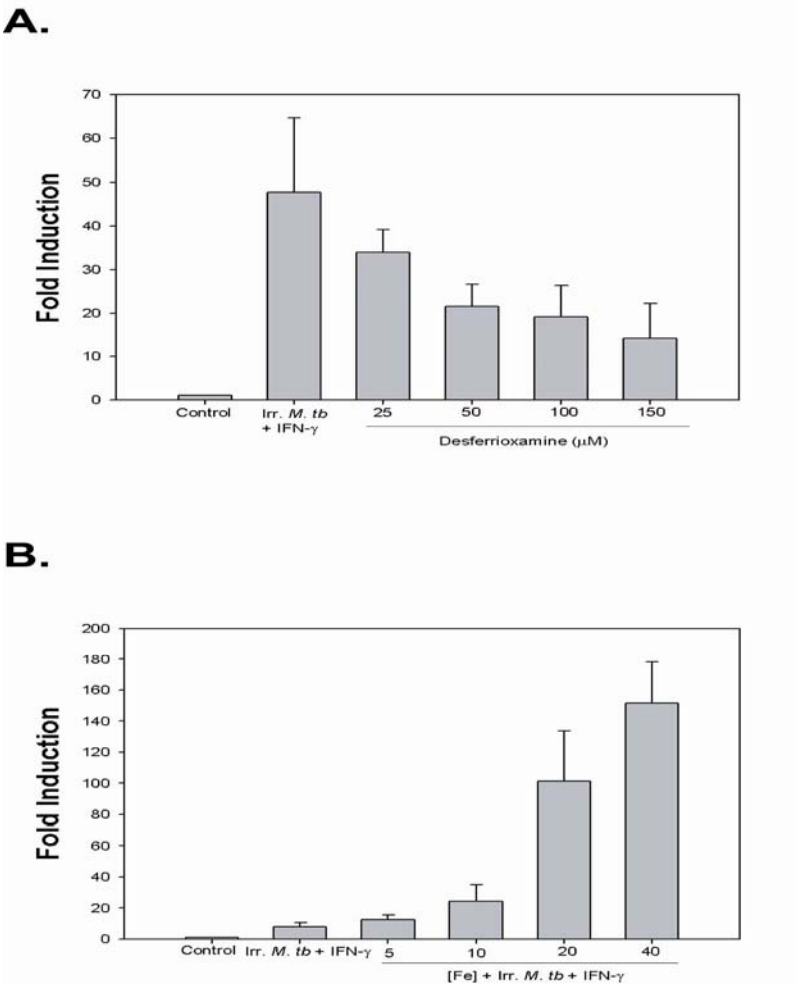

Figure 10: Effect of iron levels on the expression of ferroportin1 mRNA. A. RAW264.7 cells were pretreated with varying concentrations of desferrioxamine one hour prior to being treated with 100 μg/ml irradiated *M. tuberculosis* and stimulation with 200 Units/ml IFN-γ for 8 hours. B. RAW264.7 cells were pretreated with varying amounts of iron one hour prior to treatment with 100 μg/ml irradiated *M. tuberculosis* and stimulation with 200 Units/ml IFN-γ for 8 hours. RNA was isolated and mRNA expression of ferroportin1 determined by real-time RT-PCR. The results were normalized to GAPDH and expressed as the fold-increase in ferroportin1 expression compared to control unstimulated cells. The results are the mean +/- S.D. of three independent experiments.

