IN VIVO EVALUATION OF GENETIC AND PHARMACOLOGICAL TARGETING OF THE *LEISHMANIA DONOVANI* POLYAMINE BIOSYNTHETIC PATHWAY

By

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CERTIFICATE OF APPROVAL

This is to certify that the Master's thesis of

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I will miss all the good times in the Ullman Lab, but hopefully there will be plenty of post-degree visits!

Abstract

Protozoan parasites of the genus *Leishmania* are the causative agents of leishmaniasis, which can manifest as a single cutaneous ulcer, chronic cutaneous or mucocutaneous lesions, or visceral disease. Leishmaniasis is endemic in nearly 90 countries, and there are an estimated 12 million people currently infected with *Leishmania* parasites. Two million new cases are thought to occur annually, including 500,000 cases of visceral leishmaniasis. Because of this burden, leishmaniasis is now considered a disease that impedes socioeconomic development.

There is a lack of effective vaccines for preventing parasitic diseases, leaving chemotherapy as the only means for treatment and prevention. However, current drugs used in the treatment of leishmaniasis are far from ideal. There is a high risk of toxicity, some treatments require multiple administrations for extended periods of time, there is an emergence of resistance, and availability is hindered by cost.

One of the biochemical pathways of *Leishmania* that has shown potential as a therapeutic target is the metabolism of cationic polyamines. Significant differences exist between the polyamine biosynthetic pathways of *Leishmania* and their mammalian hosts, reinforcing this pathway as a potential therapeutic target for leishmaniasis. The polyamine pathway of *Leishmania* consists of four enzymes: arginase (ARG), ornithine decarboxylase (ODC), spermidine synthase (SPDSYN), and S-adenosylmethionine decarboxylase (ADOMETDC). To characterize this pathway in detail, we employed a targeted gene replacement strategy to create a series of null mutants, each lacking one

of the four genes in the pathway. Each of the genetic lesions creates a conditional lethal mutation that can be bypassed by media supplementation with downstream metabolites *in vitro*, illustrating that an intact polyamine biosynthetic pathway is essential for promastigote survival and growth. Moreover, the *Δldarg, Δldodc, Δldadometdc*, and *Δldspdsyn* mutants are all markedly compromised in their abilities to establish infections in mice. The extents of the infectivity deficits, however, vary considerably with the nature of the genetic lesion, which we hypothesize is attributable to the differential availability of amino acid and/or polyamine pools in the mammalian phagolysosome. Furthermore, we have shown that oral administration of putrescine to *Δldodc*-infected mice can partially restore the virulence defect of *Δldodc* parasites, whereas oral administration of DFMO, an ODC inhibitor, to mice inoculated with wild-type *L. donovani* results in a partial decrease in parasite burdens, indicating that the polyamine biosynthetic machinery of *L. donovani* can be nutritionally or pharmacologically targeted.

Chapter 1 – Introduction

1-1 PARASITES

Parasitic diseases are responsible for an enormous amount of morbidity and mortality around the world. There are a large variety of organisms that fall into the category of disease-causing parasites. The classes of Trematoda and Cestoidea, the phylum Nematoda, and the subkingdom of Protozoa all contain parasites that cause human disease [1].

The protozoans contribute greatly to the overall parasitic disease burden. They are unicellular eukaryotic organisms, often possessing unique biological features and multistage lifecycles. Protozoans are geographically widespread and put billions of people at risk for disease and death. Transmission occurs via the fecal oral route or by introduction into the bloodstream by an arthropod vector, depending on the type of parasite. Frequently, the highest concentration of disease is in developing countries where access to adequate healthcare is limited and there are natural reservoirs of infectious parasites. For instance, malaria, caused by the protozoan *Plasmodium*, is considered the most deadly parasitic disease, resulting in approximately one million deaths a year [CDC].

Several diseases caused by protozoan parasites are considered “neglected tropical diseases” (NTDs) due to the fact that they have suffered from a lack of attention from the public health community. Included in this category and accounting for a significant disease burden are African and American trypanosomiasis and leishmaniasis. It is

estimated that NTDs affect a billion people worldwide. The map below shows the geographical areas where NTDs are prevalent [CDC].

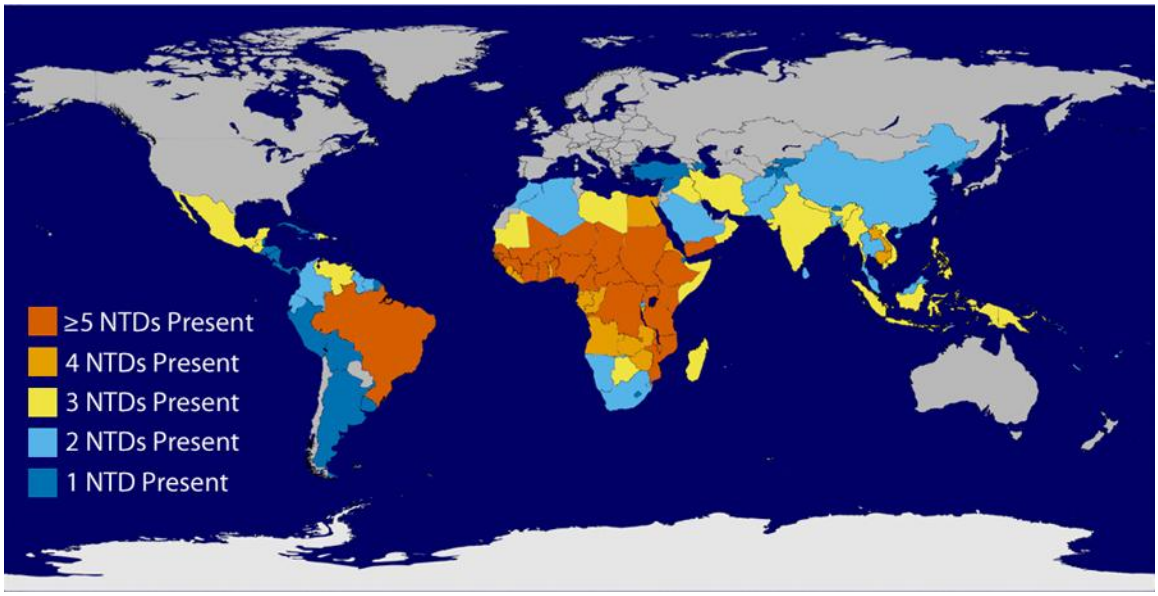

Figure 1-1 Distribution of Neglected Tropical Diseases
[http://www.cdc.gov/globalhealth/ntd/diseases/ntd-worldmap-static.html]

1-2 *LEISHMANIA*

Leishmaniasis is the disease that results from infection with *Leishmania,* a protozoan parasite genus in the Kinetoplastid order and Trypanosomatidae family. There are over 20 species of *Leishmania* that infect humans and the manifestation of disease varies depending on which species has infected the host. The spectrum of disease ranges from cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis.

Cutaneous leishmaniasis is the most common and consists of either a single or multiple skin ulcers. Mucocutaneous disease is rare and affects the nasal and oral mucosa and can be severely disfiguring. Visceral leishmaniasis is the most severe form of disease and is systemic. It results in enlargement of the liver and spleen, as well as anemia, and

is invariably fatal if not treated. It is estimated that 350 million people are at risk of *Leishmania* infection, with an overall prevalence of 12 million. Two million new cases occur annually. Of these, approximately 500,000 are visceral disease, resulting in 60,000 deaths [WHO].

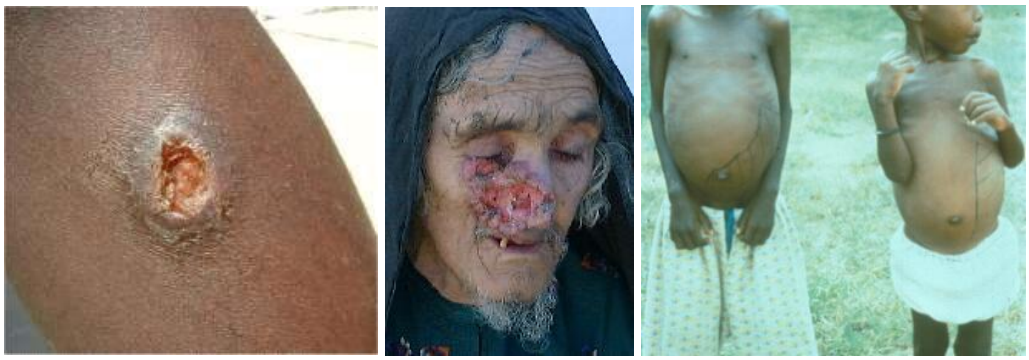

Figure 1-2 (left to right) Cutaneous, mucocutaneous, and visceral leishmaniasis [Armed Forces Pest Management Board, AFPMB]

The World Health Organization considers leishmaniasis to be endemic in 88 countries, covering four continents. The map below represents the global distribution of this disease. The majority of the visceral leishmaniasis cases occur in Bangladesh, Brazil, India, Nepal and Sudan. The species that are responsible for visceral disease are primarily *L. donovani* in the Old World and *L. chagasi* in the New World. Mucocutaneous disease is concentrated in Bolivia, Brazil and Peru, and is most often caused by species in the *L. braziliensis* complex. Cutaneous leishmaniasis is somewhat more widespread, with 90% of the cases occurring in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria. The causative agents of cutaneous disease are primarily *L. major* and *L. tropica* in the Old World, and *L. braziliensis*, *L. mexicana*, and related species in the New World [WHO].

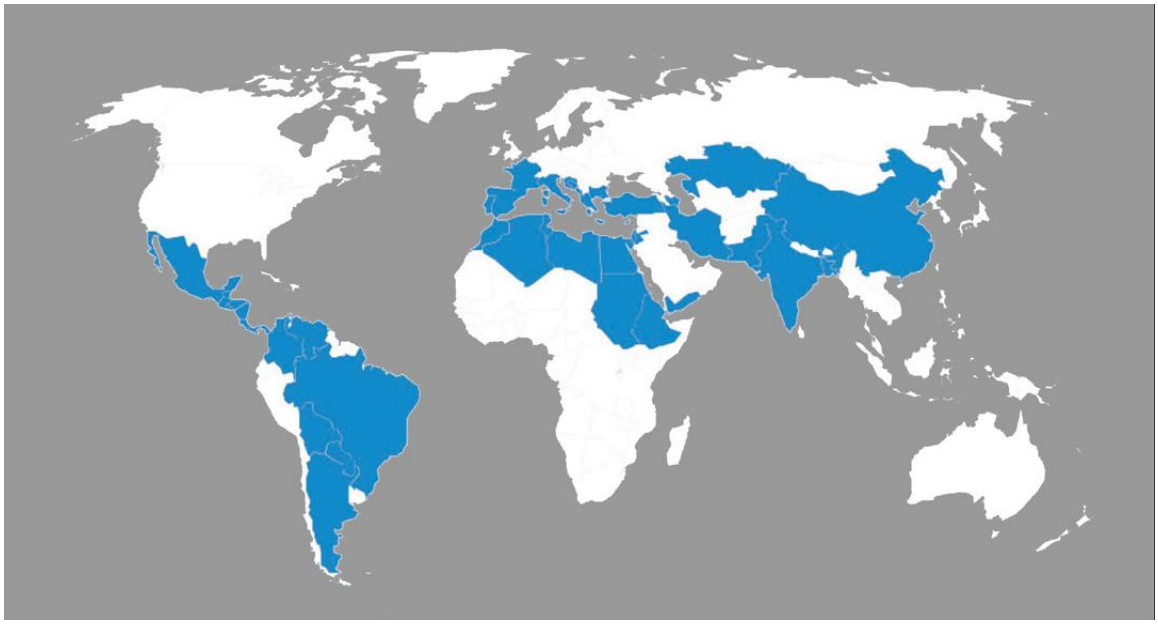

Figure 1-3 Distribution of leishmaniais [BIO Ventures for Global Health]

1-3 TRANSMISSION AND LIFECYCLE

Figure 1-4 Sand fly [AFPMB]

Leishmania species are transmitted via phlebotomine sand flies. There are over 400 species of sand flies, but only about 30 have been confirmed to transmit *Leishmania*. The sand flies that transmit *Leishmania* in the Old World are in the genus *Phlebotomus*. In the New World the sand flies belong to the genus *Lutzomyia*. Interestingly, there seems to be specificity in the species of *Leishmania* that the different species of sand fly will carry and transmit. Transmission occurs when female insects bite and take a blood meal from a mammalian host, which is important for the development of the insects' eggs.

