MECHANISMS OF IMMUNITY AND IMMUNE EVASION IN CUTANEOUS LEISHMANIASIS

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By
Neeti Bhardwaj, M.D.

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The Ohio State University
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Master's Examination Committee:
Dr. Abhay R. Satoskar, Adviser
Dr. Richard F. Mortensen
Dr. Paula W. Bryant
Dr. William P. Lafuse

Approved by

Adviser
Graduate Program in Microbiology
Leishmania are obligate intracellular parasites that infect macrophages and cause a wide range of diseases such as cutaneous, mucocutaneous and visceral leishmaniasis in many parts of the world. Although macrophages are the primary target cells for Leishmania, they are also involved in host defense against this parasite by their ability to kill Leishmania by producing nitric oxide and super oxide. Additionally, macrophages function as antigen-presenting cells, and produce cytokines such as TNF-α and IL-12, which play a critical role in development of innate and acquired immunity against Leishmania. Previous studies have demonstrated that Leishmania donovani attenuates STAT1-mediated signaling in macrophages; however it is not clear whether other species of Leishmania, which cause cutaneous disease, also interfere with macrophage IFN-γ signaling. Therefore, we determined the effect of L. major and L. mexicana infection on STAT1-mediated IFN-γ signaling pathway in J774A.1 and RAW264.7 macrophages. We found that both Leishmania major and Leishmania mexicana suppressed IFNγRα (α subunit of interferon gamma...
receptor) and IFN-γRβ (β subunit of interferon gamma receptor) expression, reduced levels of total Jak1 and Jak2, and down-regulated IFN-γ-induced Jak1, Jak2 and STAT1 activation. The effect of *L. mexicana* infection on Jak1, Jak2 and STAT1 activation was more profound when compared to *L. major*. Although tyrosine phosphorylation of STAT1α was decreased in IFN-γ stimulated macrophages infected with *L. major* or *L. mexicana*, those infected with *L. mexicana* showed a significant increase in phosphorylation of the dominant negative STAT1β. These findings indicate that *L. major* and *L. mexicana* attenuate STAT1-mediated IFN-γ signaling in macrophages. Furthermore, they also demonstrate that *L. mexicana* preferentially enhances tyrosine phosphorylation of dominant negative STAT1β, which may be one of the several survival mechanisms used by this parasite to evade the host defense mechanisms.

We further investigated the role of STAT1 signaling in protection against cutaneous leishmaniasis caused by *L. major*. Previous studies have demonstrated that STAT1 is crucial to the development of protective immunity against *L. major*. We further dissected the role of STAT1 mediated signaling in lymphocytes by performing lymphocyte transfer from STAT1<sup>+/+</sup> and STAT1<sup>−/−</sup> mice to Rag2<sup>−/−</sup> mice (deficient in T and B cell development). The Rag2<sup>−/−</sup> mice reconstituted with cells form STAT1<sup>−/−</sup> mice developed large, ulcerating lesions as compared to those transferred with STAT1<sup>+/+</sup> cells. Also footpad and lymph node parasite burdens were found to be higher in the former category. IFN-γ levels
were much lower in the mice receiving STAT1\textsuperscript{−/−} cells indicating that progressive disease in these mice can be attributed to a failure to develop Th1 response. We further investigated the role of STAT1 signaling in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. We found that STAT1 in CD4 T cells is critical to the control of \textit{L. major} infection and that STAT1 in macrophages alone is not sufficient to contain the infection. These data together point towards the critical role that STAT1 plays in immune response to \textit{Leishmania} and also the clever strategies the parasite has evolved to survive inside the host. Taken together, these findings may have implications for development of a vaccine against the parasite.
Dedicated to my husband and daughter
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VITA

January, 1999.................. Bachelor of Medicine & Bachelor of Surgery (M.B.B.S)

June, 2002...................... Doctor of Medicine (M.D)

Sept 2002- present............ Graduate Teaching & Research Associate, The Ohio State University

PUBLICATIONS


FIELDS OF STUDY

Major Field: Microbiology
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CHAPTER 1

INTRODUCTION

Leishmaniasis is a vector-borne disease caused by obligate intracellular protozoa of the genus *Leishmania*. About 21 to 30 species that infect mammals infect humans. The protozoa are transmitted by the bite of a tiny 2 to 3 millimeter-long insect vector, the phlebotomine sandfly. The disease is prevalent in areas where enough sandfly vectors and mammalian reservoirs exist to permit frequent transmission (Herwaldt, 1999). The leishmaniases comprise one of the six tropical afflictions on the World Health Organization/Tropical Disease Research list of most important diseases. The increased interest in leishmaniasis in the industrially developed world is the result of the travel medicine in this era of facilitated international travel and the inclusion of visceral leishmaniasis as a component of AIDS in Mediterranean countries where the two infections co-exist (Berman, 2001). There are two morphological stages of the parasite: promastigote form which is the flagellated form found in sandflies and culture, and the amastigote form which is the nonflagellated tissue form and replicates in macrophage phagosomes in mammalian hosts. Only the female sandfly
transmits the protozoa, infecting itself with the *Leishmania* parasites contained in the blood it sucks from its human or mammalian host. Leishmaniasis is endemic in areas of the tropics, subtropics and southern Europe (Herwaldt, 1999).

**1.1 Historical information**

Russell described cutaneous leishmaniasis as a clinical entity in 1856. It was Cunningham, working in India in 1885, however, who correctly described the *Leishmania* organism he observed in a fixed section of a skin lesion (Despommier et al., 1994). Finally Adler and Theodor demonstrated that sandflies infected in the laboratory could transmit *Leishmania tropica* (causative agent of cutaneous leishmaniasis) to human volunteers. Carini in 1911 differentiated mucocutaneous lesions from the cutaneous types. The following year Vianna identified and named *Leishmania braziliensis* as the causative agent of mucocutaneous leishmaniasis. The causative agent of visceral leishmaniasis was discovered simultaneously in 1903 by two physicians, Leishman and Donovan, while they were working in India. Sir Ronald Ross named the genus and species after them in recognition of their landmark discovery. Swaminath and colleagues working in India in 1942 proved in human volunteers that the infection was transmitted by the *Phlebotomus* flies (Despommier et al., 1994).
Figure 1.1 The life-cycle of *Leishmania*. The sandfly vector becomes infected when feeding on the blood of an infected individual or an animal reservoir host. The *Leishmania* parasites live in the macrophages as round, non-motile amastigotes (3-7 micrometers in diameter). The macrophages are ingested by the fly during the blood-meal and the amastigotes are released into the stomach of insect. The amastigotes transform into the motile, elongated (10-20 micrometers), flagellate promastigote form. The promastigotes then migrate to the alimentary tract of the fly, where they live extracellularly and multiply by binary fission. Four to five days after feeding the promastigotes move forward to the oesophagus and the salivary glands of the insect. When the sandfly next feeds on a mammalian host, the *Leishmania* promastigotes are transferred to the host along with the saliva. Once in the host the promastigotes are taken up by the macrophages where they rapidly revert to the amastigote form. The *leishmania* multiply inside the macrophages, eventually leading to the lysis of the macrophages. The released amastigotes are taken up by additional macrophages and so the cycle continues.
Figure 1.1
1.2 Life Cycle

The life-cycle of the parasite is depicted in Figure 1.1. The sandfly is the vector of leishmaniasis. It is a small dipteran, whose flight range is short (Despommier et al., 1994). Only the adult females bite, and the blood they ingest is used for egg production. Sandflies become infected by biting an infected individual. The amastigotes (1-3um in diameter present in the macrophages) are ingested by the fly and become disrupted in its midgut. Thus the amastigotes are released and almost immediately transformed into promastigotes. The promastigotes migrate to the gut and divide by binary fission. Ultimately, the promastigotes migrate to the proboscis, where they differentiate from a noninfective to an infective form, sometimes referred to as a metacyclic stage. Humans acquire leishmaniasis when the metacyclic stage of the parasite enters the individual during a sandfly bite (Despommier et al., 1994).

