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MYCOBACTERIUM AVIUM INFECTION OF MOUSE MACROPHAGES INHIBITS INTERFERON-GAMMA JAK-STAT SIGNALING AND GENE INDUCTION BY DOWN-REGULATION OF INTERFERON-GAMMA RECEPTOR

DISSertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Shabbir Hussain, D.V.M., M. Sc.(Hons.)

The Ohio State University

1999

Dissertation Committee:
Professor William Lafuse, Adviser
Professor Bruce Zwilling
Professor Marshall Williams
Professor Fredika Robertson

Approved by

Professor William Lafuse, Adviser
Department of Medical Microbiology and Immunology
ABSTRACT

Macrophage activation is required to control the growth of intracellular pathogens. Recent data indicate that macrophages become functionally deactivated during mycobacterial infection. Macrophage deactivation was studied by examining expression of a panel of IFN-γ-inducible genes and activation of JAK-STAT pathway in *M. avium* infected macrophages. Reduced expression of IFN-γ-inducible genes: MHC class II gene Eβ; MHC class II transactivator (CIITA); interferon regulatory factor-1 (IRF-1); and Mg21, a gene coding for a GTP-binding protein, was observed in *M. avium* infected macrophages. Decreased tyrosine phosphorylation and DNA binding activity of STAT1 was observed in *M. avium* infected macrophages stimulated with IFN-γ. Tyrosine phosphorylation of JAK1, JAK2 and IFN-γRα was also reduced in infected cells. Northern and western blot analyses showed that a down-regulation of IFN-γR α- and β-chains mRNA and protein occurred in *M. avium* infected macrophages. The down-regulation of IFN-γR and STAT1 inhibition was time dependent and required at least 4 hours of infection. A decreased expression of IFN-γ-inducible genes and down-regulation of IFN-γR α- and β-chains was also observed in macrophages treated with cell free supernatant from *M. avium* infected.
macrophages. The supernatant was found to contain a potent inhibitory factor(s). A 1:5 dilution was sufficient to mimic the inhibitory effects of 10:1 (bacteria:macrophage) ratio of infection. The inhibitory factory is neither TGF-β nor IL-10 as neutralization of these cytokines with antibodies did not abrogate the inhibitory effect. The production of the inhibitory factor(s) was also indomethacin resistant, suggesting that the factor(s) is not prostaglandin. The supernatants generated in the presence of cycloheximide did not inhibit the expression of IFN-γ-inducible genes, indicating that the inhibitory factor(s) is protein in nature.

These findings suggest that *M. avium* infection inhibits induction of IFN-γ-inducible genes in mouse macrophages by down-regulating IFN-γR, resulting in reduced phosphorylation of IFN-γRα, JAK1, JAK2, and STAT1.
Dedicated to my Parents
ACKNOWLEDGMENTS

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VITA

September 19, 1965........Born - Kotmoman, Punjab, Pakistan

1987........................................D.V.M., University of Agriculture, Faisalabad, Pakistan

1989........................................M. Sc.(Hons.), Veterinary Clinical Medicine and Surgery,
University of Agriculture, Faisalabad, Pakistan

1989-1991..............................Lecturer, Department of Veterinary Clinical Medicine and
Surgery, University of Agriculture, Faisalabad, Pakistan

1991-1993..............................Graduate Scholar, The Ohio State University, Columbus, OH

1993-present............................Graduate Teaching and Research Associate, The Ohio State
University, Columbus, OH

PUBLICATIONS

Research papers:


**Abstracts:**


**FIELDS OF STUDY**

**Major Field:** Medical Microbiology and Immunology

**Minor Field:** Infection and Immunity
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<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>Cox</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CR</td>
<td>Complement receptor</td>
</tr>
<tr>
<td>CIITA</td>
<td>Class II transactivator</td>
</tr>
<tr>
<td>CIS</td>
<td>Cytokine inducible SH2 protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetate</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
</tr>
<tr>
<td>GAS</td>
<td>Gamma interferon activation site</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GBP-1</td>
<td>Guanylate binding protein-1</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IFN-γRα</td>
<td>Interferon gamma receptor alpha</td>
</tr>
<tr>
<td>IFN-γRβ</td>
<td>Interferon gamma receptor beta</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
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<td>LP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IRF-1</td>
<td>Interferon regulatory factor-1</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency associated protein</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Leukocyte function-associated antigen-1</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, normal T cell Expressed and Secreted</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology domain 2</td>
</tr>
<tr>
<td>SHP-1</td>
<td>Src homology tyrosine phosphatase-1</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SSI</td>
<td>STAT-induced STAT-inhibitor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
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<tr>
<td>W.B.</td>
<td>Western blotting</td>
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CHAPTER 1

INTRODUCTION

*Mycobacterium avium* is a facultative intracellular pathogen and causes severe pulmonary and disseminated disease in immunocompromised hosts, especially in individuals with acquired immunodeficiency syndrome (AIDS). Recent data indicate that 40 to 50% of all patients with AIDS are infected with *M. avium* which is refractory to conventional antibiotics and antimycobacterial drugs (32, 67, 81, 90).

Mycobacteria are taken up by macrophages by phagocytosis and reside within phagosomes of these cells (12, 13, 90, 168). Infected macrophages initiate a cell mediated immune response by processing and presenting antigen to T lymphocytes in context of major histocompatibility (MHC) cell surface molecules. The activated T lymphocytes then secrete interferon gamma (IFN-γ), which activates macrophages and increases expression of MHC class II molecules and other costimulatory molecules on the cell surface (15, 35, 145, 190, 191, 195).

Stimulation of macrophages with IFN-γ results in activation of the JAK/STAT signal transduction pathway (35, 61, 192, 200, 236). IFN-γ binds to its cell surface receptor consisting of two heterodimeric subunits, IFN-γRα and IFN-γRβ, which are
associated with Janus kinases, JAK1 and JAK2 respectively (18, 39, 122). IFN-γ binding results in receptor dimerization/oligomerization and phosphorylation of JAK1 and JAK2. Phosphorylated JAK1 and JAK2 are responsible for phosphorylation of IFN-γRα, recruitment of STAT1, and its phosphorylation (18, 39, 102, 122, 235, 247). Phosphorylated STAT1 dimerizes and translocates to the nucleus where it binds to gamma activation sites (GAS) of IFN-γ-inducible genes, including CIITA and IRF-1 genes (158, 177, 205).

Virulent mycobacteria are able to survive and multiply within macrophages for extended periods of time. The ability of mycobacteria to evade being killed within phagocytic cells contributes to their success as pathogens. Macrophages infected with mycobacteria also have poor responses to IFN-γ, including reduced expression of MHC class II molecules, decreased capacity to present antigen, and decreased ability to inhibit microbial growth (55, 96, 117, 130, 173, 175, 176, 195, 226). Macrophages infected with mycobacteria also secrete immunosuppressive and macrophage deactivating cytokines (TGF-β and IL-10), and prostaglandin E2 (PGE2) (95, 111, 117, 226, 268). However, the mechanism by which M. avium infection affects the expression of IFN-γ-inducible genes remains unknown.

The purpose of this study was to investigate the mechanism(s) involved in the unresponsiveness to IFN-γ of M. avium infected macrophages by studying the IFN-γ signal transduction pathway and the role of immunosuppressive and macrophage deactivating cytokines (TGF-β and IL-10) and PGE2 produced by infected macrophages.
CHAPTER 2

LITERATURE REVIEW

2.1. Macrophage Biology.

Macrophages are highly versatile cells and constitute the effector arm of the cellular immune system (2, 3, 14). Eli Metchnikoff was the first to use the term "macrophage" to describe large mononuclear phagocytic cells in tissues (14). Macrophages originate in the bone marrow from pluripotent stem cells and are distributed to tissues of the body through blood circulation (93). Under normal conditions monocytes migrate to various body tissues and cavities where they differentiate into tissue macrophages (25). According to their localization in body tissues and cavities, they have different names, e.g., alveolar macrophages in the pulmonary airways, Kupffer cells in the liver, mesangial cells in the kidney, and microglial cells in the central nervous system. Alveolar macrophages, Kupffer cells, and splenic macrophages are the major macrophage populations that cope with infection. Alveolar macrophages are mainly involved in the clearance of air borne pathogens such as M. tuberculosis (81, 113). Kupffer cells and splenic macrophages clear blood borne pathogens (89).
Macrophages survive in tissues for 1-3 months. During local infection or inflammation, monocytes migrate into the affected site under the influence of chemotactic factors and other mediators of inflammation, such as proinflammatory cytokines and eicosanoids (25). Adhesion molecules on monocytes and endothelial cells are responsible for adherence of monocytes to endothelial cells and their subsequent transendothelial migration (25). At the site of infection, the monocytes differentiate into exudate macrophages. Macrophages are highly differentiated cells and exhibit a wide range of morphological, functional, and phenotypic heterogeneity that reflects their adaptation to a wide range of environmental constraints, maturation stage, distinct myeloid progenitor cells, and differential hemopoietic stimulation (14, 225).

Macrophage express a variety of cell surface receptors. Mannose receptor, Fc receptors and complement receptors (CR1, CR3 and CR4) are involved in phagocytosis (14, 59, 71, 113, 127, 223, 240). CD14, which is a receptor for lipopolysaccharide (LPS), is also used by virulent *M. tuberculosis* for phagocytosis (201, 231, 282). Macrophage also possess receptors for cytokines and chemokines. Cytokines interact with specific receptors and modulate the functions of these cells. Interleukin-2 (IL-2) enhances the tumoricidal and microbicidal activities of macrophages through the induction of tumor necrosis factor alpha (TNF-α) production and generation of reactive oxygen intermediates (27). IL-10 and TGF-β down-regulate the production of proinflammatory cytokines by macrophages (50, 72, 179, 280). IFN-γ induces or upregulates MHC class II molecules on the surface of macrophages (2, 3, 35, 98). Chemokines such as MCP-1, -2, and -3 (macrophage chemotactic protein-1, -2, and -3); macrophage inflammatory protein-1α and
-β (MIP-1α and β); and RANTES (Regulated upon Activation, normal T cell Expressed and Secreted) attract the monocytes to the site of infection (137, 171, 220, 233, 273).

In addition to their phagocytic and immunomodulating properties, macrophages also secrete many mediator substances including complement components, arachidonic acid metabolites, proteases, various growth factors and cytokines which have the potential for regulating diverse cellular functions such as wound healing (14). Macrophages regulate wound healing by releasing cytokines and growth factors, which stimulate migration and proliferation of epidermal keratinocytes and fibroblasts, neurovascularization, and synthesis of connective tissue proteins and extracellular matrix.

2.2. Mycobacterium Biology.

The genus Mycobacterium contains more than 50 species but only a few are human pathogens (22, 81, 99). *Mycobacterium tuberculosis* is the major cause of tuberculosis around the world and was initially described by Robert Koch in 1882. According to an estimate of the World Health Organization (WHO), 1700 million people have been infected with *M. tuberculosis*, 8 million develop disease each year, and 2.9 million die from it each year. *M. africanum* infection is limited to northwestern Africa. *M. bovis* can also cause tuberculosis but the incidence has declined due to pasteurization and boiling of consumed milk. *M. avium* and *M. intracellulare* are usually not human pathogens, but can cause severe pulmonary disease in immunocompromised hosts. With the introduction of Acquired Immunodeficiency Syndrome (AIDS) in the beginning of the last decade *M. avium* infection has become a major human health problem (32, 67, 91).
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 related to the cell wall of gram positive bacteria, but contain a high lipid content and can only be stained with acid fast stain (99). The cell wall consists of four layers. The innermost layer consists of peptidoglycan, a polymer of N-acetylglucosamine, and N-acetylmuramic acid, common to almost all bacteria. This backbone structure is covered by a second layer composed of arabinogalactan, a branched macromolecule consisting of arabinose and galactose. The side chains of these polysaccharides are esterified at distal ends with mycolic acids and constitute the third layer of the cell wall. This thick mycolic acid layer is believed to be responsible for the characteristic “acid fast” staining reaction (22). The fourth and the outermost layer of the cell wall is the capsule, which contains various complex molecules including phenolic glycosides, glycolipids, and peptidoglycolipids termed mycosides. These molecules are involved in the virulence of the pathogen. Lipoarabinomannan (LAM) is the most important cell wall glycolipid having complex immunomodulating properties (19, 81, 251, 252).

2.3. Interaction between Mycobacterium and Macrophages.

Adhesion of pathogens to the host cell surface is the first and most critical step for entry of intracellular pathogens into cells. The initial binding and uptake of microorganisms by macrophages can occur by both opsonic and nonopsonic phagocytosis. Once mycobacteria are ingested by macrophages, they may either start to
divide within the phagosome or they are destroyed by the defense mechanisms of the macrophage.

