The Role of Parasite-Derived Arginase in Murine *Leishmania major*

by

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Abstract

The outcome of infection with *Leishmania major* depends in part on the balance between arginase and inducible nitric oxide synthase in macrophages. These enzymes compete for the substrate L-arginine. *Leishmania major* also encodes an arginase gene but, the role of this parasite-derived enzyme in infection remains unclear. We hypothesize that parasite-derived arginase influences parasite survival and host immune response to *L. major*. To examine this hypothesis, we employed an arginase deficient null mutant *L. major* in *in vitro* and *in vivo* experiments. Our results show that deficiency of parasite-derived arginase impaired parasite proliferation and disease pathogenesis. Increased arginase activity however neither affected nitric oxide production, nor did it correlate with IL-4 production. Primary infection of normally resistant hosts causes a chronic infection and does not protect them against re-infection. Thus, parasite-derived arginase is of nutritional importance to *L. major*, but is not a feasible therapeutic drug target.

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Dedication

This work is dedicated to my heroes:

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List of Abbreviations

AAMϕ	Alternatively activated macrophage
APC	Antigen presenting cell
BMDM	Bone marrow derived macrophages
CAMϕ	Classically activated marcophage
CFSE	Carboxyfluorescein succinimidyl ester
CL	Cutaneous leishmaniasis
DC	Dendritic cell
DMEM	Dubelco's modified eagle medium
FBS	Fetal bovine serum
IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-4	Interleukin 4
iNOS	Inducible nitric oxide synthase
LPG	Lipophosphoglycan
NK	Natural killer
NO	Nitric oxide
ODC	Ornithine decarboxylase
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
PKDL	Post-kal-azar dermal leishmaniasis
RPMI-1640	Roswell Park Memorial Institute culture media
scid	Severe combined immunodeficiency
SLA	Soluble leishmanial antigen
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
VL	Visceral leishmaniasis

1. Introduction

1.1 Background to Leishmaniasis

i. Leishmaniasis: Forms of Disease

The leishmaniases represent a spectrum of diseases ranging from relatively short-lived, localized and self-healing lesions to systemic fatal disease. There are three forms of the disease characterized by the pathological outcome: cutaneous, visceral and muco-cutaneous leishmaniasis. *Leishmania major, mexicana, tropica* and *aethiopica* cause cutaneous lesions. Beginning as a small papule within two months after infection, the inflammation grows in size and eventually ulcerates. After several months the lesions self-heal and the individual has life-long protection from re-infection. Protection in cutaneous leishmaniasis is due to the prescence of a small number of persistent parasites that are kept from spreading by the appropriate immune response [2-9] - a phenomenon that will be discussed in later sections. If patients experience suppression of the immune system after healing from leishmaniasis, for example HIV, the cutaneous disease can relapse [10-13]. Though cutaneous leishmaniasis is not fatal, the lesions are prone to secondary infection and leave unsightly scars. In some cases, patients develop an improper immune response due to exhaustion of the immune system – anergy [14-16] . This type of disease is called diffuse cutaneous leishmaniasis and results in the spread of disease from one localized lesion to multiple wounds all over the body. Cutaneous leishmaniasis is divided into Old and New World diseases based on geographical distribution of the causative parasite species [17]. New and Old World diseases have different biological characteristics that translate into more severe disease in New World species than in Old World.

Leishmania donovani, chagasi and *infantum* cause visceral leishmaniasis in which parasites infect organ systems most notably the liver and spleen. Following an incubation period ranging from two weeks to several months, symptoms of visceral leishmaniasis appear and include fever, sweating, weakness, weight loss, hepatosplenomegaly and anaemia [18, 19]. Enlargement of the spleen and liver occurs because of infiltration of lymphocytes and macrophages, Kupffer cells in the liver, engorged with amastigotes [18]. If untreated, visceral leishmaniasis is fatal however, even after successful treatment, some patients suffer reactivation of disease termed post-kal-azar dermal leishmaniasis (PKDL) [20].

Leishmania braziliensis causes muco-cutaneous leishmaniasis, when the parasites invade the mucous membranes. Muco-cutanous leishmaniasis generally involves the mucous membranes of the face including the mouth, pharynx and larynx [21]. Untreated, this form of the disease can cause breakdown of the palate of the mouth and nose leaving severe disfiguration [21]. Muco-cutaneous leishmaniasis initially follows the same pathology as cutaneous leishmaniasis but can re-occur in the mucous membranes several years after the initial infection has healed due to failure of the immune system [22, 23]. Mucocutaneous leishmaniasis can also be the result of an over-active immune response where the majority of parasites are eliminated by the inflammatory response that continues damaging the host tissues [5].

ii. Life Cycle

Leishmania are masters of immune evasion because these obligate intracellular parasites are able to commandeer the very cells programmed for their destruction. The

leishmania life cycle begins by infection of the insect vector (Figure 1). When sandflies of the *Phlebotomus* species take a blood meal from an infected host, infected macrophages are also ingested carrying with them a small number of parasites. These parasites move from the proboscis of the fly into the midgut where the digestive enzymes and pH change signal them to differentiate into procyclic promastigotes. Procyclics have an elongated sausage shape and flagellum. This form of the parasite attaches to the midgut wall and rapidly divides by binary fission [24].

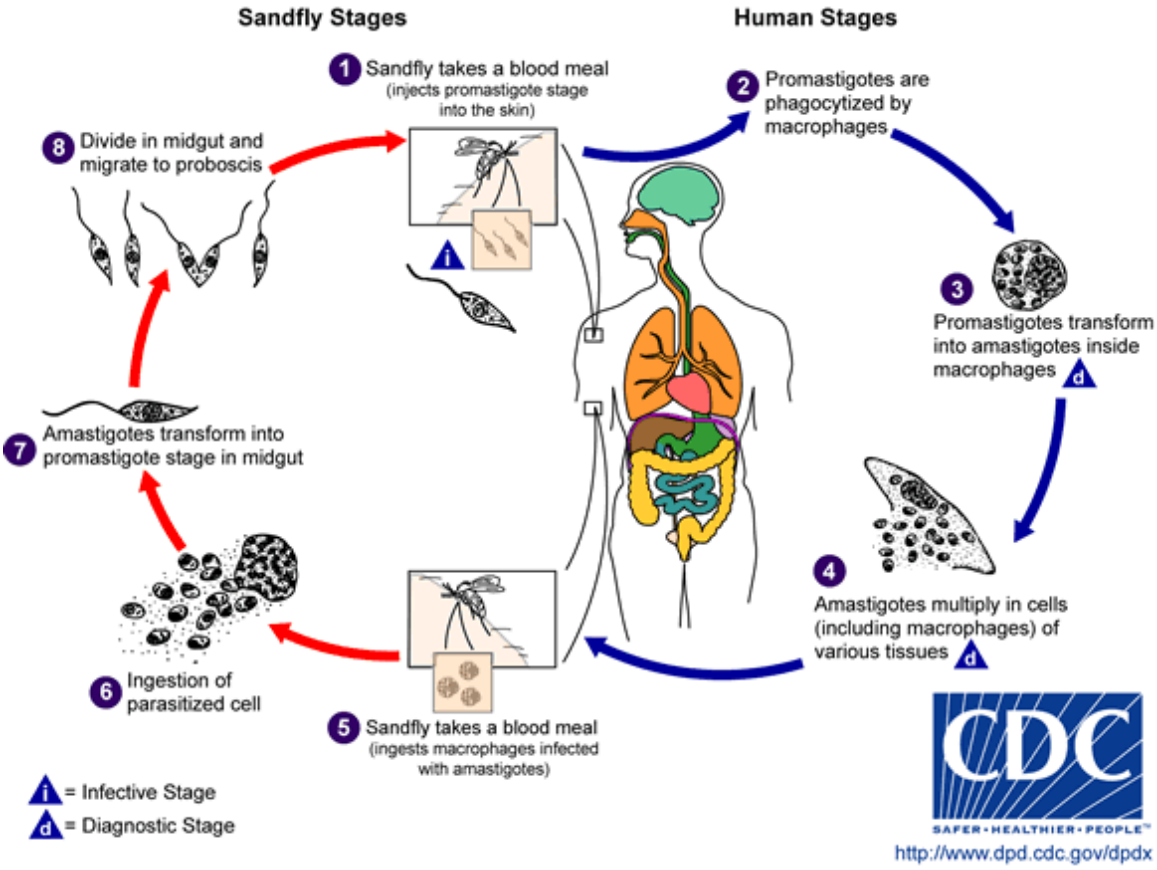

Figure 1. The life cycle of *Leishmania major*.
http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Leishmaniasis_il.htm

Following this period of rapid proliferation begins the process of metacyclogenesis in which the parasites acquire the glycocalyx essential for their survival in the mammalian host [25]. The glycocalyx is a dense surface matrix of carbohydrate moieties that provides protection from immune effector molecules especially the complement system. Lipophosphoglycan (LPG), in particular, is of key importance in promastigote evasion from host complement [26]. LPG functions by binding C3b which causes the C5b-9 complexes to form far from the parasite surface and bars the membrane attack complex from inserting in the parasitic membrane [27, 28]. In fact, the glycocalyx of Trypanosomatidae is arguably their most important virulence factor and the secret of their success as pathogens [25, 27, 29]. The metacyclic promastigote is the non-dividing infectious form of leishmaniasis and is regurgitated from the midgut back to the proboscis where it is transmitted to the host during the next blood meal [27, 30, 31].

Following injection of metacyclic promastigotes into the mammalian host, local inflammation at the site of the bite recruits cells of the innate immune response. Neutrophils are the first cells to take up the parasites followed two days later by a wave of macrophages and dendritic cells [32], the preferred host cells of these intracellular parasites. It is proposed that in the true sandfly mediated infection, although perhaps not the experimental inoculation by syringe, the initial neutrophil uptake of parasites has a dominant role in the outcome of infection [32, 33]. Phagocytosis of metacyclics is mediated by bound C3b and iC3b on the surface of the parasite binding CR1 and Mac-1 on the macrophage surface respectively [34-36]. Complement independent mechanisms of parasite phagocytosis also occur. For example, LPG can also bind the macrophage mannose-fucose lectin receptor [37]. Although the glycocalyx provides expert protection

for metacylics outside the host cell, once inside the host cell *Leishmania* must develop efficient immune evasion and subversion techniques to overcome the microbicidal effector molecules inside macrophages.