1.3 Various forms of leishmaniasis

The leishmaniases are parasitic diseases with a wide range of clinical symptoms: cutaneous, mucocutaneous and visceral.

**Cutaneous** leishmaniasis results from multiplication of *Leishmania* in phagocytes of the skin. The disease is caused primarily by members of the *L. mexicana* complex (*L. mexicana mexicana, L. mexicana amazonensis, L. mexicana venezuelensis*) and *L. braziliensis* complex (*L. brazilienses braziliensis, L. braziliensis panamensis, L. braziliensis guyanensis*) in the New World, and *L.*
tropica and L. major in the Old world (Berman, 2001). Cutaneous forms of the
disease normally produce skin ulcers on the exposed parts of the body such as
the face, arms and legs. The disease can produce a large number of lesions-
sometimes up to 200- causing serious disability and invariably leaving the patient
permanently scarred, a stigma which can cause serious social prejudice.

**Mucocutaneous** leishmaniasis can lead to partial or total destruction of the
mucous membranes of the nose, mouth and throat cavities and surrounding
tissues. Mucosal disease is characteristically non-curing, slowly evolving over a
mean of 3 years before first being brought to medical attention. Causative agents
of mucocutaneous leishmaniasis are *L. mexicana*, *L. amazonensis*, and *L.
venezuelensis* (Berman, 2001).

**Visceral** leishmaniasis, also known as kala azar, results from multiplication of
*Leishmania* within the systemic reticuloendothelial system. The disease is
primarily caused by *L. donovani* in the Indian subcontinent and Africa, *L.
infantum* in Mediterranean regions, and *L. chagasi* in the New World. Classical
visceral leishmaniasis is characterized by irregular bouts of fever, substantial
weight loss, swelling of the spleen and liver, and anemia. If left untreated, the
fatality rate can be as high as 100% in the developing countries (Berman, 2001).

### 1.4 Magnitude of the problem

There are 88 Leishmaniasis-endemic countries (Herwaldt, 1999). The disease is
endemic from northern Argentina to southern Texas, in southern Europe, Asia
(not southeast Asia), the Middle East, and Africa (particularly east and North Africa), but not in Australia and Oceania. An estimated 350 million people are at risk of infection. The estimated annual number of new cases of visceral leishmaniasis is about 500,000; over 90% of worldwide cases are in Bangladesh, northeastern India (particularly Bihar state), Nepal and Sudan (old world), and in northeastern Brazil (new world). There are about 1.5 million new cases of cutaneous leishmaniasis every year (Herwaldt, 1999).

In a particularly ominous trend, co-infection with leishmaniasis and HIV has been reported in 34 countries in Africa, Asia, Europe, and South America (http://www.who.int/leishmaniasis/burden/hiv_coinfection/burden_hiv_coinfection/en/). In southern Europe, 70% of adult cases of visceral leishmaniasis are associated with HIV infection. The two infections co-exist in a deadly synergy. Where leishmaniasis occurs in urban areas, conditions often favor explosive epidemics. In persons infected with HIV, leishmaniasis accelerates the onset of AIDS by cumulative immunosuppression and by stimulating replication of the virus. Moreover, co-infection activates disease in parasite carriers.

1.5 Treatment Options

The two pentavalent antimonial compounds, sodium stibogluconate and meglumine antimoniate were first introduced in the 1940s and have since been used as the first line chemotherapeutic agents against all forms of leishmaniasis including visceral leishmaniasis
The drugs are administered parenterally. Potential side-effects include nausea, vomiting, diarrhea and convulsions. Despite considerable toxicity and the need for hospitalization during the 4-week treatment, antimony therapy has remained the first line treatment in most endemic areas. However the spread of drug resistance is threatening to render antimony treatment ineffective. If treatment with pentavalent antimonials is unsuccessful, a preparation of an aromatic diamidine, Pentamidine is used. It is administered parenterally. The drug binds to the liver and kidneys and is excreted slowly in urine and feces. Amphotericin B is another drug of second choice used in the treatment of leishmaniasis. It is a polyene microlide antibiotic and acts on cell membrane sterols and phospholipids of leishmania spp. This drug is now available as a lipid associated formulation. The idea is that the lipid component will recognize receptors on specific cells and so the drug can be targeted to particular sites of infection, hence more drug will be available to interact with the parasite ergosterol, and less to react with human cholesterol and so reducing its side-effects. Miltefosine, an alkylphospholipid orally administered anti-cancer drug has been found to be effective against leishmania species both in vitro and in vivo. In a randomized trial in India, oral miltefosine was found to be effective and safe for treatment of visceral leishmaniasis but it remains largely inaccessible to people in the developing countries because of its being too expensive (Sundar et al., 2002). Immunotherapy has been used to improve antileishmanial drug action. One of
the methods employed is the use of interferon gamma to treat visceral leishmaniasis. It enhances the intracellular killing of the leishmania parasites and reduces the dose of antimony required for inhibition and killing of leishmania.

1.6 Immunology

The immunoregulation of leishmaniasis is being studied to improve understanding of the immune response to intracellular pathogens in general and leishmania in particular. The fundamental principle of immunoregulation is that the parasite, which replicates in quiescent macrophages, is killed by activated macrophages. Murine models of *L. major* disease exemplify the Th1/Th2 paradigm, in which the outcome of the disease is determined by the nature and magnitude of the T cell and cytokine responses early in infection. Two functionally distinct subsets of CD4+ T cell subsets, T helper 1 (Th1) and T helper 2 (Th2) have been described (Mosmann and Coffman, 1989). These are distinguishable by the pattern of cytokines they produce upon stimulation. Th1 cells are characterized by secretion of IFN-γ which is known to activate host defenses against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13 (Cherwinski at al., 1987) that favor the development of humoral responses protecting against extra cellular pathogens (Abbas et al., 1996). In infected inbred mice, production of interferon gamma by Th1 and natural killer cells mediates resistance, whereas expansion of the interleukin-4-producing Th2 cells confers susceptibility. Interleukin 12 has a key role in the development of cell-
mediated immunity by inducing naïve T cells to differentiate into Th1 cells and by inducing T cells and natural killer cells to produce interferon gamma (Herwaldt, 1999). The T cell and cytokine responses in infected human beings are more complex and less polarized than in mice, and the immune responses differ among leishmanial syndromes and species. Interferon gamma seems to be important for cure of human disease. The presence of interleukin 10 is associated with disease process in visceral leishmaniasis; interleukin 4 also contributes to disease progression. An understanding of the immune response to leishmania could help find out whether manipulations of the immune system may be therapeutic, and to rationalize vaccine development.
CHAPTER 2

INVESTIGATION OF IMMUNE EVASION MECHANISMS IN
CUTANEOUS LEISHMANIASIS

2.1 Rationale

*Leishmania* are obligate intracellular parasites that infect macrophages and cause a wide range of diseases such as cutaneous, mucocutaneous and visceral leishmaniasis in many parts of the world. Although macrophages are primary target cells for *Leishmania*, they are also involved in host defense against this parasite by their ability to kill *Leishmania* by producing nitric oxide and super oxide (Buchmuller-Rouiller and Mauel, 1987). Additionally, macrophages function as antigen-presenting cells (APC), and produce cytokines such as TNF-α and IL-12, which play a critical role in development of innate and acquired immunity against *Leishmania* (Louzir et al., 1998).