2.3.1. Opsonic phagocytosis.

Humoral factors are important in the initial attachment and uptake of intracellular pathogens by mononuclear phagocytic cells (28, 71, 263). Complement receptors CR1, CR3, and CR4 expressed on mononuclear cells are important in the binding and phagocytosis of mycobacteria opsonized with complement components (71, 113, 238). A synergistic role of CR1 and CR3 in binding and phagocytosis of M. avium has been described (248). Preincubation of monocytes with monoclonal anti-CR1 or anti-CR3 antibody alone reduced M. avium uptake by 20-40% and 10-20% respectively. However, incubation with both antibodies reduced phagocytosis by 50-80%.

Complement receptors differ in structure and their complement component binding abilities. CR1 is a monomeric transmembrane protein that binds only C3b and C4b complement components (7, 71). CR3 and CR4 are heterodimeric proteins of the integrin superfamily containing identical β-subunits and distinct α-subunits. Both CR3 and CR4 receptors bind C3bi (28, 113, 238). In addition to C3b and C3bi binding to complement receptors, pathogenic mycobacteria can also use the complement fragment C2a present in serum to form a C3 convertase and generate opsonically active C3b in the absence of early activation components of the alternative and classical pathways (242).

Antibodies against mycobacterial components also enhance phagocytosis by opsonization of the pathogen and binding to phagocytic cells through Fc receptors.
Antibodies against glycopeptidolipid (GPL) of *M. avium* have been detected in 44% of homosexual men positive for HIV virus (148). Natural antibodies present in sera from nonimmune hosts have also been shown to enhance complement fixation and binding of mycobacteria to phagocytes (238).

### 2.3.2. Nonopsonic phagocytosis.

Nonopsonic mechanisms of mycobacterial uptake by phagocytic cells involve binding of mycobacteria to a variety of receptors including vitronectin (αVβ3), fibronectin, and mannose receptors (1, 127, 212, 237, 239, 240). The macrophage mannose receptor is a monomeric transmembrane protein expressed only on mature macrophages. Human monocyte-derived macrophage phagocytize virulent *M. tuberculosis* via mannose receptors by interacting with terminal mannosyl units of the lipoarabinomannan (LAM) (239, 240). Besides phagocytosis of whole mycobacteria, the mannose receptor also mediates delivery of LAM to endocytic compartments and facilitates mycolic acid and lipoglycan antigen presentation through CD1b to T cells (209).

Nonopsonic phagocytosis of *M. avium* complex by human monocytes and alveolar macrophages by complement receptors (CR1 and CR3), Fc receptor, and transferrin receptor has also been described (223). CR3 contains a β-glucan binding site near the C-terminus (59, 267). Mycobacteria use their capsular polysaccharides to interact with this β-glucan binding site. Binding of mycobacteria to CR3 can be inhibited by laminarin.
(Seaweed β-glucan), N-acetylglicosamine, and mechanical extraction of capsular polysaccharides from mycobacteria (59).

CD14, a phosphatidylinositol glycan-linked membrane protein, is the well characterized receptor for lipopolysaccharides of gram negative bacteria (231, 282). A recent report also indicates that virulent *M. tuberculosis* uses CD14 on the surface of microglial cells for attachment and phagocytosis (201). Binding of *M. tuberculosis* to scavanger receptors on human monocyte-derived macrophages has also been reported (71). However, the role of the scavanger receptors in phagocytosis is not clear.

2.4. **Macrophage activation.**

Macrophage activation by IFN-γ is quite complex. Macrophage activation by IFN-γ requires binding of IFN-γ homodimer to the IFN-γ receptor, which activates the IFN-γ JAK-STAT signal transduction pathway (18, 61). This results in a cascade of gene expression as some of the early induced genes are required for the induction of other genes that are expressed late after IFN-γ stimulation (159, 205, 219). For example, macrophages stimulated with IFN-γ express high levels of MHC class II genes, which require prior induction of another IFN-γ-inducible gene, MHC class II transactivator (CIITA). CIITA is a transcription factor that activates transcription without DNA binding (158, 219). Recently a binding site of IRF-1, which is also induced by IFN-γ, has been reported in the promoter of CIITA (177).
2.4.1. Interferon gamma.

IFN-γ which is also known as type II interferon, was initially identified based on its antiviral activity which was present in the culture supernatant of virus infected human leukocytes (35, 78). IFN-γ is produced by both CD4+ and CD8+ T lymphocytes and natural killer cells (78, 133). IFN-γ production by macrophages has also been recently reported (82, 92, 163). The human IFN-γ gene has been mapped to chromosome 12 and encodes a 20-25 kDa glycoprotein. The mouse IFN-γ gene is located on chromosome 10 and codes for a 19 kDa glycoprotein. Receptor binding studies (104) suggest that the IFN-γ receptor binds IFN-γ as a homodimer and that both the C- and N-termini are important in the ligand-receptor interaction.

Besides its antiviral and antiproliferative properties (35, 78, 250), IFN-γ has been reported to enhance humoral and cellular immune responses by promoting B cell differentiation and by promoting differentiation of T lymphocytes into the Th1 phenotype (15, 35, 78). IFN-γ also augments microbicidal and tumoricidal activities of macrophages by modulating expression of cell surface molecules, and increasing nitric oxide production and oxidative burst activity (3, 35, 87, 97, 98, 101, 259, 264).

The importance of IFN-γ in controlling the growth of intracellular pathogens became more apparent following the creation of IFN-γ and IFN-γR knockout mice (57, 60, 88, 121, 279). The mutant mice were unable to inhibit the growth of mycobacteria and suffered fatal infection (57, 60, 88).
2.4.2. IFN-γ Receptor.

IFN-γ receptor was initially characterized in the beginning of the last decade using a radio-ligand binding assay (213, 230). The receptor is expressed on most types of primary cells and cultured tumor cells (18, 78). The number of cell surface of receptors expressed varies between 200-25,000 receptors per cell. The binding of IFN-γ to its receptor is specific and is not inhibited by IFN-α/β. Moreover, human and murine IFN-γ bind to their respective receptors in a species specific manner and induce biological response only in species matched cells (18, 78).

The IFN-γ receptor consists of two subunits (Fig. 2.1). The multicomponent structure of IFN-γR was first proposed based upon genetic studies with somatic cell hybrids (112, 126). In these studies, stable murine:human somatic cell hybrids were created containing the full complement of murine chromosomes and a random assortment of human chromosomes. All hybrids containing human chromosome 6 bound human IFN-γ with high affinity (125, 203). However, biological response to IFN-γ was observed only in hybrids containing both human chromosomes 6 and 21 (86). Similar studies were also conducted with hamster:murine somatic cell hybrids (112). These studies provided the first evidence that the IFN-γ receptor consists of at least two species-matched subunits. The first subunit of the IFN-γ receptor is responsible for ligand-binding in a species specific manner (203). The second species-matched subunit is required for induction of biological activity (112, 228). The multicomponent structure of IFN-γ receptor was then confirmed by purification and cloning of receptor subunits. Purification and cloning of the ligand-binding component of the receptor was completed.
in 1987-1988 (5, 6, 44, 54, 100, 109, 143, 186). A few years later, in 1994, the second subunit of the IFN-\(\gamma\) receptor was simultaneously identified by two different laboratories using complementation cloning approaches (110, 255).

The nomenclature of the IFN-\(\gamma\) receptor has not yet been formally established (200). Currently, the ligand binding subunit of the receptor is designated as IFN-\(\gamma\)R\(\alpha\) or IFN-\(\gamma\)R1. Similarly, the second component initially identified as accessory factor-1(AF-1) is referred to as IFN-R\(\beta\) or IFN-\(\gamma\)R2. The human and mouse IFN-\(\gamma\)R \(\alpha\)-chain genes were mapped to human chromosome 6 and mouse chromosome 10, respectively (160, 203). Transcription of both human and mouse \(\alpha\)-chain genes gives rise to 2.3 kb mRNAs. IFN-\(\gamma\)R \(\alpha\)-chain is synthesized in the endoplasmic reticulum. The receptor then moves to the Golgi where N-linked glycosylation takes place. The binding of ligand to the receptor results in ligand-receptor complex formation and internalization within the cell, where the ligand gets degraded by lysosomes and the uncoupled receptor \(\alpha\)-chain recycles back to the cell surface (46, 83). The IFN-\(\gamma\)R \(\beta\)-chain gene is localized on human chromosome 21 and mouse chromosome 16 (56, 203, 255). Transcription of the \(\beta\)-chain gene gives rise to 1.8 and 2.0 kb transcripts in human and mouse, respectively.

Both IFN-\(\gamma\)R \(\alpha\)- and \(\beta\)-chains belong to the cytokine receptor family (123). Like other members of this family, the intracellular domains lack intrinsic tyrosine kinase activity. The extracellular domain of IFN-\(\gamma\)R \(\alpha\)-chain is required for ligand binding. The function of the extracellular domain of the IFN-\(\gamma\)R \(\beta\)-chain is not clearly known, except that it stabilizes the ligand bound to the \(\alpha\)-chain (18). The intracellular domain of the
Figure 2.1. Structure of the IFN-γ receptor. The IFN-γR consists of two polypeptide chains α and β. The α-chains are required for ligand binding and signaling, whereas the β-chain is only involved in signal transduction. Two intracellular domains of the α-chain, LPKS and YDKPH are involved in JAK1 binding and STAT1 binding, respectively. The intracellular domain of the β-chain contains two closely associated sequences that are required for JAK2 binding.
IFN-γRα-chain contains two functionally important sequences. A membrane proximal L<sup>266</sup>PKS<sup>269</sup> motif is required for JAK1 binding. A second site, consisting of a five residue sequence (Y<sup>440</sup>DKPH<sup>444</sup>) that is close to the carboxyl terminus, provides the docking site for STAT1 following the phosphorylation of Y<sup>440</sup> (76, 77). This sequence is conserved in mouse and human receptors. The intracellular domain of the IFN-γR β-chain contains two closely spaced sequences that are required for JAK2 association (17, 141). The cytoplasmic domains of both α- and β-chains can be interchanged between species without loss of biological activity (180), suggesting that species-specific interaction of the IFN-γR α- and β-chains involves only the extracellular domains of the two subunits.

2.4.3. Janus Kinases.

The Janus kinases (JAKs) are highly related intracellular protein tyrosine kinases with apparent molecular weights of about 120-140 kDa (85, 283, 285). JAK kinases do not have Src homology domains (SH2 and SH3) typical of other tyrosine kinases. They do possess five blocks of sequence homology (JH3 through JH7) and two c-terminal catalytic domains (JH1 and JH2) (Fig. 2.2A). With the exception of JH1, which has catalytic activity, the function of the other regions is not clearly understood.

To date four mammalian members of the Janus kinase family have been discovered (85, 283, 285). JAK1, JAK2 and Tyk2 were identified through different cDNA cloning approaches and are expressed ubiquitously and constitutively (85, 285). A
Figure 2.2. Structure of JAK and STAT proteins. (A). JAKs have two kinase domains (JH1 and JH2) at the C-terminus. Only JH1 has catalytic activity. There are also five homology regions numbered JH3-JH7 from C-terminus to N-terminus. These JAK homology regions are involved in binding to signaling receptors. (B). STAT proteins contain a SH2 domain, a DNA binding domain, and a conserved tyrosine residue at the C-terminus whose phosphorylation allows STAT dimerization. The SH2 domain is involved in binding to the phosphorylated docking site of receptors. At the N-terminus, heptad leucine repeats facilitate interaction with other STATs.
fourth member, JAK3, was cloned on the basis of similarity and was found to be
predominately expressed in hematopoietic cells (283). The IFN-γ receptor utilizes JAK1
and JAK2 for its phosphorylation. JAK1 and JAK2 are also associated with other
members of the cytokine receptor family (185, 199, 222). Functional studies of JAK2
deletion mutants indicate that the N-terminal region is required for the association with
the IFN-γR β-chain. Studies using chimeric JAK constructs (JAK2/JAK1) also showed
that the JH7 and JH6 regions of JAK2 are involved in the association with the IFN-γRβ-
chain. On the other hand, the whole N-terminal half of JAK1 (JH7-JH3) was found to be
necessary for its interaction with the IFN-γR α-chain (140).