The intracellular form of *leishmania* is the amastigote which differs from the promastigote both morphologically and in surface molecule composition. Whereas the promastigote form is spindle shaped and flagellated, amastigotes are spherical and non-flagellated. Within the macrophage host, amastigotes reside in the phagolysosome, which is a fusion of the phagosome and secondary lysosomes. Since lysosomes carry destructive materials, amastigotes exert numerous changes on macrophage function to impair the cell's ability to destroy the parasites [38]. Many macrophage parasite-killing mechanisms are regulated by protein phosphorylation which *Leishmania* subverts by altering host cellular kinases and phosphatases or by expressing its own phosphatases [36]. Infected macrophages display reduced CD40 and MHCII expression [39], are less prone to apoptosis [40], and produce lower amounts of IL-12 [41, 42] and nitric oxide [43] than uninfected macrophages.

Once successfully established inside macrophages, amastigotes undergo replication by binary fission eventually overcoming the cell completely. Macrophage lysis follows and the released amastigotes are in turn phagocytosed by subsequent macrophages via FcIg and CR3 receptors [44]. All of the pathology associated with leishmaniasis is due to this cell destruction and the bystander damage therein. During this period, a bite from an uninfected sandfly repeats the cycle. Whether a macrophage will succumb to *Leishmania* infection or survive depends on the balance between two

inducible enzymes nitric oxide synthase II and arginase [45-47]. These enzymes are under the control of Th1 and Th2 immune responses respectively [48-50].

iii. Clinical Aspects of Disease

Leishmaniases are a severe public health problem. Endemic in 88 countries (Figure 2), 90% of cutaneous leishmaniasis cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria [16]. The global prevalence is 12 million and 1.5 to 2 million new cases occur every year (1 – 1.5 million for CL and 500,000 for VL) with an additional 350 million people at risk [51]. These numbers are estimates because most cases go unreported; leishmaniasis is a notifiable disease in only 33 of the 88 endemic countries.

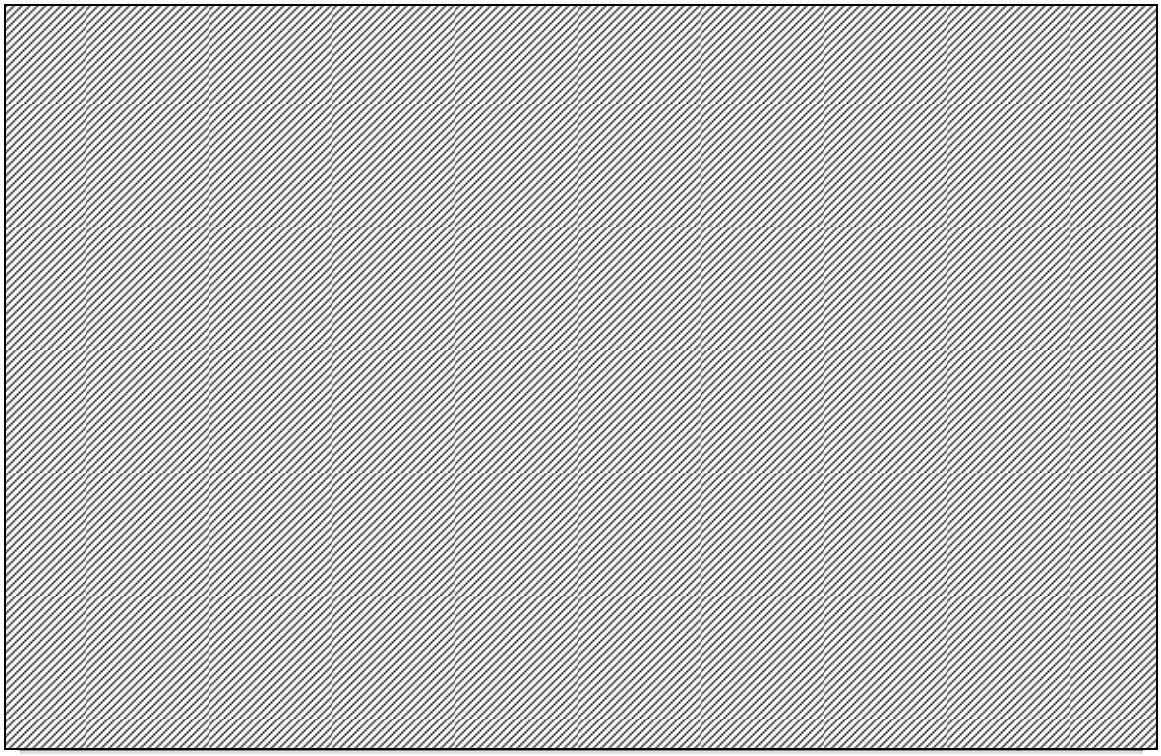

Figure 2. Distribution of Old World and New World Cutaneous Leishmaniasis.
Red indicates an endemic area. To view this figure please go to the website http://www.who.int/leishmaniasis/leishmaniasis_maps/en/index.html

Leishmaniasis-related disabilities create a heavy social burden. In 2001 the global disability-adjusted life years was estimated at 2.4 million years [16]. Furthermore, Leishmaniasis carries a social stigma due to the deformities and disfigurement caused by the lesions and resulting scars [16]. Not only does the disease impair the lifestyle of the mostly impoverished victims but also, the extended time, physical side-effects and cost of treatment (for VL anywhere from USD 30 for generic sodium stibogluconate to USD 70 for pentamidine [16] make leishmaniasis a barrier to socioeconomic development. In addition, patients may interrupt treatment because of lengthy hospitalization, logistic difficulties in traveling to the treatment facility, and poor communication, resulting in increased drug resistance [16]. Epidemics therefore deplete the labor force and significantly hinder agricultural and industrial development programs.

Diagnosis of Leishmaniasis is made by visualizing the amastigote form by microscopy from lesion samples. Biopsies, tissue scrapings, aspirates or impression smears from lesions are applied to microscope slides, fixed, stained using Giemsa or H & E and examined for amastigotes. In addition, material from the lesion can be cultured to encourage parasite multiplication *in vitro* and confirm a diagnosis particularly when the pathology is that of leishmaniasis but the amastigotes cannot be found by microscopy. Culture of parasites in this way is also useful for typing by isoenzyme analysis. Serological tests for *leishmania*-specific antibodies are not useful because the antibodies will be undetectable or present in low titre [52]. A Leishmanin skin test analogous to the tuberculin test detects cell-mediated immunity but this test cannot distinguish between past and present infection and so is not useful clinically [53].

In recent years, molecular biological techniques have been applied to the diagnosis of leishmaniasis [6]. Polymerase chain reaction is considered by many to be the gold standard for diagnosis. The disadvantage of PCR as a diagnostic tool is the lack of availability of equipment in many endemic countries as well as the problem of false-positive results because of poor technique. In addition, like the Leishmanin test, PCR cannot distinguish between past and present infection. PCR applied to scarred lesions will also detect parasite DNA due to the persistent nature of the parasites even after successful chemotherapy [6, 54].

Although cutaneous leishmaniasis is self-healing, lesions can take several months to a year to heal [55], are always unsightly and are sometimes located in unfortunate areas like the face or hands. Open sores on the body also present a concern for secondary bacterial infection. "Treatment is given to reduce scarring and to prevent parasite dissemination or relapse. It is commonly used for persistent (>6 months duration), multiple or large lesions, for facial or disabling lesions near joints." [17].

The conventional treatment for CL is pentavalent antimonials but these drugs carry significant side-effects and the requirement of daily parenteral administration presents practical problems particularly in the third world. Thus, there is urgent need for alternative therapies particularly effective oral or topical treaments [56, 57]. Pentavalent antimonials have been in use since the 1940s despite their high level of toxicity [58, 59]**.** Two preparations currently in use are sodium stibogluconate (SSG; Pentostam, GlaxoSmithKline, London UK) or meglumine antimoniate (Glucantime, Rhone-Poulenc, Rorer France) which are similar in efficacy and toxicity but differ in their antimony concentrations, 100 mg/ml versus 85 mg/ml respectively. Both drugs must be

administered intravenously or intramuscularly. Their mechanism of action is unknown but may be related to inhibition of ATP synthesis [22] or glycolysis and fatty acid oxidation in *Leishmania* [60]. Unfortunately, effective use of either of these drugs requires daily injections Sb, 20 mg/kg/day, approximately 15 to 20 ml, for 20 days which necessitates hospitalization for most patients. Side-effects of pentavalent antimonials include pancreatitis, hepatitis, headache, nausea, anorexia, myalgia, arthralgia, mild leucopenia, thrombocytopenia, fatigue, and elevations in serum aminotransferases [22, 23, 61-63]. Although all of these side-effects are reversed at the end of treatment, the invasiveness, cost and side-effects of pentavalent antimonial therapy result in many patients failing to complete the course of treatment [56]. Intralesional SSG injections are an alternative to systemic treatment and a primary therapy in Old World CL in endemic countries. In New World CL, this therapy runs a risk of causing mucosal disease. The advantage of intralesional therapy is that a high concentration of the drug targets the site of infection at a lower cost and without the systemic side-effects [17]. Due to the limitations of pentavalent antimonials, a number of other therapies for CL are under development or in use with varying success.

Pentamidine is an aromatic diamidine which is also effective against African trypanosomes. Pentamidine is mostly used in New World leishmaniasis particularly in French Guyana against *L. guyanensis* (responsible for over 90% of cases) [22]. Pentamidine also carries a high side-effect load including nephrotoxicity, hypotension and arrhythmias, nausea, vomiting and neurological conditions such as confusion and hallucinations.