It is widely accepted that IFN-γ plays a critical role in control of *Leishmania* infection by inducing macrophage leishmanicidal activity as well as favoring Th1 development (Murray et al., 1982; Belosevic et al., 1989). The biological functions of IFN-γ are mediated via IFN-γR (Interferon gamma receptor) mediated
pathway involving receptor-associated kinases JAK1/JAK2 and STAT-1 (Greenlund et al., 1994; Igarashi et al., 1994; Greenlund et al., 1995; Sakatsume M et al., 1995). Binding of IFN-\(\gamma\) to its receptor activates JAK1/JAK2 kinases and phosphorylates STAT-1, which translocates to the nucleus and enhances transcription of IFN-\(\gamma\)-induced genes to increase macrophage microbicidal activity (Figures 2.1 and 2.2).

*Leishmania* has evolved several strategies to alter host macrophage function and circumvent host immunity (Reiner et al., 1988; Kwan et al, 1992). For example, infection of bone marrow derived macrophages with *Leishmania* promastigotes inhibits macrophage IL-12 production required for the induction of protective Th1 response (Carrera et al., 1996). Furthermore, a previous study has shown that infection of macrophages with *L. donovani* promastigotes dysregulates the IFN-\(\gamma\)-signaling pathway by attenuating tyrosine phosphorylation of JAK1, JAK2 and STAT1\(\alpha\) molecules and blocking biological functions of IFN-\(\gamma\) (Nandan and Reiner, 1995).

In the current study, we examined the effect of *L. major* and *L. mexicana* infection on STAT1 mediated IFN-\(\gamma\) signaling by measuring levels of IFN-\(\gamma\)R\(\alpha\), as well as total and phosphorylated Jak1, Jak2 and STAT1 by western blot analysis in *L. major* or *L. mexicana* -infected J774A.1 and RAW264.7 macrophages following in vitro IFN-\(\gamma\) activation and compared them to those in similarly stimulated uninfected macrophages.
Figure 2.1 Cytokine signaling by the JAK/STAT pathway. Cytokine-induced clustering of receptors leads to JAK-mediated phosphorylation of the receptor chains, attachment of inactive STATs, phosphorylation of the bound STATs (also by the JAKS), dimerization of STATs and migration to the nucleus, and stimulation of gene transcription.
Figure 2.2 Activation of STAT1 in IFN-$\gamma$ and IFN-$\alpha$ signaling. IFN-$\gamma$ signaling involves ligand-induced oligomerization of the IFN-$\gamma$ receptor subunits (IFNGR1 and IFNGR2) leading to the phosphorylation and activation of Jak1, Jak2, IFNGR1 and STAT1. STAT1 homodimers translocate to the nucleus and bind to GAS elements. The IFN$\alpha$/\beta mediated association of IFNAR1 and 2 stimulates the phosphorylation and activation of Tyk2, Jak1, IFNAR1, STAT1 and STAT2. STAT1-2 heterodimers, in conjunction with p48, form the trimeric complex ISGF3, which translocates to the nucleus and binds to ISRE elements. Alternatively, STAT1 homodimers and STAT1-2 heterodimers translocate to the nucleus and bind to GAS elements.
2.2 Materials and Methods

2.2.1 Parasites

*Leishmania major* (LV39) was maintained by serial passage of amastigotes inoculated subcutaneously into footpads of BALB/c mice. *L. mexicana* (MNYC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated subcutaneously into shaven rumps of 129Sv/Ev mice. Amastigotes isolated from the lesions of infected mice were grown to stationary-phase promastigotes as described previously (Satoskar et al., 1997).

2.2.2 Cell culture and in vitro infection of RAW264.7 and J774A.1 cells

The macrophage cell lines RAW264.7 and J774A.1 were obtained from the American Type Culture Collection (Manassas, VA) and cultured in suspension in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 10U penicillin/ml, 100µg streptomycin/ml and 0.05 mM 2-mercaptoethanol at 37°C in the presence of 5 % CO₂ in 75 cm² cell culture flasks. Macrophages were seeded in six-well plates at 5 x 10⁶ cells/ well. Cells were cultured for 6 h before infection with *L. major* and *L. mexicana* promastigotes (at a parasite-to-macrophage ratio of 10:1). After overnight incubation at 37°C in a humidified atmosphere of 5% CO₂-95% air, non-infected promastigotes were removed by washing with PBS. Cells were treated with IFN-γ (100 U/ml) for 45 minutes. Following treatment with IFN-γ, cells were solubilised in lysis buffer containing 20mM Tris-HCl, pH 8, 1% Triton X-100, 137mM NaCl,
10% glycerol and 20 µl/ml Protease Arrest (GenoTech, St. Louis, MO). Cell debris were removed by centrifugation at 4°C at 14,000 × g for 15 min. Protein concentration was determined by the Bradford method using a Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA).

2.2.3 Western blot analysis

Samples were separated by SDS-PAGE (40 µg of protein was loaded in each well) using 8% acryl amide mini-gels (National Diagnostics, Atlanta, GA), followed by transfer to PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blocked in milk protein blocker (GenoTech) in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 1.5 h and incubated with primary antibodies overnight. The detection step was performed with peroxidase-coupled anti-mouse IgG and anti-rabbit IgG antibodies (GenoTech; 1:5000). Primary antibodies were polyclonal anti-phospho STAT1 (dilution 1:2000), total STAT1 (dilution 1:2000), JAK1 (dilution 1:750), JAK2 (dilution 1:750), p-JAK1 (dilution 1:750), p-JAK2 (dilution 1:750), IFNγRα (dilution 1:750) and IFNγRβ (dilution 1:1000). All antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA and diluted in 5% milk protein blocker (GenoTech) suspension prepared in TBS with 0.1% Tween 20. Blots were developed with femtoLucent detection system (GenoTech).

The blots from three independent experiments for each protein were analyzed with Sigma Scan Pro (Sigma, GA). The total intensity (in gray levels)
was measured and plotted as percent increase in intensity, taking the percent expression in control (uninfected) cells as hundred percent (for each protein analyzed).

2.2.4 Statistical Analysis

For each of the proteins examined in the study, the densitometric analysis data from three independent experiments was used to calculate mean % increase ± SE. Statistical analysis was performed by One-way Anova (Analysis of Variance) using Sigma Stat Statistical Software Version 2.0 (Sigma, GA) to compare expression of protein in uninfected cells versus L. major- or L. mexicana- infected cells for each cell line.

2.3 Results

2.3.1 Interferon gamma receptor α subunit expression is attenuated in Leishmania-infected macrophages. We used Western blot analysis to compare the expression of IFN-γRα (interferon gamma receptor α subunit) in RAW264.7 and J774A.1 macrophages infected with L. major and L. mexicana promastigotes. Approximately 70-80% of the RAW264.7 and J774A.1 macrophages were infected with both the parasites, the number of amastigotes per cell being 3-4, as examined microscopically after Giemsa staining of the cells (data not shown). We found that IFN-γRα expression was markedly attenuated in both cell lines following Leishmania infection (Figure 2.3). Both L. major and L.
mexicana were equally efficient in decreasing IFNγ-Rα expression in both cell
tlines. Moreover, densitometric analysis of the blots revealed that the levels of
IFN-γRα Leishmania infected J774A.1 and RAW264.7 cells were significantly
lower as compared to uninfected cells (Figure 2.3). Similar results were also
observed in the experiments that were performed using dead (formalin fixed) L.
major and L. mexicana promastigotes (data not shown).

2.3.2 L. major and L. mexicana infection decreases IFN-γRβ expression in
macrophages. Next, we determined the effect of Leishmania infection on
interferon-gamma receptor beta (IFN-γRβ) expression in RAW264.7
macrophages infected with live or dead L. major and L. mexicana promastigotes
(Figure 2.4). In two independent experiments, although no apparent differences
were notable on the blot itself, densitometric analysis revealed that both live as
well as dead L. major and L. mexicana down regulated IFN-γRβ expression in
RAW264.7 cells. Furthermore, there was no difference in IFN-γRβ expression
between cells infected with live and dead parasites (Figure 2.4).