Cycles of infection can be sylvatic or domestic. In sylvatic cycles there is an animal reservoir for *Leishmania* that can maintain transmission indefinitely without human disease. Sporadic or epidemic disease occurs when people encroach on the sylvatic setting. In domestic cycles, humans or dogs make up the primary or only reservoir. In some areas, visceral leishmaniasis transmission is zoonotic (dog – sand fly – human), and in others it is anthroponotic (human - sand fly - human) [WHO, CDC].

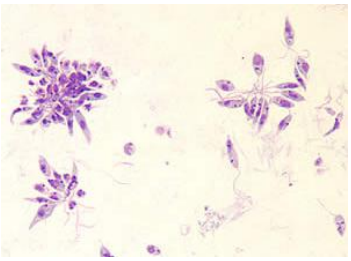

Figure 1-5 *Leishmania* promastigotes

Leishmania parasites are digenetic organisms, having very distinct lifecycle stages depending on whether they are in a mammalian host or the sand fly vector. When the sand fly ingests a blood meal, it may be taking in mammalian host macrophages that are infected with *Leishmania* parasites in the intracellular amastigote form. When the amastigotes exit the macrophage and encounter the environment of the sand fly gut, they transform into an elongated motile promastigote form. Promastigotes remain extracellular and proliferate in the midgut of the sand fly. The promastigotes then move to the alimentary tract of the sand fly and are regurgitated onto the vertebrate host when the fly takes another blood meal.

After infectious promastigotes are deposited at the sand fly bite site, they will be taken up by the phagocytic cells of the mammalian host, primarily macrophages or dendritic cells. It has recently been shown that after inoculation neutrophils can take up the *Leishmania* parasites and then be quiescently phagocytosed by macrophages and dendritic cells [2, 3]. Macrophages seem to be the most heavily infected cell type.

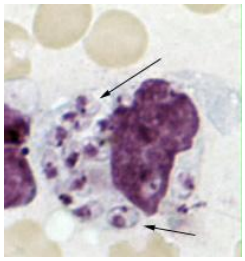

Figure 1-6 *Leishmania* amastigotes inside a macrophage

Inside mammalian host cells *Leishmania* parasites are contained inside of an acidic phagolysosome and transform into the non-motile, intracellular amastigote form. The parasites will proliferate in this form, burst out of the host cell and spread to infect other nearby macrophages. Below is a diagram that outlines the complete lifecycle of *Leishmania* parasites from a sand fly ingesting infected blood, development of the promastigote form of the parasite, and then introduction into a new human host.

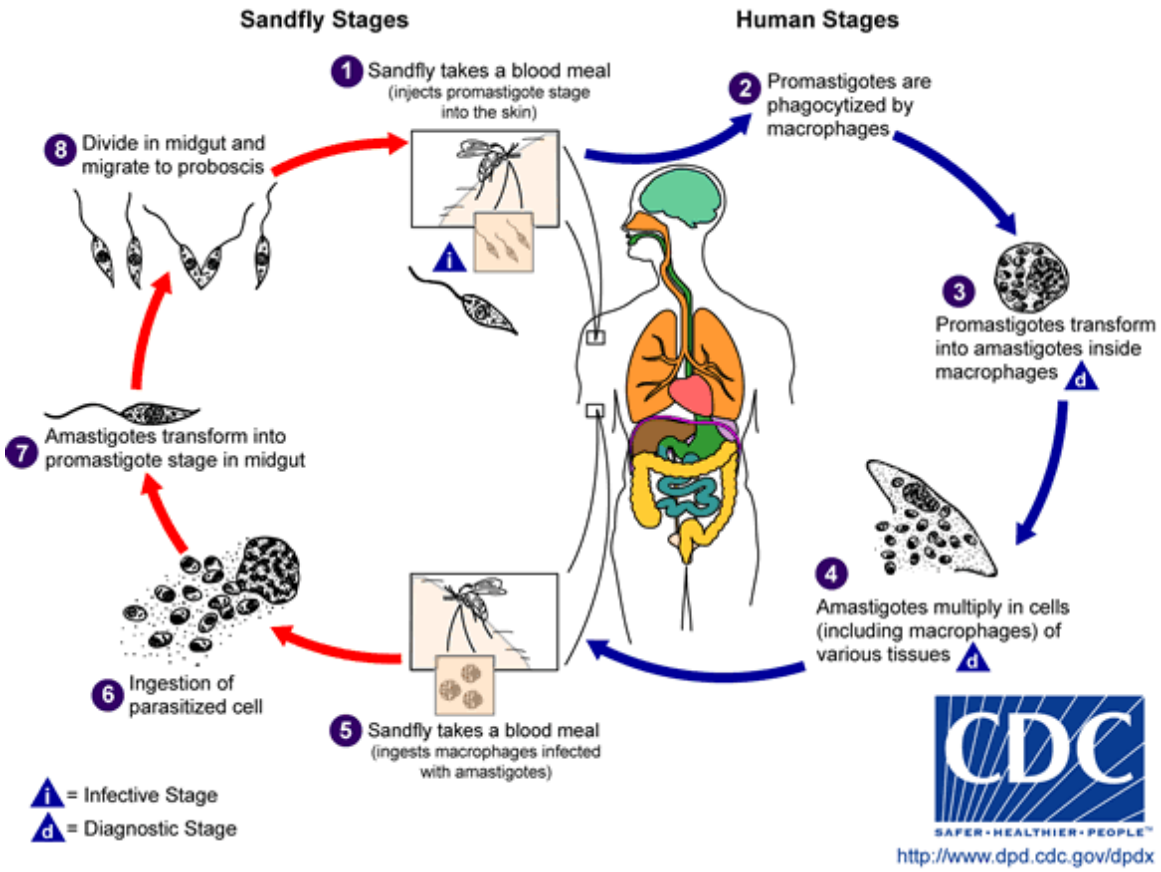

Figure 1-7 Leishmania lifecycle [CDC]

1-4 HOST IMMUNE RESPONSE

Much of what is known about the host response to *Leishmania* infection has come from mouse models of infection and disease. The two most widely used models for *Leishmania* infection are BALB/c and C57BL/6. BALB/c is considered a "susceptible" mouse strain in which infection is readily established and parasites reach high titers. C57BL/6 mice are considered "resistant" and readily control parasite burdens. However, a few cutaneous *Leishmania* species have been shown to produce non-healing lesions in this strain. The disease pathology resulting from *Leishmania* infection varies, as was stated earlier, depending on the species that infects a host, and there are multiple factors that seem to influence whether a human host will develop severe disease or be asymptomatic and potentially clear the infection. The activation status of an infected macrophage, the inflammatory environment and the type of immune response generated are all important components that influence disease outcome.

Studies have shown that different receptors on macrophages can be used to induce phagocytosis of *Leishmania* parasites and different pathways may be activated in the macrophage that will influence the overall immune response to the infection. Factors expressed by the parasites correlate with the type of interaction that exists with the macrophage. The receptors that have been reported to be involved with internalization of *Leishmania* include the third complement receptor (CR3), the first complement receptor (CR1), mannose receptor (MR), Fc Gamma receptors (FcyRs), and fibronectin receptors. Either the classical or the alternative activation pathways will then be induced in the macrophage, resulting in differential L-arginine metabolism. The two

important enzymes in this process are inducible NO synthase (iNOS) and arginase. During alternative activation arginase hydrolyzes L-arginine to form urea and ornithine. Ornithine is the substrate for the first step of the biosynthesis of polyamines, which are small cationic molecules that are essential for growth and proliferation of cells, including *Leishmania* parasites. Classical activation of macrophages results in increased iNOS activity, converting L-arginine to NO, which is a potent cytotoxin and will aid in clearance of *Leishmania [4-6]*.

After the innate response has been initiated, the adaptive immune response to *Leishmania* infection will become polarized to either a Th1 response, which is associated with clearing the infection, or a Th2 response, which is associated with disease progression. In the case of cutaneous leishmaniasis, susceptibility to disease has been associated with the dominance of a TH2 response and elevated levels of IL-4. Conversely, resistance to disease was determined to correspond with the dominance of a TH1 response with high levels of IFN-γ and TNF-α [3, 7-10]. INF-γ is a potent immune-modulator, activating antigen presenting cells (APC), promoting proliferation of cytotoxic CD8+ T cells, and inducing pathogen-killing activities of macrophages. IFN-γ acts to amplify the production of IL-12 in dendritic cells and macrophages, and these two cytokines act in a positive feedback cycle to amplify a TH1 response. IFN-γ can also be inhibitory to other responses, blocking B cell activation and TH2 cytokines, including IL-4. These TH1 responses are characteristic during infections with intracellular pathogens. The TH2 response is typically associated with the production of IL-4, IL-5, IL-6, IL-10 and IL-13, and the inhibition of IL-2, IFN-γ and IL-12. TH2 involvement is

characteristic of an infection with extracellular pathogens and generally promotes a humoral response. The presence of this type of response during murine *Leishmania* infection is unusual given that these parasites are intracellular pathogens. [3, 7, 9, 11].

Less is known about the immune response during visceral leishmaniasis. The research that has been done with mouse models and in limited human studies has suggested *L. donovani* infection does not induce a TH2 response, but is associated with elevated levels of IL-10, which often plays a suppressive role and helps regulate immunopathology [7, 8, 12-16]. A recent study in patients suffering from visceral leishmaniasis demonstrated that increased IL-10 levels correlated with increased parasite loads [16]. Models of cutaneous leishmaniasis have also been associated with elevated IL-10[7, 17-19]. This cytokine, along with TGF-β, has been implicated to have a role in the *in vivo* function of regulatory T cells (Tregs) as immune suppressive agents. It has been suggested that Tregs may promote *Leishmania* survival by dampening effector T cell responses. Moreover, recent studies have shown that higher proportions of regulatory T cells are present in mouse models of cutaneous leishmaniasis and in skin lesions from patients suffering from post kala-azar dermal leishmaniasis (a manifestation of *L. donovani* that can occur after visceral disease) [14, 16, 17, 19, 20]. *Leishmania*-specific expansion of Tregs has been described in experimental infection with *L. major* (a species that manifests as cutaneous disease), and while Treg expansion has been implicated in visceral disease, it has not been directly examined in experimental *L. donovani* infection [15, 21].

1-5 CLINICAL MANIFESTATIONS

The clinical manifestations of cutaneous leishmaniasis can are categorized into 5 groups: localized, disseminated, recidivans, diffuse, and post–kala-azar dermal leishmaniasis.

The most common manifestation by far is localized leishmaniasis (also known as "Oriental sore" or "Baghdad boil"). This form exhibits a slowly developing inflammatory skin sore that appears at the site of the sand fly bite after two to eight weeks. It typically heals spontaneously within a year, leaving a scar. The lesion begins as an asymptomatic nonspecific erythematous papule that looks like an insect bite that slowly enlarges. It may be nodular, plaque-like, or ulcerative. As the lesion progresses the center usually softens and may start to ooze, with a crusting that comes off and is replaced recurrently, resulting in a well circumscribed, shallow ulcer. Lesions have been described to have various appearances, including hyperkeratotic, zosteriform, erysipeloid, sporotrichoid, eczematoid, and verrucous. Disseminated cutaneous leishmaniasis shows spreading from the initial localized lesion. There may be multiple daughter nodules, sporotrichoid subcutaneous nodules that track toward regional lymphatics, or localized adenopathy [22] [WHO, CDC].

Leishmaniasis recidivans (caused mostly by *L. tropica*) is rare variant that presents as a chronic non-healing or relapsing lesion on or near the edge of a scar from a previous cutaneous ulcer. The lesion is usually seen within two years of the original ulcer, however, this type of relapse has been reported as happening much later following trauma or topical steroid use. Because of its resemblance to *lupus vulgaris*, leishmaniasis recidivans has sometimes been called "chronic lupoid leishmaniasis".

Diffuse cutaneous leishmaniasis, mainly caused by *Leishmania aethiopica* in Africa and *Leishmania mexicana amazonensis* in the New World, is an unusual form of the infection. There is profuse parasitization and a lack of an appropriate inflammatory response. Post–kala-azar dermal leishmaniasis (PKDL) is sometimes exhibited by patients that have previously had visceral leishmaniasis due to *Leishmania donovani*. This manifestation consists of symmetric macules, papules, or nodules primarily on the face.

Mucocutaneous leishmaniasis initially manifests as a cutaneous ulcer, but spreads to the nasal and oral mucosa, causing degeneration of the cartilage and soft tissue. Necrosis and secondary bacterial infection commonly occur with this form of leishmaniasis. The lips, palate, and pharynx are often affected and the condition can last for several years [22] [WHO, CDC]..