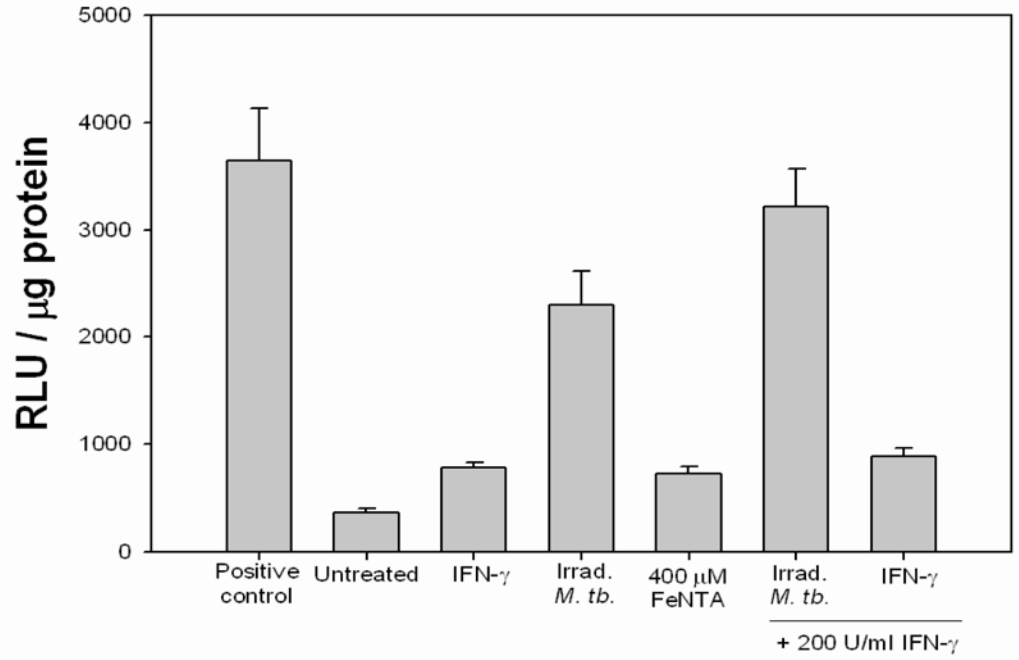

Figure 11: IFN-γ and *Mycobacterium tuberculosis* synergistically induce ferroportin1 promoter activity. RAW264.7 cells were transiently transfected with the full length ferroportin1 promoter region clone and were then stimulated with 100 μg/ml gamma-irradiated *M. tuberculosis*, 400 μM FeNTA, and stimulated with 200 Units/ml IFN-γ for 8 hours. Cellular lysates were prepared, luciferase activity was measured, and protein concentrations were determined. The results are expressed as relative light units (RLU)/μg protein.