Amphotericin B desoxycholate is an antifungal agent that is also effective against *Leishmania* species. Although the pure drug has significant side-effects, lipid formulations are more easily tolerated and are highly effective in treatment of VL making them attractive candidates for CL [64, 65]. Amphotericin B appears to be a solid candidate as a substitute for pentavalent antimonials but more trials with larger study sizes are necessary. Topical formulations appear effective against Old World *L. major* and well tolerated with particular benefits when the patients are children but those studies presented here are all from one location [17, 66-68]. Species specificity and different responses in other endemic areas may affect the results. [66, 67, 69-71]

Miltefosine was originally developed as an anti-cancer drug but has been found highly effective against visceral leishmaniasis, particularly in India [72], with recent reports demonstrating its use against CL [73]. The major benefit of miltefosine is its oral administration route – at a dosage of 2.5 mg/kg/day for 21 to 28 days – and mild side-effects compared to antimony treatment including nausea, vomiting and minor creatinine elevations. Miltefosine as a drug against CL shows strong species specificity. The results of a placebo-controlled study in Colombia and Guatemala indicate that miltefosine as an oral agent is effective against CL caused by *L. panamensis* in Colombia but not by *L. braziliensis* in Guatemala where it is associated with ML. [74]. Clearly more research into miltefosine's use against CL is needed particularly in Old World species. Drug resistance is already a problem [75].

Anti-fungal compounds known as azoles are used to combat leishmaniasis because they are convenient – administered orally - and well tolerated by most patients. In general they are not the most efficacious drugs against CL and routinely fail [23]. In

addition, results are species specific and due to conflicting results in clinical trials more study is required. Azoles include itraconazole, ketoconazole and fluconazole and all have anti-leishmanial activity *in vitro* [76, 77] but clinical trials have shown varying results due to inappropriate controls, low study sizes or focus on different species of parasite [78, 79]. In 2002, a randomized double-blind placebo-controlled trial for efficacy of fluconazole (Diflucan, Pfizer, New York) against *Leishmania major* was carried out in Saudi Arabia [55]. This was the first study to examine the efficacy of fluconazole against CL and more so against only one species of *leishmania*. The researchers concluded that oral fluconazole is useful and well tolerated as a treatment for cutaneous leishmaniasis caused by *L. major* because it improves the chances that lesions will heal more rapidly. This study highlights the necessity of proper controls and of studying one species of parasite at a time in one endemic region.

Paromomycin phosphate is an aminoglycoside antibiotic that has been used systemically against both CL and VL. Paromomycin can also be used topically as a formulation with 15% paromomycin phosphate combined with 12% methylbenzethonium chloride (MBCL) in soft white paraffin. This formula contains a concentration of 15mg/ml paromomycin which is vastly higher than the serum concentration after paromycin injection (10 μg/ml) meaning very little of the agent needs to penetrate the lesion for effective treatment [54]. Applied twice daily, paromomycin is effective against *L. major* [80, 81] but less so against New World species where results are species and region specific[82, 83]. Use of paromomycin in the New World is overshadowed by the risk of enhancing mucosal disease particularly if reliable species identification is not available [22].

As shown, current chemotherapies against cutaneous leishmaniasis are fraught with challenges; they carry significant side effects, do not work for all species, or have yet to be adequately studied. In the arms race against this pathogen, drug resistance is a continued barrier to successful treatment of CL. For these reasons, a successful vaccine remains the best approach to widespread public health. However, and as the next section of this work will show, infection with *Leishmania* causes a complicated and multifaceted immune response that we are only just beginning to understand.

1.2 Experimental *Leishmania major*

i. Murine Models of *Leishmania major*

Mice provide an excellent model to study the immunology of *Leishmania major* infection. Most strains of mice are resistant to subcutaneous *Leishmania major* infection but definitions of resistance or susceptibility can vary between different papers. In some, resistance means a transient ability to control lesion development despite chronic infection while in others chronic infection defines susceptibility. In this work, resistance means that when experimentally infected by a subcutaneous or intradermal route, mice develop lesions that heal over time and confer life-long immunity to re-infection. Immunity is accompanied by the presence of a small number of persistent parasites that if removed result in the loss of protection [8, 9].

Unlike most other inbred strains, BALB/c mice are susceptible to *L. major* infection [84]. Following subcutaneous challenge, the mice develop progressive, non-resolving lesions. Parasite burden increases as parasites disseminate from site of infection to the viscera leading to systemic disease and death [85]. This susceptibility is mediated by early, high and sustained production of IL-4 by CD4+ T cells in response to parasite antigen. In other strains of mice, for example C57BL/6, resistance to *L. major* infection is mediated by IFN-γ produced by CD4+ T cells that activate infected macrophages to kill the intracellular parasites. The balance between IFN-γ and IL-4 defines a key parameter determining the outcome of *L. major* infection that will be further reviewed in the next section.

ii. Th1/Th2 and *Leishmania major*

CD4+ T helper type 1(Th1) and type 2 (Th2) cell populations were first described in 1986 [86] and classified based on their cytokine profiles and effector functions. Current dogma dictates that the decision of a naïve T cell to differentiate to Th1 or Th2 is based on the priming environment and cytokines produced by antigen presenting cells (APC), primary dendritic cells. Dominant IL-12 production by DCs directs T cells to differentiate along a Th1 pathway subsequently producing IFN-γ and TNF and supporting cell-mediated immunity. Alternatively, interactions between DCs and T cells involving monocytic chemotactic protein 1 (MCP1) and OX40 ligand (OX40L) [87] direct T cells to differentiate along a Th2 pathway where they produce IL-4 and IL-13 and support humoral or antibody immune responses. IL-4 production likely by mast cells, eosinophils, basophils and NKT cells [88, 89] also promote Th2 cell differentiation.

Th1 and Th2 lineage decisions may not be an irreversible endpoint in T cell differentiation [90, 91]. However, Th1 and Th2 pathways do reciprocally regulate each other – IL-4 inhibits Th1 development and IFN-γ inhibits Th2 development [92]. The selective exclusivity of Th1 and Th2 responses coupled with clonal expansion of naïve T cells following activation, not only allows predominantly Th1 or Th2 responses to develop in response to specific diseases, but also allows tissues to direct immune responses along quiescent phenotypes for the protection of the host [93]. For example, Th1 responses are generally associated with viral infections and autoimmune disorders while Th2 responses are associated with allergy and clearance of extracellular parasites.

The Th1/Th2 paradigm of resistance/susceptibility to intracellular infection is largely supported by investigations using *L. major* [94]. Murine *Leishmania major* was

one of the first *in vivo* demonstrations of the impact of Th1 versus Th2 immune responses on the outcome of infection [95, 96]. Comparison of susceptible mouse strains found consistent expression of IL-4 correlating with parasite burden [97]. Furthermore, successful passive transfer of resistance to disease depended on transfer of CD4+ T cells producing Th1 cytokines while Th2 cytokine producing cells caused disease exacerbation [95, 98]. The target of Th1 or Th2 cytokines in *L. major* is the host cell, macrophages. The Th1 cytokine IFN-γ induces classical activation of macrophages leading to parasite killing [99] while the Th2 cytokine IL-4 induces alternative activation of macrophages resulting in parasite proliferation [46]. The interplay between classical and alternative macrophage activation in *Leishmania major* is the basis of this work and will be discussed at length in subsequent sections.

The preceding paragraph presents cytokine regulation of *L. major* infection as a very simplistic left or right directive. In fact, at the whole animal level, Th1 and Th2 immune responses to *L. major* infection do not occur in isolation. Resistant C57BL/6 mice also induce a Th2 response to *L. major* infection and T cells capable of protecting recipient mice have been isolated from susceptible BALB/c mice [100, 101]. The timing, amounts and quality of cytokine produced in each case has more bearing on the outcome of infection than the mere presence of one cytokine over another.

Interleukin-13

The results observed in IL-4 knock-out mice following *L. major* infections are different from those obtained in infected IL-4Ra knock-out mice [102-105]. For example, whereas IL-4Ra-/- mice are resistant to *L. major* infection (strain IR173) with a

lesion size development similar to C57BL/6 mice, IL-4-/- mice show only a decreased susceptibility. These results suggest that another cytokine which signals through the same receptor plays a role in susceptibility to *L. major*. Interleukin-13 (IL-13) signals through IL-4Ra as does IL-4 [106]. In *L. major* infection, both these cytokines appear to work in concert because susceptible mice unable to signal through both IL4 and IL-13 were rendered less susceptible than mice unable to signal through IL-4 alone [104, 107-109].

Although some studies report protective roles for IL-13 in *L. major* infection [110, 111] the consensus is that IL-13 is a Th2 cytokine that promotes disease exacerbation [104, 105, 109, 110]. For example in a recent study, transgenic C57BL/6 mice that overexpress IL-13 cannot resolve *L. major* lesions and this susceptibility was IL-4 independent demonstrating the additive effect of these cytokines [105, 109]. Surprisingly, murine lymphocytes do not express any IL-13 receptors [112], indicating that the effects of IL-13 on immune responses are mediating through other cell types, most likely APCs. Indeed IL-13 has been found to downregulate such macrophage functions as IL-12 [113], iNOS [114, 115], and TNF-α [116, 117] production. IL-13 may also mediate some if its effects indirectly. For example, IL-13 can upregulate PGE_2 production [118] which can in turn inhibit IL-12Rβ2 expression [119] and is a susceptibility factor in *L. major* infection [120, 121].

Interleukin-10

The cytokine interleukin-10 (IL-10) functions to suppress the immune system to avoid collateral damage to the host due to excessive immune responses [122]. The

importance of IL-10 is best shown by the results of studies in mice deficient in IL-10. For example, IL-10-/- mice or IL-10Rβ-/- mice develop spontaneous colitis [123, 124].