2.4.4. Signal Transducers and Activators of Transcription.

Cytokines and growth factors induce gene expression by activating a family of
latent cytoplasmic transcription factors called STATs (signal transducers and activators of
transcription). Ligand induced tyrosine phosphorylation of the STATs promotes their
dimerization and subsequent nuclear translocation (18, 39, 102, 151, 192). Six
mammalian STATs (STAT1, STAT2, STAT3, STAT4, STAT5, and STAT6) have been
identified so far and are numbered in order of their discovery (151). STATs contain a Src
homology 2 domain (SH2), a DNA binding domain, and a conserved tyrosine residue at
the C-terminus (Fig. 2.2B). Phosphorylation of the tyrosine residue results in STAT
dimerization (235, 249). The SH2 domain is also required for binding to the
phosphorylated docking sites on receptors. The N-terminus region contains heptad
leucine repeats that facilitate interaction with other STAT family members.
STAT1 and STAT2 were the first identified members of this family (235, 249). In response to IFN-α, STAT1 and STAT2 are phosphorylated at conserved tyrosine residues, form heterodimers, and translocate to the nucleus (235). In the nucleus they interact with a 48 kDa DNA binding protein and form a complex called ISGF3 (interferon stimulated gene factor3) (91). This complex binds to ISRE (interferon stimulated response element) in the promoters of IFN-α -inducible genes. IFN-γ-stimulation of cells results in STAT1 tyrosine phosphorylation and homodimerization (249). The STAT1 homodimers then enter the nucleus and bind to the gamma interferon activation sequence (GAS) in the promoters of IFN-γ-inducible genes (35, 177, 205). GAS consists of 9 nucleotides with a consensus sequence of “TTNCNNNAA” that activates transcription of IFN-γ-inducible genes (18, 119). STAT3 is expressed in many cell types and binds to GAS sequences as either a homodimer or a heterodimer with STAT1 (151, 292, 293). STAT3 tyrosine phosphorylation takes place in response to IL10 and cytokines, such as IL-6, IL-11 and LIF, whose receptors share a common signaling component, gp130 (123, 151, 292). STAT4 expression is limited to thymus, spleen, and testis. STAT4 is phosphorylated only in response to IL-12 (151, 293). Stat5 is expressed in mammary tissues as well as cells of the hemopoietic lineage, and is activated by various cytokines (IL-2, IL-9, IL-15 etc.), growth hormone, and prolactin (151, 277). STAT6 is also widely expressed and is tyrosine phosphorylated in response to IL-4 and IL-13 (151, 265).
2.4.5. **JAK-STAT Pathway.**

In general, binding of cytokine to its receptor results in recruitment and activation of STAT protein(s). The activated STAT protein(s) then migrates into the nucleus and binds to DNA, and consequently induces transcription of cytokine specific genes. In IFN-γ cell signaling, signal transduction is initiated by the interaction of the IFN-γ homodimer with the IFN-γR α-chain (Fig. 2.3). The interaction between IFN-γ and its receptor results in receptor dimerization/oligomerization (18, 35). The receptor dimerization/oligomerization, in turn, results in activation of JAK1 and JAK2 kinases and the phosphorylation of the IFN-γR α-chain (18, 35). The phosphorylated IFN-γR α-chain creates two juxtaposed binding sites for the SH2 domains of latent STAT1. Phosphorylation of STAT1 by the JAK kinases leads to a rapid dissociation of the receptor-STAT1 complex and formation of STAT1 homodimers (18, 35, 249). The STAT1 homodimers migrate into the nucleus and bind to GAS sequence in the promoters of IFN-γ-inducible genes (35, 177, 205). After signaling, the IFN-γ-receptor complex is internalized into the endocytic/lysosomal pathway where the IFN-γ dissociates from the receptor α-chain. IFN-γ is degraded in the lysosomes and the receptor recycles back to the cell surface (46, 83).
Figure 2.3. IFN-γ signal transduction pathway. Binding of IFN-γ homodimer to the IFN-γR α-chain results in receptor dimerization/oligomerization, JAK1 and JAK2 activation, and phosphorylation of IFN-γR α-chain. The phosphorylation of the receptor α-chain creates two juxtaposed binding sites for the SH2 domains of latent STAT1. Following binding to the receptor α-chain, STAT1 is phosphorylated by the JAK kinases, homodimerizes, and translocates into the nucleus. In the nucleus STAT1 homodimers bind to GAS sequences and activate IFN-γ-inducible gene transcription.
2.5. Macrophage Deactivation:

Macrophage activation is required for the elimination of intracellular pathogens (128, 218). Among macrophage activating stimuli, IFN-γ plays a key role in enhancing microbicidal and tumoricidal activities of these cells (2, 3, 35, 57, 60, 88). IFN-γ-stimulation eradicates or inhibits the growth of pathogens by enhancing antigen presentation, nitric oxide production and oxidative burst activity of mononuclear phagocytes (2, 3, 35, 78, 87, 97, 101, 264). Infected macrophages are partially or completely unresponsive to IFN-γ and other macrophage activating stimuli (65, 117, 173, 175, 217). Macrophage deactivation mainly results from infection with intracellular pathogens and interaction with immunosuppressive molecules.

2.5.1. Deactivation by infection with intracellular pathogens.

Macrophage deactivation by intracellular infection with a wide range of microorganisms including bacteria, parasites, and fungal pathogens has been reported (49, 51, 84, 129, 130, 134, 243, 245, 287). Macrophages infected with mycobacteria or treated with LAM have been shown to be poor responders to IFN-γ-stimulation and are defective antigen presenting cells (48, 55, 84, 96, 117, 130, 173, 175, 176, 195, 226, 252). The defect in antigen presentation was shown to be the result of reduced MHC class II expression at both the mRNA and protein level (48, 117, 130, 173). Recently Hmama et al. (117) found that reduced expression of Class II molecules on M. tuberculosis infected monocytes was a result of intracellular sequestration of immature MHC class II heterodimers. A reduced expression of ICAM-1, LFA-1, B7-2 and HLA-DR molecules
was also observed in *M. avium* infected human monocytes (175). This reduction in the cell surface expression of the accessory molecules was not mediated by IL-10 or PGE₂ production. Infection in the presence of either anti-IL-10 neutralizing antibodies or indomethacin, a prostaglandin synthesis inhibitor, did not abrogate the inhibitory effect of infection. Moreover, the inhibitory effect was dose dependent and was more pronounced at 100:1 bacteria to monocyte ratio of infection. A reduction in B7 expression has also been reported in *M. tuberculosis* infected macrophages (226). Pourshafie and Sonnefeld (208) also observed an increase in the expression of LFA-1 in *M. intracellulare* infected macrophages previously primed with IFN-γ. However, there was no increase in the expression of LFA-1 when *M. intracellulare* and IFN-γ were added together, suggesting that *M. intracellulare* infection down-regulates the IFN-γ-induced LFA-1 expression.

Reduced expression of non-IFN-γ-induced genes has also been observed in macrophages infected with intracellular bacteria. A down-regulation of mitochondrial cytochrome c oxidase gene has been observed in *M. tuberculosis* infected macrophages (210). A low transcription of the mouse Golgi mannosidase gene by *Salmonella typhimurium* and *Listeria monocytogenese* infected J774A.1 cells has also been reported (244). Mannose receptors are also selectively down-regulated in mouse macrophages infected with *Mycobacterium* and *Leishmania* species (24, 73)

Macrophages incubated with the mycobacterial cell wall associated glycolipid, LAM, also become refractory to the activating properties of IFN-γ for induction of intracellular microbicidal activity, expression of MHC class II molecules, and killing of
tumor cells (251, 252). Exposure of cells to LAM also attenuates the production of TNF-α and IL-12 and the expression of MHC class II molecules (48, 138).

Ingestion of *Yersinia pestis* also results in macrophage deactivation (51). *Y. pestis* proliferate extracellularly within the reticuloendothelial system, but can also be found within macrophages in tissues from infected hosts. *Y. pestis* is sensitive to killing by oxidizing compounds (H₂O₂ and O₂⁻), but it survives inside phagocytic cells by either blocking the oxidative burst or by rapid removal of the oxidizing compounds.

Macrophages infected with intracellular parasites are also unresponsive to IFN-γ-stimulation (94, 134, 150, 156, 207). Macrophages infected with *Leishmania* species are poor antigen presenting cells; the expression of IFN-γ-induced costimulatory molecules is reduced, as is LPS-induced IL-1 production (94, 144, 215-218). *Leishmania* infected macrophages respond poorly to phorbol ester induced c-fos gene expression and oxidative burst activity (43, 65).

Although the malarial parasite *Plasmodium falciparum* does not directly infect macrophages, phagocytosis of plasmodium-infected erythrocytes by macrophages results in macrophage deactivation and macrophage dysfunction (245). The infected macrophages also become refractory to phagocytosis and show impaired tumoricidal and bactericidal activities. Uptake of the malarial pigment hemozoin, a covalently polymerized heme derived from erythrocyte hemoglobin, by macrophages also results in impaired phagocytosis (84). The infected and hemozoin containing macrophages become unresponsive to phorbol ester induction of oxidative burst.
Infection of macrophages with *Histoplasma capsulatum* alters oxidative burst activity of these cells (287). Both viable and heat killed *H. capsulatum* yeast cells fail to induce release of reactive oxygen metabolites by unprimed elicited mouse macrophages. Moreover, macrophages containing *H. capsulatum* were rendered refractory to subsequent stimulation with zymosan or phorbol 12-myristate 13-acetate (PMA). *Cryptococcus neoformans* also inhibits nitric oxide (NO) production by murine peritoneal macrophages stimulated with IFN-γ and LPS (129). This inhibition in NO production was not mediated by IL-10 and TGF-β production.

Studies have shown that alteration in cell signaling pathways is one of the mechanisms by which macrophages become refractory to IFN-γ-stimulation (150, 183, 189, 218). Protein kinase C (PKC) is known to be involved in cell signaling in various cell types in response to IFN-γ and a variety of other biologically active substances (45, 75, 136). LAM purified from *M. tuberculosis* has been shown to inhibit PKC activity partially purified from macrophages and to attenuate the IFN-γ-induced expression of HLA-DR in intact macrophages (48). Sulfatide, another mycobacterial cell wall component, has also been shown to antagonize certain functional properties of macrophages, including priming by either LPS, IL-1, TNF-α or IFN-γ for enhanced oxidative burst responses to phorbol esters (40, 194). These responses were related to altered intracellular signaling in that the phosphorylation of individual cellular proteins was found to be either increased or decreased in LPS-stimulated cells that had been previously exposed to sulfatide, when compared to the cells treated with LPS alone. Moreover, exposure of intact macrophages to sulfatide resulted in 62-75% reduction in
total PKC activity. However, sulfatide did not directly inhibit the activity of partially purified PKC in vitro, suggesting a different mechanism of action from that observed with LAM.

Defective stimulus-response coupling in human monocytes infected with *Leishmania donovani* has also been found associated with altered activation and translocation of PKC (189). This study provided direct evidence of intracellular infection mediated inhibition of PKC activity and defective cell signaling. Another study from the same laboratory showed that *L. donovani* infected human monocytes and U937 cells are defective in the JAK-STAT cell signaling pathway as a result of reduced tyrosine phosphorylation of JAK kinases and STAT1 protein in response to IFN-γ-stimulation (183). Recently, Lee and Rikihisa (150) reported similar results from *Ehrlichia chaffeensis* infected monocytes. Both of the above studies reported the presence of intact IFN-γ receptors on infected monocytes, but differed in time required to obtain the inhibitory effect, suggesting different mechanisms of action. *Leishmania donovani* infection required 16 hours to exhibit inhibitory effect; whereas, in the *Ehrlichia chaffeensis* infection the inhibitory effect was immediate and did not require phagocytosis of the pathogen.

Protein tyrosine phosphatases may be responsible for reduced tyrosine phosphorylation of JAK kinases and STAT1. Tyrosine phosphatases are associated with cytokine and growth factor receptors and act as both positive and negative regulators of cellular functions (23, 38, 107, 164, 234, 290). Positive regulatory effect of the protein tyrosine phosphatase, SHP-1, has been reported in enhancing the activation of STAT1 in
epidermal growth factor and IFN-γ-stimulated HeLa cells (290). Bassal et al. (23) observed that priming of bone marrow derived macrophages with LPS, TNF-α or GM-CSF enhances respiratory burst activity of these cells by suppressing the cellular PTP activity. A negative regulatory effect of protein tyrosine phosphatases has also been reported by several researchers (10, 107, 164). Inhibition of IFN-γ-induced STAT1 tyrosine phosphorylation by UV light was thought to be mediated by protein tyrosine phosphatase as vanadate treatment of cells, which inhibits tyrosine phosphatase activity, antagonized the inhibitory effect (10). Haque et al. (107) also reported a constitutive activation of cytokine signaling pathways by treatment of myeloblastoid and fibroblast cell lines with pervanadate. Similar results were published by another laboratory using primary mice astrocytes (164). In that study, treatment of cells with IFN-γ in the presence of vanadate prolonged STAT1 activation and transcription of the IRF-1 gene. Similar results were obtained using astrocytes from moth-eaten mice, which lack the protein tyrosine phosphatase SHP-1. In a recent study Knutson et al. (138) found that LAM from virulent M. tuberculosis promotes tyrosine dephosphorylation of multiple cellular proteins and attenuates PMA-induced activation of mitogen-activated protein kinase. They also showed that treatment of monocytes with LAM resulted in increased tyrosine phosphorylation of SHP-1 and its translocation to the membrane. Both the in vivo and in vitro assays indicated that LAM treatment of monocytes increases the phosphatase activity of SHP-1.