Visceral leishmaniasis is the most severe manifestation of *Leishmania* infection. The incubation period for this type of disease typically ranges from 2 to 6 months, but can be a little as a few weeks and as long as several years. As the infection progresses to disease infected individuals will develop fever, weight loss, and enlarged organs that persist for months. Splenomegaly is very apparent and soft on palpation. Complications from splenomegaly can include infarction or spontaneous subcapsular bleeding. Enlargement of the liver, or hepatomegaly, may also occur but is less marked. Some symptoms of disease are specific to the particular endemic regions. For instance, lymphadenopathy is usually only observed in Sudan, and darkening of the skin is found in South Asia, (where the Hindi name kala-azar, meaning black fever, originated). Other

symptoms associated with visceral leishmaniasis include anemia, thrombocytopenia, and neutropenia, resulting from bone marrow suppression and splenic sequestration. Patients may exhibit hyperglobulinemia, cachexia, edema, congestive heart failure, and bleeding. In advanced disease there is often significant liver dysfunction .

While visceral leishmaniasis can be quite severe, some infected individuals may remain asymptomatic. In some endemic regions, up to 30% of the population may exhibit asymptomatic infection. However, these individuals can still be a source for disease transmission. Additionally, HIV co-infection is a major emerging problem. Contracting HIV can activate an asymptomatic *Leishmania* infection or make a person more susceptible to new *Leishmania* infection [23] [24] [CDC, WHO].

1-6 PREVENTION AND CONTROL

Vaccine strategies against parasitic diseases have not been successful, leaving chemotherapeutics and vector control efforts as the main defenses against leishmaniasis. Sand flies are most active from dusk until dawn, and remaining indoors or covered during these times can help reduce exposure to sand fly bites. Bed nets are important as well and should be a small weave and treated with repellent. Insecticides and repellents are can be used around dwellings to prevent sand flies from establishing themselves in these areas.

Because infected people constitute such a large reservoir of *Leishmania*, early diagnosis and treatment may be the most important means of controlling future infections. However, current drugs used for the treatment of leishmaniasis are far from ideal, as evidenced

by their high risk of toxicity and often their requirement for multiple administrations over extended periods of time.

The most widely used chemotherapeutics agents used to fight *Leishmania* infection are the pentavalent antimonial drugs, sodium stibogluconate and meglumine antimoniate. The dosing of these drugs consists of 20 mg/kg/day of antimony intravenously or intramuscularly over 20-30 days. For visceral leishmaniasis, resistance to antimoial drugs is on the rise, particularly in the region of Bihar, India, where resistance levels have escalated to approximately 60%. The most effective alternative for treating visceral leishmaniasis is liposomal amphotericin B (Ambisome). The current treatment regimen of this drug consists of 3 mg/kg on days 1-5, 14 and 21. For individuals with HIV-*Leishmania* co-infection a higher total dose (e.g., 40-60 mg/kg total dose) and a longer course is usually recommended as well as post-treatment secondary prophylaxis. Conventional amphotericin B deoxycholate has been shown to be effective against *Leishmania*, but it is associated with high risk of renal toxicity and other side effects. The regimens for this drug include 0.75 to 1.0 mg/kg daily for 15 to 20 days or 0.75 to 1.0 mg/kg every other day for 30 to 40 days.

Other treatments that are available for cutaneous leishmaniasis include oral antifungal drugs (fluconazole, ketoconazole, itraconazole) with results varying depending on the *Leishmania* species and geographic location. The following treatment schemes have shown efficacy in clinical trials: fluconazole 200 mg daily for 6 weeks for cutaneous leishmaniasis due to *L. major*, and ketoconazole 600 mg daily for 28 - 30 days for cutaneous leishmaniasis due to *L. mexicana*, *L. panamensis* and *L. major*. There is

potential for a higher dose of fluconazole (8 mg/kg for 4-6 weeks) to have efficacy against *L. braziliensis*, but the most effective treatment for this species seems to be the pentavalent antimonial drugs. Liposomal amphotericin B also appears to have efficacy against several cutaneous *Leishmania* species, but data are limited and the optimal dose regimen has not been established. Other treatment modalities that have shown some efficacy for cutaneous leishmaniasis due to some *Leishmania* species include topical paromomycin ointment, oral miltefosine, thermotherapy, and intralesional pentavalent antimonial drugs, but efficacy data are limited. There are also attempts at developing combination therapies that would help reduce the length of treatment and combat the emergence of drug resistance [WHO].

In addition to long treatment courses and relatively high toxicity profiles of current treatments, the problem of cost and availability is still a major hindrance to effective therapeutic control of leishmaniasis. Consequently, the need for new and more efficacious antileishmanial drugs is acute and identifying and validating new targets in parasites is a critical component of this process.

1-7 POLYAMINES

One of the biochemical pathways of *Leishmania* that has shown potential as a therapeutic target is the metabolism of polyamines, small cationic molecules. The precise functions of polyamines are largely unknown, but numerous studies have shown that they are crucial for proliferation and differentiation of many eukaryotic cells, including *Leishmania*. [7] The polyamine biosynthetic pathway in protozoan parasites differs significantly from the human host pathway. Differences have been observed in

the variety and amounts of polyamines and their metabolites, enzymatic machinery employed in biosynthesis and degradation, functions of polyamine-containing compounds, and polyamine enzyme half-life and turnover. [8-9] The four enzymes of the polyamine biosynthetic pathway of *Leishmania* are: 1) arginase (ARG) that generates ornithine and urea; 2) ornithine decarboxylase (ODC) that catalyzes the decarboxylation of ornithine to form putrescine; 3) S-adenosylmethionine decarboxylase (ADOMETDC) that produces decarboxylated S-adenosylmethionine (dcAdoMet) for aminopropylylation of putrescine; and 4) spermidine synthase (SPDSYN) that transfers the aminopropyl moiety from dcAdoMet to putrescine.

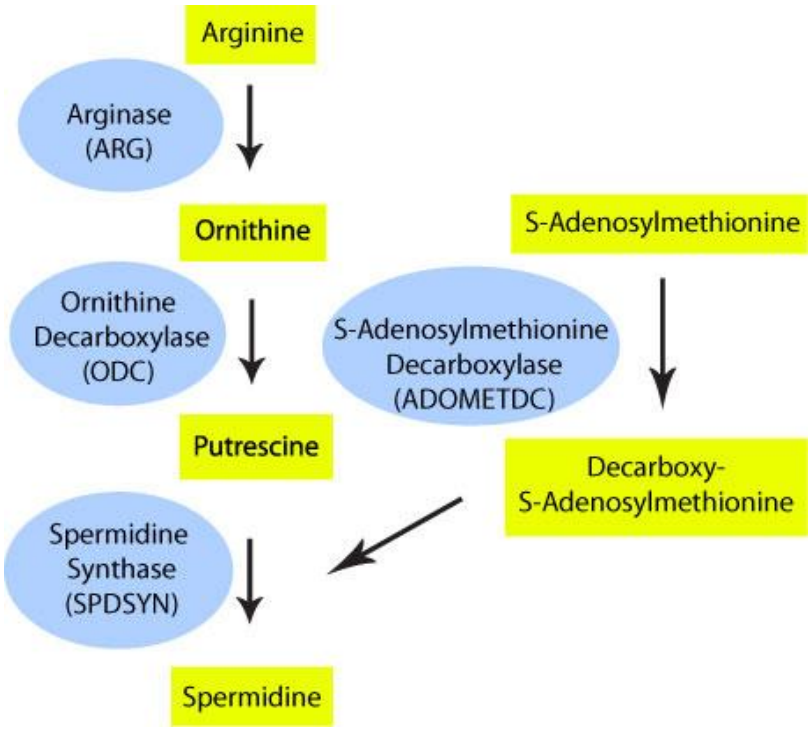

Figure 1-8 Polyamine Biosynthetic Pathway in *Leishmania*

There is an additional step in polyamine biosynthesis that exists in mammalian cells, but is absent in *Leishmania*. The enzyme spermine synthase (SPMSYN) catalyzes the transfer of an additional aminopropyl group to spermidine to generate spermine, which seems to be one of the more abundant polyamines in mammalian cells. There is no synthesis or utilization of spermine in *Leishmania* parasites. Another

difference between the two systems is that mammalian cells have a catabolic pathway and can interconvert polyamines. This capacity does not exist in *Leishmania*. A major function of polyamines in *Leishmania* is to provide the precursor necessary to the production of trypanothione. Trypanothione is a conjugate of glutathione and spermidine, and it is an essential thiol involved in the redox metabolism of the parasite.

There are many specific inhibitors of polyamine enzymes, most of which have been developed as anti-cancer agents. (Nearly all cancer cells contain elevated concentrations of polyamines). One of the first rationally designed anti-tumor agents is α-difluoromethylornithine (DFMO), a suicide inhibitor of ODC. [11-12] This drug gave the first indication that inhibitors of polyamine metabolism could impact the growth of protozoan parasites. It was shown that DFMO could eradicate T*rypanosoma brucei brucei* infections in mice. [13] It has also been shown that 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (MDL 73811), an irreversible inhibitor of ADOMETDC, is active *in vitro* and in vivo against *T. b. rhodesiense* and *T. b. brucei* at much lower concentrations than DFMO. [14] Other ADOMETDC inhibitors that have antiparasitic activity include methylglyoxal bis, pentamidine, and berenil [15], although our laboratory has demonstrated that only MDL7381 has an effect on intact *L. donovani* parasites. [16-17] Additionally, previous work has shown that *L. donovani* in which the genes encoding polyamine biosynthesis enzymes have been knocked out are auxotrophic for polyamines in culture. These knockout parasites require exogenous polyamine supplementation to survive and proliferate. However, these knockouts were

generated in non-infectious parasites and the *in vivo* consequences of the lack of polyamine biosynthesis could not be determined.

Altogether, the differences between the mammalian and parasite polyamine pathways and the evidence of polyamine enzyme inhibitors provide a solid basis for further validation of the polyamine biosynthetic pathway as a drug target in *Leishmania donovani*. Prior to the work described in this thesis, the Ullman laboratory successfully knocked out the *ODC* gene from an infectious strain of *L. donovani* (LdBob strain) and determined that the infectivity level of the knockout parasites was drastically reduced compared to wild type parasites. This background led to my work and the continued validation of the *L. donovani* polyamine biosynthesis pathway as a drug target.

1-8 GOALS OF THIS THESIS

The overall goal of this thesis is to provide evidence that multiple components of the *L. donovani* polyamine pathway are required for successful infection of a mammalian host, and are therefore potential drug targets. To accomplish this goal I have employed several tools and techniques, including *Leishmania* cell culture, genetic manipulation, molecular biology, and animal models. I have employed the BALB/c mouse model of infection to demonstrate genetically, pharmacologically, and nutritionally that targeting polyamines *in vivo* affects the infection level of *Leishmania donovani* parasites.

Chapter 2 – Manuscript 1

Oral putrescine restores virulence of ornithine decarboxylase-deficient *Leishmania donovani* in mice

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Abbreviations: DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase

2-1 INTRODUCTION

Leishmania donovani, the etiologic agent of visceral leishmaniasis, is a digenetic parasite that exists as the extracellular promastigote in its insect vector and as the intracellular amastigote within the phagolysosome of macrophages and other reticuloendothelial cells of the mammalian host. The current arsenal of drugs that is used to treat visceral and other forms of leishmaniasis is far from ideal. The mainstay drugs include pentavalent antimonials, pentamidine, amphotericin B, and miltefosine, and the chemotherapeutic regimens that employ these drugs are complicated by toxicity, often invasive routes of administration, and the emergence of drug refractoriness/resistance. Consequently, the need for new and more efficacious antileishmanial drugs is acute, and identifying and validating new targets for leishmaniasis is a critical component of this process.

One pathway that has stimulated considerable interest among scientists and clinicians alike for the treatment of parasitic diseases is that for the biosynthesis of polyamines, aliphatic cations that play vital roles in key cellular processes such as growth, differentiation, and macromolecular synthesis. The first intimation that inhibitors of polyamine biosynthesis could significantly impact the viability of protozoan parasites was the observation by Bacchi and coworkers that α-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC) [25], could eradicate *Trypanosoma b. brucei* infections in mice [26]. This seminal contribution was followed by the demonstration that DFMO was an extremely effective agent (>95% cure) against early- and late-stage African sleeping sickness caused by *T. b. gambiense*, including

many arsenical-refractory cases that would otherwise have proven fatal [27, 28]. Subsequent studies by Wang, Coffino, and coworkers revealed that the selectivity of DFMO toward African trypanosomes could be ascribed to the extremely rapid rate of turnover of the mammalian ODC compared to the trypanosome counterpart, a lability that is conferred by a 39 amino acid COOH-terminal extension rich in PEST sequences [29].