In *L. major*, IL-10 is involved in regulation of effector mechanisms of infected macrophages during the primary infection and, in resistant mice, the anti-parasite Th1 response during infection-induced immunity. Experimentally, mAb treatment blocking IL-10 does not change the outcome of infection in resistant or susceptible strains [125]. Genetic manipulations on the other hand shed light on the role of IL-10 in this infection. Transgenic mice on a resistant B6 background that over express IL-10 under control of the MHCII promoter were unable to control infection with *L. major,* despite having similar IFN-γ and IL-4 cytokine profiles to wild-type mice [126]. This finding highlights that though IFN-γ was produced it was unable to render the appropriate response in infected macrophages. Similar transgenic mice under control of the IL-2 promoter however were able to control lesion development [127] highlighting this cytokine's effects in primary infection are mediated directly by antigen presenting cells. IL-10-/- mice on a susceptible BALB/c background can control infection for up to 80 days post infection however, it is unclear whether these mice achieved infection-induced immunity [128]. After healing of primary infection or in cases of chronic infection, IL-10 produced by regulatory T cells dampens the Th1 response enough to allow parasites to persist in low numbers thus maintaining infection-induced immunity [129].

iii. Resistance and Susceptibility to *L. major*

Early Immune Responses

Within the first three days post infection, BALB/c mice develop a robust IL-4 response to *L. major* infection [5]. IL-4 is responsible for initial priming of naïve T cells to differentiate to a Th2 phenotype. During an *L. major* infection, the bulk of IL-4 production is by these Th2 cells which maintain their own population in a paracrine fashion while influencing other cells. Since high production of IL-4 early after infection was associated with susceptibility, researchers began to investigate possible antigenic causes of this cytokine's production. A *Leishmania* homolog of receptors for activated C kinase (LACK) was first identified as a possible vaccine candidate because when administered with IL-12 before infection it provided some protection to BALB/c mice after challenge [130]. LACK recognized directly through the TCR of Vβ4Vα8CD4+ T cells with low affinity for peptide/MHC complexes causes these T cells to produce IL-4 [131] leading to Th2 polarization. Subsequent studies however found that *in vivo* LACK actually induced IL-4 responses early after infection and may be the cause of the initial high burst of IL-4 [132]. Furthermore, mice tolerized to LACK could produce a successful Th1 response to subsequent challenge [132]. Subsequent studies provided strong evidence that LACK reactive T cells are responsible for the early production of IL-4 "driving" susceptibility to *L. major*; however most of these studies were performed only in BALB/c mice [132-134]. Investigations of resistant C57BL/6 mice revealed that early production of IL-4 in response to LACK antigen occurs in both resistant and susceptible strains of mice [135, 136].

The dichotomy of immune responses in *L. major* outcome led researchers to investigate the kinetics and regulation of IL-4 over the course of infection. IL-4 initiates the type-2 response and, in susceptible mice, maintains this response later in infection [137]. Surprisingly, both susceptible and resistant mice begin the immune response to *L. major* with a predominantly Th2 cytokine profile including IL-4 [138], IL-13 and IL-2. IL-4 mRNA in draining lymph node cells of infected susceptible and resistant mice peak at four days post infection but in resistant mice, this response gradually decreases and IFN-γ levels increase steadily taking over the immune response [100].

Experimental evidence has shown that in the C57BL/6 model of murine *L. major* infection, IFN-γ is not produced until two weeks after infection [5]. Traditional dogma dictated that susceptible strains of mice produced IL-4 and resistant strains IFN-γ and the two were mutually exclusive but it has been shown that both BALB/c and C57BL/6 mice initially produce near identical levels of IL-4 early in infection [100]. Only mice that are able to overcome the initial IL-4 response are able to resolve the infection. If the late IFN-γ response is diminished, recurrence of the infection will occur transiently or permanently depending on the cause of IFN-γ reduction. Studies with knockout mice illustrate the importance of IFN-γ. C57BL/6 mice with a homologous disruption of the IFN-γ gene such that they cannot make IFN-γ were completely susceptible to challenge with *L. major* and disease in these mice progressed like in BALB/c mice [139]. CD4+ T cells in these knockout mice displayed all characteristics of Th2 cells when re-stimulated *in vitro*. In contrast, CD4+ T cells from infected mice genetically deficient in the IFN-γ receptor (IFN-γ-R-/-) could still take on a Th1 phenotype upon *in vitro* stimulation [140].

This was despite the fact that the IFN-γ-R-/- mice were completely susceptible to the infection indicating that lack of the receptor does not abolish production of this cytokine.

Parasite Dose

Surprisingly, BALB/c mice infected with a low dose of parasites (less than 1,000) can resolve infection [141, 142]. Resolution of low dose infection in BALB/c mice is due to induction of a cell-mediated immune response characterized by higher amounts of IFN-γ production than IL-4 production [142]. In contrast, although C57BL/6 mice can heal both low and high dose infections, cells from low-dose infected mice produce substantially more IL-4 [143].

Low dose murine infection also revealed a role for CD8+ T cells in *L. major* infection. C57BL/6 mice deficient in CD8+ T cells are unable to heal from low dose infection [144]. Only transfer of CD8+ T cells that make IFN-γ can restore resistance in these mice [143]. These results suggest that IFN-γ-producing CD8+ T cells have a role in regulating the development of Th1 CD4+ T cells perhaps through promoting IL-12 production by DCs [145] or enhancing chemokine production [5, 146, 147]. In BALB/c mice, the CD8+ T cell response may be defective or overcome by the prominent Th2 response.

What Mediates Susceptibility of BALB/c Mice?

Oddly, treatment of susceptible BALB/c mice with recombinant IFN-γ does not result in resistance to infection [148]. Treatment with anti-IL-4 antibodies however, results in complete resistance (in 85% of the mice treated in the study) that is adoptively transferable. This seemingly confounding result is evidence that the presence of IFN-γ

alone is not sufficient to induce classical macrophage activation. Most likely, cell-to-cell interactions between Th1 T cells and macrophages are necessary for effective classical activation. The caveat of this interpretation is that treatment with rIFN-γ did not modulate physiologic IFN-γ production; possibly the recombinant protein did not reach the site of infection or was used up by cells close to the administration site. This hypothesis is corroborated by the work of Soong et al [149] who found that stimulation of infected peritoneal macrophages with IFN-γ alone did not result in measurable nitric oxide production by Griess assay. The work of Dalton et al. [150] is regarded by some to be the definitive proof that IFN-γ is an inducer of iNOS [151]. However in this work IFN-γ was used in combination with LPS to produce NO from macrophages isolated from IFN-γ-/- mice. LPS signaling through TLR4 is a distinct pathway from IFN-γ signaling. Another work by Ding et al. [152] reported a measurable production of nitrates by Griess assay induced by recombinant IFN-γ treatment of peritoneal macrophages. Furthermore, Liew et al. [99] found that treatment of infected peritoneal macrophages with rIFN-γ induced NO-mediated parasite killing over 3 days post-infection. Importantly, the latter two studies were conducted in resistant strains of mice while Soong et al. [149] conducted their studies in susceptible BALB/c mice. Iniesta et al. [47] showed that infected BMDM from resistant and susceptible strains produce different amounts of NO and induce different levels of arginase activity in response to stimulants like IL-4 or IFN-γ and LPS. Therefore, the ability to find measurable NO after rIFN-γ stimulation may be strain specific.

The interaction between DCs and T cells in the presence of IL-12 (produced by dendritic cells), results in Th1 differentiation of naïve T cells into Th1 cells capable of

producting large scale IFN-γ. IL-12 acts through the IL-12 β2 receptor (IL-12Rβ2) and JAK/STAT-4 pathway leading to induction of the AP-1 site in the IFN-γ promoter and subsequent gene transcription [153-155]. STAT-4 independent pathways also exist [156]. IL-12 is essential for resistance to *L. major* [157, 158]. IL-12Rβ2-/- mice on a resistant background are completely susceptible to disease and display a Th2 cytokine profile [159] as do mice lacking bioactive IL-12 [160]. Despite its importance in mediating resistance, transcripts of IL-12 p40 mRNA cannot be found earlier than 7 days post infection [100]. Late induction of IL-12 production may be associated with the infection of macrophages with the amastigotes [100].

IL-12 is also essential for maintaining a Th1 response in resistant mice. Resistant mice infected with *Leishmania major* and treated with anti-IL-12 antibodies display a transient Th2 response that is resolved when IL-12 levels return to normal [135, 161]. Surprisingly, the necessity of IL-12 for resolution of *Leishmania* lesions may be somewhat species specific. When infected with *Leishmania mexicana,* C57BL/6 mice develop chronic non-healing lesions. In this system, Buxbaum et al. found that IL-12 plays no role in control of disease because mice treated with rIL-12 or infected IL-12p40-/- mice have the same disease as wild-type mice [162]. This finding is controversial however because in a similar work by Torrentera et al. [163] IL-12 was found to be critical for immune responses to *L. mexicana* late in infection.

Susceptible BALB/c mice given recombinant IL-12 daily for the first seven days of infection show enhanced resistance to *L. major* infection even after a secondary challenge [164]. The protection induced by IL-12 therapy is completely IFN-γ mediated because mice given a single treatment of anti-IFN-γ antibodies at the time of infection

revert to total susceptibility even with IL-12 therapy. Taken together with the fact that rIFN-γ treatment does not rescue BALB/c mice [148], this finding highlights the ability of BALB/c mice to produce Th1 cells. The efficacy of these cells to mediate protection indicates that the defect in this strain of mouse leading to their inherent susceptibility is less likely in the T cell compartment and more likely in the dendritic cell compartment. Without intervention – i.e., exogenous IL-12 – DCs are unable to prime naïve T cells along the appropriate antigen-specific Th1 pathway. Further evidence for this theory is the demonstration that protective Th1 effector cells can be isolated from infected BALB/c mice and when transferred, protect severe combined immunodeficiency (*scid*) mice upon challenge [101].