2.3.3 Interferon gamma induced tyrosine phosphorylation of Jak1 and Jak2
is attenuated in Leishmania-infected macrophages. To determine the effect
of Leishmania infection on Jak1/Jak2 phosphorylation, we used western blot to
measure levels of total as well as phosphorylated Jak1 and Jak2 in cell lysates of
RAW264.7 and J774A.1 cells infected with L. major and L. mexicana. We found
that *L. major* and *L. mexicana* reduced levels of total Jak1 and Jak2 in both cell lines (Figures 2.5 and 2.6). Jak2 attenuation was much more profound as compared to Jak1. Moreover, *L. mexicana* produced greater decrease in Jak1 and Jak2 expression in both cell lines (Figures 2.5 and 2.6). We further analyzed the effect of *Leishmania* infection on phosphorylation of Jak1 and Jak2 in response to IFN-γ. There was very marked attenuation of Jak1 and Jak2 phosphorylation in RAW264.7 and J774A.1 cells upon infection with *L. major* as well as *L. mexicana*.

2.3.4 Interferon gamma induced phosphorylation of phospho-STAT1β is up regulated in macrophages infected with *L. mexicana*. We used Western blot analysis to measure levels of total as well as phosphorylated p-STAT1α and p-STAT1β in *Leishmania*-infected RAW264.7 and J774A.1 macrophages. Following activation with IFN-γ, both *L. major* and *L. mexicana* infected RAW264.7 and J774A.1 macrophages displayed a decrease in tyrosine phosphorylation of p-STAT1α (Figure 2.7). Similarly, *L. major*-infected cells also showed a decrease in phosphorylation of dominant negative p-STAT1β, but those infected with *L. mexicana* displayed increased levels of phosphorylated p-STAT1β (Figure 2.7). Furthermore, the effect observed was more profound in J774A.1 than RAW264.7 cells. In addition, experiments were done to compare the effect of formalin-fixed dead parasites with that of live parasites on STAT1 phosphorylation in RAW 264.7 cells. Interestingly, dead *L. major* and *L.*
*mexicana* also induced similar alterations in the levels of phosphorylated p-STAT1α and p-STAT1β in RAW 264.7 cells (data not shown).
Figure 2.3 *Leishmania major* and *Leishmania mexicana* infection down regulates IFN-γRα expression in macrophages. A. J774A.1 and RAW264.7 macrophages were infected overnight with *L. major* and *L. mexicana* promastigotes at 10:1 parasite/macrophage. Cells were then stimulated with IFN-γ (100U/ml) for 45 minutes. Cell lysates were analyzed for IFN-γRα expression by western blot. The blots represent one of the three experiments with similar results. B. Densitometry analysis was plotted as percentage increase in IFN-γRα expression relative to control cells (the percent increase in expression in control cells is taken as 100) when stimulated with IFN-γ. Data shown in the graphs is combined from three independent experiments and shown as mean % increase ± SE. * = P<0.05.
IFN-γRα in J774A.1

<table>
<thead>
<tr>
<th></th>
<th>No IFN-γ</th>
<th>IFN-γ++</th>
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<tbody>
<tr>
<td>Uninfected</td>
<td>L. major</td>
<td>L. mexicana</td>
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<tr>
<td>Uninfected</td>
<td>L. major</td>
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IFN-γRα in RAW264.7

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<tr>
<td>Uninfected</td>
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<tr>
<td>Uninfected</td>
<td>L. major</td>
<td>L. mexicana</td>
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**Figure 2.3**
Figure 2.4 *Leishmania major* and *Leishmania mexicana* infection down regulates the expression of IFN-γRβ. Also there is no difference in expression between live and dead parasites. **A.** RAW264.7 macrophages were infected overnight with live and dead *L. major* and *L. mexicana* promastigotes at 10:1 parasite/macrophage. Cells were then stimulated with IFN-γ (100U/ml) for 45 minutes. Cell lysates were analyzed for IFN-γRβ by western blot. The blots represent one of the three experiments with similar results. **B.** Densitometry analysis was plotted as percent increase in IFN-γRβ expression relative to control cells (the percent increase in expression in control cells is taken as 100) when stimulated with IFN-γ. Data shown in the graphs is combined from three independent experiments and shown as mean % increase ± SE. n.s. = not significant, * = P<0.05.
Uninfected cells
L. major live
L. major dead
L. mexicana live
L. mexicana dead

Figure 2.4
Figure 2.5 *Leishmania major* and *Leishmania mexicana* infection down regulates phosphorylation of Janus kinase 1 (JAK1) in macrophages. A. J774A.1 and RAW264.7 macrophages were infected overnight with *L. major* and *L. mexicana* promastigotes at 10:1 parasite/macrophage. Cells were then stimulated with IFN-γ (100U/ml) for 45 minutes. Cell lysates were analyzed for total JAK1 and p-JAK1 by western blot. The blots represent one of the three experiments with similar results. B. Densitometry analysis was plotted as percentage increase in total JAK1 and p-JAK1 relative to control cells (the percent increase in expression in control cells is taken as 100) when stimulated with IFN-γ. Data shown in the graphs is combined from three independent experiments and shown as mean % increase ± SE. n.s. =not significant, * = P<0.05.
Total Jak1 in J774A.1

No IFN-γ | IFN-γ++
---------|-------
Uninfected | L. major | L. mexicana | L. major | L. mexicana

pJAK1 in J774A.1

JAK1 & pJAK1 Expression in J774A.1

% Increase

0 20 40 60 80 100 120

JAK1  pJAK1

Total Jak1 in RAW 264.7

No IFN-γ | IFN-γ++
---------|-------
Uninfected | L. major | L. mexicana | L. major | L. mexicana

pJAK1 in RAW264.7

JAK1 & pJAK1 Expression in RAW264.7

% Increase

0 20 40 60 80 100 120

JAK1  pJAK1

Figure 2.5
Figure 2.6 *Leishmania major* and *Leishmania mexicana* infection down regulates phosphorylation of Janus kinase 2 (JAK2) in macrophages. A. J774A.1 and RAW264.7 macrophages were infected overnight with *L. major* and *L. mexicana* promastigotes at 10:1 parasite/macrophage. Cells were then stimulated with IFN-γ (100U/ml) for 45 minutes. Cell lysates were analyzed for total JAK2 and p-JAK2 by western blot. The blots represent one of the three experiments with similar results. **B.** Densitometry analysis was plotted, as percentage increase in total JAK2 and p-JAK2 relative to control cells (the percent increase in expression in control cells is taken as 100) when stimulated with IFN-γ. Data shown in the graphs is combined from three independent experiments and shown as mean % increase ± SE. n.s. =not significant, *=P<0.05.
Total JAK2 in J774A.1

No IFN-γ | IFN-γ++
---|---
Uninfected | L. major | L. mexicana | Uninfected | L. major | L. mexicana

pJAK2 in J774A.1

JAK2 & pJAK2 Expression in J774A.1

% Increase

Control | L. major | L. mexicana
---|---|---
JAK2 | pJAK2

Total JAK2 in RAW264.7

No IFN-γ | IFN-γ++
---|---
Uninfected | L. major | L. mexicana | Uninfected | L. major | L. mexicana

pJAK2 in RAW264.7

JAK2 & pJAK2 Expression in RAW264.7

% Increase

Control | L. major | L. mexicana
---|---|---
JAK2 | pJAK2

Figure 2.6
Figure 2.7 *Leishmania major* and *Leishmania mexicana* infection downregulates p-STAT1α but *L. mexicana* infection increases phosphorylation of p-STAT1β. A. J774A.1 and RAW264.7 macrophages were infected overnight with *L. major* and *L. mexicana* promastigotes at 10:1 parasite/macrophage. Cells were then stimulated with IFN-γ (100U/ml) for 45 minutes. Cell lysates were analyzed for p-STAT1α and p-STAT1β by western blot. The blots represent one of the three experiments with similar results. B. Densitometry analysis was plotted as percent increase in p-STAT1α and p-STAT1β relative to control cells (the percent increase in expression in control cells is taken as 100) when stimulated with IFN-γ. Results in the blots are representative of three separate experiments. Data shown in the graphs represents mean and standard errors from three separate experiments. n.s. = not significant, * = P<0.05
Figure 2.7
2.4 Discussion