DFMO is also effective against *Leishmania* promastigotes propagated axenically *in vitro* [30]. However, DFMO appears to be much less effective against the infectious amastigote form of the parasite. DFMO can reduce parasite loads in short-term experimental *Leishmania* infections in mice and hamsters but is not curative [30-32]. For instance, Keithly and Fairlamb reported that DFMO in the drinking water could partially suppress the parasitemia in affected organs of Balb/c mice infected with either *L. donovani* or *L. braziliensis* and that the antiparasitic effects of DFMO could be synergistic, additive, or have no effect when combined with other antileishmanial agents [30]. A later study by Gradoni *et al.* [32] showed that oral DFMO administration markedly suppressed *L. infantum* infections in Balb/c mice. Additionally, Mukhopadhyay and Madhubala [31] reported that a 2% DFMO solution provided to Syrian golden hamsters 2-6 days post-infection with *L. donovani* suppressed the number of parasites in liver and spleen by 90% and 99%, respectively. In none of these experimental infections did DFMO achieve elimination of the parasite, but it should be noted that the studies were preliminary and the time courses of drug administration limited. As a consequence, it has been generally viewed that ODC and the other

enzymes of the polyamine biosynthetic pathway are not realistic drug targets for curing leishmaniasis, primarily due to the notion that the phagolysosome in which the parasite resides is replete with salvageable polyamines that can bypass a pharmacological block of ODC.

This assumption that ODC and the polyamine pathway are not valid drug targets has been challenged by the recent observation that a *Δodc* lesion, which renders the parasite auxotrophic for polyamines, profoundly impairs the ability of *L. donovani* to sustain an infection in Balb/c mice [33]. Parasite burdens in liver and spleen were reduced by six and two orders of magnitude, respectively, in mice inoculated with *Δodc* knockout lines compared to mice injected with wild type *L. donovani* [33]. Moreover, complementation of the *Δodc* with an *ODC*-containing plasmid rescued the virulence defect and restored polyamine prototrophy. The finding that a *Δodc* lesion profoundly compromises *L. donovani* virulence in a manner that can be circumvented by episomal complementation provides compelling genetic evidence that ODC is a potential therapeutic target for leishmanial infections. These results do not, however, prove that the damaging effect of an *ODC* lesion on *L. donovani* virulence is due to a consequence of a deleterious effect on polyamine biosynthesis. It is plausible that *ODC* may have an additional essential function in the amastigote that is unrelated to its acknowledged metabolic role and that the avirulence of the *Δodc* lines is due to the loss of that second function.

2-2 RESULTS

In order to authenticate that the mechanism of avirulence in the *Δodc* parasites is attributable to a loss of polyamine biosynthetic capacity in amastigotes, we evaluated whether dispensing putrescine as a 1% solution (w/v) in the drinking water could rescue the detrimental effects of a *Δodc* null mutation on parasite infection in mice. This approach mirrors that taken by Bacchi and coworkers when they demonstrated that polyamine administration could antagonize DFMO-elicited cures of *T. brucei* infections in mice [34]. Oral putrescine was chosen as a route of administration since the bioavailability of polyamines in the gut is proficient [35], and 1% putrescine in the drinking water has been provided to mice for periods up to 10 weeks without side effects [36, 37]. The four week course of oral 1% putrescine increased the parasite burden of livers of mice infected with *Δodc L. donovani* by almost three orders of magnitude, while the parasite loads in mouse spleens were augmented approximately 10-fold (Fig. 1). Putrescine had no effect on parasite numbers in mice infected with wild type *L. donovani* (data not shown). The substantial reversal of the virulence deficit of *Δodc* parasites by putrescine indicates that the orally administered diamine can access the phagolysosome of infected macrophages in liver and spleen. Furthermore, this partial virulence reversal provides a proof-of-concept that the loss of virulence in the null parasites is due to the inability of the mutant parasite to synthesize sufficient polyamines *de novo* and not to an alternative *ODC* function in the *L. donovani* amastigote. Furthermore, these data imply that *Δodc L. donovani* amastigotes fail to thrive because they cannot retrieve host polyamines in adequate quantities for survival

and growth under physiological conditions. It should be noted, however, that infectivity rates of *Δodc* parasites were not restored to wild type levels by oral administration of putrescine (Fig. 1). The explanation for this, although uncertain, could be ascribed to limitations in putrescine absorption or pharmacokinetics that prevent unhindered access of the amastigote sequestered in the phagolysosome of macrophages in liver and spleen to the orally administered diamine.

To determine whether *L. donovani* ODC could be pharmacologically targeted by DFMO in an experimental murine infection using a regimen that more closely imitates the experimental paradigm used for the *Δodc* parasites ([33] and Fig. 1) than the previous short-term rodent studies with DFMO [30-32], Balb/c mice infected with wild type *L. donovani* were treated with a 2% DFMO solution in the drinking water for three weeks. DFMO is known to be well-absorbed (50% oral bioavailability) after oral administration, although the half-life in serum is short [38], and this DFMO concentration and route of administration is curative for *T. brucei* infections after three days [26, 34] and dramatically reduces *L. donovani* parasitemias in hamster spleen and liver after four days [31]. Furthermore, it is known that DFMO is a particularly well-tolerated agent in experimental animals, and provision of DFMO as a 2% solution in the drinking water for up to 28 days shows no visible signs of toxicity in mice [39, 40]. The effects of oral administration of DFMO on parasite burdens in mice infected with wild type parasites are shown in Fig. 2. Whereas the negative *Δodc* control parasites exhibited the expected dramatically reduced parasite loads in both liver and spleen, the three week exposure of mice inoculated with wild type *L. donovani* to DFMO only reduced parasite

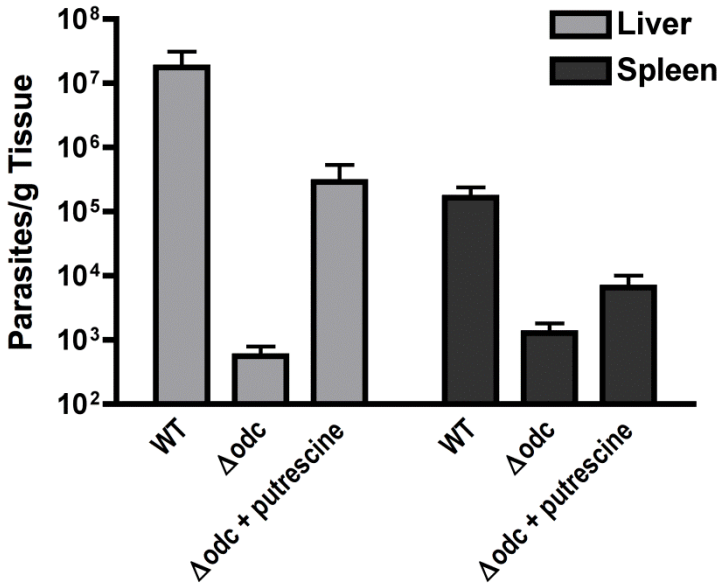
levels by an order of magnitude compared to untreated controls (Fig. 2) and had no effect on the splenic amastigote numbers. These data are similar to those reported previously for experimental DFMO treatments of *Leishmania* infections [30-32]. The discrepancies in the extents by which genetic ablation and pharmacologic inhibition of ODC affect *L. donovani* virulence can be ascribed to limited DFMO bioavailability or to the lack of DFMO access to the intracellular parasite and indicate that there is little rationale for exploiting DFMO as an antileishmanial drug. The data do not, however, negate ODC as a drug target for leishmaniasis and encourage the discovery of potent inhibitors with better therapeutic properties as a valid therapeutic paradigm.

2-3 Conclusions

Collectively, these data reveal that the avirulent phenotype of the *Δodc L. donovani* [33] can be ascribed to a loss of metabolic function rather than to an unknown complementary function for the *ODC* gene and ODC protein. The partial restoration of *Δodc* virulence by putrescine suggests that the orally supplied diamine can reach the phagolysosome of macrophages in liver and spleen, the milieu in which *L. donovani* resides. This further suggests that *L. donovani* amastigotes in the normal physiological setting cannot salvage adequate amounts of host polyamines to bypass a genetic or pharmacologic deficiency in ODC and that the *Δodc* avirulent phenotype cannot be ascribed to polyamine transport down regulation in the amastigote as has been reported for the *L. major* polyamine transporter [41]. These results further validate ODC as a potential therapeutic target, although DFMO is clearly not a leading candidate drug, and reinforce efforts to discover additional ODC inhibitors. Finally, these findings provide a foundation for subsequent investigations to determine whether genetic lesions in other components of the polyamine pathway, e.g., ARG, ADOMETDC, and SPDSYN, impact virulence of *L. donovani* and are, therefore, putative therapeutic candidates [42, 43].

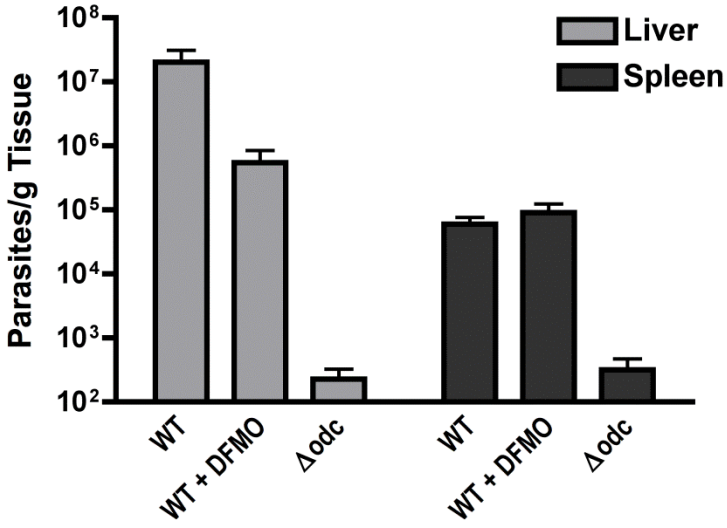
2-5 FIGURES AND METHODS

Figure 2-1



Putrescine partially reverses avirulent phenotype of *Δodc L. donovani*. Three cohorts of five 6-7 week old female Balb/c mice were inoculated with 5 X 10^6 stationary phase promastigotes by tail vein injection. One group was injected with wild type parasites and the other two with *Δodc L. donovani* knockout. One of the groups of mice injected with *Δodc* parasites was given a 1% putrescine solution to drink for 28 days while the remaining groups were provided with unadulterated drinking water. Mice were weighed and fluid intake measured at weekly intervals. No differences in body weights or fluid consumption were observed among the three cohorts, reflective of negligible toxicity triggered by the putrescine solution. Mice were sacrificed at four weeks and livers and spleens harvested and processed by standard surgical techniques. Single cell suspensions of mouse organs were prepared by passage through a 70 µm cell strainer (BD Falcon), and parasitemias were then determined in 96-well microtiter plates using the standardized limiting dilution assay of Buffet *el al.* [44]. For this infectivity study, parasite strains were cycled back and forth between promastigotes and axenic amastigotes just prior to animal inoculation in order to ensure optimal infectivity. This experiment has been repeated one time with similar findings. Data presented are the average results and standard error from five animals.

Figure 2-2



Oral administration of DFMO reduces parasite burden in livers but not spleens of Balb/c mice infected with *L. donovani*. Two groups of five 6-7 week old female Balb/c mice were infected with 5 X 10^6 wild type promastigotes and one set of five mice was inoculated with an equivalent number of the *Δodc* mutant via tail-vein injection. Seven days post-infection, one of the two groups of mice infected with the wild type line was provided with a 2% (w/v) solution of DFMO in the drinking water for three additional weeks. The other group of mice injected with wild type parasites, as well as the group inoculated with the *Δodc* mutant, was given water for 28 days. Mice in each cohort were monitored weekly for weight and fluid intake with no significant differences observed between groups. At 28 days post-infection, mice were harvested as described in Fig. 1, the livers and spleens excised, and parasitemias determined by the limiting dilution method [44]. Data presented are the average results and standard error from five animals.

Chapter 3 – Manuscript 2

Spermidine Synthase is Required for Virulence of *Leishmania donovani*

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3-1 INTRODUCTION

Leishmania donovani, a digenetic protozoan parasite, is the etiological agent of visceral leishmaniasis, a devastating and invariably fatal disease when left untreated. This unicellular eukaryote lives as the extracellular, flagellated promastigote within its insect vector, the phlebotomine sand fly, and resides as the intracellular, non-motile amastigote within the phagolysosomes of infected macrophages and other reticuloendothelial cells of the mammalian host. There is no reliable vaccine for leishmaniasis, and, consequently, drugs are the only avenue by which the disease can be treated. Regrettably, the existing collection of antileishmanial drugs is far from ideal, primarily due to their lack of selectivity toward the metabolic machinery of the parasite. Drug toxicity, as well as increased drug resistance [45, 46], reinforces the need to develop new therapeutics and to identify and validate new drug targets.