Recently the genes encoding negative regulators of JAK-STAT pathway have been cloned simultaneously by three different laboratories (68, 165, 166, 181, 258). They
have different names but have similar structures with a SH2 domain and a C-terminal domain, the CIS homology domain (165, 166). JAB (JAK binding protein), also called SOCS-1 (suppressor of cytokine signaling) and SSI-1 (STAT-induced STAT inhibitor-1) was induced by IL-4, IL-6, Leukemia inhibitory factor (LIF), G-CSF, in murine myeloid leukemia cells (M1 cells) (68, 181, 258). CIS1 (cytokine inducible SH2 protein1) was also induced by various cytokines in cytokine dependent hematopoietic cell lines and attenuated the JAK-STAT5 pathway by interacting with STAT5 docking sites on the IL-3 and erythropoietin receptors (166). CIS2 and CIS3 (named SOCS-2 and SOCS-3 in the mouse) are induced by IL-6 in mouse liver and IFN-γ, EPO, GM-CSF and IL-3 in mouse bone marrow cells and human cytokine dependent hematopoietic cell lines (165). CIS3, like JAB, bound the JAK2-JH1 domain, although the interaction was much weaker. Although the physiological functions of these negative regulators of cytokine signaling are not clearly defined, the studies suggest that these proteins act in a negative feedback loop to regulate cytokine signaling. Their expression in infected macrophages remains to be studied.

Immune complexes were shown to inhibit IFN-γ-induced expression of MHC class II and FcyR1 genes in human monocytes by inhibiting tyrosine phosphorylation of JAK kinases and STAT1 (80, 274). Ligation of FcyR by immune complex on macrophages also resulted in reduced expression of LPS-induced IL-12, both at mRNA and protein levels (262). The down-regulation of IL-12 was found to be due to influx of extracellular calcium. Morth and Kelsall (162) reported that ligation of CR3 with
antibody on the surface of human monocytes resulted in the down-regulation of IL-12 production and reduced tyrosine phosphorylation of STAT1.

2.5.2. Deactivation by immunosuppressive cytokines and prostaglandins.

2.5.2.1. Immunosuppressive Cytokines.

Cytokines play an important role in modulation of the host’s immune response to mycobacterial infection as well as other intracellular pathogens (9, 32, 64, 81, 271, 278). Intracellular pathogens are potent inducers of cytokine production by mononuclear phagocytes. Some of the cytokines produced, such as TGF-β and IL-10, have macrophage deactivating properties.

Transforming growth factor-β.

Three highly homologous isoforms of TGF-β (TGF-β1,3) genes are localized to different chromosomes in both human and mouse (152). TGF-β1 is secreted by all immune cells and is highly regulated in response to disease signals (131, 152, 257, 276). Besides immune cells, TGF-β is also secreted by many other cell types and exhibits a wide spectrum of immunomodulatory functions (152, 257, 276). The major immunomodulatory functions of TGF-β include down-regulation of proinflammatory cytokines (IL-1, IL-6 and TNF-α) and IFN-γ production (50, 72, 115, 179, 257). TGF-β also inhibits T and B cell proliferation and attenuates the cytotoxic activity of NK cells and T cells (115, 116, 131, 132, 224, 257, 261).
TGF-β is secreted by cells as a biologically inactive complex of 25 kDa homodimer of active TGF-β bound noncovalently to latency associated protein (LAP) (152, 257). Binding of TGF-β to its receptor requires dissociation of LAP, which is accomplished by interaction with proteolytic enzymes or alteration of the carbohydrate structure of LAP by glycosidases or sialidases (157, 174). Alveolar macrophages of rabbits injected with BCG have increased sialidase activity and secrete active TGF-β (105, 204). Extremely acidic or alkaline pH conditions also activate TGF-β (41).

Supernatant from monocytes of patients with active tuberculosis, which were stimulated in vitro with crude *M. tuberculosis* antigen, contained high concentrations of bioactive TGF-β (116). TGF-β is also expressed by Langerhans and epitheloid cells in the tuberculous pulmonary granulomas (268). Moreover, the spontaneous release of bioactive TGF-β is higher in monocytes from patients with active tuberculosis than in monocytes from healthy tuberculin reactors (115). An increase in mycobacterial counts has been observed by exogenous TGF-β treatment in both *in vivo* and *in vitro* experimental systems (62, 114, 196).

TGF-β has also been linked to the pathogenesis of other intracellular pathogen infections (20, 21, 29, 31). Mouse macrophages infected with *Mycobacterium avium*, *leishmania amazonensis*, *Trypanosoma cruzi*, and *Toxoplasma gondii* produce biologically active TGF-β (20, 29, 31). A direct correlation between the amount of TGF-β produced and virulence of the pathogens has been reported (21, 253). Enhanced
replication of *Trypanosoma cruzi* has been observed in infected mouse macrophages treated with TGF-β (253).

A suppressive effect of TGF-β on IFN-γ-induced expression of cell surface molecules, such as HLA-DR, ICAM-1, and VCAM-1, has been observed in various cell types (154, 182, 241). TGF-β knockout mice had up-regulation of proinflammatory cytokines and enhanced expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and MHC class II molecules (182). TGF-β also reduces the expression of Fc receptors on macrophages (154). The suppressive effect of TGF-β was more pronounced when used before or during IFN-γ-stimulation (154, 241).

A reduced expression of MHC class II molecules both at mRNA and protein level has been reported by many laboratories in mycobacteria infected monocytes and monocytes treated with TGF-β (149, 154, 197). Recent work by two different laboratories showed that the reduced expression of the HLA-DR gene in TGF-β treated monocytes in response to IFN-γ is due to selective attenuation of CIITA mRNA expression by TGF-β (149, 184). CIITA is an IFN-γ-inducible transcription factor required for HLA-DR transcription (158, 177, 219). Treatment of mononuclear phagocytes with rTGF-β did not effect the IFN-γ JAK-STAT cell signaling pathway nor induction of IRF-1, GBP-1, or ICAM-1 mRNA expression. These studies suggest the involvement of a TGF-β-sensitive accessory pathway in IFN-γ induction of CIITA mRNA (184, 197).
**Interleukin-10.**

IL-10 is another immunosuppressive and macrophage deactivating cytokine produced by a variety of cells (120). Monocytes are the predominant cell type responsible for the synthesis of IL-10 in the blood (53). IL-10 has been shown to negatively regulate macrophage functions in a variety of experimental systems (36, 134, 159, 161, 178, 193). It inhibits TNF-α production, a costimulatory cytokine required for IFN-γ-induced macrophage activation (193). IL-10 also inhibits proinflammatory cytokine (IL-1β, IL-6 and TNF-α) gene transcription in human peripheral blood mononuclear cells (280). These cytokines are required for clearance of mycobacterial infection (9, 64, 146).

IL-10 down-regulates the macrophage expression of MHC class II, ICAM-1 and B7 molecules that are required for antigen presentation to T lymphocytes (66, 159, 256, 286). IL-10 also decreases the respiratory burst and microbicidal activity of macrophages (36, 193). It has also been reported to reduce the production of nitric oxide by mycobacteria infected and LPS stimulated macrophages (161). Production of IL-10 has been observed in mycobacterial infection and infection with other intracellular pathogens (30, 134, 139, 254, 266). Both *in vivo* and *in vitro* studies indicate that neutralization of IL-10 with antibodies enhances the mycobacteriostatic activity of macrophages (30, 63). A study, by Murray *et al.* (178), found that IL-10 transgenic mice were unable to clear mycobacterial infection.
2.5.2.2. Prostaglandins.

Prostaglandins are produced by many cell types (42, 70, 284). Prostaglandin E\(_2\) (PGE\(_2\)), an immunosuppressive molecule, is produced from arachidonic acid metabolism by cyclooxygenase-1 and -2 (cox-1 and cox-2) isozymes (11, 58, 284). Both cox-1 and cox-2 are expressed in macrophages. Cox-1 expression is constitutive; whereas, cox-2 is induced by inflammatory stimuli such as LPS (11, 58, 70, 269). The immunosuppressive effects of PGE\(_2\) have been studied in many experimental models by either blocking endogenous PGE\(_2\) synthesis or by adding exogenous PGE\(_2\) (8, 111, 198, 221).

Endogenous PGE\(_2\) synthesis can be inhibited by pharmacological agents, such as indomethacin, that blocks cyclooxygenase activity (8, 111). Infection by mycobacteria and other intracellular and extracellular parasites induces PGE\(_2\) production (8, 69, 95, 111, 167, 211, 281). The increased levels of PGE\(_2\) enhance disease progression by reducing the expression of costimulatory molecules and production of NO and TNF-\(\alpha\) by macrophages (95, 103, 108, 124, 170, 226, 227). Deficiency in dietary essential fatty acids enhances cell mediated immune response by reducing PGE\(_2\) synthesis (4).

Besides down-regulation of iNOS expression, studies also indicate a stimulatory effect of PGE\(_2\) on NO production (108, 170). The stimulatory and inhibitory effects of PGE\(_2\) on iNOS expression are both time and dose dependent. Addition of exogenous PGE\(_2\) before or at the time of LPS-stimulation inhibited NO production, but a 3 hour delay had no effect. PGE\(_2\) in a dose range of 1-10 ng/ml increases iNOS expression; whereas, doses >50 ng/ml decrease NO synthesis (170). This effect was also observed in the J774.1 macrophage cell line with LPS-stimulation (108). A suboptimal dose of LPS
(0.1 µg/ml) induces endogenous PGE$_2$ synthesis that stimulates iNOS activity. However, higher LPS doses (1 µg/ml) produced higher levels of PGE$_2$ that reduced the levels of iNOS. Studies also indicate that LPS-induced NO production also regulates prostaglandin synthesis by down-regulating cox-2 at both the mRNA and protein level (106). A feedback regulatory suppression of cox-2 expression has also been observed in which elevated levels of cAMP induced by PGE$_2$ inhibits cox-2 induction (198).

A complex cross regulation between cytokines and PGE$_2$ has also been observed (11, 260). IFN-γ and TNF-α alone induce a moderate level of cox-2 mRNA in mouse macrophages and this effect is enhanced by stimulating IFN-γ primed macrophages with LPS or TNF-α (11). On the other hand, PGE$_2$ inhibits LPS-stimulated induction of TNF-α. This inhibitory effect of PGE$_2$ was mediated by IL-10 production as IL-10 neutralization by antibodies abrogated the inhibitory effect (260). Effect of PGE$_2$ is also cell type specific. It inhibits IL-12 production by macrophages but enhances production by dendritic cells (221). In dendritic cells, PGE$_2$ synergises with TNF-α to increase for IL-12 production. Effect of TGF-β on PGE$_2$ production is controversial since it enhances LPS-induced PGE$_2$ synthesis in rat microglial cells and inhibits PGE$_2$ synthesis in LPS-stimulated mouse macrophages (172, 214).
CHAPTER 3

MATERIALS AND METHODS

3.1. Mice.

Male BALB/c mice were purchased from Charles River (Wilmington, MA) at 5-6 weeks of age. The mice were housed in groups of five in isolation cages (Lab Products Inc., Maywood, NJ) and were provided food and water *ad libitum*.

3.2. Reagents.

Phosphatase inhibitors sodium orthovanadate (\(\text{Na}_3\text{VO}_4\)) and sodium fluoride (NaF); protease inhibitors aprotinin, ethylene diamine tetra-acetate (EDTA) and phenylmethylsulfonyl fluoride (PMSF); prostaglandin synthesis inhibitor indomethacin; and protein synthesis inhibitor cycloheximide were purchased from Sigma (St. Louis, MO). Protease inhibitors leupeptin and pepstatin were obtained from Boehringer Mannheim (Indianapolis, IN). Monoclonal antibodies to STAT1, and STAT5 were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibodies to STAT3 was obtained from Zymed Laboratories, Inc. (San Francisco, CA). Phospho-specific STAT1 antibody was from New England BioLabs, Inc.
(Beverly, MA). Anti-phosphotyrosine monoclonal antibody 4G10, rabbit anti-human JAK1, and rabbit anti-mouse JAK2 were obtained from Upstate Biotechnology (Lake Placid, NY). Affinity purified IFN-γRα polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies to IFN-γRβ, MOB47 and MOB55, were a gift from Dr. R. D. Schreiber, Washington University, St. Louis, MO. Rabbit anti-human TGF-β1 and goat anti-mouse IL-10 neutralizing antibodies were purchased from Promega Corporation (Madison, WI) and Sigma (St. Louis, MO), respectively. Peroxidase-conjugated affinity purified goat anti-hamster IgG was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY). Peroxidase-linked donkey anti-rabbit and sheep anti-mouse antibodies were obtained from Amersham (Arlington Heights, IL).