Previous studies have shown that IFN-γ plays a critical role in mediating protective immunity against intracellular bacteria, viruses and parasites (Cooper et al., 1993; Flynn et al., 1993; Wang et al., 1994; Harty and Bevan, 1995). The protective role of IFN-γ has been attributed to its ability to activate macrophages and increase their microbicidal activity via IFN-γR mediated STAT1 dependent pathway (Boehm et al., 1997). The critical role of IFN-γR in development of IFN-γ-mediated host immunity is evident in studies showing that IFN-γR-/- mice are highly susceptible to pathogens such as *Mycobacterium avium* (Alvarez et al., 2003), *Listeria monocytogenes* (Harty and Bevan, 1995), *Candida albicans* (Balish et al., 1998) and *Plasmodium berghei* (Amani et al., 2000). Furthermore, we and others have shown that IFN-γR-/- mice are highly susceptible to high as well as low dose *L. major* infection indicating that IFN-γR is essential for control of cutaneous leishmaniasis (Rosas et al., 2003). IFN-γR is comprised of IFN-γRα and IFN-γRβ chains. While IFN-γRα chain plays a critical role in ligand binding, IFN-γRβ is required for IFN-γ signal transduction (Boehm et al., 1997). Several studies have demonstrated that certain bacterial and viral pathogens evade host immunity by down regulating IFN-γRα expression on effector cells (Hussain et al., 1999; Fukuda et al., 2001; Gehring et al., 2003 ;). In addition, *L. donovani* has also been shown to attenuate IFN-γR expression in human monocytes (Nandan and Reiner, 1995). Similarly, in the present study, we found that J774A.1 and
RAW264.7 macrophages infected in vitro with *L. mexicana* or *L. major* promastigotes displayed significantly lower levels of IFN-γRα as compared to uninfected control cells (Fig. 1). These results demonstrate that both *L. major* and *L. mexicana* interfere with IFN-γ signaling in macrophages by suppressing IFN-γRα expression. The IFNγRβ chain is 332 and 337 amino acids in length in mouse and human respectively and represents the signal transducing component of the IFN-γR (Bach et al., 1997). Both *L. major* and *L. mexicana* also down regulated the expression of IFN-γRβ in RAW264.7 macrophages when compared to uninfected cells. Also, there was no difference in expression between cells infected with the live and formalin-fixed parasites. Taken together, these findings suggest that *L. major* and *L. mexicana* not only decrease expression of the ligand binding component of IFN-γR but also reduce its signal transducing ability in macrophages by down-regulating IFN-γRβ levels.

The Janus protein tyrosine kinases Jak1 and Jak2 play a critical role in signal transduction through IFN-γR (Ihle et al., 1995; Taniguchi, 1995). We found that J774A.1 as well as RAW264.7 cells infected with *L. major* or *L. mexicana* displayed significantly reduced levels of total as well as phosphorylated Jak1 and Jak2). The phosphorylation of JAK1 and JAK2 was markedly abrogated in both cell lines by both the parasites. These findings demonstrate that *L. major* and *L. mexicana* can block IFN-γ induced macrophage activation by attenuating Jak1/Jak2 activation. This is perhaps not surprising, as previous studies have
shown that *L. donovani* selectively blocks IFN-\(\gamma\) induced tyrosine phosphorylation of Jak1/Jak2 as well as triggers dephosphorylation of Jak2 by activating macrophage SHP1 tyrosine phosphatase (Blanchette et al., 1999).

Signal transducer and activator of transcription -1, also known as STAT-1 is a cytoplasmic protein which plays a critical role in signaling of several cytokines such as IFN-\(\gamma\) (Boehm et al., 1997), IFN-\(\alpha/\beta\)30 and IL-27 (Lucas et al., 2003). Activated Jak1/Jak2 catalyze phosphorylation of STAT1 which then translocates to nucleus to initiate the gene transcription (Darnell et al., 1994). Due to alternative RNA splicing, STAT1 exists in two forms, STAT1\(\alpha\) (p91) and STAT1\(\beta\) (p84). Stat1\(\alpha\) has 38 aa at the C terminus that are absent in STAT1\(\beta\) (Schindler et al., 1992). As C-terminus of STAT1 is essential for its interaction with transcriptional co-activator CREB-binding protein (CBP)/p300 (Zhang et al., 1996), only STAT1\(\alpha\) is capable of initiating transcription of IFN-\(\gamma\) induced genes whereas STAT-1\(\beta\) functions a dominant-negative inhibitor (Bromberg et al., 1996; Muller et al., 1993). We have previously demonstrated that STAT1-mediated pathway is indispensable for the development of protective immunity against *L. major* (Rosas et al., 2003). Furthermore, others have shown that *Leishmania donovani* also attenuates IFN-\(\gamma\) induced STAT1-phosphorylation in human macrophages and may use this strategy to circumvent host immunity (Nandan and Reineer, 1995). In the present study, infection of J774A.1 as well as RAW264.7 cells with *L. major* and *L. mexicana* resulted in marked attenuation of IFN-\(\gamma\)-induced STAT-1 phosphorylation. While infection of J774A.1 and
RAW264.7 cells with both parasites resulted in a marked decrease in level of phospho-STAT1α, phospho-STAT1β expression was markedly reduced only in L. major-infected cells. Interestingly, L. mexicana –infected J774A.1 and RAW264.7 cells displayed significantly higher levels of phosphorylated STAT1β following IFN-γ activation as compared to those infected with L. major and un-infected controls. Furthermore, pSTAT1α/pSTAT1β ratio was markedly decreased (approximately 0.8) in L. mexicana-infected cells of both cell lines. This decrease was significant compared to the uninfected control cells (for which the ratio was 1.5-2) and L. major-infected cells (the ratio, in this case, was more than 2). These observations demonstrate that both L. mexicana and L. major can block IFN-γ induced macrophage activation by inhibiting STAT1 phosphorylation. However, perhaps the most interesting observation in our study is that infection of murine macrophages with L. mexicana results in preferential phosphorylation and activation of the dominant negative STAT1β. Therefore, it is likely that L. mexicana may use this mechanism as one of the strategies to evade host immune response and prolong its survival within host macrophages. Moreover, our experiments with formalin-fixed dead L. major and L. mexicana demonstrated that dead parasites produce the same effects as live parasites (data not shown). This suggests that some components in the parasites are responsible for the observed effects on IFN-γ mediated signaling pathway. This is not surprising as lipophosphoglycan, a major component of the Leishmania surface coat has been shown to be the major ligand for macrophage adhesion and a key factor
mitigating host responses by deactivation of macrophage signaling pathways (Spath et al., 2000). A recent study has found that activation of TLR2 using TLR2 agonists leads to an increase in the tyrosine phosphorylation of STAT1β (Alvarez et al., 2003). Therefore, it may be hypothesized that TLR-2 mediated pathway may play a role in increased phosphorylation of STAT1β observed in L. mexicana- infected macrophages. Nevertheless, in the preliminary studies we have found that TLR2/- mice remain highly susceptible to L. mexicana suggesting that TLR-2 mediated pathway is not involved in mediating susceptibility to L. mexicana (data not shown).