Clearly, IL-4 is a major susceptibility factor in murine models of *L. major,* resistant mice made transgenic for expression of IL-4 are susceptible to disease [165]. In addition, the ability of IL-4 to mediate susceptibility has been demonstrated by administration of antibodies against IL-4 which allowed susceptible mice to become resistant [104, 148]. Furthermore, susceptible BALB/c mice deficient in IL-4 show decreased susceptibility to disease in a dose dependent manner [107]. Heterozygous IL-4 +/- mice were intermediate in their level of susceptibility between wild-type BALB/c and IL-4-/-. Deficiency in IL-4 however does not result in relatively increased production of IFN-γ indicating that it is the suppressive actions of IL-4 on Th1 development that prevent resistance in susceptible animals not an impaired ability to produce IFN-γ [101, 104]. This fact is further supported by the finding that susceptible BALB/c mice can effectively clear a low-dose infection (10^2 parasites) with *L. major* but this resistance is

reversed by treatment with IL-4 or IL-10 plasmids [166] or antibodies against IL-12 or IFN-γ [167].

iv. Memory Responses

Both human and murine infections with *L. major* result in life-long protection against re-infection, which is dependent on the presence of persistent parasites. For centuries, this phenomenon has been exploited by those living in endemic regions through the process of leishmanization [175-176]. The practice involves injection of a small number of parasites in an inconspicuous part of the body. Upon healing of this infection, the patient is protected against subsequent infections on the face or hands that would result in disfiguring scarring. Despite knowledge of this practice for centuries, our understanding of the correlates and mediators of protection against *L. major* remains limited.

Two populations of CD4+ T cells have been found to play key roles in immune memory of *L. major* [5]. Upon secondary infection, a population of activated ($CD44^{high}$) effector memory cells (T_{eff}) migrates to the site of infection and produces IFN-γ rapidly after antigen stimulation, within three days post infection. These cells have low expression of L-selectin ($CD62L^{low}$) which is thought to aid their migration to the tissues. These cells mediate delayed type hypersensitivity (DTH) responses but are relatively short-lived [177-178]. A second population of central memory cells (T_{cm}) remains in draining and non-draining lymph nodes after primary infection ($CD62L^{high}$) and remains activated ($CD44^{high}$). Upon secondary infection, these cells initially express IL-2 but upon antigen stimulation they will proliferate and downregulate CD62L expression.

These cells then acquire the T_{eff} phenotype, produce IFN-γ and migrate to the site of infection. Thus, T_{cm} are able to repopulate the T_{eff} population and mediate protection. Adoptive transfer of $CD62L^{low}$ or $CD62L^{high}$ populations separately demonstrated that both populations of cells can protect but T_{eff} respond faster than T_{cm} [4]. It is important to note that not all the T_{cm} cells are converted to the effector phenotype during challenge infection. A number of T_{cm} cells remain circulating between draining and non-draining lymph nodes ready to replenish the effector cells upon challenge.

Although the behaviour of T_{eff} and T_{cm} after infection is increasingly well-characterized, the pathways that generate these subsets remain unclear. Early studies proposed that T_{cm} are T cells that proliferate during the primary infection but do not become effector cells [180-181]. This theory is the divergent model of memory differentiation because it implies that memory T cells are generated directly. The alternative linear model proposes that memory T cells are residual effector cells that somehow acquire the ability to remain in circulation [168]. Proliferation alone does not define if T cells will become T_{eff} or T_{cm} as the work by Zaph et al. has shown an important role for CD62L [4]. A caveat to the classification of T_{eff} and T_{cm} as $CD44^{high}CD62L^{low}$ or $CD44^{high}CD62L^{high}$ is that naïve T cells upon initial activation will also modulate these markers. In this case, proliferation, IL-2 and IFN-γ expression may also aid in differentiating between these cells.

Cytokines also play a role in maintenance of memory T cells. IL-7 is important for maintenance of naïve and memory T cells [183] but in most T cells, the receptor (IL-7Rα) is downregulated after activation. In a small number of cells, IL-7Rα is re-expressed later in the infections and these cells become memory cells. The re-expression

of IL-7 may depend on the strength of the initial IL-2 signal during T cell priming [184]. The importance of IL-7 in CD4 memory cannot be understated as IL-7 deficient or receptor knock-out mice cannot generate CD4 memory [185-186]. IL-15 also aids in memory CD4+ T cell maintenance but to a lesser extent than in CD8 and NK cell memory [187].

In contrast to viral and bacterial infections, development of immune memory in *L. major* does not require pathogen clearance. In fact, clearance of parasites results in loss of infection-induced immunity [6-7]. Surprisingly, infection with avirulent strain can still result in significant protection. Dihydrofolate reductase-thymidylate synthase deficient parasites (*dhfr-ts-)* can only survive in mice for six to eight weeks [169]. Despite this loss of persistent parasites, parasite burden in mice challenged at 25 weeks post infection was significantly lower than in naïve mice but, two logs higher than in mice that healed a wildtype infection [4]. This protection was associated with CD62L ^{high} T_{cm} cells but the results show that without persistent parasites a full protective response cannot be generated. As such, the ideal vaccine against *L. major* may be an attenuated parasite that can persist without causing pathology. Such mutant parasites are under study. Lipophosphoglycan (LPG) is one of the molecules that make up the dense glycocalyx coating the surface of *L. major* and protecting the parasites from complement fixation [189]. When injected into normally susceptible BALB/c mice, *lpg2*$^{-}$ mutants can protect against virulent challenge by suppression of IL-4 and IL-10 [190] but protection in resistant C57BL/6 mice required use of CpG-containing oligodeoxynucleotides as an adjuvant [170].

1.3 Classical and Alternative Macrophage Activation

i. Definitions of Classical and Alternative Macrophage Activation

Janeway et al. [171] define macrophages as "effector cells in humoral and cell-mediated immunity" with "a crucial role in host defense". As members of the innate immune system, macrophages are among the first line of host defense against invading pathogens. The interaction between a macrophage and a pathogen results in a chain of events leading to macrophage activation which is generally accepted to be within a spectrum of phenotypes. The two extremes of this spectrum are defined as classical and alternative macrophage activation and in combination with dendritic cells, they direct the nature of the adaptive immune response to follow. Thus, it may be argued that macrophages represent the beginning and the end of immunity.

Macrophage activation is regulated in part by Th1/Th2 cytokines. Classical activation is caused by stimulation of macrophages with IFN-γ initially produced by NK cells and later by Th1 cells. In addition, TNF produced by APCs and acting by both paracrine and autocrine mechanisms, promotes this type of activation [172]. Classically activated macrophages (CAMϕ) are produced during cell-mediated immune responses and this type of activation is essential in host defence against intracellular bacteria, protozoans, fungi, helminthes, and viruses [173]. These cells are highly microbicidal due to production of reactive oxygen and nitrogen intermediates and produce large amounts of pro-inflammatory cytokines like IL-1, IL-6 and IL-23 [172]. A key enzyme mediating the microbicidal activity of CAMϕ, is inducible nitric oxide synthase which uses the substrate L-arginine to make the pathogen-killing molecule nitric oxide (NO). Although essential in immune responses, the pro-inflammatory cytokines and reactive nitrogen and

oxygen species released by CAMϕ can cause significant bystander damage in host tissues.

The Th2 cytokines IL-4 and IL-13 cause macrophages to convert to alternative activation. In contrast to the microbicidal phenotype of CAMϕ, alternatively activated macrophages (AAMϕ) produce low amounts of pro-inflammatory cytokines and exhibit a proliferative phenotype, due to collagen and polyamine production, which promote proliferation of cells such as fibroblasts [154, 193, 195]. Under control of IL-4 and IL-13, AAMϕ express a number of characteristic proteins such as eosinophil chemotactic factor Ym1 [196], resistin-like molecule-α (RELMα) [196], macrophage mannose receptor (MMR) [174] and the enzyme arginase which uses the substrate L-arginine to produce polyamines [154, 198]. Despite their role in regulating wound-healing, AAMϕ can damage the host when dysregulated. In chronic schistosomiasis for example, AAMϕ have been linked to pathological tissue fibrosis [199]. Alternative macrophage activation also plays a role in airway disease where polyamine production and proline production lead to collagen deposition and airway remodeling [200].

Since both arginase and iNOS use the substrate L-arginine, the generation of classically or alternatively activated macrophages depends on the outcome of L-arginine metabolism. L-arginine is derived from diet, endogenous synthesis and protein turnover but is classified as a conditionally-essential amino acid because its production by biosynthesis is insufficient for its uses in the body during times of stress or infection. Arginine is the substrate of the enzymes arginase, arginine decarboxylasearginase, nitric oxide synthases, arginine:glycine amidinotransferase and arginyl-tRNA synthetase [175]. These pathways produce ornithine and urea, agmatine, nitric oxide, creatine, and protein

respectively. For many years, arginine was known primarily for its role in the final reaction in urea biogenesis. More recently, L-arginine has been shown to be of particular importance due to its products nitric oxide and polyamines. Nitric oxide is an essential signaling molecule in all cell types and a potent microbicidal agent [202-203]. Polyamine production is essential for cell proliferation, for example, it plays a role in pregnancy facilitating growth of the fetus [176] and in cancer in the proliferating tumor cells [177]. Arginine enters macrophages via the cationic amino acid transport system which is composed of four transporters of which CAT2B is essential for baseline activity of both iNOS and arginase [178].

ii. Arginase and Alternative Macrophage Activaion

Arginase functions as a homotrimer and is a metalloenzyme with each subunit requiring binding of manganese to function [179]. The basic reaction hydrolyzes L-arginine to produce urea and L-ornithine. L-ornithine is in turn converted by ornithine decarboxylase (ODC) to putrescine leading to production of the polyamines spermine, and spermidine and by ornithine aminotransferase (OAT) leading to proline and collagen production [178, 180, 181].