3.3. Macrophages.

The RAW 264.7 mouse macrophage cell line was obtained from American Type Culture Collection (ATCC TIB 71, Rockville, MD). Peritoneal macrophages were obtained by lavage of mice that had been injected with 4% thioglycollate broth (Difco laboratories, Detroit, MI) 4 days previously. Both peritoneal macrophages and RAW 264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 5mM sodium pyruvate, and penicillin-streptomycin at 37 °C in 5% CO₂. Culture media and all supplements were purchased from Gibco BRL (Gaithersburg, MD).
3.4. *Mycobacteria.*

*M. avium* (ATCC 35713) was grown in Middlebrook 7H9 broth supplemented with OADC (Difco Laboratories, Detroit, MI) at 37 °C in 5% CO$_2$ until mid-log phase. Bacteria were frozen in one ml aliquots in 10 % glycerol at -80 °C at the concentration of 2.38 x 10$^8$ cfu/ml. Frozen aliquots were thawed and briefly sonicated before each use. Heat killed bacteria were prepared by autoclaving at 121 °C for 20 minutes.

3.5. *Mycobacterium infection and IFN-γ-stimulation.*

The expression of IFN-γ-inducible genes was examined by Northern blot hybridization. RAW 264.7 cells or peritoneal macrophages were cultured in 6 well tissue culture plates for 6 hours at 5x10$^6$ cells per well. The cells were washed with Hank's balanced salt solution (HBSS) and infected overnight with *M. avium* at 5:1 to 50:1 bacteria:macrophage ratio in complete DMEM without antibiotics and fetal bovine serum. Non-phagocytized bacteria were removed by washing with HBSS and fresh antibiotic free DMEM was added to cells. The macrophages were stimulated with IFN-γ (100 units/ml) for 20 hours before isolation of RNA by the acid guanidinium isothiocyanate phenol chloroform extraction method of Chomczynski and Sacchi (52). For electrophoretic mobility shift assays and western blot analyses of protein, cells that were infected overnight were stimulated with IFN-γ (100-500 units/ml) for the times indicated in each experiment.
3.6. Preparation of cell free Supernatant from \textit{M. avium} infected macrophages.

RAW 264.7 macrophages were infected overnight at 10:1 bacteria to macrophage ratio in serum free media. Next morning, culture media was collected and mycobacteria were removed by filtering through a 0.22 \(\mu\)m filter. To inhibit ongoing protein synthesis in macrophages, cycloheximide (5 \(\mu\)g/ml) was added to the culture media during \textit{M. avium} infection \cite{37}. After overnight infection, the supernatant was collected and filtered. To inhibit prostaglandin E\(_2\) synthesis, indomethacin (10 \(\mu\)g/ml), which inhibits cyclooxygenase activity (IC\textsubscript{50} for cox-1 = 740 nM & for cox-2 = 970 nM), was added to the culture media during \textit{M. avium} infection \cite{8}. Supernatant was collected and filtered after overnight infection. The filtered supernatants were stored at -20 °C until further use.

3.7. IFN-\(\gamma\)-stimulation of macrophages treated with cell free supernatant from \textit{M. avium} infected macrophages.

RAW 264.7 cells were cultured in 6 well tissue culture plates at 5 \(\times\) 10\(^6\) cells per well for six hours. The cells were washed with HBSS and culture media was replaced with cell free supernatant from \textit{M. avium} infected cells. The cells were stimulated with IFN-(100 U/ml) for 20 hours following 30 minutes incubation with cell free supernatant from M. avium infected cells. RNA was then isolated for Northern blot analysis. To determine the minimum amount of supernatant from \textit{M. avium} infected macrophages that could inhibit the expression of IFN-\(\gamma\)-induced genes, cells were treated with various dilutions of supernatant and stimulated with IFN-\(\gamma\) (100 U/ml) for 20 hours. The macrophages were treated in a similar manner with supernatant generated in the presence
of cycloheximide and indomethacin. For immunoprecipitation and western blot analysis, cells were cultured overnight in presence of 1:4 dilution of supernatant from *M. avium* infected macrophages and then lysed with lysing buffer.

### 3.8. Neutralization of IL-10 and TGF-β in cell free supernatant from *M. avium* infected macrophages.

Polyclonal neutralizing anti-IL10 (50 μg/ml) and anti-TGF-β (10 μg/ml) antibodies were added to the supernatant from *M. avium* infected macrophages and incubated at 37 °C for 30 minutes before adding to macrophage culture. The cells were then stimulated with IFN-γ (100 U/ml) for 20 hours and RNA was isolated for Northern blot hybridization.

### 3.9. Northern blot hybridization.

RNA (10-25 μg/lane) was size fractionated in 1% formaldehyde agarose gel and transferred by capillary blotting onto Hybond-N+ membranes (Amersham). RNA ladder (0.24-9.5 Kb, Gibco BRL) was included in each gel and stained with ethidium bromide for RNA size determination. Northern blot hybridization were performed as described previously (147). Briefly, membranes were prehybridized for 2 hours at 42 °C in 50% formamide, 5X SSPE, 5X Denhart's solution, 0.5% SDS, and 100 μg/ml herring sperm DNA. Hybridization was completed overnight under the same conditions as prehybridization except that the hybridization solution contained 10% dextran sulfate and 1.5 x 10^6 cpm/ml of radiolabeled probes. The membranes were washed twice at 42 °C in
2X SSPE and 0.50% SDS. A third wash was performed at 65 °C in 0.50X SSPE and 0.50% SDS. Blots were exposed to X-rays films at -80 °C for appropriate times.


Probes to IFN-γR α and β chain genes, CIITA and IRF-1 were derived by RT-PCR of IFN-γ-stimulated macrophages using the following primers:

IFN-γRα sense: 5' -GGTTCCCTGGACTGATTCCCTGCACC- 3'
Anti-sense: 5' -AGTTCTTTCCCTGTCTGCTGCTTCGG- 3'
IFN-γRβ sense: 5' -TACACTTCTCCCTCCTCCCTTTTG-3'
Anti-sense: 5' -ACATCATCTCGCTCCTTTTCT- 3'
CIITA sense: 5' -CAAGTCCCTGAAGGATGTGGA- 3'
Antisense: 5' -ACGTCCATCACCAGGAGGAC- 3'
IRF-1 sense: 5' -CCAAGAGGAGCTGTGGAGGAG- 3'
Anti-sense: 5' -CAGCAGGCTGTCCATCCACATG- 3'

The IFN-γR α-chain primers were designed using PC/GENE (IntelliGenetics, Inc., Mount View, CA). The IFN-γRβ, IRF-1, and CIITA primer sequences were described previously (155, 169, 184). All the primers were synthesized by Gibco BRL. Identity of each probe was confirmed by DNA sequencing. cDNA inserts of Mg21, MHC class II gene Eβ, and G3PDH were isolated from a subtraction library of IFN-γ-stimulated macrophages. The probes were labeled with 32P-dCTP by high prime DNA labeling system (Boehringer Mannheim, Indianapolis, IN).
3.11. Nuclear extraction and electrophoretic mobility shift assay.

Nuclear extracts were prepared as described previously (187). Macrophages (1x10^7 cells per treatment) were washed twice with ice cold phosphate buffered saline (PBS) and incubated on ice for 15 minutes in 400 μl hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.10 mM EDTA, 1 mM DTT, 0.50 mM PMSF, 1 μg/ml aprotinin, and 1 mM sodium orthovanadate. The cells were then lysed by adding 25 μl of 10% Nonidet P-40 and brief vortexing. Nuclei were pelleted and extracted on ice for 15 minutes in 100 μl of buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinin, and 1 mM Na_3VO_4. Nuclear extracts were recovered from supernatants after centrifugation at 10,000 x g for 15 minutes. Protein concentration was determined by Bradford method using Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA). The extracts were assayed immediately for STAT1 activity or stored at -80 °C until further use.

Electrophoretic mobility shift assays were performed in 20 μl binding reactions containing 3 μg of nuclear extract, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 50 mM DTT, 5 mM MgCl_2, 10% glycerol, 0.20% NP-40, 1 μg poly (dI-dC), and 70,000 cpm of 32P-dCTP labeled GAS probe radiolabeled by filling with klenow DNA polymerase. The GAS probe (5′ AGCCATTTCCAGGAATCGAAA 3′) was derived from sequence of the Mg21 promoter (Lafuse, unpublished data) and contains a GAS site identical to the optimum GAS sequence (TTCCSGGAA) for STAT1 binding (119). Binding reactions were incubated for 20 minutes at room temperature and then subjected to electrophoresis on 5% polyacrylamide gels in 0.5X TBE buffer. The gels were dried and analyzed by
autoradiography. In competition assays, 100X unlabeled GAS or ISRE (Interferon Stimulated Response Element) probes were added along with radiolabeled GAS probe. The ISRE oligonucleotide (5' GATCGGAAAGGGAAACCGAACTGAAGC 3') was derived from the sequence of the ISG15 promoter (202). In supershift assays, 1 µg of STAT1 monoclonal antibody was incubated with binding reactions for 20 minutes prior to the addition of radiolabeled GAS probe.

3.12. Immunoprecipitation.

Immunoprecipitation of IFN-γR (α- and β-chains) and Janus kinases (JAK1 and JAK2) were performed as previously described with slight modifications (39, 184). For IFN-γ receptor immunoprecipitation, 2 x 10⁷ RAW264.7 cells were lysed on ice in lysing buffer consisting of 0.5% Nonident P-40 (NP-40), 50 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 1 mM Na₃VO₄, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 3 µg/ml aprotinin. The immunoprecipitation buffer for JAK kinases consisted of 1% Triton X-100, 50 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 50 mM NaF, 5 mM sodium pyrophosphate plus phosphatase and protease inhibitors as mentioned above. The samples were centrifuged at 10,000 x g for 10 minutes. The supernatants were cleared for 2-6 hours at 4 °C with recombinant protein G agarose (GIBCO BRL, Gaithersburg, MD) preincubated with normal rabbit serum. After removal of protein G agarose by centrifugation, the lysates were incubated with antibodies indicated in each experiment and protein G agarose for 6-18 hrs at 4 °C. The protein G agarose was then collected by brief centrifugation and washed four times with lysis
buffer. The immunoprecipitated proteins were then removed by boiling the washed protein G agarose with SDS sample buffer. For standard western blot analysis of STAT1 protein, $1 \times 10^7$ cells per treatment were lysed in buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol and phosphatase and protease inhibitors at 4°C.

3.13. Western blot analysis.

The samples were resolved by 8% SDS-PAGE, transferred with the Trans blot Semi Dry Transfer Cell (Bio-Rad) to PVDF membranes (Bio-Rad). The membranes were then blocked in 3% BSA in TBS containing 0.5% Tween-20 (TBST). The membranes were washed and incubated with primary antibodies STAT1 (1:2,000), Phospho-STAT1 (1:500), IFN-γRα (1:1,000), IFN-γRβ (1:1,000), JAK1 and JAK2 (1:4,000), and Phosphotyrosine 4G10 (1:2,000) followed by 1:5,000 dilution of anti-mouse, anti-rabbit or anti-hamster HRP-conjugated antibodies. The blots were developed using chemiluminescence kit (Amersham Arlington Heights, IL).
CHAPTER 4

RESULTS

4.1. Effect of *M. avium* infection on the expression of IFN-γ-inducible genes and the IFN-γ JAK-STAT signal transduction pathway.

4.1.1. *M. avium* infection inhibits expression of IFN-γ-inducible genes in mouse macrophages. We examined the expression of a panel of IFN-γ-inducible genes including MHC class II gene Eβ, CIITA, IRF-1, and Mg21, a gene coding for an intracellular protein with a GTP-binding motif (147). IFN-γ-stimulation of macrophages infected with *M. avium* resulted in the reduced expression of the IFN-γ-inducible genes when compared to non-infected cells. *M. avium* infection inhibited the expression of IFN-γ-inducible genes in both RAW 264.7 cells and peritoneal macrophages (Fig. 4.1). We found that the inhibitory effect of *M. avium* infection on expression of IFN-γ-inducible genes was dose dependent. A 10:1 mycobacteria to macrophage ratio was sufficient to inhibit the expression of IFN-γ-inducible genes in RAW 264.7 macrophages (Fig. 4.2). However, a higher mycobacteria to macrophage ratio of infection (50:1) was
required to inhibit expression of IFN-γ-inducible genes in peritoneal macrophages (Fig. 4.1).