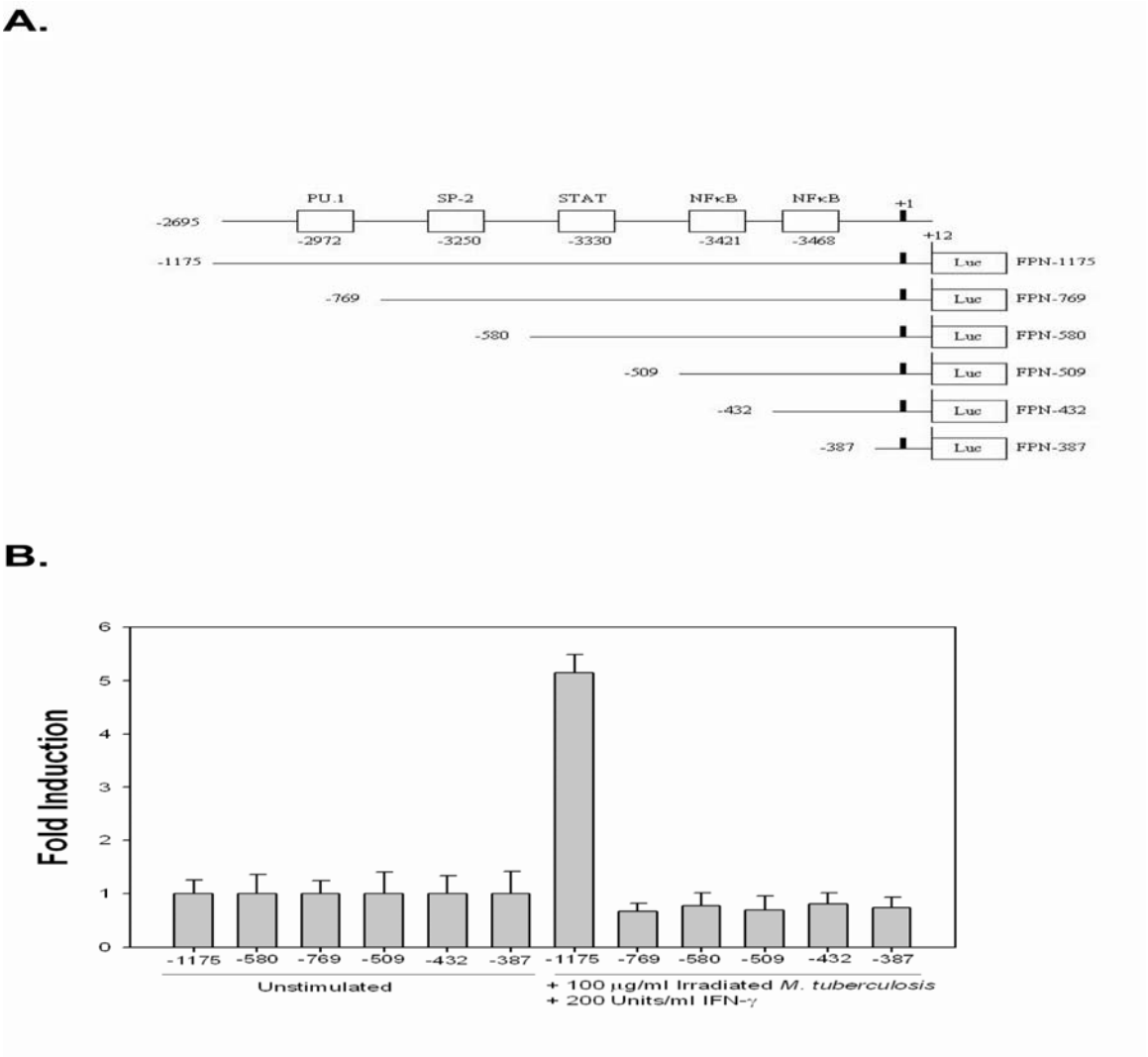

Figure 12: Analysis of ferroportin1 promoter activity following stimulation of macrophages with IFN-γ and *M. tuberculosis*. (A) Schema of promoter fragment-luciferase reporter gene constructs (Luc). (B). The indicated promoter fragment-luciferase reporter gene constructs (Luc) were transfected into RAW264.7 macrophages overnight and then stimulated with IFN-γ and gamma-irradiated *M. tuberculosis* for 8 hours. Luciferase activity was determined and normalized to the protein level of the lysate. Results are representative of three separate experiments. RLU, relative light units.