In mammals, arginase exists in two isoforms, arginase I and II which are cytosolic and mitochondrial respectively [182, 183]. Though biochemically identical, the isoforms are distinct in function and genetic location and are induced by different activities [178, 183]. Tissue localization of the isoforms is complex, particularly in humans where experimental means are limited, because of their different subcellular locations and differential induction in response to hormones and cytokines [178]. The urea cycle,

which takes place completely in hepatocytes, is mediated by the isoform Arginase I [183]. However, this isoform is also expressed in immune cells including human platelets [184], human neutrophils [185], human alveolar macrophages [186] and murine bone-marrow derived macrophages and dendritic cells [187]. In these cells, Arginase I is upregulated by Th2 cytokines, GM-CSF, prostaglandins and catecholamines [188] and mediates production of polyamines which are important for regulating cell growth and differentiation and for stabilizing nucleic acid and membranes [189]. Mitochondrial arginase II is expressed at low levels in a wide variety of tissues [190] and also in murine bone-marrow derived macrophages and dendritic cells [187]. While murine arginase II is constitutively expressed, the arginase I gene (ARGI) is flanked by regions that control transcription in response to IL-4, IL-13, cAMP, TGF-β, dexamethasone, LPS and STAT6 [115, 190].

Both isoforms of arginase are found in murine macrophages [187, 191, 192] and upregulation of Arginase I characterizes alternative activation and demonstrates a proliferative phenotype due to the production of polyamines. In macrophages, both isoforms of arginase respond to antigen challenge; arginase II can be activated by LPS, cAMP or intact bacteria but only arginase I is induced by Th2 cytokines and inhibited by Th1 cytokines [187, 193] . In a number of disease states and during inflammation in general, prostaglandins, in particular PGE1 and 2, can also induce arginase activity in macrophages [194] causing them to deplete arginine from the microenvironment [195]. This has also been shown experimentally where induction of macrophage arginase I causes arginine depletion from the culture media [181]. Tumor cells have taken advantage of this pathway by producing prostaglandins in the tissue microenvironment

which in turn increases arginase activity in a special subset of myeloid suppressor cells similar to alternatively activated macrophages [195]. T cells that might have initiated immune responses against the tumors are thus limited from proliferating due to arginine scarcity which also reduces CD3ζ chain expression impairing T cell function [196]. Similar regulation of T –cell hyporesponsiveness has been reported in pregnancy [197] and tuberculosis [198].

In murine *L. major* infection, the Th2 cytokines IL-4, IL-10, and TGF-β increase macrophage arginase 1 activity and promote intracellular parasite proliferation in infected bone marrow derived macrophages from BALB/c and C57BL/6 mice [45, 46]. This response was specific to arginase because treatment of infected cells with a specific arginase inhibitor blocked all growth achieved in response to cytokine stimulation. Interestingly, these cytokines were more effective in BALB/c cells than C57BL/6 cells presenting one possibility for the differences in susceptibility between these two strains. The induction of arginase activity by IL-4 administration was further increased by infection of macrophages from BALB/c mice with *L. major* [45]. In fact, infection of macrophages with *L. major* increased L-arginine catabolism, intracellular ornithine and spermine more than IL-4 induction alone.

iii. Inducible Nitric Oxide Synthase and Classical Macrophage Activation

Nitric oxide synthase (NOS) hydroxylates L-arginine to N^{ω}-hydroxy-L-arginine (NOHA) and then oxidizes this intermediate to produce L-citrulline and NO [199]. NOS exists in three isoforms two of which are constitutively active neuronal NOS (nNOS) and endothelial and epithelial NOS (eNOS) and one which is inducible (iNOS also known as

NOS2). All are calmodulin binding dependent enzymes but only iNOS can work independently of calcium. Constitutive NOS are regulated by mechanical, chemical and electrical stress while iNOS is regulated by cytokines (IL-1β, TNF-α, IFN-γ), NFκB, cAMP and cGMP [200]. Interestingly, the cytokines responsible for upregulating iNOS, particularly TNF-α, down-regulate constitutive NOS [201]. Constitutive NOS produces a low amount of NO which is likely to mediate its effects directly. In contrast, iNOS produces NO in the millimolar range and mediates its effects via reactive nitrogen oxide species RNOS [202], which are effective microbicides. Though the half-life of NO is only 1-5 seconds, iNOS expressing cells will produce NO several hours after induction and continue for days as long as the molecule is stable and the substrate available [200, 203] . High RNOS can lead to lipid peroxidation, DNA damage, oxidation of thiols, nitration of tyrosine residues and production of peroxynitrite if NO reacts with a superoxide anion radical [202, 203] . Therefore, utilization of RNOS can have significant detrimental bystander damage. Overproduction of NO has been implicated in multiple sclerosis, HIV dementia, Huntington's disease, Alzheimer's disease, Parkinson's disease and stroke [203].

Of most importance in infectious disease is macrophage iNOS expressed under the control of Th1 cytokines [204, 205]. iNOS-derived NO in this context is an essential microbicide [206, 207] in extracellular infections like *Trypanosoma congolense* [208] and intracellular infections like *Toxoplasma gondii* [209], *Mycobacterium tuberculosis* [210] and *Leishmania major* [99]. Experimentally, iNOS-/- mice are susceptible to *S. enterica serovar Typhimurium* [211] and have a significantly higher mortality rate following injection of LPS [212].

In *L. major* NO is essential in the primary infection to mediate parasite control [47, 204, 205, 213] but iNOS activity is maintained in healed mice to limit the spread of persistent parasites [7]. When iNOS is blocked experimentally by administration of the inhibitor L-N^6-iminoethyl-lysine (L-NIL), the infection was reactivated and parasite burdens in the footpad and lymph nodes of previously healed mice increased substantially [7].

However, as previously mentioned there are serious side-effects to use of NO as a microbicide and in some infectious diseases it has proven a double-edge sword. For example, NO can lead to apoptosis in *Listeria monocytogenes* infection [214] and systemic immunosuppression in *Sporothrix schenckii* [215]. Even in *Toxoplasma gondii* NO production as a protective molecule may depend on the stage of the infection [216]. Furthermore, in experimentally induced colitis models, iNOS-/- had decreased intestinal inflammation than WT controls [217]. Thus while NO is an essential antimicrobial molecule, CAMϕ must be in physiological balance with AAMϕ to ameliorate the harmful side-effects. While NO can cause apoptosis, AAMϕ promote cell proliferation. The balance between these types of activation is mediated by cytokines.

iv. Reciprocal Regulation Between Classical and Alternative Macrophage Activation

As has now been shown, arginase and iNOS not only compete for substrate but also, have opposing roles in disease pathogenesis and immune function. As such, "depending on their role in host defense and pathology, immune cells typically synthesize NOS or arginase but not both" [218]. These enzymes are reciprocally regulated at many levels (Figure 3).

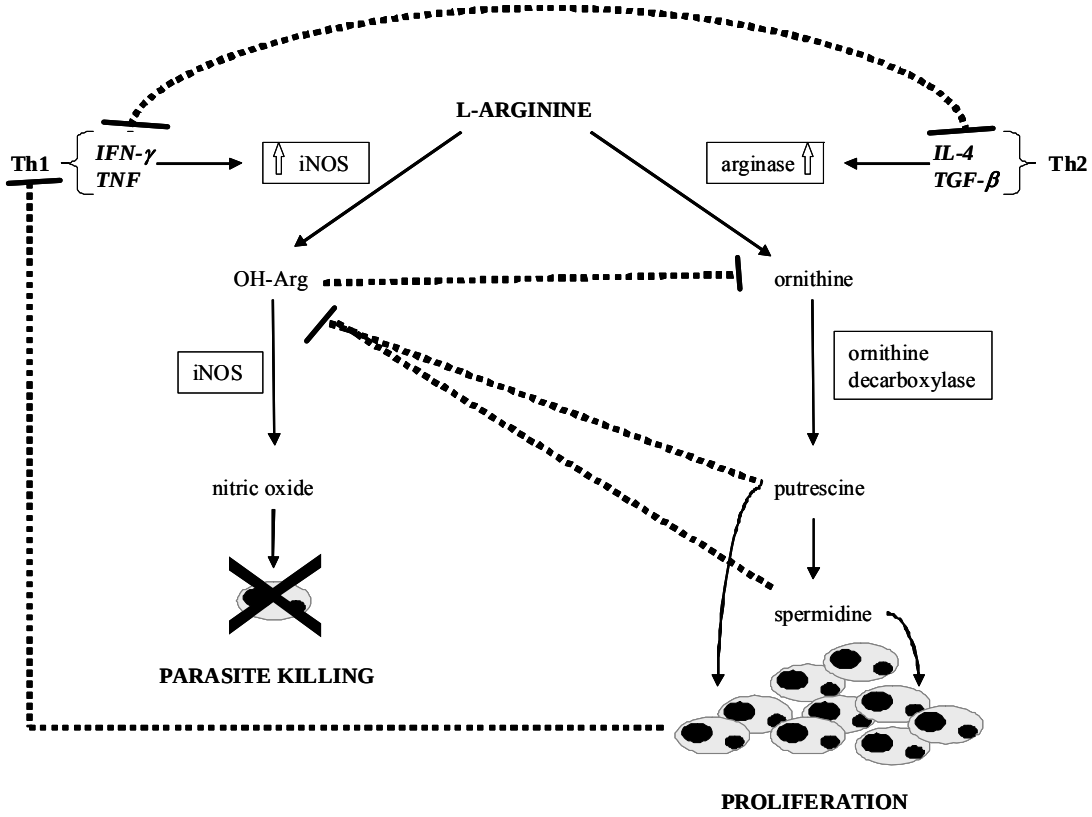

Figure 3. Metabolism of L-Arginine in Macrophages and Consequences during *L. major* infection. Under the regulation of Th1 and Th2 cytokines, iNOS or arginase are induced (open arrows). Solid arrows show the enzymatic pathways, dashed arrows show inhibition. Outlined words are enzymes. iNOS = inducible nitric oxide synthase.