In conclusion, the results presented in this study show that L. major and L. mexicana interfere with IFN-γ signaling in macrophages by attenuating expression of IFN-γR and activation of several signal transduction molecules that are critical components of STAT1-mediated IFN-γ signaling pathway. In addition, these findings also demonstrate that stimulation of L. mexicana- infected macrophages with IFN-γ leads to preferential activation of the dominant negative STAT1β, which is a negative regulator of transcription.
CHAPTER 3

INVESTIGATION OF THE ROLE OF STAT1 SIGNANLING IN T CELLS IN IMMUNE RESPONSE AGAINST CUTANEOUS LEISHMANIASIS

3.1 Rationale:

Experimental infection with *Leishmania major* in inbred mice of different genetic backgrounds can reproduce distinct features of the spectrum of clinical manifestations seen in patients with cutaneous leishmaniasis (Behin et al., 1979; Mitchell et al., 1981). Mice from the majority of inbred strains (C3H/He, CBA, C57BL/6, and 129Sv/Ev) develop locally cutaneous lesions which spontaneously resolve. These mice belong to the resistant phenotype. Mice from a few strains (BALB/c) develop severe ulcerating lesions without becoming immune to reinfection. Such mice represent the susceptible phenotype (Mitchell et al., 1981). The murine model of leishmaniasis has been used to characterize the immune responses developing in the resistant and susceptible strains of mice.

Two functionally distinct subsets of CD4⁺ T cells have been described: T helper 1 (Th1) and T helper 2 (Th2) (Mosmann and Coffman, 1989). The two
types of cells are distinguished by the pattern of cytokines they produce upon stimulation. Th1 cells are characterized by the production of IFN-γ to activate host defences against intracellular pathogens, while Th2 cells produce IL-4, IL-5, and IL-13 that favor the development of humoral responses protecting against extracellular pathogens (Cherwinski et al., 1987). *L. major* is a well-characterised model for studying the in vivo differentiation and function of CD4⁺ T cells. This model provided the first correlation between the development of protective immunity and an expansion of Th1 CD4⁺ T cells in resistant mice, and progressive disease and the development of Th2 response in susceptible mice (Locksley et al., 1987; Scott et al., 1988; Heinzel et al., 1989).

*Leishmania* are obligate intracellular parasites of the macrophages that survive within the severe environment of the phagolysosome and evade the defense mechanisms induced during host immune responses. (Ray et al., 2000). IFN-γ is the most potent cytokine for the induction of macrophage activation for leishmanicidal and microbicidal activity in macrophages (Murray et al., 1982; Belosevic et al., 1989). The protective role of IFN-γ in cutaneous leishmaniasis has been attributed to its ability to favor Th1 differentiation of CD4⁺ T cells as well as to induce nitric oxide (NO) production by increasing NO synthase 2 (NOS2) gene expression and enhancing macrophage leishmanicidal activity (Liew and O'Donnell, 1993; Reiner and Locksley, 1995). It is widely accepted that the majority of pleiotropic effects of IFN-γ are mediated via the JAK-STAT1 signaling pathway (Igarashi et al., 1994; Greenlund et al., 1994; Sakatsume M et al., 1995;
Greenlund et al., 1995). Binding of IFN-γ to its receptor (IFN-γR) leads to dimerisation of the receptor, and trans-phosphorylation of the receptor-associated kinases Jak1 and Jak2. Activation of these kinases induces phosphorylation of the cytoplasmic tail of the receptor itself. The cytoplasmic molecule STAT1α is then recruited to the activated receptor complex, is phosphorylated, becomes homodimer, and then translocates to the nucleus to enhance transcription of IFN-γ-induced genes (Lucas et al., 1998).

Rosas et al. investigated the role of STAT1 in protective immunity against Leishmania major. They found that L. major-infected STAT1−/− mice developed larger lesions with more parasites as compared to similarly infected STAT1+/+ mice. Moreover, the inability of STAT1−/− mice to control infection was due to impaired Th1 response associated with reduced production of IFN-γ, IL-12 and nitric oxide. Their findings demonstrated that STAT1-mediated IFN signaling is indispensable for the development of protective immunity against cutaneous L. major infection. The purpose of the following set of experiments was to further dissect the role of STAT1 in T cells and macrophages and investigate how critical STAT1 signaling is separately in T cell populations and macrophages for immunity against L. major infection.
3.2 Materials and Methods

3.2.1 Mice

Six-to-eight-week-old male and female STAT1\(^{-/-}\), STAT1\(^{+/+}\) and Rag2\(^{-/-}\) mice on C57BL/6 background were used in the study. They were maintained in the SPF facilities at the Ohio State University animal facilities in accordance with the institutional guidelines.

3.2.2 Adoptive transfer

Spleens were removed aseptically from STAT1\(^{-/-}\) and STAT1\(^{+/+}\) mice and cell suspensions prepared by gentle teasing of the tissue in cold RPMI 1640 supplemented with 10% fetal bovine serum, 100 units of penicillin/streptomycin and 2 mM glutamine (GIBCO, BRL Grand Island, NY). Following washing to remove tissue debris, cell numbers were enumerated by standard protocols. Rag2\(^{-/-}\) mice were injected i.p. with 2 \times 10^7 spleen cells resuspended in the medium. The mice were rested for 3 weeks post-reconstitution before infection with parasites. In a subsequent experiment, cell suspensions were prepared from spleens of STAT1\(^{-/-}\) and STAT1\(^{+/+}\) C57BL/6 mice as described above and passed through Nylon Wool Fiber Columns (Polysciences, Inc. PA). The non-adherent T cells were collected from the columns. CD4+ T cells and CD8+ T cells were purified from the T cell suspensions by immunomagnetic cell depletion using Dynabeads Mouse CD8 (Lyt2) and Dynabeads Mouse CD4 (L3T4) respectively (Dynal Biotech, Inc. WI). The cell counts of each of the T cell populations were adjusted to 2 \times 10^6 cells/ml by trypan blue exclusion method.
Four sets of T cell populations were prepared: STAT1+/+CD4+ STAT1+/+CD8+, STAT1+/CD4+ STAT1+/−CD8+, STAT1+/+/CD4+ STAT1+/−CD8+, STAT1+/− CD4+ STAT1+/+/CD8+. Four groups of mice with four mice per group were injected i.p. with 2X10^6 cells of each of the four sets of T cell populations. A fifth group of mice injected i.p. with phosphorus buffered saline served as control. The mice were rested for three weeks before infection with *L. major* in the hind footpad as described below.

### 3.2.3 Parasites and infection protocol

*Leishmania major* (LV39) was maintained by serial passage of amastigotes inoculated subcutaneously into footpads of BALB/c mice. Amastigotes isolated from the lesions were grown to stationary phase as described previously. Mice were injected in the hind footpad with 2X10^6 stationary phase promastigotes. Disease progression was monitored by measuring the increase in thickness of the infected footpad using a dial-gauge micrometer at weekly intervals and comparing this to the thickness of the contra lateral uninfected footpad. Relative parasite burdens in the infected footpads and draining lymph nodes were determined by limiting dilution analysis as described previously (Monteforte et al., 2000).
3.2.4 Antibody ELISA

Peripheral blood was obtained from *L. major*-infected Rag2\(^{-/-}\) mice by tail snapping at various time points after infection. Plasma samples were obtained following centrifugation at 200xg, and were used to analyze the levels of Th2-associated LmAg-specific IgG1as well as Th1-associated IgG2a as described previously (Stamm et al., 1998).

3.2.5 Cell preparations and cytokine assays

The draining lymph nodes were removed in sterile conditions from the reconstituted Rag2\(^{-/-}\) mice 8 weeks postinfection with *L. major*. Single suspensions were prepared by gentle teasing in complete RPMI 1640. Viable cells were counted using trypan blue exclusion and adjusted to 3 X 10\(^6\) cells/ml in the same medium. Aliquots (100\(\mu\)l) were placed into 96-well flat-bottom culture plates and stimulated with *L. major* antigen (LmAg; 20 ug/ml). Supernatants were collected after 60 h of incubation at 37°C, and the levels of cytokines (IL-12, IFN-\(\gamma\), IL-4 and IL-10) were measured using sandwich ELISA methods according to the manufacturer’s instructions (Pharmingen, San Diego, CA).