One metabolic pathway that has drawn considerable attention in proposing new antiparasitic therapies is that for the synthesis of polyamines, ubiquitous aliphatic cations (putrescine, spermidine, spermine) that are known to play critical roles in a variety of fundamental cellular processes such as growth, differentiation, and macromolecular synthesis [47-50]. Polyamines also act as precursors for the production of trypanothione, a molecule present in trypanosomatids that is involved in combating oxidative stress [51]. The root of this interest in the polyamine biosynthesis pathway originates from the observation that DL-α-difluoromethylornithine (DFMO) has proven effective in curing West African sleeping sickness caused by *Trypanosoma brucei gambiense*, a protozoan parasite phylogenetically similar to *Leishmania* [52-56]. DFMO

is a suicide inhibitor of ornithine decarboxylase (ODC), the enzyme that catalyzes the conversion of ornithine to putrescine [57]. This drug is also effective at killing other genera of protozoan parasites *in vitro* [58-61], including *Leishmania* promastigotes [61-65], and markedly ameliorates but does not eliminate short-term *L. donovani* infections in mice [32, 63, 66] and hamsters [67]. In addition, inhibitors of a second enzyme in the polyamine pathway, *S*-adenosylmethionine decarboxylase (ADOMETDC), the enzyme that produces the decarboxylated S-adenosylmethionine substrate for the spermidine synthase (SPDSYN) reaction, are also effectual antitrypanosomal agents [59, 68-74].

The polyamine biosynthetic pathway of *Leishmania* is comprised of four enzymes: arginase (ARG), ODC, ADOMETDC, and SPDSYN. The genes encoding all four enzymes have been cloned, and conditionally lethal gene knockouts have been created in *L. mexicana* and *L. major* (*Δarg* mutant only) [75-78] and in *L. donovani* (*Δodc*, *Δspdsyn*, and *Δadometdc* mutants) [79-82] via double targeted gene replacement. Growth studies with the mutant promastigotes revealed that each knockout was auxotrophic for polyamines as a consequence of the gene deletion events and that this nutritional deficiency could be circumvented by propagation in medium supplemented with an appropriate source of polyamine or polyamine precursor. Thus, an intact polyamine biosynthetic pathway is essential for the viability and growth of the promastigote stage of the *Leishmania* parasite.

Despite the extensive genetic and biochemical characterization of the null mutants in their promastigote stage, only recently have polyamine gene functions been assessed in the infectious amastigote stage of the parasite. The evaluation of polyamine gene

function in amastigotes has also been hampered by the fact that the initially characterized *Δodc*, *Δadometdc*, and *Δspdsyn L. donovani* knockouts were created within an attenuated wild type strain of *L. donovani* that had lost its capacity to infect macrophages or rodents, therefore precluding functional evaluation of these genes as virulence determinants [79-81]. Thus, an effort to reconstruct the genetic lesions in polyamine biosynthesis genes in a virulent *L. donovani* background was initiated.

Creation of a *Δodc* lesion in a wild type clone of this virulent strain dramatically compromised the ability of the parasite to infect mouse livers and spleens [82]. Moreover, episomal complementation of the chromosomal lesion in *ODC* restored parasite burdens in infected organs to near wild type levels, proving that the virulence defect in the *Δodc* knockout was triggered by the polyamine gene deletion event [82]. Recent studies also found that exogenous administration of putrescine to mice infected with *Δodc L. donovani* partially restored virulence in the mutant parasites [66], further substantiating that the virulence defect in the *Δodc* knockout is due to a lack of polyamine biosynthetic capacity and not to a secondary unknown function of *ODC*. The fact that a defect in the *ODC* gene so profoundly compromises the virulence of *L. donovani* suggests that other enzymes in the polyamine biosynthesis pathway might be critical virulence determinants as well.

To address the fundamental question whether other components of the polyamine biosynthesis pathway are essential for *L. donovani* to trigger an infection in mammals, we have recapitulated the *Δspdsyn* genotype within a virulent strain of *L. donovani* and evaluated the capacity of the *Δspdsyn* mutant to infect mice. Here we report that

parasite burdens in livers and spleens of mice infected with the knockout parasites are significantly lower than those infected with the wild type strain. These studies demonstrate that *L. donovani* amastigotes require SPDSYN activity to sustain a robust infection in mice and indicate that SPDSYN and perhaps all of the enzymes in the polyamine pathway are realistic therapeutic targets for the treatment of leishmaniasis.

3-2 RESULTS

Molecular characterization of *Δspdsyn* lines in LdBob *L. donovani*. Although *Δspdsyn L. donovani* promastigotes have been described previously, the genetic lesion was created within an avirulent strain that precluded ascertaining whether *SPDSYN* was an indispensable virulence determinant [79]. Thus, the *Δspdsyn* lesion was reconstructed in LdBob, a virulent *L. donovani* strain that is capable of establishing robust visceral infections in mice [82-85]. Two independent *Δspdsyn* clones were isolated after double targeted gene replacement and designated *Δspdsyn1* and *Δspdsyn2*. In order to authenticate the deletion of both *SPDSYN* alleles, Southern blot analysis was performed on genomic DNA prepared from wild type, *Δspdsyn1* and *Δspdsyn2* parasites using probes to the *SPDSYN* gene coding region (Fig. 1A, 1B). Ethidium bromide staining confirmed equal loading of chromosomal DNA and the Southern analysis demonstrated the expected genetic lesion. To further substantiate the gene knockout, expression of the SPDSYN protein was examined by Western blot in the same three cell lines as well as an add-back line *Δspdsyn1*[pXG-BSD-*SPDSYN*] (*Δspdsyn1*[p*SPDSYN*]) consisting of the *Δspdsyn1* mutant with a multi-copy episome containing the *SPDSYN* open reading frame (Fig. 1C). Polyclonal antisera specific for SPDSYN recognized the expected ~33-kDa protein corresponding to the predicted *SPDSYN* translation product [79] in both wild type and *Δspdsyn1*[p*SPDSYN*] lysates while this band was not detected in extracts prepared from *Δspdsyn1* and *Δspdsyn2* lysates (Fig. 1C). The blot was also probed with anti-tubulin antibody as a loading control.

***Δspdsyn* parasites are polyamine auxotrophs.** A growth assay was performed to determine the reliance of the *Δspdsyn* knockout cell lines on polyamines, and as anticipated, *Δspdsyn1* promastigotes were auxotrophic for polyamines. While wild type parasites had undergone approximately 10 – 11 cell doublings during the course of the growth experiment, the mutant cell line was unable to proliferate in polyamine-deficient medium (Fig. 2). Visual inspection of the *Δspdsyn1* promastigotes incubated under these nonpermissive conditions at the end of the experiment revealed a few surviving parasites that were misshapen and lethargic. These parasites did not recover when maintained for longer periods of time under nonpermissive conditions. The polyamine auxotrophy of the *Δspdsyn1* null mutant was circumvented genetically in the *Δspdsyn1*[p*SPDSYN*] promastigotes by complementation with the covering *SPDSYN* plasmid or nutritionally by supplementation of the culture medium with spermidine (Fig. 2). The polyamine auxotrophy could not, however, be bypassed by the addition of putrescine, the product of ODC and a substrate of SPDSYN (Fig. 2). The *Δspdsyn2* clone displayed a growth phenotype identical to that of *Δspdsyn1* but was not examined in as great of detail (data not shown).

***Δspdsyn L. donovani* has reduced virulence *in vivo*.** Virulence studies in Balb/c mice were carried out to determine the effects of a genetic deficiency of *SPDSYN* and to further characterize the SPDSYN enzyme as a potential therapeutic target. Groups of five BALB/c mice were inoculated with either wild type, *Δspdsyn1*, or *Δspdsyn1*[p*SPDSYN*] promastigotes and after four weeks parasite loads were quantified in livers and spleens, the two organs most affected by a visceralizing *Leishmania*

infection. The ability of the *Δspdsyn1* knockout parasites to infect mice was severely compromised (Fig. 3). The average parasite burdens in livers and spleens of mice infected with wild type parasites were three and two orders of magnitude higher, respectively, than those of *Δspdsyn* parasites. The virulence defect of the knockout lines was rescued by episomal complementation with parasitemias in livers and spleens of *Δspdsyn1*[p*SPDSYN*]-infected mice equivalent to those obtained in mice inoculated with wild type parasites (Fig. 3). It should be noted that the discrepancy in the absolute numbers of wild type parasites between livers and spleens on a per-gram basis is typical for *L. donovani* infections [86-89].

3-3 CONCLUSIONS

Previous studies with *Leishmania* null mutants have established that each component of the polyamine biosynthetic pathway, ARG, ODC, ADOMETDC, and SPDSYN, is indispensable for the viability and growth of the promastigote stage of the parasite [76, 77, 79-81]. Whether all of these enzymes are also essential for the amastigote to maintain an infection is less clear, because virulence data with knockout strains from several species have not offered a consistent conclusion. Parasite burdens and lesion sizes of mice infected with *Δarg L. mexicana* [78] or *Δarg L. major* [75, 77] were only somewhat lower than in mice infected with the corresponding wild type line from which these mutants were derived. In contrast, parasite loads in mice infected with *Δodc L. donovani* [82] were dramatically reduced by many orders of magnitude compared to mice inoculated with the wild type progenitor. Whether the differences in the impact of the genetic lesions can be ascribed to the nature of the genetic lesion, to species differences, or to differences in the cutaneous and visceral environments in which the species reside is unknown. Resolution of this fundamental question requires the evaluation of mutations of the same polyamine pathway gene in the different *Leishmania* species, i.e. a comparison of a *Δodc* mutation in *L. donovani*, *L. mexicana* and *L. major*.

To establish whether null mutations in the *L. donovani* polyamine biosynthetic pathway other than *ODC* could also affect the virulence of the organism, a null mutant at the *SPDSYN* locus was created in a virulent wild type background. The *Δspdsyn* mutation conferred polyamine auxotrophy (Fig. 2), and caused a substantial reduction in parasite

burdens in mice when compared to wild type (~ 3 orders of magnitude reduction in livers and 2 orders of magnitude reduction in spleens) (Fig. 3), although the extent of the virulence defect in mice was less than that previously observed for *Δodc L. donovani* [82].

It should be noted that livers and spleens from mice inoculated with *Δspdsyn* parasites retained a small population of persistent parasites, ~10^3 per liver and spleen, after the 4 week infectivity experiment (Fig. 3). The mechanism of this persistence is unclear and is difficult to analyze at the molecular level since the persistent parasites can only be resurrected under permissive growth conditions *in vitro* (see Fig. 2). One could speculate that this persistence is analogous to the dying but enduring *Δspdsyn* parasites observed under nonpermissive growth conditions *in vitro* (Fig. 2), and infer that it takes >4 weeks to kill amastigotes by polyamine starvation *in situ*. However, whether these persistent *Δspdsyn* amastigotes would eventually expire is difficult to assess because BALB/c mice eventually clear *L. donovani* parasites with parasitemias beginning to decrease 4 – 8 weeks post-infection [90]. A more suitable model for assessing long-term virulence of this parasite is the Syrian Golden hamster, a rodent capable of mimicking the human form of visceral leishmaniasis [88]. Testing Δ*spdsyn* virulence in hamsters is a logical next step in SPDSYN target validation.

The reason for the discrepancy in the virulence deficit triggered by the *Δodc* and *Δspdsyn* lesions in an otherwise isogenic background is not known but could be ascribed to differences in the putrescine and spermidine contents of the phagolysosome in which visceral macrophages reside. It is likely that *L. donovani* amastigotes require both

putrescine and spermidine for optimal proliferation as has been shown for promastigotes [81]. If, for example, the phagolysosomal environment contains pools of spermidine but not putrescine, then a *Δodc* lesion would be expected to have far more deleterious consequences to the parasite than a *Δspdsyn* defect, because *L. donovani* lacks a back conversion pathway from spermidine to putrescine but can readily synthesize spermidine from putrescine through ODC. The conjecture that the putrescine and spermidine pools of the phagolysosome are different can be further explored by determining the virulence properties of *Δadometdc L. donovani*, which like *Δspdsyn* parasites, require spermidine but not putrescine for survival [80].

It should be noted that *Δodc L. donovani* promastigotes are much more susceptible to putrescine withdrawal than *Δspdsyn L. donovani* promastigotes are to spermidine withdrawal (personal observations). This intriguing observation is mirrored in *T. brucei*, where genetic investigations showed that depletion of putrescine results in a more rapid cell death than spermidine depletion [91]. Although the cellular functions of polyamines are not completely understood, it appears evident that putrescine is more than just a precursor for the production of spermidine, which could account for the discrepancy in the magnitude of the virulence deficit caused by the *Δodc* and *Δspdsyn* lesions.