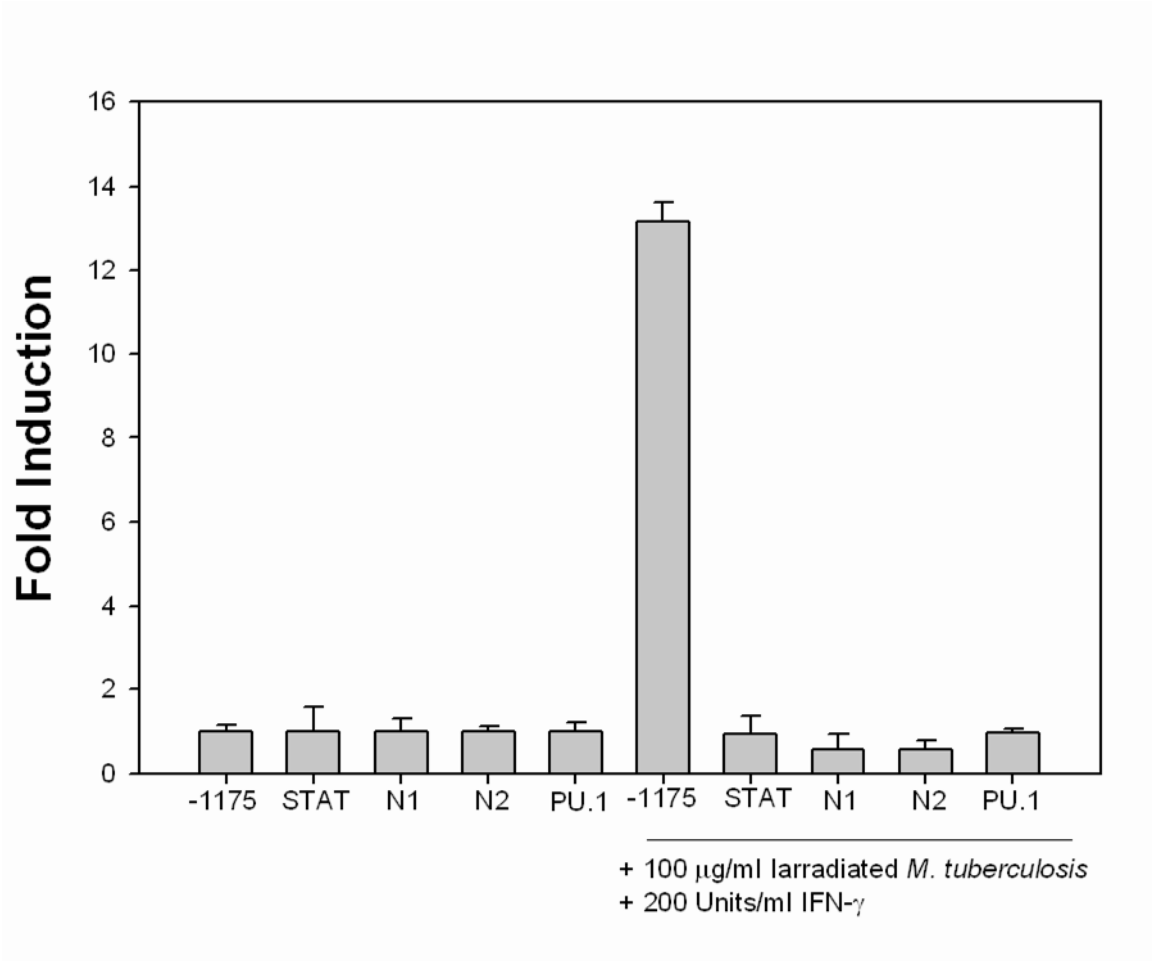

Figure 13: Effect of site directed mutagenesis on the promoter activity of the ferroportin1. Site directed mutations in the STAT site and two NFκB sites were constructed. The indicated mutations-luciferase reporter gene constructs (Luc) were transfected into RAW264.7 macrophages overnight and then stimulated with IFN-γ and gamma-irradiated *M. tuberculosis* for 8 hours. Luciferase activity was determined and normalized to the protein level of the lysate. Results are representative of three separate experiments. RLU, relative light units.

CHAPTER 4

CONCLUSIONS:

Since no studies have examined the expression of ferroportin1 in the context of a mycobacterial infection, I sought to provide a better understanding of the regulation of expression of the mRNA and protein, as well as the regulatory mechanisms controlling the expression of the protein during mycobacterial infection.

The first part of my work suggests that ferroportin1 mRNA expression is upregulated in response to a combination of IFN-γ and mycobacteria (*M. avium*, gamma-irradiated *M. tuberculosis* and live *M. tuberculosis*). The increase in expression of ferroportin1 mRNA is absolutely dependent upon both IFN-γ and *M. tuberculosis,* as either one alone is not enough to increase expression. In these experiments, we treated the macrophages simultaneously with IFN-γ and *M. tuberculosis*. However, we have pre-treated with IFN-γ for 16 hours prior to infection and obtained the same level of increased ferroportin1 mRNA expression as when the macrophages were treated simultaneously. Although we did not pre-treat the macrophages with mycobacteria, our laboratory and others have shown that *M. tuberculosis* inhibits IFN-γ-induced gene expression, and *M. tuberculosis* is particularly effective at inhibiting IFN-γ-induced gene expression at low levels of IFN-γ. Thus, *in vivo,* during the early stages of an *M. tuberculosis* infection, when macrophages infected with *M. tuberculosis* are

predominantly resident macrophages and levels of IFN-γ are low, we would expect to observe inhibition of IFN-γ signaling and no increase in ferroportin1 mRNA expression. However, during the middle stages of infection, i.e., the period of infection when both the naïve monocytes are infiltrating the site of infection and IFN-γ-producing T-cells have arrived from the draining lymph nodes, we would expect to observe an increase in ferroportin1 mRNA expression induced by IFN-γ and *M. tuberculosis,* as the newly arriving monocytes would more likely be exposed to IFN-γ prior to exposure to *M. tuberculosis* or, at least, be exposed simultaneously.

The increase of ferroportin1 mRNA expression also appears to be transient with maximum effect at 8 hours post-exposure and then back to baseline by 24 hours. In examining the levels of ferroportin1 protein, we also found that there was only a 1.7 fold increase in protein expression. One possibility for the rapid decline in the levels of mRNA could be that the infection is decreasing the stability of ferroportin1 mRNA as well as blocking the translation of the mRNA. Thus, when treated with IFN-γ, which results in lowered iron levels within the cell, IRPs would bind to the IRE present in the 5' UTR of the ferroportin1 mRNA, thus resulting in decreased translation, and subsequent to that, would be less protein expression. In addition, there are putative binding sites for miRNA (micro RNA) in the 3' end of the ferroportin1 mRNA. Induction of miRNA by infection and binding of miRNA to the 3' end of the ferroportin1 mRNA could also contribute to the relative instability of the mRNA and blockage of translation.