Reciprocal regulation of iNOS and arginase in macrophages is largely influenced by Th1 and Th2 cytokines respectively. Numerous *in vitro* studies have shown that pre-treatment of bone marrow-derived and peritoneal macrophages with Th2 cytokines impairs their ability to take on a CAMϕ phenotype when stimulated with Th1 cytokines and vice versa [48, 115, 219] . In addition, pre-treatment of *L. major* infected murine peritoneal macrophages with IL-4 blocked parasite killing when the cells were subsequently stimulated with IFN-γ [220]. Reciprocal regulation by cytokines has also been shown in an *ex vivo* study of *L. major* where IL-4 from immunosuppressive T cells blocked protective IFN-γ responses in susceptible BALB/c mice [92]. In addition to the dogmatic IFN-γ and IL-4 cytokines, TNF-α [221, 222] and TGF-β [223, 224] also regulate CAMϕ and AAMϕ respectively. In a murine model of *L. major*, TGF-β was found to inhibit IFN-γ mediated production of NO while TNF worked synergistically with IFN-γ in an autocrine manner and amplified NO production [225].

Furthermore, NO itself may modulate the immune response. The results of iNOS-/- studies in infectious disease models, presented in the last section, may be confounded by the fact that in the absence of iNOS signaling, macrophages produce more IL-12 [226]. Thus IL-12 and IFN-γ form part of a feedback loop whereby IL-12 production by APCs generates Th1 cells that produce IFN-γ which in turn classically activates macrophages inducing them to produce more pro-inflammatory cytokines perpetuating the cycle. In this context, NO may act to limit excessive Th1 cytokine production at the transcriptional level [226] without which the immune response is overwhelmed and the detrimental disease responses cited above become evident.

Because of their mutual dependence on L-arginine as a substrate, iNOS and arginase are reciprocally regulated in terms of enzyme activity and mRNA expression. Although the K_m of iNOS is 2-20μM and that of arginase is 2-20mM, the V_{max} for arginase (1400 μM /min/mg) is 1000 times higher than that of iNOS (1mM/min/mg) indicating the biochemical basis for substrate competition [175, 178]. As a result, only a small fraction of arginine in cells is used by iNOS with the majority used by arginase [227]. Since arginase reduces NO synthesis due to substrate depletion [48, 228] , arginase may be the cause of the arginine paradox. The arginine paradox refers to the "dependence of cellular NO production on exogenous L-arginine concentration despite the theoretical saturation of NOS enzymes with intracellular L-arginine." [203]. In *L. major* and other infections, "The concentration of L-arginine is crucial in determining the effect of NO-dependent parasite killing by macrophages" therefore experimental observations of reduced killing efficiency could be a result of substrate depletion [193]. Many studies have shown that inhibition or deficiency of arginase activity leads to increased NO production, but it is important to note that baseline arginase activity may not be high enough to mediate substrate competition and the type of cell stimulation will determine the outcome of reciprocal regulation.

In addition to substrate competition, the enzymatic pathways of arginase and iNOS each contain reciprocal inhibitors (Figure 3). The NO intermediate OH-arg is a major inhibitor of arginase [229, 230] , NO inhibits ODC, the first rate-limiting enzyme in polyamine biosynthesis [231], and NO negatively regulates polyamine uptake [232], and polyamines inhibit iNOS induction [233]. Furthermore, iNOS dimerization cannot occur without arginine and substrate scarcity, possibly resulting from high arginase

activity, results in production of an inactive enzyme [234]. Arginase can also inhibit translation of iNOS mRNA – but this may also be arginine-dependent [203]. As previously mentioned, the outcome of reciprocal regulation has major implications in a number of diseases and infections. For example, in asthma NO is thought to be helpful due to its broncho- and vasodilatory effects [235]. When arginase is induced however, substrate competition leads to low-level production of NO believed to be detrimental because it favours production of destructive peroxynitrites [235]. In addition, arginase induction in asthma has been linked to the collagen deposition leading to airway remodeling [236].

1.4 Arginase and *Leishmania major* Infection

i. Host-Derived Arginase

Host-derived arginase 1 induction supports parasite proliferation *in vivo* [45, 138] . In susceptible BALB/c mice levels of arginase protein and mRNA are induced after infection leading to a significant correlation between parasite load and footpad arginase activity. Even in resistant CBA mice, arginase activity – though drastically lower than in susceptible mice - mirrors parasite load [45, 138]. This result is not solely due to genetics because susceptible IL-12Rβ2 -/- mice on a C57BL/6 background expressed high footpad arginase activity again correlating with high parasite burden [45]. Furthermore, in susceptible mice, inhibition of host arginase with NOHA ameliorated lesions and significantly lowered parasite burden without inducing Th1 responses *in vivo* and *in vitro* [45, 138]. It is important to note that these two studies used a different dose of nor-NOHA (100μg [45] vs 10μg [138]) and route of administration (i.p. vs at site of infection). When given at the site of infection, nor-NOHA treatment can only control lesion development for the first three weeks of infection after which time lesions return to normal. The side-effects of intraperitoneal administration of nor-NOHA were not discussed by Kropf et al. [45] but may be notable given the unknown tissue distribution of arginase 1. Finally, induction of host arginase following infection with *L. major* and the subsequent arginine depletion results in impaired parasite-specific T-cell responses [188].

ii. Parasite-derived Arginase

In the same way that polyamines are important in cell proliferation, they are also essential for proliferation of many intracellular pathogens [237, 238] . In particular, "Trypanosomatids depend on spermidine for growth and survival" [47, 239] and have evolved a system of enzymes to derive the necessary polyamines de novo or transporters to acquire them from host cells. The discovery of DL-α-difluoromethylornithine (DFMO) in 1980 [240] and its later widespread use as an efficient drug against late and early stage human African trypanosomiasis (*Trypanosoma brucei gambiense* [241]) has led to increased study into polyamine pathways as drug targets for protozoan diseases [242]. DFMO is a suicide inhibitor for ornithine decarboxylase (ODC) and its use exploits the relatively long half-life of trypanosome-derived enzyme compared to the short half-life of human-derived ODC. A number of site-directed mutagenesis studies have demonstrated the necessity of polyamine metabolism enzymes in *Leishmania.* In *L. donovani* for example, parasites deficient in ODC, s-adenosyl-methionine decarboxylsase, or spermidine synthase cannot survive in axenic cultures in polyamine-deficient medium [243-245]. Experiments *in vitro* demonstrated that spermidine in particular is vital for optimal parasite growth [237].

Due to the requirement of polyamines for cell proliferation, the arginase enzyme family is "highly conserved across kingdoms" [193]. Furthermore, the catalytic centre where Mn^{2+} and L-arginine binding occur is conserved in all arginases [246]. Though host arginase promotes parasite proliferation, findings of null mutant experiments in *Leishmania* have shown that in contrast to *in vitro* results [218, 237], *in vivo* scavenging of host polyamines is insufficient to promote parasite proliferation at least early in the

infection [218, 247] Thus, parasites have evolved other mechanisms to ensure successful growth. Arginase is expressed by a number of pathogens including *Helicobacter pylori* [248], *Bacillus anthracis* [249], and members of *Leishmania* [237, 250, 251] .

Leishmania depends on an intact and functional arginase enzyme for growth *in vitro*. Arginase null mutants have been characterized in *L. major* and *L. mexicana*. When grown in polyamine-free media, both mutants ceased proliferation or died [237]. In *L. mexicana* optimal growth of these mutants could be restored by addition of exogenous putrescine but less so by addition of ornithine which required a greater concentration to reach optimal growth levels [237]. Similar results were found in *L. major* where spermidine and agmatine were also tested and could restore parasite proliferation [218]. This study [237] suggests that the sole function of parasite-derived arginase is to facilitate polyamine synthesis, but in the *in vivo* environment under the influences of cytokines this conclusion may not hold true [218].

Parasite-derived arginase is essential in *L. infantum* infected macrophages as treatment of bone marrow-derived macrophages with the arginase inhibitor LOHA (analogous to NOHA) controlled intracellular parasite growth [47]. This response was specific to arginase inhibition as LOHA is not a substrate for iNOS [252] and its addition to cultures did not increase NO production. This inhibitor impairs both parasite-derived and host-derived arginase since it decreased parasite proliferation regardless of the infected macrophages being in AAMϕ or CAMϕ priming environments (i.e., IL-4 or IFN-γ/LPS treated).

Unlike mammals, *Leishmania* contain only a single arginase [237]. The primary amino acid sequence of arginase from *L. amazonensi*s shows 58% similarity to human

arginase I and 54% similarity to arginase II [250]. The amino acid sequence of *L. mexicana* arginase shares 38.5% and 32.6% sequence identity similarity to human arginase I and II respectively [237]. Importantly, amastigotes of *L. amazonensis* have also been shown to express parasite-derived arginase at the same levels as promastigotes of this strain [250], suggesting that enzyme levels and activity are not stage specific and that gene expression is maintained when inside the host cell. Genomic characterization of arginase from *L. amazonensis, L. mexicana* and *L. major* revealed that this enzyme shows some species specificity perhaps across Old versus New World lines [250]. Arginase from *L. Mexicana* bore sequence similarity to *L. amazonenesis*-derived arginase but neither was as similar to *L. major*-derived arginase. The similarity between *Leishmania*-derived arginase and human arginase I suggests many tantalizing hypotheses about its regulation. As summarized above, iNOS and arginase are tightly regulated by cytokines, but what is the effect of arginase on the immune system? Since arginase and iNOS are reciprocally regulated, does parasite-derived arginase serve as a virulence factor, subverting destructive immune responses by depriving iNOS of its one and only substrate?

2. Project Rationale

The outcome of *Leishmania major* infection is regulated by the balance between the products of L-arginine metabolism in macrophages. L-arginine can be oxidized by host iNOS to produce nitric oxide (NO), which contributes to parasite killing or hydrolysed by host arginase providing polyamines, which can support parasite proliferation. Futhermore, *Leishmania* encode their own arginase, which if expressed in high levels, may alter this balance influencing infectivity and disease pathogenesis. Significant research has explored the role of host arginase in infection with *Leishmania major* but less research has examined the role of parasite-derived arginase. Although arginase deficient *Leishmania* mutants have been characterized *in vitro*, little is known about the role of parasite-derived enzyme in disease pathogenesis in an *in vivo* infection model. It has been shown that parasite-derived arginase plays an essential role for parasite in intra-macrophage nutrition by increasing available polyamines for proliferation [218, 237]. What remains unclear is whether parasite-derived arginase also serves as a virulence factor or an immune evasion mechanism during infection by lowering the availability of arginine from iNOS and thereby limiting microbicidal NO production and/or influencing the overall immune host immune response by some unknown pathway.