Another explanation for the *Δodc* and *Δspdsyn* virulence discrepancy is the possibility that the amastigote form of the parasite exhibits differing capabilities in transport of putrescine versus spermidine. *Leishmania* promastigotes express robust polyamine transport activities [92], which can account for the ability of putrescine to rescue *Δodc*

parasites [82] and spermidine to enable *Δspdsyn* parasite growth (Fig. 2), but less is known about transport in the amastigotes stage. It should be noted that the only leishmanial polyamine transporter that has been identified at the molecular level is enormously down-regulated in the amastigote stage of the parasite [41]. However, axenic amastigotes of *Δodc L. donovani* were able to proliferate in 200 μM putrescine [82], and *Δspdsyn L. donovani* axenic amastigotes survive in 200 μM spermidine (personal observation), suggesting that the polyamine transport machinery remains somewhat functional in the infectious stage of the parasite, but the extent is unknown. The crippling effects of *Δodc* or *Δspdsyn* mutations on the establishment of virulence by *L. donovani*, together with the at least minimally functional polyamine transport activities in the amastigote stage, suggest that the phagolysosomal compartment in which the parasite resides does not provide sufficient exogenous polyamine to bypass a genetic defect in polyamine biosynthesis.

A pairwise alignment of the *L. donovani* and human SPDSYN primary structures revealed a ~ 56% identity [79], and the amino acids in the human crystal structure that make contact with the substrate [93] are conserved in the parasite enzyme. However, similarities in the active site or ligand-binding pockets of the *L. donovani* and human SPDSYNs do not preclude this enzyme from potential drug targeting. For example, DFMO, the specific ODC inhibitor used in West African sleeping sickness chemotherapy, displays selectivity for the metabolic machinery of *T. brucei* due to discrepant stabilities of the parasite and human ODC enzymes [94-96] and not to disparities in the affinity of DFMO for the two proteins [97]. Furthermore, differential susceptibility to potential

inhibitors could be caused by the presence of a parasite-specific allosteric site on the leishmanial SPDSYN or to other discrepancies in the parasite polyamine pathway, such as the lack of the mammalian back-conversion pathway that transforms spermine to spermidine [98].

The finding that an additional lesion in the polyamine biosynthetic pathway dramatically moderates *L. donovani* virulence implies that the virulence deficit of *Δodc L. donovani* is not specific to the lesion but rather to a more generalized polyamine biosynthetic defect. Moreover, the fact that two independent genetic lesions in the polyamine biosynthesis pathway both cause a striking decrease in the capacity of *L. donovani* to infect mouse livers and spleens provides strong support that the cause of the avirulence is due to a lack of polyamine biosynthetic capacity and not to a secondary unknown function of the *ODC* or *SPDSYN* genes in the amastigote. This study demonstrates that Δ*spdsyn* parasites are greatly reduced in their ability to infect a mammalian host and further validates additional enzymatic components of the polyamine biosynthetic pathway as potential targets for the treatment of visceral and perhaps other forms of leishmaniasis.

3-4 Materials and Methods

Parasite cell culture. Parasites used in this study were derived from the wild type LdBob strain of *L. donovani* [85] obtained from Stephen M. Beverley (Washington University, St. Louis, MO). LdBob promastigotes were routinely cultivated at 26°C, pH 6.9 in previously described media [85] with 10% chicken serum or 5% Serum Plus (Gibco Cell Culture, Carlsbad, CA). Wild type parasites were cultured in the absence of supplementation, while the *Δspdsyn* mutants were supplemented with either 100 µM spermidine alone or with 100 µM spermidine to which 50 µg/ml hygromycin and 20 µM puromycin were added to maintain appropriate selective pressure. The "add-back" strain, *Δspdsyn1*[pXG-BSD-*SPDSYN*], was routinely propagated in 20 µg/ml blasticidin to maintain the multicopy episomal plasmid containing the full-length *SPDSYN* gene.

Construction of *Δspdsyn* knockouts. The cloning and sequencing of *SPDSYN* and its adjacent flanking regions from an *L. donovani* cosmid library have been reported [79]. The *SPDSYN/spdsyn* heterozygotes were constructed using the same drug resistance cassette, pX63-HYG-*Δspdsyn*, employed in the derivation of the *Δspdsyn* mutants in the previously described avirulent DI700 strain of *L. donovani* [79]. The *Δspdsyn* null mutants were created using the drug resistance cassette pX63-PAC-*Δspdsyn*, which was constructed using the same methods as pX63-HYG-*Δspdsyn* [79]. These targeting vectors contain the hygromycin phosphotransferase and puromycin n-acetyltransferase markers, respectively, flanked by sequences derived from the 5' and 3' untranslated regions of *SPDSYN*. Linear targeting DNAs were excised from plasmid by restriction digest with *Hind*III and *Bgl*II and gel purified prior to transfection as described [81]. Wild

type *L. donovani* promastigotes were transfected via electroporation with linearized pX63-HYG-*Δspdsyn* according to standard protocols using a GenePulser XCell (Bio-Rad) system and previously described parameters [99]. Transfected parasites were plated on semisolid agar containing 50 μg/ml hygromycin, colonies were expanded, and homologous recombination was confirmed by Southern blotting. One *SPDSYN/spdsyn* heterozygote clone was then subjected to a second round of transfection using the excised targeting construct from pX63-PAC-*Δspdsyn* and plated on semi-solid agar containing 50 μg/ml hygromycin, 20 μM puromycin, and 100 μM spermidine. Colonies were expanded and Southern blot analysis employed to confirm the deletion of both wild type *SPDSYN* alleles. Two independent *Δspdsyn* clones, *Δspdsyn1* and *Δspdsyn2*, were chosen for further analysis.

Creation of the *Δspdsyn*[p*SPDSYN*] "add-back" parasites. The *Δspdsyn1* knockout line was functionally complemented by transfecting a chimeric plasmid containing the *L. donovani SPDSYN* open reading frame ligated into the pXG-BSD leishmanial expression plasmid [100]. The add-back line was selected in medium containing 20 μg/ml blasticidin and lacking polyamine, and was designated *Δspdsyn1*[pXG-BSD-*SPDSYN*] according to the generally accepted genetic nomenclature for *Leishmania* [101].

Southern and Western blot analyses. For Southern blot analysis genomic DNA from logarithmic growth phase wild type and Δ*spdsyn* parasites was prepared using the DNAeasy kit (Qiagen Inc.) according to the manufacturer's protocol. Genomic DNA was digested with either *Sal*I and probed with a 1.0-kb fragment of the *SPDSYN* coding

region or digested with *Xho*I and probed with a 1.2-kb fragment of the *SPDSYN* 5' flanking region as described previously [79].

For Western blot analysis, parasite lysates were prepared from logarithmic-phase wild type, *Δspdsyn*, and *Δspdsyn1*[pXG-BSD-*SPDSYN*] cell lines and were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [102], and blotted onto Immun-Blot PVFD membranes (Bio-Rad). The membranes were probed with monospecific polyclonal antibody raised to the purified *L. donovani* SPDSYN [79, 103] or with commercially available anti-α-tubulin mouse monoclonal antibody (DM1A) (Calbiochem).

Growth phenotypes of *Δspdsyn* promastigotes. Wild type, *Δspdsyn1*, and *Δspdsyn1*[pXG-BSD-*SPDSYN*] promastigotes were inoculated at a density of 5×10^4 cells/ml into growth media containing 5% Serum Plus and appropriate drug selections. Cultures were maintained at 26ºC with 5% CO_2. *Δspdsyn1* parasites were starved of exogenous polyamines 2 days prior to growth phenotyping, and upon inoculation, 100 μM spermidine or 100 μM putrescine was added as appropriate. Parasites were enumerated after 4 days by hemacytometer. The inoculation density was subtracted from the final cell density, as cell growth and multiplication were of primary interest.

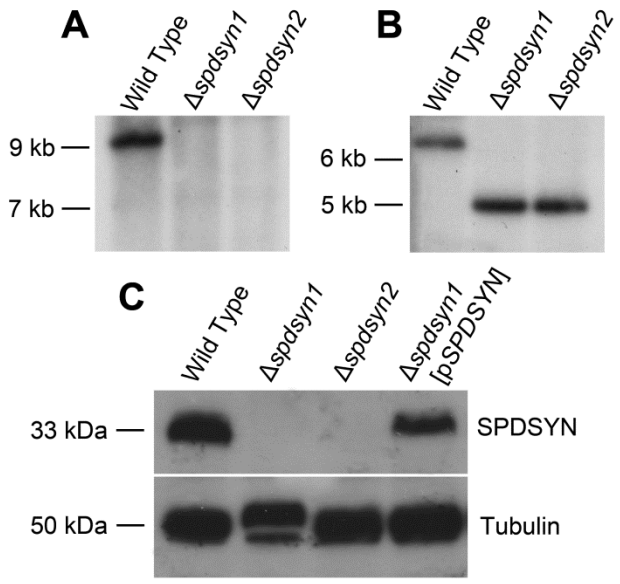
Infectivity studies with mice. Groups of five 6-week-old female BALB/c mice (Charles River Laboratories) were each inoculated by tail vein injection with 5×10^6 of either wild type, *Δspdsyn1*, or *Δspdsyn1*[pXG-BSD-*SPDSYN*] stationary-phase promastigotes grown in medium containing 10% chicken serum. Prior to the mouse injections, each of the three strains were passaged through mice for 10 days in order to eliminate parasites

that had become attenuated in response to prolonged culture. At four weeks post-injection, livers and spleens were harvested and passaged through 70-µm cell strainers to create single-cell suspensions. To quantify parasite loads, limiting dilution assays were performed as described [104]. Serial four-fold dilutions of liver and spleen homogenates in 96-well microtiter plates were cultivated at 26ºC with 5% CO_2 in medium containing 5% Serum Plus with 100 µM spermidine supplementation for the Δ*spdsyn* strain. Growth in individual wells was assessed after 3 weeks by visual inspection, and graphs were constructed displaying mean parasite burdens and standard deviations from five animals.

Note: all mouse studies were carried out in compliance with guidelines established by the Institutional Animal Care and Use Committee.

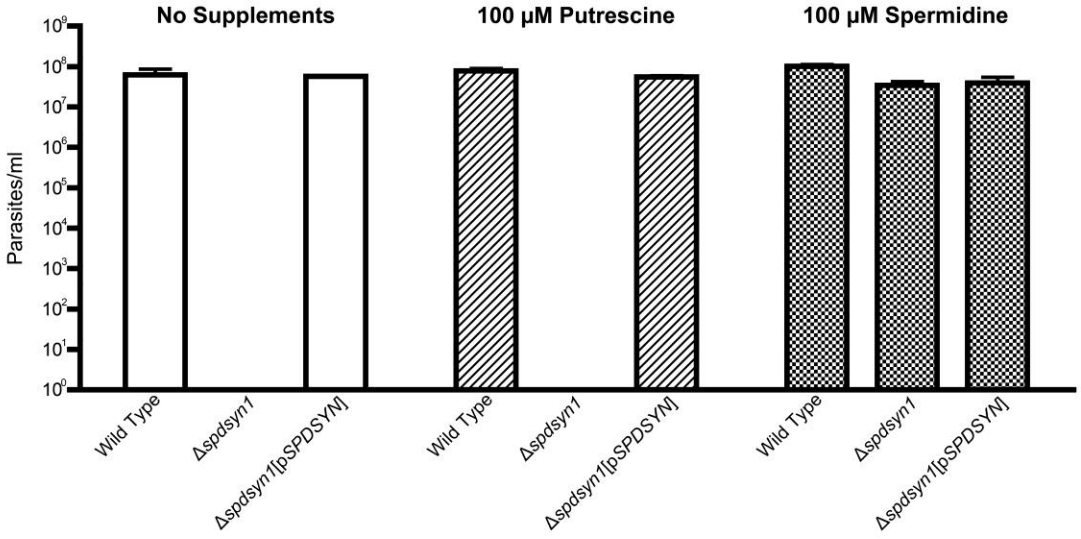
3-5 Figures

Figure 3-1



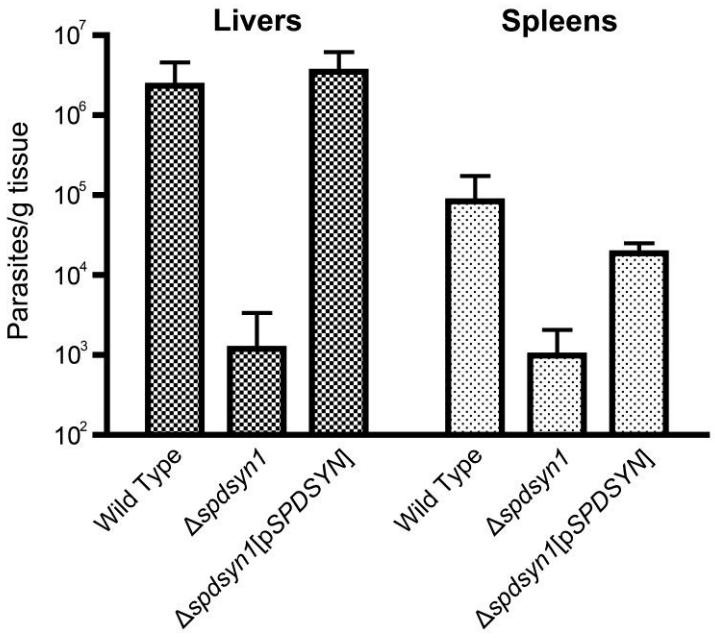
(A,B) Molecular characterization of the *SPDSYN* locus and SPDSYN expression in the *Δspdsyn* knockouts. 2 µg of genomic DNA from wild-type, *Δspdsyn1*, or *Δspdsyn2* parasites was digested with (A) *Sal*I and hybridized to a 1.0-kb fragment of the *SPDSYN* coding region, or (B) *Xho*I and hybridized to a 1.2-kb fragment of the 5' flanking region. Molecular size markers are indicated and equal loading of DNA was verified via ethidium bromide staining. (C) For protein expression analysis, protein lysates from 1.0×10^6 wild-type, *Δspdsyn1*, *Δspdsyn2*, and *Δspdsyn1*[pXG-BSD-*SPDSYN*] (*Δspdsyn1*[p*SPDSYN*]) promastigotes were fractionated by SDS PAGE, blotted, and probed with polyclonal antibodies against *L. donovani* SPDSYN. Tubulin expression was analyzed as a loading control.