4.1.2. Live and heat killed mycobacteria equally inhibit expression of IFN-γ-inducible genes. We also examined the effect of live vs heat killed *M. avium* on the expression of IFN-γ-inducible genes. Heat killed bacteria were as effective as viable mycobacteria in inhibiting the expression of IFN-γ-inducible genes (Fig. 4.3). To rule out the possibility that phagocytosis alone might be responsible for the inhibitory effect, we incubated RAW264.7 cells with sterile latex beads prior to stimulating the cells with IFN-γ. Phagocytosis of latex beads did not inhibit expression of IFN-γ-inducible genes (Fig. 4.4).

4.1.3. *M. avium* infection blocks STAT1 activation and tyrosine phosphorylation. Binding of IFN-γ to its cell surface receptor activates the JAK/STAT cell signaling pathway that leads to expression of IFN-γ-inducible genes (35, 61, 158, 177, 205). We examined the effect of *M. avium* infection on STAT1 activation and phosphorylation. Nuclear extracts prepared from mock infected cells and *M. avium* infected RAW 264.7 cells were analyzed for STAT1 activation by EMSA gels (Fig. 4.5A). Reduced STAT1 binding to the GAS element was observed in *M. avium* infected cells. The specificity of the GAS-STAT1 complex was confirmed by supershift analysis with STAT1 monoclonal antibody and by competing STAT1 binding with 100X unlabeled GAS probe. A nonspecific competitor ISRE, did not compete for binding. We also examined STAT1 activation in macrophages incubated with heat killed *M. avium*.
We found that heat killed bacteria equally inhibited binding of STAT1 to the GAS element (Fig. 4.5B). Experiments using thioglycollate elicited mouse peritoneal macrophages gave similar results (Fig. 4.6). However, an extra GAS binding activity appeared in *M. avium* infected thioglycollate elicited mouse macrophages. Mycobacteria infected macrophages secrete cytokines that also phosphorylate other STATs (33, 34, 47, 79). IL-6 preferentially induces phosphorylation of STAT3 (151). Recently activation of STAT5 by GM-CSF in LPS stimulated monocytes has been reported (288). However, our EMSA gels indicate that the extra band was not shifted by STAT3 or STAT5 monoclonal antibodies (Fig. 4.7 A&B).

Since phosphorylation of STAT1 is required for its activation, we also investigated the effect of *M. avium* on STAT1 phosphorylation. Western blot analysis with phospho-specific STAT1 antibody, which recognizes only phosphorylated STAT1, showed that phosphorylation of STAT1 was reduced. Western blot analysis, using STAT1 antibody on the same cell lysate, showed that there was a slight increase in total STAT1 protein expression in infected cells (Fig. 4.8).
Figure 4.1. *M. avium* infection inhibits induction of IFN-γ-inducible genes in mouse macrophages. RAW 264.7 cells and mouse peritoneal macrophages were infected with *M. avium* at 10:1 (bacteria:macrophages) or a 50:1 (mycobacteria:peritoneal macrophages) ratios of infection. After overnight infection, macrophages were stimulated with IFN-γ (RAW 264.7 cells, 100 U/ml and peritoneal macrophages, 50 U/ml) for 20 hours. RNA was isolated and Northern blots hybridized with Mg21, Eβ, CIITA, IRF-1, and G3PDH probes.
Figure 4.2. Inhibition of IFN-\(\gamma\)-inducible genes by \textit{M. avium} infection depends on mycobacteria to macrophage ratio of infection. RAW 264.7 macrophages were infected overnight with 5:1 to 50:1 bacteria to macrophage ratios and then stimulated with IFN-\(\gamma\) (100 U/ml) for 20 hours. RNA was isolated and Northern blots hybridized with Mg21, E\(\beta\), and G3PDH probes. Percent inhibition was calculated after densitometric analyses and normalization to levels of G3PDH mRNA.
Figure 4.3. Infection of mouse macrophages with live or heat killed M. avium resulted in a reduced expression of IFN-\(\gamma\)-inducible genes. RAW 264.7 macrophages were infected with live and heat killed M. avium overnight and then stimulated with IFN-\(\gamma\) (100 U/ml) for 20 hours. RNA was isolated and Northern blots hybridized with Mg21, Eβ, and G3PDH probes.
Figure 4.4. Phagocytosis of latex beads did not inhibit expression of IFN-γ-inducible genes. Sterile latex beads (2 μl/ml) were added to the RAW 264.7 cells overnight. Following removal of non-phagocytized beads, the cells were stimulated with IFN-γ (100 U/ml) for 20 hours. RNA was isolated and Northern blots probed with Mg21, IRF-1, and G3PDH probes.
Figure 4.5. Infection of RAW 264.7 macrophages with M. avium results in the inhibition of STAT1. (A). RAW 264.7 cells infected overnight with live M. avium were stimulated for 45 min with IFN-γ (100 U/ml). Nuclear extracts were prepared and incubated with $^{32}$P-labeled GAS sequence and binding assayed by EMSA gels. For competition experiments, nuclear extracts were incubated with 100-fold excess of unlabeled oligonucleotides as indicated. Nuclear extracts were preincubated with anti-STAT1 monoclonal antibody for 20 minutes prior to addition of the radiolabeled probe in order to identify the STAT1 protein. (B). RAW 264.7 cells were infected overnight with either live or heat killed M. avium and then stimulated with IFN-γ (100 U/ml) for 45 minutes. Nuclear extracts were prepared and binding to the $^{32}$P-dCTP labeled GAS sequence assayed by EMSA gel. Competition and antibody shift experiments were done as indicated. STAT1 in cells infected with either live or heat killed bacteria resulted in a reduced binding to the GAS sequence.
Figure 4.5
Figure 4.6. *M. avium* infection of thioglycollate elicited mouse peritoneal

macrophages inhibit STAT1 activation. Thioglycollate elicited mouse peritoneal

macrophages were infected overnight with *M. avium* at 50:1, bacteria to macrophage ratio

of infection. The cells were then stimulated with IFN-γ (50 U/ml) for 45 minutes.

Nuclear extracts were prepared and incubated with $^{32}$P-dCTP labeled GAS sequence and

binding assayed by EMSA gels. For competition experiments, nuclear extracts were

incubated with 100-fold excess of unlabeled oligonucleotides as indicated. Nuclear

extracts were pre-incubated with anti-STAT1 monoclonal antibody for 20 minutes prior

to addition of the radiolabeled probe in order to identify the STAT1 protein. Besides

STAT1, another GAS binding activity also appeared in peritoneal macrophages.
Figure 4.6
Figure 4.7. STAT3 or STAT5 did not shift the extra GAS binding activity in thioglycollate elicited mouse peritoneal macrophages. (A). Thioglycollate elicited mouse peritoneal macrophages were infected overnight with *M. avium* (50:1, bacteria to macrophage ratio) and the stimulated with IFN-γ (50 U/ml) for 45 minutes. Nuclear extracts were prepared and binding to the $^{32}\text{P}$-dCTP labeled GAS sequence assayed by EMSA gel. Antibody shift experiments were done as indicated. STAT1 in cells infected with *M. avium* resulted in a reduced binding to the GAS sequence. The extra GAS binding activity observed in thioglycollate elicited macrophages (Fig. 6) was not shifted by anti-STAT3 monoclonal antibody. (B). Thioglycollate elicited mouse peritoneal macrophages infected overnight with *M. avium* were stimulated for 45 min with IFN-γ (50 U/ml). Nuclear extracts were prepared and incubated with $^{32}\text{P}$-dCTP labeled GAS sequence and binding assayed by EMSA gels. For competition experiments, nuclear extracts were incubated with 100-fold excess of unlabeled oligonucleotides as indicated. Nuclear extracts were preincubated with anti-STAT1 or anti-STAT5 monoclonal antibodies for 20 minutes prior to the addition of the radiolabeled GAS probe in order to identify the STAT1 or STAT5.
IFN-γ
M. avium
STAT1

GAS binding activity?

Figure 4.7
Figure 4.8. *M. avium* infection reduced tyrosine phosphorylation of STAT1. RAW 264.7 cells were infected overnight with *M. avium* and then stimulated with IFN-γ (100 U/ml) for 45 minutes. Cell lysate was prepared and tyrosine phosphorylation analyzed with a phosphospecific-STAT1 antibody. The blot was then stripped and reprobed with STAT1 monoclonal antibody. A reduction of tyrosine phosphorylation of STAT1 was observed in *M. avium* infected cells.
4.1.4. *M. avium* infection results in reduced phosphorylation of IFN-γRα and Janus kinases. STAT1 activation requires phosphorylation of upstream components of the IFN-γ signal transduction pathway including IFN-γRα, JAK1, and JAK2 (35, 61, 122). A reduction of tyrosine phosphorylation of IFN-γRα, JAK1 and JAK2 proteins in RAW264.7 cells infected with *M. avium* for 16 hrs was observed (Fig. 4.9A). This suggests that reduced phosphorylation of STAT1 was the result of reduced phosphorylation of upstream JAK kinases and IFN-γRα required for STAT1 phosphorylation.

4.1.5. *M. avium* infection down-regulates the IFN-γ receptor protein and mRNA expression. Western blot analysis of protein showed that the levels of JAK1 and JAK2 did not change in infected cells (Fig. 4.9B). However, we found that the expression of IFN-γ receptor α and β chains was reduced in infected cells (Fig. 4.9A & 4.10). Northern blot analysis also indicated a decreased expression of IFN-γR α- and β-chain mRNA in *M. avium* infected macrophages beginning 4-8 hours after infection (Fig. 4.11).

4.1.6. Correlation between IFN-γ receptor down-regulation and STAT1 activation in *M. avium* infected macrophages. STAT1 tyrosine phosphorylation and activation by IFN-γ was examined in RAW 264.7 macrophages infected with *M. avium* in time course experiments. These experiments show that at least 4 hours of *M. avium* infection was required to reduce STAT1 tyrosine phosphorylation and activation (Fig. 4.12 A & B). These results are consistent with the results of time course experiments of IFN-γ Rα.
expression in *M. avium* infected cells. At least 4 hours of infection were required before a down-regulation of IFN-\(\gamma\) R\(\alpha\) was observed (Fig. 4.13).
Figure 4.9. *M. avium* infection of RAW 264.7 macrophages blocks tyrosine phosphorylation of JAK1 and JAK2 and induces down-regulation of IFN-γ Rα chain.

(A). RAW 264.7 macrophages infected overnight with *M. avium* were stimulated with IFN-γ (500 U/ml) for 15 minutes. Cell lysate was prepared and immunoprecipitated with JAK1, JAK2, and IFN-γ Rα antibodies. Western blots were prepared from the immunoprecipitated proteins and analyzed with the phosphotyrosine specific monoclonal antibody 4G10. A reduction of tyrosine phosphorylation of JAK1, JAK2, and IFN-γ Rα was observed in *M. avium* infected cells stimulated with IFN-γ. (B). The western blots probed with anti-phosphotyrosine antibody were stripped and reprobed with respective antibodies used for immunoprecipitation. The blots reveal equal loading of protein except for IFN-γRα which was down-regulated with *M. avium* infection.
Figure 4.9
Figure 4.10. *M. avium* infection results in reduced expression of IFN-γRβ in RAW 264.7 macrophages. Cell lysates were prepared from non-infected cells and from cells infected overnight with *M. avium*. IFN-γRα and β chains were immunoprecipitated using polyclonal and monoclonal antibodies respectively (see materials and methods). Western blot was prepared and analyzed with IFN-γRβ monoclonal antibodies, MOB-47 (0.5 μg/ml) and MOB-55 (0.5 μg/ml). Expression of IFN-γRβ was reduced in *M. avium* infected cells.
Figure 4.11. The expression of IFN-γR α and β chain mRNA was reduced in *M. avium* infected RAW 264.7 macrophages. RAW 264.7 cells were infected with *M. avium* for the indicated times (0-24 hours). Total RNA was isolated and analyzed by Northern blotting. The blot was probed with IFN-γRα, IFN-γRβ, and G3PDH probes. Both chains of IFN-γR were down-regulated at 4 hours onward. Percent inhibition was calculated after densitometric analysis and normalization to G3PDH mRNA levels.
Figure 4.12. Inhibition of STAT1 activation and tyrosine phosphorylation in *M. avium* infected cells is time dependent. (A). RAW 264.7 cells were infected for indicated times (0-16 hours) and then stimulated for 45 minutes with IFN-γ (100 U/ml). Nuclear extracts were prepared and incubated with $^{32}$P-dCTP labeled GAS sequence. STAT1 activation was assessed by EMSA. STAT1 activation was inhibited at 4 hrs with maximum effect at 16 hours. Percent inhibition was calculated from densitometric analysis. (B). RAW 264.7 macrophages were infected with *M. avium* for the indicated times and then stimulated for 45 minutes with IFN-γ (100 U/ml). Western blots were prepared from whole cell lysates and analyzed with phospho-specific anti-STAT1 antibody 1:500 dilution. The blot was stripped and reprobed with STAT1 monoclonal antibody (1:1,000 dilution) to confirm equal loading of protein. Densitometric analysis was performed and percent inhibition was calculated.
Figure 4.12