In order to determine if the phenomenon of ferroportin1 induction in the RAW264.7 mouse cell line correlated with the effect induced in primary mouse macrophages, I next examined the expression of ferroportin1 mRNA in primary mouse macrophages in response to infection with mycobacteria and stimulation with IFN-γ. When resident peritoneal macrophages were infected with *M. tuberculosis* and stimulated with IFN-γ, ferroportin1 mRNA up-regulation was seen. However, the increase in the expression of ferroportin1 mRNA in these macrophages was much lower than that of the RAW264.7 macrophages. Ferroportin1 mRNA may be constitutively expressed in this cell at higher levels, which would mean that the increase in expression we are seeing is above and beyond what is already present and, thus, may appear low. Previous studies have indicated that ferroportin1 mRNA is constitutively expressed in BMDMs, and that in response to stimulation with LPS, the levels of ferroportin1 decrease. With this in mind, I next examined the induction of ferroportin1 mRNA in BMDMs in response to *M. tuberculosis* infection and stimulation with IFN-γ. I used LPS in combination with IFN-γ as a control. In contrast to what was seen with the cell lines and the peritoneal macrophages, expression of ferroportin1 appears to be down-regulated in response to mycobacterial infection and, as expected, there is also a down-regulation of expression following stimulation with LPS. One possibility for this result is that the constitutive levels of ferroportin1 mRNA are so high in these cells that we are not able to induce the expression any higher, i.e., the levels have reached a plateau**,** in which any further increase in expression is unattainable. This could be because the promoter in these cells is

constitutively active and any infectious stimuli may have no further effect on the promoter activity. One would need to do promoter analysis in these cells before and after infection to determine if the promoter is constitutively active. Another possibility is that the promoter in these cells is regulated by different transcription factors than that of the resident peritoneal macrophages and macrophage-like cell lines. Again, a closer examination of the promoter region would be needed to determine if this is the case. The down-regulation of expression induced in these cells by LPS and *M. tuberculosis* could also be due to post-transcriptional effects, such as mRNA stability and translational blockage. Interestingly, the same phenomenon, i.e., the down regulation of expression of ferroportin1 mRNA, is also seen in alveolar macrophages isolated from mice and then infected with *M. tuberculosis* and stimulated with IFN-γ. The reasons for this could be the same as the reasons given for the BMDMs.

I have shown that ferroportin1 mRNA expression is up-regulated in various macrophages including the resident peritoneal macrophages, as well as the RAW264.7 macrophage-like cell line. Thus, I next examined the trafficking of the protein during infection. Here, I have shown that ferroportin1 localizes to the phagosome after phagocytosis of *M. tuberculosis* and is not expressed on the cell membrane. The localization of ferroportin1 to the phagosome was rapid. The lack of trafficking of ferroportin1 to the cell membrane is possibly due to the low levels of iron in these cells. Previous studies (Delaby *et al*, 2005; Knutson *et al*, 2003) have shown that iron loading of macrophages induces trafficking of ferroportin1 to the plasma membrane, which

suggests that the trafficking of ferroportin1 to the plasma membrane is regulated by a signal pathway that is activated by intracellular iron levels. Thus, we speculate that since following phagocytosis of *M. tuberculosis* and stimulation with IFN-γ intracellular iron levels decrease, there is no trafficking of ferroportin1 to the plasma membrane. Trafficking of ferroportin1 to the *M. tuberculosis* phagosome is also most likely regulated by signaling pathways induced by the interaction of *M. tuberculosis* with the macrophage, since ferroportin1 localized to the phagosome following phagocytosis of *M. tuberculosis,* but not to the phagosome following phagocytosis of latex beads. The rapid trafficking of the ferroportin1 protein to the mycobacteria-containing phagosomes suggests that this protein is important for the removal of iron from the phagosome, thus limiting the availability of iron to *M. tuberculosis*. Our lab has seen that Nramp1, another iron transport protein, and ferroportin1 are co-localized to intracellular vesicles in untreated cells. While we have not examined the co-localization of the two proteins following infection with *M. tuberculosis*, one would assume that since both are rapidly recruited to phagosomes, both proteins would be present in the phagosomes at the same time. For this reason, we propose a model in which Nramp1 and ferroportin1 work together in a coordinated manner to both attack the invading pathogen and limit the availability of iron. Nramp1 serves as an iron import protein that provides the iron (Fe^{2+}) needed for the production of toxic hydroxyl radicals via the Fenton reaction. A byproduct of this reaction is the creation of Fe^{3+}, which can be utilized by *M. tuberculosis*. Thus,

there needs to be a mechanism to remove this iron from the phagosomal environment. Because ferroportin1 only exports Fe^{2+}, a ferrireductase would be needed to reduce the available levels of Fe^{3+} iron to Fe^{2+}. A possible candidate for this is the newly discovered Lcytb. Ferroportin1 could then export the iron from the phagosome where it would be loaded onto ferritin and stored.