Figure 3-2



Growth phenotype of *Δspdsyn* promastigotes. Wild type, *Δspdsyn1*, and *Δspdsyn1*[pXG-BSD-*SPDSYN*] (*Δspdsyn1*[p*SPDSYN*]) promastigotes were incubated in their respective growth medium in the absence or presence of 100 µM spermidine or 100 µM putrescine. *Δspdsyn1* parasites were starved of exogenous polyamines 2 days prior to the start of the growth assay. Parasites were inoculated at 5×10^4 cells/mL, maintained at 26ºC with 5% CO_2, and enumerated after 4 days. Reported cell densities represent final cell densities minus inoculation densities. Final cell densities of *Δspdsyn1* parasites grown under nonpermissive conditions were significantly lower than that of the initial inoculums, and thus growth was zero.

Figure 3-3



Parasite burdens in mice. Three separate cohorts of BALB/c mice were infected with either wild-type, *Δspdsyn1*, or *Δspdsyn1*[pXG-BSD-*SPDSYN*] (*Δspdsyn1*[p*SPDSYN*]) stationary-phase promastigotes as described in Materials and Methods. Mice were sacrificed after 4 weeks and parasite loads in liver (A) or spleen (B) preparations were determined by limiting dilution. The experiment was repeated three times with similar conclusions.

Chapter 4 – Unpublished work

4-1 INTRODUCTION

Recent studies, as described earlier in this thesis, have demonstrated that disrupting polyamine biosynthesis in *Leishmania donovani* parasites is detrimental to proliferation and infectivity of these parasites. Knockout parasites lines lacking the genes encoding either ornithine decarboxylase (ODC) or spermidine synthase (SPDSYN) are dramatically crippled in their abilities to establish a robust infection in BALB/c mice. Additionally, the genetic lesion of the parasites lacking *ODC* can be partially mimicked pharmacologically by the administration of DFMO to mice infected with wild type parasites. And the infectivity deficit seen with *Δodc* parasites can be rescued by the oral administration of putrescine to infected mice in their drinking water. These findings provide motivation to continue in the identification of new drug targets in *Leishmania donovani*, specifically in the same polyamine biosynthetic pathway.

My most recent work consists of examining the infectivity phenotype of an additional knockout parasite line in mice, one in which the S-adenosylmethionine decarboxylase (ADOMETDC) gene has been replaced. There are some inhibitors of ADOMETDC that have shown anti-parasitic activity in Trypanosomes and in *Leishmania,* providing further incentive to determine the viability of *Δadometdc* parasites in our animal model system.

4-2 RESULTS

Molecular characterization of *Δadometdc*. *Δadometdc L. donovani* promastigotes have been described previously in the avirulent DI700 strain, precluding the possibility of examining the *in vivo* consequences of the genetic lesion. Thus, the *Δadometdc* lesion was reconstructed in LdBob, a virulent *L. donovani* strain that is capable of establishing robust visceral infections in mice [82-85]. An independent *Δadometdc* clone was isolated after double targeted gene replacement, and an add-back line *Δadometdc*[pXG-BSD-*ADOMETDC*] (*Δadometdc*[p*ADOMETDC*]) was generated, consisting of the *Δadometdc* mutant with a multi-copy episome containing the *ADOMETDC* open reading frame. To substantiate the gene knockout, expression of the ADOMETDC protein was examined by western blot in wild type and knockout parasites as well as the *pADOMETDC* add-back (Fig. 4-1). Polyclonal antisera specific for ADOMETDC recognized the expected protein corresponding to the processed *ADOMETDC* translation product in both wild type and *Δadometdc*[p*ADOMETDC*] lysates. [79] This band was not detected in extracts prepared from *Δadometdc* (Fig. 4-1). The blot was also probed with anti-tubulin antibody as a loading control.

***Δadometdc* parasites are polyamine auxotrophs.** A growth assay was performed to determine the reliance of the *Δadometdc* knockout cell lines on polyamines, and as anticipated, *Δadometdc* promastigotes were auxotrophic for polyamines. Wild type parasites exhibited normal growth, but the mutant cell line was unable to proliferate in polyamine-deficient medium (Fig. 2). The polyamine auxotrophy of the *Δadometdc* null mutant was circumvented genetically in the *Δadometdc1*[p*ADOMETDC*] parasite line by

complementation with the covering *ADOMETDC* plasmid, or nutritionally by supplementation of the culture medium with spermidine (Fig. 4-2).

***Δadometdc L. donovani* has reduced virulence *in vivo*.** Virulence studies in Balb/c mice were carried out similarly to previous studies with knockouts of the other components of the polyamine biosynthetic pathway. The goal of this work was to determine the effects of a genetic deficiency of *ADOMETDC* and to further characterize the ADOMETDC enzyme as a potential therapeutic target. Groups of five BALB/c mice were inoculated with either wild type, *Δadometdc*, or *Δadometdc*[p*ADOMETDC*] promastigotes and after four weeks parasite loads were quantified in livers and spleens, the two organs most affected by a visceralizing *Leishmania* infection. The ability of the *Δadometdc* knockout parasites to infect mice was compromised but not to the extent that had been seen previously with *ODC* and *SPDSYN* knockouts. Unfortunately the overall infection level with wild type parasites was lower than expected, but the difference between the knockout and wild type is still marked (Fig. 4-3). The average parasite burdens in livers and spleens of mice infected with wild type parasites were approximately one and a half and one orders of magnitude higher, respectively, than those of *Δadometdc* parasites. The infectivity defect of the knockout lines was rescued by episomal complementation with parasite burdens in livers and spleens of *Δadometdc*[p*ADOMETDC*]-infected mice equivalent to those obtained in mice inoculated with wild type parasites (Fig. 4-3). It should be noted that the discrepancy in the absolute numbers of wild type parasites between livers and spleens on a per-gram basis is typical for *L. donovani* infections [86-89].

4-3 CONCLUSIONS

Previous studies with *Leishmania* null mutants have established that each component of the polyamine biosynthetic pathway, ARG, ODC, ADOMETDC, and SPDSYN, is indispensable for the viability and growth of the promastigote stage of the parasite [76, 77, 79-81]. And now we have evidence that at least three of these pathway components in *Leishmania donovani* are important for establishing robust infections in the BALB/c model system. However, the infectivity deficits exhibited by the different knockout parasites are variable. This could be ascribed to differences in the putrescine and spermidine contents of the phagolysosome in which visceral macrophages reside. It is likely that both putrescine and spermidine are required by *L. donovani* amastigotes for optimal proliferation. If, for example, the phagolysosomal environment contains pools of spermidine but not putrescine, then a *Δodc* lesion would be expected to have far more deleterious consequences to the parasite than a *Δspdsyn* or *Δadometdc* defect, because *L. donovani* lacks a back conversion pathway from spermidine to putrescine leaving a putrescine deficit [80]. Virulence data with *ARG* knockout strains is still ongoing, and may continue to enlighten the mechanism behind the differential impacts of *Δodc* , *Δspdsyn,* and *Δadometdc* parasites.

It should be noted that *Δodc L. donovani* promastigotes are much more susceptible to putrescine withdrawal than *Δspdsyn L. donovani* promastigotes are to spermidine withdrawal, and *Δadometdc* seems to fall somewhere in between (personal observations). This intriguing observation is mirrored in *T. brucei*, where genetic investigations showed that depletion of putrescine results in a more rapid cell death

than spermidine depletion [91]. It appears evident that putrescine may be more than just a precursor for the production of spermidine, which could account for the discrepancy in the magnitude of the virulence deficit caused by the *Δodc*, *Δspdsyn,* and *Δadometdc* lesions.

Another explanation for the *Δodc, Δspdsyn* and *Δadometdc* virulence discrepancy is the possibility that the amastigote form of the parasite exhibits differing capabilities in transport of putrescine versus spermidine. *Leishmania* promastigotes express robust polyamine transport activities [92], which can account for the ability of putrescine to rescue *Δodc* parasites [82] and spermidine to enable *Δspdsyn* and *Δadometdc* parasite growth, but less is known about transport in the amastigotes stage. It should be noted that the only leishmanial polyamine transporter that has been identified at the molecular level is down-regulated in the amastigote stage of the parasite [41]. However, axenic amastigotes of *Δodc L. donovani* were able to proliferate in 200 μM putrescine [82], and *Δspdsyn L. donovani* axenic amastigotes survive in 200 μM spermidine (personal observation), suggesting that the polyamine transport machinery remains somewhat functional in the infectious stage of the parasite, but the extent is unknown. The observations about the viability of knockout parasites *in vivo* suggest that the phagolysosomal compartment in which the parasite resides does not provide sufficient exogenous polyamine to bypass a genetic defect in polyamine biosynthesis.

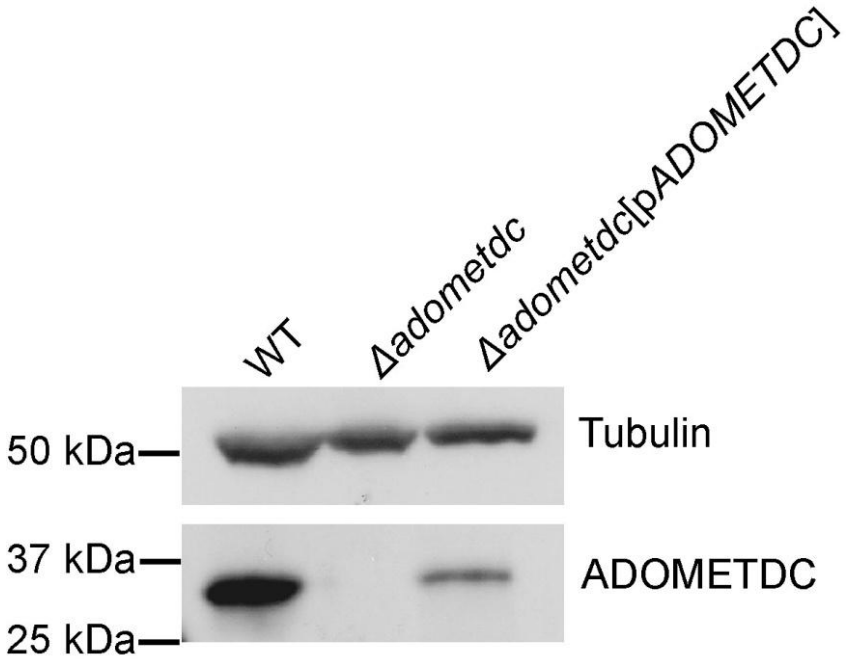
Data from other *Leishmania* species have not offered a consistent conclusion. Parasite burdens and lesion sizes of mice infected with *Δarg L. mexicana* [78] or *Δarg L. major* cutaneous strains [75, 77] were only somewhat lower than in mice infected with the

corresponding wild type line from which these mutants were derived. It may be possible to ascribe the differences in the impact of the genetic lesions to the nature of lesion, to species differences, or to differences in the cutaneous and visceral environments in which the species reside. Perhaps determination of these questions will require the evaluation of mutations of the same polyamine pathway gene in the different *Leishmania* species, i.e. a comparison of a *Δodc* mutation in *L. donovani*, *L. mexicana* and *L. major*.