A

\[
\begin{array}{cccccc}
M. avium & - & - & 0.5 & 1 & 2 & 4 \\
\text{IFN-}\gamma & - & - & 8 & 16 \\
\text{hours} & & & & & & \\
\end{array}
\]

\[
\text{STAT1} \rightarrow
\]

STAT1 % inhibition: \(0\) \(0\) \(0\) \(0\) \(39\) \(47\) \(56\)

B

\[
\begin{array}{cccccc}
M. avium & - & - & 0.5 & 1 & 2 & 4 \\
\text{IFN-}\gamma & - & - & 8 & 16 \\
\text{hours} & & & & & & \\
\end{array}
\]

\[
p\text{Tyr-STAT1} \rightarrow
\]

\[
\text{STAT1} \rightarrow
\]

pTyr-STAT1 % inhibition: \(0\) \(0\) \(0\) \(0\) \(2\) \(59\) \(55\)
**Figure 4.13. Down-regulation of IFN-γRα in *M. avium* infected RAW 264.7 macrophages is time dependent.** RAW 264.7 cells were infected with *M. avium* for the indicated times (0-16 hours). Lysates were prepared from infected cells and immunoprecipitated with polyclonal anti-IFN-γRα antibodies. Western blot analysis with anti-IFN-γRα antibodies shows time dependent down-regulation of IFN-γRα in *M. avium* infected cells.
4.1.6. Summary.

1. *M. avium* infection of RAW 264.7 macrophages and thioglycollate elicited mouse peritoneal macrophages inhibits expression of IFN-γ inducible genes. The inhibitory effect was higher at 50:1 (bacteria:macrophage) ratio of infection compared to 5:1 ratio of infection (Figs. 4.1 & 4.2).

2. Infection of RAW 264.7 cells and mouse peritoneal macrophages with live and heat killed *M. avium* inhibits expression of IFN-γ-inducible genes and activation of STAT1 (Figs. 4.3, 4.5-4.7).

3. Phagocytosis of sterile latex beads did not inhibit the expression of IFN-γ-inducible genes (Fig. 4.4).

4. *M. avium* infection of RAW 264.7 macrophages reduces the tyrosine phosphorylation of IFN-γ receptor α-chain, STAT1 and JAK kinases (Figs. 4.8 & 4.9).

5. *M. avium* infection down-regulates the expression of IFN-γ receptor both at protein and mRNA level. However, levels of STAT1 and JAK kinases did not change (Figs. 4.8-4.13).

6. At least 4 hours of *M. avium* infection is required to down-regulate the IFN-γ receptor and STAT1 inhibition (Figs 4.12 & 4.13).
4.2. Effect of cell free supernatant from *M. avium* infected macrophages on the expression of IFN-γ-inducible genes and IFN-γ receptor.

4.2.1. Cell free supernatant from *M. avium* infected macrophages reduces expression of IFN-γ-inducible genes in macrophages. In order to determine if *M. avium* infected macrophages secrete autoinhibitory molecule(s), RAW 264.7 macrophages were treated with cell free supernatants derived from *M. avium* infected macrophages and then stimulated with IFN-γ. Cell free supernatant derived from *M. avium* infected macrophages inhibited expression of IFN-γ-inducible genes (Fig. 4.14). Supernatant from cultures infected with a 50:1 mycobacteria to macrophage ratio had more inhibitory activity compared to cultures infected with a 5:1 ratio of infection (Fig. 4.15). To determine the potency of inhibitory molecule(s), macrophages were treated with various dilutions of this cell free supernatant for 30 minutes followed by stimulation with IFN-γ (100 U/ml). A 1:5 dilution (400 μl of supernatant in 2 ml of culture media) was sufficient to inhibit expression of IFN-γ-inducible genes (Fig. 4.16).

4.2.2. Supernatant from *M. avium* infected macrophages down-regulates IFN-γ receptor. The results of present studies indicate that *M. avium* infected macrophages down-regulate the IFN-γR mRNA and protein expression (Figs. 4.9-4.11, 4.13). To determine if supernatant from infected macrophages also down regulates IFN-γR expression, RAW 264.7 macrophages were treated overnight with cell free supernatant
from *M. avium* infected macrophages and IFN-γR expression was measured by immunoprecipitation and western blotting. A reduced expression of IFN-γR α- and β-chain protein expression was observed in macrophages treated with cell free supernatant from *M. avium* infected macrophages (Figs. 4.17 & 4.18).
Figure 4.14. Inhibition of IFN-γ-inducible genes by cell free supernatant from *M. avium* infected macrophages. RAW 264.7 macrophages were treated for 30 minutes with cell free supernatant from *M. avium* infected macrophages and then stimulated with IFN-γ (100 U/ml) for 20 hours. RNA was isolated and Northern blots hybridized with Mg21, Eβ, and G3PDH cDNA probes.
Figure 4.15. Potency of cell free supernatant to inhibit expression of IFN-γ-inducible genes depends on mycobacteria to macrophage ratios of infection. RAW 264.7 macrophages were treated with cell free supernatant obtained from different mycobacteria to macrophage ratios of infection as indicated for 30 minutes. The cells were then treated with IFN-γ (100 U/ml) for 20 hours. RNA was isolated and Northern blots hybridized with Mg21, Eβ, and G3PDH cDNA probes. Percent inhibition was calculated after densitometric analysis and normalization to levels of G3PDH mRNA.
Fig. 4.16. Cell free supernatant from M. avium infected macrophages has a potent inhibitory molecule(s). RAW 264.7 macrophages were treated with various dilutions of supernatant from M. avium infected macrophages as indicated for 30 minutes. The cells were then stimulated with IFN-γ (100 U/ml) for 20 hours. RNA was isolated and Northern blots hybridized with Mg21, Eβ and G3PDH cDNA probes. A 1:5 dilution is sufficient to mimic the inhibitory effect of 10:1 bacteria to macrophage ratio of infection.
Figure 4.17. Cell free supernatant from *M. avium* infected macrophages down-regulates expression of IFN-γR α-chain. RAW 264.7 macrophages were treated overnight with undiluted cell free supernatant from *M. avium* infected macrophages. Cell lysate was prepared and immunoprecipitated with IFN-γR α-chain polyclonal antibodies. The immunoprecipitated IFN-γRα was detected by western blotting using 1:1000 dilution of IFN-γRα polyclonal antibodies.
Figure 4.18. Cell free supernatant from *M. avium* infected macrophages inhibits expression of IFN-γ R β-chain. RAW 264.7 macrophages were treated overnight with cell free supernatant from *M. avium* infected macrophages. Cell lysate was prepared and immunoprecipitated with MOB-47 and MOB-55 monoclonal antibodies against IFN-γ R β-chain. The IFN-γ Rβ was then detected by western blotting using MOB-47 and MOB-55 monoclonal antibodies at 1 μg/ml concentration.
4.2.3. The inhibitory molecule(s) is not prostaglandin $E_2$. Prostaglandin $E_2$ is a macrophage deactivating molecule and is secreted by activated macrophages (42, 70, 95, 284). We examined macrophage gene induction by treating the cells with cell free supernatant generated in the presence of indomethacin (10 $\mu$g/ml). Indomethacin inhibits the activity of cyclooxygenase-1 and -2 (cox-1 & cox-2) isozymes, which metabolize arachidonic acid into prostaglandins (8, 11, 58, 284). We observed no restoration in mRNA expression of IFN-γ-inducible genes in cells treated with this supernatant, suggesting that $PGE_2$ is not involved in inhibiting the expression of IFN-γ-inducible genes (Fig. 4.19).

4.2.4. Neither TGF-β nor IL-10 are involved in inhibiting the induction of IFN-γ-inducible genes. TGF-β and IL-10 are macrophage deactivating cytokines and are secreted by mycobacteria infected macrophages (30, 115, 268). We examined involvement of these cytokines in inhibition of IFN-γ-inducible genes in mouse macrophages treated with supernatant from $M. avium$ infected macrophages. We incubated the supernatant from $M. avium$ infected macrophages with neutralizing anti-TGF-β or anti-IL-10 antibodies for 30 minutes at 37 °C before treating macrophages with it. The anti-TGF-β and anti-IL-10 neutralized supernatant did not abrogate the inhibitory effect suggesting that these cytokines are not involved in inhibition of IFN-γ-inducible genes (Fig. 4.20).
Figure 4.19. Prostaglandin E₂ is not involved in inhibiting expression of IFN-γ-inducible genes. RAW 264.7 macrophages were treated with cell free supernatant obtained from macrophages infected with M. avium in the presence of indomethacin (10 μg/ml) for 30 minutes. The cells were then stimulated with IFN-γ (100 U/ml) for 20 hours. RNA was isolated and Northern blots hybridized with Mg21, Eβ, and G3PDH cDNA probes.
Figure 4.20. *TGF-β and IL-10 are not involved in inhibiting the expression of IFN-γ-inducible genes.* RAW 264.7 macrophages were treated for 30 minutes with supernatant neutralized with anti-TGF-β (10 μg/ml) and anti-IL-10 (50 μg/ml) antibodies. The cells were then stimulated with IFN-γ (100 U/ml) for 20 hours. RNA was isolated and Northern blots hybridized with Mg21, Eβ, and G3PDH cDNA probes.
4.2.5. *Induction of inhibitory molecule(s) requires protein synthesis.* We generated cell free supernatant from *M. avium* infected macrophages cultured in the presence of cycloheximide (5 μg/ml), a protein synthesis inhibitor (37). Treatment of macrophages with this supernatant did not inhibit expression of IFN-γ-inducible genes (Fig. 4.21A). The macrophages were also treated with various dilutions of cell free supernatant generated in the presence of cycloheximide. Expression of Mg21 was not inhibited at any dilution. However, we did find a reduced expression of MHC class II gene Eβ at 1:2 dilution (Fig. 4.21B). This inhibition at 1:2 dilution is most likely due to carryover of cycloheximide since IFN-γ induction of MHC class II but not Mg21 is inhibited by cycloheximide (147).
Figure 4.21. Induction of inhibitory factor(s) requires protein synthesis. (A). RAW 264.7 macrophages were treated for 30 minutes with cell free supernatant from *M. avium* infected macrophages generated in the presence of cycloheximide (5 μg/ml). The cells were then stimulated with IFN-γ (100 U/ml) for 20 hours before RNA isolation. Northern blots were prepared and hybridized with Mg21, Eβ, and G3PDH cDNA probes. (B). RAW 264.7 cells were treated for 30 minutes with various dilutions of cell free supernatant from *M. avium* infected macrophages generated in presence of cycloheximide as indicated. The cells were then stimulated with IFN-γ (100 U/ml) for 20 hours. RNA was isolated and Northern blots hybridized with Mg21, Eβ, and G3PDH cDNA probes.
Figure 4.21
4.2.6. Summary.

1. Cell free supernatant from *M. avium* infected macrophages inhibits expression of IFN-γ-inducible genes. The culture supernatant from the infected macrophages was very potent and a 1:5 dilution was sufficient to mimic the inhibitory effect of 10:1 (bacteria:macrophage) ratio of infection (Figs. 4.14-4.16).

2. Cell free supernatant reduces the expression of IFN-γ receptor α- and β-chains (Figs. 4.7 & 4.18).

3. The inhibition of IFN-γ-inducible gene expression is not mediated by prostaglandins as supernatant generated in the presence of indomethacin did not abrogate the inhibitory effect (Fig. 4.19).

4. The inhibitory effect of cell free culture supernatant from *M. avium* infected cells is not neutralized by anti-TGF-β or anti-IL-10 antibodies, suggesting the inhibitory factor(s) is not these macrophage deactivating cytokines (Fig. 4.20).