3.3 Results

3.3.1 Rag2\(^{-/-}\) C57BL/6 mice reconstituted with STAT1\(^{+/+}\) splenocytes are highly susceptible to cutaneous *L. major* infection and develop large non-healing lesions. Course of cutaneous *L. major* infection in Rag2\(^{-/-}\) C57BL/6 mice transferred i.p. with splenocytes from wild type and STAT1\(^{+/+}\) C57BL/6 or injected
with phosphorus buffered saline (control group) was monitored by measuring the increase in thickness of the infected footpad and comparing this to the thickness of contra-lateral uninfected footpad. Rag2⁻/⁻ mice reconstituted with splenocytes from STAT1⁻/⁻ C57BL/6 mice developed lesions that were significantly bigger than those developed by Rag2⁻/⁻ mice transferred with wild type splenocytes (Figure 3.1A). Parasite burdens in the footpads and draining lymph nodes were determined day 45 post infection as shown in Figure 3.1B. The parasite burdens in Rag2⁻/⁻ mice reconstituted with STAT1⁻/⁻ splenocytes were significantly higher than those reconstituted with wild type cells. These findings demonstrate that a STAT1-mediated IFN signaling pathway is essential; for the development of protective immunity against cutaneous L. major infection.

3.3.2 Rag2⁻/⁻ mice reconstituted with wild type splenocytes produce significantly more LmAg-specific Th1-associated IgG2a than those transferred with STAT1⁻/⁻ cells. The levels of LmAg-specific Th2-associated IgG1 were found to be significantly higher in Rag2⁻/⁻ mice transferred with STAT1⁻/⁻ splenocytes when compared to those that received wild type cells (Figure 3.2). The antibody profile is reflective of the Th cell commitment which in this case appears to skew towards the Th1 pathway in mice reconstituted with STAT1⁺/⁺ cells as evidenced by higher IgG2a levels. These results indicate that a STAT1-mediated IFN signaling pathway plays a critical role in the development of Th1 response in L. major infection.
3.3.3 *Rag2*+/− mice reconstituted with wildtype splenocytes produce significantly more IFN-γ and IL-12 as compared to those reconstituted with *STAT1*+/− splenocytes. The draining lymph nodes were removed and subjected to T cell proliferation in the presence of *L. major* antigen. Cytokine levels were measured in the supernatants by doing ELISA. It was found that *L. major* antigen stimulated lymph node cells from *L. major* infected *Rag2*+/− mice transferred with *STAT1*+/− splenocytes produced higher levels of IFN-γ and IL-12 than similarly infected *Rag2*+/− mice reconstituted with *STAT1*+/− cells (Figure 3.3). Moreover, the levels of IL-10 produced by lymph node cells from *Rag2*+/− mice transferred with *STAT1*+/− cells were higher than those reconstituted with wild type cells (Figure 3.4). IL-4 levels in the control group and the group transferred with WT cells were too low to be detectable.

3.3.4 *STAT1* in CD4+ T cells is critical for control of *L. major* infection. As explained in Materials and Methods, *Rag2*+/− mice were reconstituted with CD4+ and CD8+ T cells from *STAT1*−/− and *STAT1*+/+ in four different combinations. The mice transferred with *STAT1*−/− CD4+ *STAT1*−/−CD8+ cells developed large and ulcerating lesions whereas the mice that received *STAT1*+/+CD4+ *STAT1*+/+CD8+ cells controlled the infection (Figure 3.5). And interestingly enough, the mice with *STAT1*−/− CD4+ *STAT1*+/+CD8+ developed rapidly progressive lesions while those reconstituted with *STAT1*+/+CD4+ *STAT1*−/−CD8+ cells were able to control the infection.
Figure 3.1 Rag2⁻/⁻ C57BL/6 mice reconstituted with STAT1⁻/⁻ splenocytes are highly susceptible to cutaneous L. major infection and develop large non-healing lesions. Course of cutaneous L. major infection in Rag2⁻/⁻ C57BL/6 mice transferred i.p. with splenocytes from wild type and STAT1⁻/⁻ C57BL/6 or injected with phosphorus buffered saline (control group). Mice were infected with 2 × 10⁶ stationary-phase promastigotes in the left footpad three weeks following intraperitoneal cell transfer. A. Disease progression was monitored by measuring the increase in thickness of the infected footpad and comparing this to the thickness of contra-lateral uninfected footpad. Data are presented as mean increase in footpad thickness ± SE. B. Parasite burdens in the footpads and draining lymph nodes were determined day 45 post infection. Data are expressed as the mean parasite titer ± SE. WT: mice transferred with STAT1+/+ cells; KO: mice transferred with STAT1-/- cells; control: mice transferred with PBS.
Figure 3.1
Figure 3.1
Figure 3.2 *Rag2*<sup>−/−</sup> mice reconstituted with wild type splenocytes produce significantly more LmAg-specific Th1-associated IgG2a than those transferred with *STAT1*<sup>−/−</sup> cells. Data is presented as mean reciprocal endpoint titer on log scale. Similar results were observed in two independent experiments. WT: mice transferred with *STAT1*<sup>+/+</sup> cells; KO: mice transferred with *STAT1*<sup>−/−</sup> cells; control: mice transferred with PBS.
Figure 3.3 *Rag2<sup>−/−</sup>* mice reconstituted with wildtype splenocytes produce significantly more IFN-γ and IL-12 as compared to those reconstituted with *STAT1<sup>−/−</sup>* splenocytes. Cytokine production by lymph node cells from *L. major*-infected mice were measured day 45 post infection. The draining lymph node cells were isolated and restimulated in vitro with 20 ug/ml of LmAg for 60 h. Similar results were observed in two independent experiments. ND: non-detectable; WT: mice transferred with STAT1+/+ cells; KO: mice transferred with STAT1−/− cells; control: mice transferred with PBS.
Figure 3.4 Rag2\(^{-/-}\) mice reconstituted with wildtype splenocytes produce significantly less IL-10 as compared to those reconstituted with STAT1\(^{-/-}\) splenocytes. Cytokine production by lymph node cells from *L. major*-infected mice were measured day 45 post infection. The draining lymph node cells were isolated and restimulated in vitro with 20 ug/ml of LmAg for 60 h. Similar results were observed in two independent experiments. ND: non-detectable; WT: mice transferred with STAT1\(^{+/+}\) cells; KO: mice transferred with STAT1\(^{-/-}\) cells; control: mice transferred with PBS.
Figure 3.5 *STAT1 in CD4+ T cells is critical for control of L. major infection.*

Course of cutaneous *L. major* infection in Rag2\(^{--}\) C57BL/6 mice transferred i.p. with CD4+ and CD8+ T cells in four different combinations from wild type and STAT1\(^{--}\) C57BL/6 or injected with phosphorus buffered saline (control group) was monitored. Mice were infected with 2 X 10^6 stationary-phase promastigotes in the left footpad three weeks following intraperitoneal cell transfer. Disease progression was monitored by measuring the increase in thickness of the infected footpad and comparing this to the thickness of contra-lateral uninfected footpad. Data are presented as mean increase in footpad thickness ± SE.
3.4 Discussion