The finding that an additional lesion in the polyamine biosynthetic pathway dramatically moderates *L. donovani* infectivity implies that the infectivity deficit of *Δodc L. donovani* is not specific to the lesion but rather to a more generalized polyamine biosynthetic defect. The findings described here further validate additional enzymatic components of the polyamine biosynthetic pathway as potential targets for the treatment of visceral and perhaps other forms of leishmaniasis.

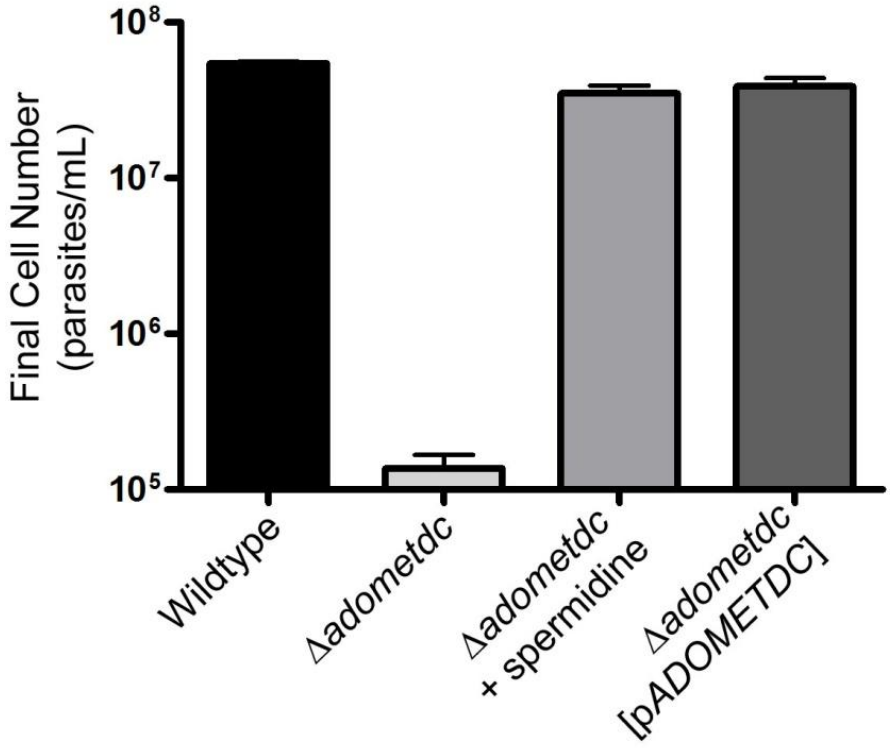
4-4 FIGURES

Figure 4-1



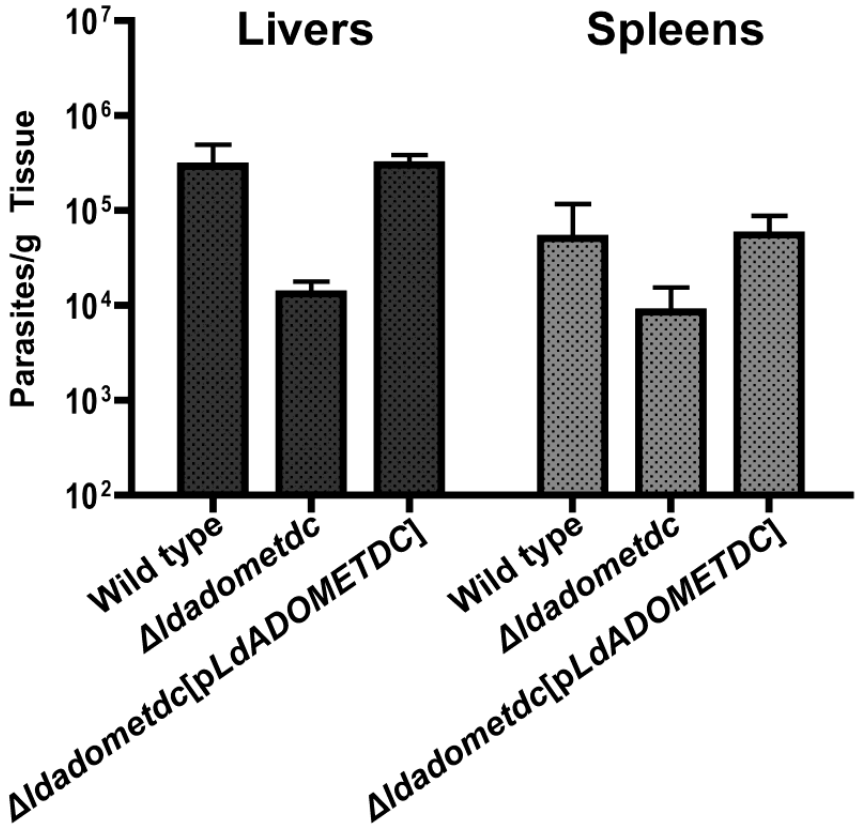
For protein expression analysis, protein lysates from 1.0×10^6 wild type (WT), *Δadometdc*, and *Δadometdc*[pXG-BSD-*ADOMETDC*] (*Δadometdc*[p*ADOMETDC*]) promastigotes were fractionated by SDS PAGE, blotted, and probed with polyclonal antibodies against *L. donovani* ADOMETDC. Tubulin expression was analyzed as a loading control.

Figure 4-2



Growth phenotype of *Δspdsyn* promastigotes. Wild type, *Δadometdc*, and *Δadometdc*[pXG-BSD-*ADOMETDC*] (*Δadometdc*[p*ADOMETDC*]) promastigotes were incubated in their respective growth medium in the absence or presence of 100 µM spermidine. Parasites were washed in PBS and inoculate at 5×10^4 cells/mL, maintained at 26ºC with 5% CO_2, and enumerated after 4 days. Reported are final cell densities.

Figure 4-3



Parasite burdens in mice. Three separate cohorts of BALB/c mice were infected with either wild-type, *Δadometdc*, or *Δadometdc*[pXG-BSD-*ADOMETDC*] (*Δadometdc*[p*ADOMETDC*]) stationary-phase promastigotes as described in Materials and Methods. Mice were sacrificed after 4 weeks and parasite loads in liver (A) or spleen (B) were determined by limiting dilution.

4-5 METHODS

Parasite cell culture. Parasites used in this study were derived from the wild type LdBob strain of *L. donovani* [85] obtained from Stephen M. Beverley (Washington University, St. Louis, MO). LdBob promastigotes were routinely cultivated at 26°C, pH 7.2 in previously described media [85] with 10% chicken serum or 5% Serum Plus (Gibco Cell Culture, Carlsbad, CA). Wild type parasites were cultured in the absence of supplementation, while the *Δadometdc* mutants were supplemented with either 100 μM spermidine alone or in combination with 50 μg/ml hygromycin and 25 μM G418 to maintain appropriate selective pressure. The "add-back" strain, *Δadometdc*[pXG-BSD-*ADOMETDC*], was routinely propagated in 20 μg/ml blasticidin to maintain the multicopy episomal plasmid containing the full-length *ADOMETDC* gene.

Construction of *Δadometdc* knockouts. The cloning and sequencing of *ADOMETDC* and its adjacent flanking regions from an *L. donovani* cosmid library have been reported. The *ADOMETDC/adometdc* heterozygotes were constructed using the same drug resistance cassette, pX63-HYG-*Δadometdc*, employed in the derivation of the *Δadometdc* mutants in the previously described avirulent DI700 strain of *L. donovani* [79]. The *Δadometdc* null mutants were created using the drug resistance cassette pX63-NEO-*Δadometdc*, which was constructed using the same methods as pX63-HYG-*Δadometdc* [79]. Linear targeting DNAs were excised from plasmid by restriction digest with *Hind*III and *Bgl*II and gel purified prior to transfection as described [81]. Wild type *L. donovani* promastigotes were transfected via electroporation with linearized pX63-NEO-*Δadometdc* according to standard protocols using a GenePulser XCell (Bio-Rad)

system and previously described parameters [99]. Transfected parasites were plated on semisolid agar containing 50 μg/ml hygromycin, colonies were expanded. One *ADOMETDC/adometdc* heterozygote clone was then subjected to a second round of transfection using the excised targeting construct from pX63-Hyg-*Δadometdc* and plated on semi-solid agar containing 50 μg/ml hygromycin, 20 μM G418, and 100 μM spermidine. One colony was picked and used for further analysis.

Creation of the *Δadometdc*[p*ADOMETDC*] "add-back" parasites. The *Δadometdc* knockout line was functionally complemented by transfecting a chimeric plasmid containing the *L. donovani ADOMETDC* open reading frame ligated into the pXG-BSD leishmanial expression plasmid [100]. The add-back line was selected in medium containing 20 μg/ml blasticidin and lacking polyamine, and was designated *Δadometdc*[pXG-BSD-*ADOMETDC*] according to the generally accepted genetic nomenclature for *Leishmania* [101].

Western blot analyses. For Western blot analysis, parasite lysates were prepared from logarithmic-phase wild type, *Δadometdc*, and *Δadometdc*[pXG-BSD-*SPDSYN*] cell lines and were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [102], and blotted onto Immun-Blot PVFD membranes (Bio-Rad). The membranes were probed with monospecific polyclonal antibody raised to the purified *L. donovani* ADOMETDC [79, 103] or with commercially available anti-α-tubulin mouse monoclonal antibody (DM1A) (Calbiochem).

Growth phenotypes of *Δadometc* promastigotes. Wild type, *Δadometdc*, and *Δadometdc*[pXG-BSD-ADOMETDC] promastigotes were inoculated at a density of 5×10^4

cells/ml into growth media containing 5% Serum Plus and appropriate drug selections. Cultures were maintained at 26ºC with 5% CO_2. *Δadometdc* parasites were washed in phosphate-buffered saline prior to inoculation, and 100 μM spermidine was added as appropriate. Parasites were enumerated after 4 days by hemacytometer.

Infectivity studies with mice. Groups of five 6-week-old female BALB/c mice (Charles River Laboratories) were each inoculated by tail vein injection with 5×10^6 of either wild type, *Δadometdc*, or *Δadometdc*[pXG-BSD-ADOMETDC] stationary-phase promastigotes grown in medium containing 10% chicken serum. Prior to the mouse injections, each of the three strains were passaged through mice for 14 days in order to eliminate parasites that had become attenuated in response to prolonged culture. At four weeks post-injection, livers and spleens were harvested and passaged through 70-μm cell strainers to create single-cell suspensions. To quantify parasite loads, limiting dilution assays were performed as described [104]. Serial four-fold dilutions of liver and spleen homogenates in 96-well microtiter plates were cultivated at 26ºC with 5% CO_2 in medium containing 5% Serum Plus with 100 μM spermidine supplementation for the *Δadometdc* strain. Growth in individual wells was assessed after 3 weeks by visual inspection. Note: all mouse studies were carried out in compliance with guidelines established by the Institutional Animal Care and Use Committee.

CONCLUSIONS

There are several major conclusions that can be drawn from the investigations described in this thesis. The work undertaken here has shown that genetic lesions in the polyamine biosynthetic pathway of *Leishmania donovani* create conditionally lethal mutations *in vitro*. More importantly, a genetic lesion in any component of the polyamine pathway compromises the infectivity of *L. donovani* parasites in mice. The variation in the infectivity deficits that were observed may result from a number of factors. There may be differences in the amounts of the various polyamines available for salvage by *Leishmania*, or the ability of the parasites to transport the exogenous polyamines may be different. Further characterization of polyamine transport in the amastigote stage of *Leishmania* parasites may help to further clarify why there are differences in the infectivity defects seen with different knockout parasites.

The observation that administration of putrescine to *Δodc*-infected mice can partially restore infectivity further confirmed the conclusion that the infectivity deficit measured in our *in vivo* model was due to a lack of polyamine biosynthesis, and not an artifact of the genetic manipulation. This also seems to suggest that normal levels of putrescine in our mouse model, without extra nutritional supplementation, are insufficient to overcome a putrescine biosynthesis deficit in *L. donovani* parasites.

Our determination that administration of DFMO to mice infected with wild type *L. donovani* can partially decrease parasite burden leads us to the conclusion that ODC, at least, is amenable to therapeutics. This proof of concept experiment supports our

hypothesis that the *Leishmania donovani* polyamine biosynthetic pathway is a druggable target. To further confirm our conclusions, infectivity and virulence studies should be continued and expanded into more clinically relevant animal models, potentially hamsters, as they are susceptible to developing visceral disease. Additionally, efforts to identify compounds that inhibit the polyamine pathway in *Leishmania* should continue, especially targeting ODC, which we have shown to be amenable to therapeutics and appears to be the most promising target in the *Leishmania* polyamine biosynthetic pathway.

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