Since there are no studies that have examined the regulation of the promoter of the ferroportin1 gene during infection, I next examined the regulation of the gene. As *M. tuberculosis* is primarily recognized by TLR2, I first examined the expression of ferroportin1 mRNA following stimulation of a RAW264.7 dominant negative cell line, which has had 14 amino acids removed from the cytoplasmic TIR domain, thus rendering it unable to signal. Coinciding with this experiment, I also examined the requirement for MyD88, a TLR signaling adaptor protein, utilizing a MyD88 DN RAW264.7 cell line. My data suggest that both TLR2 and MyD88 are required for an increase in the expression of ferroportin1 mRNA. This suggests that TLR4 plays no role in the increased expression of ferroportin1, as the RAW264.7 TLR2 DN cell line has intact MyD88 and TLR4. This is important in that while *M. tuberculosis* recognition mostly involves TLR2, several studies have suggested that the *M. tuberculosis* can also be recognized by TLR4. Because I have shown that IFN-γ in combination with *M. tuberculosis* is responsible for the increase in the expression of ferroportin1 mRNA, I next examined the expression in a RAW264.7 cell line in which the STAT proteins have been silenced. Not unexpectedly, in this cell line, treatment with *M. tuberculosis* and IFN-γ resulted in no increase in the

expression of ferroportin1 mRNA. Taken together, these results support the hypothesis that both IFN-γ signaling (via STAT1 homodimers) and *M. tuberculosis* recognition (via TLR2) are required for expression of ferroportin1.

In the literature, it has been shown that the expression of ferroportin1 mRNA and protein are both regulated by iron levels. The ferroportin1 gene contains a 5' iron response element in its mRNA that regulates the translation of the protein. My results confirm that, indeed, iron does effect the expression of ferroportin1 mRNA in RAW264.7 macrophages stimulated with *M. tuberculosis* and IFN-γ, as chelation of iron results in decreased expression and exogenously added iron increases the expression of the mRNA. When RAW264.7 macrophages transfected with a 5' promoter region clone were treated with iron prior to infection, there was no effect on the promoter region activity. Because iron has previously been shown to regulate protein expression, and here I have shown it does not affect promoter activity, my results suggest that both ferroportin1 mRNA and protein are affected by iron levels at the post-transcriptional level. One possible explanation is that iron is acting post-transcrptionally by influencing ferroportin1 mRNA stability, such that under low iron conditions ferroportin1 mRNA is less stable. This could occur by binding of IRPs to the ferroportin1 mRNA not only blocking translation, but also increasing degradation of the mRNA.

Our promoter analysis also showed that treating the transfected cells with *M. tuberculosis* and IFN-γ resulted in promoter activity, again, suggesting the importance of these two signals in the increase in the expression of ferroportin1. Examining the

promoter region of ferroportin1, we found that there was a PU.1 binding site, a STAT binding site, and two NFκB binding sites. Using deletion models, I next examined the contribution of each binding site to the promoter activity of the ferroportin1 gene. These deletional studies indicated that PU.1 is essential as the deletion construct with PU.1 removed resulted in a complete loss of promoter activity. Unfortunately, the PU.1 site is the most distal from the start site, so site directed mutagenesis (SDM) was employed to examine the contributions of the other sites. Utilizing SDM, we found that all four sites were required for the promoter activity of the ferroportin1 gene, as a loss of any of the four resulted in a complete loss of promoter activity. It is currently not known what triggers the unfolding of the chromatin during mycobacterial infection. However, based on our observations, one could assume that there is some signal, following infection with *M. tuberculosis* that triggers the acetylation of the histones, thus remodeling the chromatin to increase access to promoter sequences for binding of the transcription machinery. This signal may be the same signal that induces the expression of other genes during mycobacterial infection, or it may be one that is dependent upon iron levels within the cell, as I have shown that iron has been shown to regulate ferroportin1 mRNA expression.

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