5. The inhibitory factor(s) in the culture supernatant of *M. avium* infected macrophages is protein in nature as supernatant generated in the presence of cycloheximide lacks the inhibitory effect (Fig. 4.21).
CHAPTER 5

DISCUSSION

5.1. Effect of M. avium infection on the expression of IFN-γ-inducible genes and the JAK-STAT cell signaling pathway.

The results of this investigation demonstrate a novel mechanism by which mycobacteria may avoid immune surveillance and establish a chronic infection. The results show that M. avium infection of mouse macrophages inhibits expression of IFN-γ-inducible genes by down-regulation of the IFN-γR required for activation of the JAK-STAT signal transduction pathway (18, 35, 61). Our observations that M. avium infection inhibits expression of IFN-γ-inducible genes are consistent with previous findings that the expression of MHC class II genes and protein is reduced in lipoarabinomannan (LAM) treated and mycobacterium infected macrophages (48, 55, 117, 130, 173, 175, 195). We found the expression of MHC class II gene Eβ to be most sensitive to the inhibition that resulted from M. avium infection. This was probably a cumulative effect since expression of IFN-γ-inducible genes CIITA and IRF-1, required for the induction of the MHC class II gene Eβ, are inhibited by M. avium infection (118, 158, 219). CIITA is required for MHC class II gene transcription (158, 219) and a binding site for IRF-1 in the promoter
of CIITA has been described recently (177). A GAS sequence required for IFN-γ induction is also present in the promoters of both IRF-1 and CIITA (177, 205).

The global inhibition of IFN-γ-inducible genes by *M. avium* infection suggests that the inhibition may lie somewhere in the IFN-γ cell signaling pathway. IFN-γR (α and β chains), JAK kinases (JAK1 & JAK2), and STAT1 are the key components of this pathway (18, 35, 61, 122, 192). IFN-γ binds to IFN-γR α chain but signaling only occurs in presence of an intact β chain (16, 18). The α-chain has been shown to exist in macrophages both on the cell surface and in a large intracellular pool (18). IFN-γ binding to cell surface receptors results in internalization of receptor-ligand complex. This complex then enters an acidified compartment where the complex dissociates and free IFN-γ is trafficked to the lysosomes for degradation (83). The uncoupled receptor α-chain enters a large intracellular pool of α-subunits and eventually recycles back to the cell surface (46). Using western blot analysis we showed that there was a decrease in IFN-γR α- and β- chain protein in *M. avium* infected macrophages. It is possible that *M. avium* infection of macrophages may interfere with recycling of IFN-γR or enhance its degradation. We also found that *M. avium* infection reduces mRNA levels of both IFN-γR α- and β- chains. Thus, infection may also result in altered transcription of IFN-γR genes or in the altered stability of their mRNA's.

JAK1 and JAK2 are cytoplasmic tyrosine kinases and are associated with IFN-γ receptor cytoplasmic domains. The levels of tyrosine phosphorylation of JAK1 and JAK2 decreased in *M. avium* infected macrophages concomitant with a down-regulation
of the IFN-γR. However, there was no change in the level of JAK1 and JAK2 expression. IFN-γ stimulates only the autophosphorylation of JAK1 and JAK2 that are associated with cytoplasmic domains of IFN-γR. This observation is consistent with the association of JAK1 and JAK2 with cytoplasmic domains of other cytokines and growth factor receptors (185, 199, 222), that may not be affected by M. avium infection.

Recently negative regulators of JAK-STAT pathway have been identified (68, 165, 166, 181, 258). They have SH2 domains which can interfere with the STATs binding sites on the cytokine receptors (165, 166). JAB (JAK binding protein), also called SOCS-1 (suppressor of cytokine signaling) and SSI-1 (STAT-induced STAT inhibitor-1), was induced by IL-4, IL-6, Leukemia inhibitory factor (LIF), and G-CSF in murine myeloid leukemia cells (M1 cells) (68, 181, 258). Another JAK binding protein, CIS1 (cytokine inducible SH2 protein 1) was also induced by various cytokines in cytokine dependent hematopoietic cell lines and attenuated the JAK-STAT5 pathway by interacting with STAT5 docking sites on the IL-3 and erythropoietin receptors (166). CIS2 and CIS3 (named SOCS-2 and SOCS-3 in the mouse) are induced by IL-6 in mouse liver and IFN-γ, EPO, GM-CSF and IL-3 in mouse bone marrow cells and human cytokine dependent hematopoietic cell lines. CIS3, like JAB, bound the JAK2-JH1 domain, although the interaction was much weaker than observed with JAB. Although the physiological functions of these negative regulators of cytokine signaling are not clearly defined, the studies suggest that these proteins act in a negative feedback loop to regulate cytokine signaling. Induction of these negative regulators by M. avium remains to be determined. However, mycobacteria infected macrophage secrete IFN-γ, GM-CSF,
and IL-6 which are known to induce JAK binding proteins in murine myeloid leukemia cells and bone marrow cells (165). In the present studies, the cytokines produced by the infected macrophages might be responsible for induction of JAK binding protein(s) which might have some inhibitory effect on the JAK-STAT signaling.

There is very little information about negative regulation of the IFN-γ receptor. Studies have shown that cytokines can alter the expression of IFN-γR in various cell types (142, 246, 270, 289). IL-1, IL-6 and TNF-α, which are produced by infected macrophages and are involved in controlling mycobacterial growth (9, 64, 146), have been shown to enhance IFN-γR expression on human monocytes (142, 229, 270). However, macrophages have been shown to respond to IFN-γ in presence of these cytokines (74, 153, 188, 272). Mycobacteria infected macrophages can also produce IFN-γ (82). IFN-γ enhances the expression of IFN-γR on the U937 monocytic cell line (246), but suppresses the expression of IFN-γR β-chain on T lymphocytes (16, 228). A reduction in expression of the IFN-γR together with enhanced production of IFN-γ has also been observed in peripheral blood mononuclear cells of individuals suffering from chronic renal disease (289). A similar negative correlation in production of a cytokine (TNF-α) and the expression of its receptor on splenocytes from M. avium infected mice has also been observed (47). Whether enhanced production of IFN-γ and TNF-α are involved in down-regulation of respective receptors is not known.

Infection of macrophages by other intracellular pathogens has also been shown to affect responses to IFN-γ (129, 135, 144, 150, 183, 243, 287). Cryptococcus neoformans infection of mouse macrophages results in inhibition of nitric oxide production following
stimulation with IFN-γ and LPS (129). Prior ingestion of heat killed *Histoplasma capsulatum* also renders macrophages unresponsive to IFN-γ-stimulation (287). A reduced expression of MHC class I and MHC class II genes has been observed in *Listeria monocytogenes* infected mouse macrophages (243). *Leishmania donovani* infected monocytes show decreased expression of MHC class II protein and reduced tyrosine phosphorylation of STAT1 and JAK kinases (183, 216). A similar inhibitory effect of IFN-γ-induced tyrosine phosphorylation of STAT1 and JAK kinases has been reported in *Ehrlichia chaffeensis* infected human monocytes (150). The inhibitory effect in that study was immediate and did not require IFN-γR down-regulation and phagocytosis of the pathogen. Our findings indicate that the inhibition of JAK-STAT signaling by *M. avium* infection was a result of the decreased expression of IFN-γR. *Trypanosoma cruzi* infection of human PBMC has also been reported to induce a down-regulation of IFN-γR in B lymphocytes without affecting expression of MHC class II antigen (135). The reduced expression of the IFN-γR was observed at 3 hrs and lasted at least for 24 hrs. These data are consistent with our observation that down-regulation of IFN-γR occurs by 4 hrs in *M. avium* infected macrophages and can be observed for at least 24 hours.

Although down-regulation of IFN-γR expression following *M. avium* infection appears to be a major cause for the attenuation of the JAK-STAT pathway, participation of phosphotyrosine phosphatases cannot be ruled out. The protein tyrosine phosphatase (SHP-1) is associated with several cytokine receptors and has been implicated with down-regulation of ligand-induced signaling through dephosphorylation of the activated JAKs (107, 164). A recent study by Knutson *et al.* (138) indicates that LAM, a mycobacterial
cell wall glycolipid, promotes tyrosine dephosphorylation and inhibition of mitogen activated protein (MAP) kinase activity in human monocytes. This dephosphorylation of proteins was the result of activation of SHP-1. LAM has also been known to inhibit IFN-\(\gamma\)-mediated macrophage activation (251), but its role in down-regulation of IFN-\(\gamma\)R has not been reported.

5.2. Effect of cell free supernatant from M. avium infected macrophages on the expression of IFN-\(\gamma\)-inducible genes and the expression of IFN-\(\gamma\)receptor.

The results of this study describe an inhibitory factor(s) released by M. avium infected macrophages into the culture supernatant, which reduces the expression of IFN-\(\gamma\) inducible genes and IFN-\(\gamma\) receptor \(\alpha\)- and \(\beta\)-chains. These results are consistent with a previous finding where cell free supernatant from T. cruzi cultures containing trypanosomal immunosuppressive factor (TIF) reduced the expression of IFN-\(\gamma\) receptor on B lymphocytes (135). In contrast, the cell surface expression of HLA-DR molecules did not change in that study. This difference in MHC class II expression can be explained by MHC class II expression being constitutive in B cells and IFN-\(\gamma\)-inducible in macrophages (26).

Macrophages infected with intracellular pathogens secrete many immunosuppressive factors (167, 232, 275). Most of these factors suppress mitogen induced T cell blastogenesis, but their macrophage deactivating properties have never been tested. Cytokines (TGF-\(\beta\) and IL-10) and prostaglandin E\(_2\) are the only known immunosuppressive factors which are secreted by infected macrophages and have
macrophage deactivating properties (232). TGF-β and IL-10 exert their immunosuppressive effect by reducing the production of proinflammatory cytokines and expression of costimulatory molecules (50, 66, 152, 154, 159, 182, 256, 280, 286). The production of TGF-β and IL-10 by infected macrophages may account for our observations (184, 197). TGF-β has been shown to reduce IFN-γ binding to macrophages (206). However, we found that neutralization of TGF-β and IL-10 with antibodies did not abrogate the inhibitory effect of M. avium infection on macrophage gene expression. This suggests that the inhibitory factor is not TGF-β or IL-10. This finding is consistent with previous studies that showed that treatment of human U937 monocytic cells and rat astrocytes with recombinant TGF-β does not alter the phosphorylation of JAK1, JAK2, and STAT1 following treatment with IFN-γ (184, 197). TGF-β was shown in those studies to completely inhibit the induction of CIITA and MHC class II mRNA, without affecting IFN-γ induction of GBP-1, IRF-1, or ICAM-1 gene expression. Another study described reduced IFN-γ-induced expression of indoleamine 2, 3-dioxygenase and tryptophanyl-tRNA synthetase genes in human fibroblasts by TGF-β without affecting the JAK-STAT signal transduction pathway (291). The observation by Song et al. (256), that treatment of human monocytes with rIL-10 does not alter the phosphorylation of STAT1, also supports our observation that IL-10 is not responsible for the effect of mycobacterial infection on IFN-γ-induced gene expression.

PGE₂ induces immunosuppression by reducing the expression of costimulatory molecules, and reducing the production of nitric oxide and TNF-α (95, 108, 170, 226,
The results of the present study suggest that the inhibitory factor released by *M. avium* infected macrophages is not PGE$_2$, as supernatant generated in the presence of indomethacin did not abrogate the inhibitory effect. Release of an indomethacin resistant suppressive factor by *M. leprae* infected monocytes has also been described previously (232). The results of the present study using cycloheximide as protein synthesis inhibitor also suggest that the inhibitory factor secreted by *M. avium* infected macrophages is a protein.

**Conclusion.**

We have demonstrated that *M. avium* infection inhibits expression of IFN-γ-inducible genes in mouse macrophages. Moreover, the infected macrophage secretes inhibitory factor(s) that mimic the inhibitory effect of *M. avium* infection. This inhibitory effect is due in part to the down-regulation of the IFN-γ receptor resulting in decreased JAK-STAT signaling. However, the involvement of other inhibitory components of the JAK-STAT pathway, tyrosine phosphatases and JAK-binding protein(s), can not be ruled out. Proposed defective JAK-STAT pathway by *M. avium* infection is presented in figure 5.1. Our findings represent one mechanism by which mycobacteria are capable of avoiding immune surveillance and establishing chronic infection.
Figure 5.1. Model for *M. avium* inhibition of the IFN-γ JAK-STAT pathway and gene induction. *M. avium* infection reduces the expression of IFN-γ receptor α- and β-chains that results in reduced tyrosine phosphorylation of JAK kinases and STAT1. Reduced STAT1 phosphorylation results in reduced STAT1 homodimerization and binding to the GAS sequences in the promoters of the IFN-γ-inducible genes. The highest inhibitory effect we observed in the induction of MHC class II gene Eβ, is believed to be the cumulative inhibitory effect of IRF-1 and CIITA induction. Both IRF-1 and CIITA are involved in the induction of MHC class II genes. At present the negative regulatory role played by tyrosine phosphatases and JAK-binding protein(s) in IFN-γ JAK-STAT signal transduction pathway is not known.
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