Experimental infection with *L. major* in mice of different genetic backgrounds has been used to characterize the immune responses developing in both resistant and susceptible mice. IFN-γ is the most potent cytokine for the induction of macrophage activation for leishmanicidal activity (Belosevic et al., 1989; Murray et al., 1982). Both IFN-α/β and IFN-γ signal via a STAT1-mediated pathway and play an important role in the development of protective immunity against *L. major* (Liew et al., 1993; Reiner et al., 1995). While IFN-γ is essential for promoting IL-12 induced Th1 differentiation of CD4+ T cells and for inducing macrophage leishmanicidal activity that is required for successful control of infection, the protective role of IFN-α/β has been attributed to its ability to regulate innate immune responses during *L. major* infection (Diedenbach et al., 1998). The course of *L. major* infection in STAT1+/- and STAT-/- mice on C57BL/6 background has previously been examined in the same lab where the present study was done (Rosas et al., 2003). It was demonstrated then that STAT1+/- mice quickly resolved their lesions, while STAT1-/- mice developed large lesions containing significantly more parasites. Moreover, the inability of STAT1-/- mice to control infection was attributed to the reduced production of IL-12, and IFN-γ which are associated with Th1 development. Hence, it was established that development of protective immunity against *L. major* infection is dependent on STAT1-mediated IFN signaling pathway. The purpose of the present study was to investigate the role of STAT1 in lymphocytes for protection against cutaneous
leishmaniasis. Towards this end, Rag2\(^{-/-}\) mice (deficient in T cell and B cell development) were used and transferred with splenocytes from STAT1\(^{+/+}\) and STAT1\(^{-/-}\) mice intraperitoneally. The course of *L. major* infection in the reconstituted mice was monitored. It was found that Rag2\(^{-/-}\) mice that received STAT1\(^{-/-}\) lymphocytes developed much larger lesions when compared to similarly infected Rag2\(^{-/-}\) mice that received STAT1\(^{+/+}\) cells (Figure 3.1A). In addition, the parasite loads in the former group were also significantly higher than in the latter (Figure 3.1B). This implies that STAT1-mediated IFN-\(\gamma\) signaling pathway is critical for defense against cutaneous *L. major* infection.

In addition, it was found that *L. major* antigen (LmAg)-stimulated lymph node cells from *L. major*-infected Rag2\(^{-/-}\) mice reconstituted with wild type splenocytes produced higher levels of IFN-\(\gamma\) and IL-12 as compared to the mice transferred with STAT1\(^{-/-}\) cells (Figure 3.3). The levels of IL-10 and IL-4 were found to be higher in the latter category (Figure 3.4). The levels of all the cytokines in the Rag2\(^{-/-}\) mice not transferred with any splenocytes (control group) were too low to be detectable. The cytokine profile indicates that the inability of Rag2\(^{-/-}\) mice reconstituted with STAT1\(^{-/-}\) cells to control the *L. major* infection may be due to a failure of Th1 development associated with reduced production of IFN-\(\gamma\) and IL-12. Furthermore, Rag2\(^{-/-}\) mice that received wild type cells produced significantly more LmAg-specific Th1-associated IgG2a (Figure 3.2). The titer of Th2-associated IgG1 was found to be higher in the group of mice that were reconstituted with STAT1\(^{-/-}\) splenocytes.
Next we wanted to investigate the role of CD4+ and CD8+ T cells in immune response against *L. major*. Towards this end, I performed adoptive transfer of four different groups of T cells to Rag2<sup>−/−</sup> mice: STAT1<sup>+/+</sup>CD4+ STAT1<sup>+/+</sup>CD8+, STAT1<sup>−/−</sup> CD4+ STAT1<sup>−/−</sup>CD8+, STAT1<sup>+/+</sup>CD4+ STAT1<sup>−/−</sup>CD8+, STAT1<sup>−/−</sup> CD4+ STAT1<sup>+/+</sup>CD8+. The mice were then infected with *L. major* following a rest of three weeks and the course of infection was monitored as explained in Materials and Methods. The mice transferred with STAT1<sup>−/−</sup> CD4+ STAT1<sup>−/−</sup>CD8+ T cells developed much larger lesions as compared to the group of mice that received STAT1<sup>+/+</sup>CD4+ STAT1<sup>+/+</sup>CD8+ cells (Figure 3.5). Even more interesting was the observation that the mice reconstituted with STAT1<sup>−/−</sup> CD4+ STAT1<sup>+/+</sup>CD8+ cells developed very large ulcerating lesions whereas the group of mice that received STAT1<sup>+/+</sup>CD4+ STAT1<sup>−/−</sup>CD8+ cells controlled the infection and developed small lesions. These results demonstrate that STAT1 mediated signaling in CD4+ T cells is critical for protection against *L. major* infection and that the presence of STAT1 in macrophages alone is not sufficient to control *L. major* infection.

Many cell types in the immune system participate in combating infection, but CD4 T cells critically determine the outcome of any given infection. These cells direct the ongoing immune response through the secretion of cytokines. Th1 cells secrete IFN-γ, IL-2, TNF-α and TNF-β, which are critical for eradication of intracellular pathogens like *Leishmania major* (Szwabo et al., 2003). Th2 cells produce IL-4, IL-5, IL-6, and IL-13, which are essential for elimination of extracellular organisms (Szwabo et al., 2003). The immune system has evolved
a multitude of signal transduction pathways involved in the development of a Th1- or Th2-type response. IFN-\(\gamma\) is a pleiotropic cytokine that is considered to play a pivotal role in the development of Th1 response. A critical stage of Th1 differentiation process of naïve CD4 T cells occurs through the induction of a recently identified transcription factor, T-bet (T-box expressed in T cells). In CD4 T cells, T-bet is rapidly and specifically induced in developing TH1 but not Th2 cells. T-bet expression appears to be controlled by both the TCR and IFN-\(\gamma\)/STAT1 signal transduction pathways (Afkarian et al., 2002; Lighvani et al., 2001). A regulatory circuit involving IFN\(\gamma\)R signaling via STAT1 maintains high level T-bet expression in developing Th1 cells. Thus IFN-\(\gamma\) from diverse sources such as NK cells, macrophages, and dendritic cells, as well as naïve CD4 T cells themselves, induces expression of T-bet, which can cause chromatin remodeling of the IFN-\(\gamma\) locus (Mullen et al., 2001) and transactivation of the IFN-\(\gamma\) gene (Szwabo et al., 2000). This results in a local increase in IFN-\(\gamma\) and crates a positive feedback loop driving Th1 differentiation. T-bet also induces IL-12R\(\beta\)2 chain expression (Afkarian et al., 2002; Mullen et al., 2001), allowing IL-12/STAT4 signaling to optimize IFN-\(\gamma\) production, thereby completing the Th1 developmental commitment process. The results from the present study show that STAT1-mediated IFN-\(\gamma\) signaling pathway is crucial for the control of \(L.\) major infection. The lack of STAT1 signaling pathway impedes T-bet expression in CD4 T cells. As a result, there is failure of Th1 development. This may explain the inability of Rag2\(^{-}\) mice reconstituted with STAT1\(^{-}\) cells to contain \(L.\) major
infection. These results demonstrate that a STAT1-mediated IFN signaling pathway up regulates IL-12 as well as IFN-γ production and plays a critical role in the development of Th1 response during *L. major* infection. In conclusion, Rag2−/− mice reconstituted with STAT1−/− splenocytes are extremely susceptible to *L. major* and develop large non-healing lesions following infection with the parasite. Moreover, susceptibility of these mice to *L. major* may be due to a lack of Th1 development associated with impaired IFN-γ production. These results indicate that STAT1-mediated IFN signaling pathway is essential for the development of protective immunity against *L. major*. It will be interesting to investigate the role of STAT1 in macrophages in response against *L. major* infection. This could be achieved by performing T cells transfers in Rag2−/−STAT1−/− (double knockout mice). These mice will be deficient in STAT1 in macrophages (in addition to being deficient in T and B cells). The immune response of such mice reconstituted with different sets of T cells and then infected with *L. major* could give a valuable insight into the relative roles of STAT1 pathway in T cells and macrophages.


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http://www.who.int/leishmaniasis/burden/hiv_coinfection/burden_hiv_coinfection/en/