. Recently, an arginase deficient *L. major* (on LV39 strain) was created by double targeted gene replacement of the arginase gene (ARG) [218]. These mutants are auxotrophic for putrescine in culture but otherwise survive normally. Also created was an add-back mutant (*arg*$^-$ /+ARG**)** in which the arginase gene was reintroduced in the arginase deficient mutant by stable transfection with a vector. Using these mutants, we

have addressed several unanswered questions regarding the role of parasite-derived arginase in disease pathogenesis and modulation of host immune response *in vivo* in both the highly susceptible BALB/c and resistance C57BL/6 mice.

Global Hypothesis:

Parasite derived arginase influences parasite survival and host immune response to *L. major*

Overarching Goal:

To determine whether parasite-derived arginase influences pathogenesis and quality of host immune response to *Leishmania major* infection in mice**.**

Objectives:

To investigate whether deletion or increased expression of arginase gene in *L. major* influences the:

1) Arginase activities in *L. major*-infected macrophages *in vitro* and *in vivo*

2) Growth and/or proliferation of *L. major* in macrophages *in vitro* and *in vivo*

3) Production of nitric oxide by infected macrophages following IFN-γ and/or LPS stimulation

4) Primary immune response to the parasites (Th1 vs Th2) *in vivo*

5) Quality of memory (secondary) anti-*Leishmania* immunity following secondary challenge

3. Materials and Methods

Reagents

Unless otherwise stated, all chemicals were purchased from Sigma- Aldrich, St. Louis MO and all antibodies are from eBiosciences, San Diego, CA. Media, penicillin/streptomycin, FBS, and glutamine were purchased from Invitrogen, Carlsbad, CA.

Mice

Six to eight week old female BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Montreal, Canada) or Central Animal Care Services, University of Manitoba. Female Thy1.1 C57BL/6 mice (6 to 8 weeks old) were purchased from The Jackson Laboratories, Bar Harbor MI. All mouse experiments were approved by the University of Manitoba Animal Care Committee in accordance with the regulation of the Canadian Council on Animal Care.

Parasites and Infection

All parasites were derived from the wild-type *Leishmania major* line LV39cl5 (RHO/SU/59/P). Homozygous null mutant derivatives lacking the *LPG2* Golgi GDP-mannose transporter (*lpg2*$^-$) were described previously [253]. The generation and biochemical characteristics of homozygous null mutants lacking arginase Δ*arg*::*HYG*/Δ*arg*::*PAC*, referred to as *arg*$^-$, and its complemented line Δ*arg*::*HYG*/Δ*arg*::*PAC*/+pXG-ARG (referred to *arg*$^-$ /+*ARG*) are described elsewhere [218]. Parasites were grown at 25°C in M199 medium (Hyclone, Logan, UT) supplemented with 10% heat-inactivated FBS, 2 μg/ml biopterin, 100 U/ml penicillin,

100 μg/ml streptomycin, 5 μg/ml hemin, 1 μg/ml biotin, 0.1mM adenine, 40 mM HEPES (pH 7.4), 20 mM L-glutamine and 50 mM Putrescine (for *arg*$^-$-*L. major*). All media additives were purchased from Sigma-/Aldrich (Oakville, ON, Canada). For infection, 7-day stationary phase promastigotes were washed three times in PBS and 2 million parasites (suspended in 50 μl PBS) were injected into the right hind footpad. After infection, the development and progression of footpad lesion was monitored weekly by measuring the diameter of infected footpad with calipers. Uninfected contralateral footpads served as controls. In some experiments, mice were challenged in the contralateral footpad with 2 million parasites 16 weeks after the primary infection and footpad thickness was measured in the challenged footpad three days later to quantify the delayed type hypersensitivity response.

Adoptive Transfer

Mice were sacrificed at least 16 weeks after primary infection and single cell suspensions were made of the spleen. Four hundred million spleen cells were resuspended in 1 ml of sterile PBS and 100 μl were injected into the tail vein of recipient mice. To monitor proliferation and behaviour, donor cells were sometimes labeled with CFSE prior to transfer or Thy 1.1 recipients were used. Mice were infected as described above 24 hours after cell transfer.

Estimation of Parasite Burden

At various times after infection, mice were sacrificed and parasite burden in the footpads of infected mice was quantified by limiting dilution analysis as previously described [254, 255]. Briefly, infected feet were rinsed in chlorhexiderm and ethanol and

homogenized by hand in a glass homogenizer in PBS containing 2% Pen/Strep. The homogenate was first centrifuged at low speed (500 rpm for 5 min) to remove large tissue debris and the supernatant was then centrifuged again at high speed (3000 rpm for 15 min) to pellet the amastigotes. The pellet was resuspended in 2 ml of PBS and serially diluted (1:10) in 96-well plates (Falcon, VWR Edmonton, AB, Canada) in complete Schneider's medium (supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). If the footpad was from mice infected with *arg*$^-$*-L. major*, the media was also supplemented with 50 mM putrescine. Plates were incubated at 28°C and the presence or absence of parasites was quantified after 7 days by microscopy.

Cytokine ELISAs and Flow Cytometry

The levels of IL-4, IL-13, IL-10 and IFN-γ in dLN or spleen cell culture supernatants were determined by sandwich ELISA using antibody (Ab) pairs (BD biosciences San Jose CA) according to the manufacturer's suggested protocols. At sacrifice, single cell suspensions of lymph nodes draining the infection site (dLN) or spleens were cultured in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin), in 24-well tissue culture plates (Falcon, VWR Edmonton, AB, Canada). In some cases, cells were stimulated with soluble leishmanial antigen (SLA, 50 μg/ml) for 72 hr and the culture supernatants were collected and stored at –20 ^{o}C until assayed for cytokines by ELISA.

For flow cytometry, cells were used either directly *ex vivo* or after 3-day culture as described above. Cells were washed in flow buffer (10% FBS in PBS with 0.1%

NaN_3). To prevent non-specific binding of antibodies to the Fcγ receptor, culture supernatant from 2.4G2 cells (ATCC, Manassas, VA) was added as a source of monoclonal antibodies against FcRII. Cells were then stained using antibodies against surface molecules like Thy1.2, CD4, CD44 and C62L before being fixed with 2% paraformaldehyde and resuspended in flow buffer. For intracellular cytokine detection, cells were stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml) and BFA (10 μg/ml) for 4-6 hr before processed as above. Cells were then permeabilized with 0.1% saponin and intracellular cytokines stained with antibodies against IL-4, IL-10 and/or IFN-γ as previously described [4]. Acquisition was performed using a BD FACS Calibur or BD FACS Canto (Becton Dickinson, Franklin Lakes, NJ). In some adoptive transfer experiments, Thy1.2 was used to identify donor cells.

Griess Assay

Nitric oxide levels in culture supernatants of BMDMs were measured at 24, 48 and 72 hours post infection by the Griess assay as previously described [152]. Briefly a solution of 1 part sulfanilamide (1%) in and 1 part Napthylethylenediamine dihydrochloride (0.1%) in 2.5% H_3PO_4 is mixed with the sample in a 1:1 ratio. The nitrite in the sample react with the solution and the product of this reaction has a fuchsia colour, the intensity of which is determined spectrophotometrically at a wavelength of 600 nm. In some experiments, NO was also measured in 3-day culture supernatant fluids of lymph node cells from infected mice that had been stimulated with SLA.

in vitro Infection of Bone Marrow Derived (BMDM) and Peritoneal Macrophages

Bone marrow cells were isolated from the femur and tibia of mice and differentiated into bone marrow-derived macrophages (BMDMs) as described previously [256]. After depletion of erythrocytes with ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA), marrow cells were resuspended in macrophage medium (complete RPMI 1640 containing 30% L929 cell supernatant as a source of M-CSF), seeded in Petri dishes at 4 x10^5/ml (10 ml/Petri dish) and allowed to differentiate at 37°C in CO_2 incubator. The culture media were changed at day 3 and adherent macrophages were harvested by gentle scraping on day 7, washed, resuspended in complete medium (10^6/ml) and used for *in vitro* experiments.

Peritoneal macrophage cells were obtained by injecting 10 ml of RPMI 1640 medium (GIBCO Life Technologies, Burlington, ON, Canada) into the peritoneal cavity of euthanized mice using a 21-gauge needle. The peritoneal cavity was rocked gently several times for about 3 minutes and the media was aspirated into a syringe. The cell suspension was washed, counted and resuspended at 10^6/ml in complete medium for use in experiments.

For infection, aliquots (500 μl) of BMDMs or peritoneal cells in 5 ml polypropylene tubes (Falcon, VWR Edmonton, AB, Canada) were incubated with stationary phase promastigote parasites (day 7) at a ratio of 1:10 (cell : parasite). After 6 hr, the free parasites were washed away with fresh medium (spun at 500 rpm for 5 min, 3 times) and infected cells were cultured in complete medium in the presence or absence of IL-4 (20 ng/ml), CpG ODN (10 μg/ml), anti-mouse CD40 antibodies (10 μg/ml), IFN-γ (100 U/ml), LPS (10 μg/ml) or IFN-γ and LPS. At different times after infection,

cytospin preparations were made, stained with Giemsa and infection was determined by microscopy.

Arginase Activity Assay

Arginase activity *in vitro* was quantified as previously described by Corraliza et al. [257]. First, at least 1 x 10^6 cells were lysed in 0.1% Triton-X100. To maintain pH, 100 µl of 25 mM Tris-HCl was added. To provide the cofactor manganese, 10 µl of $MnCl_2$ was added to the preparation before incubation in 56°C for ten minutes to activate the enzyme. One hundred microliters of L-arginine (pH 9.7) was added to provide the substrate and the reaction was left to take place for 2 hours at 37°C. The reaction was stopped by addition of 800 µl of an acid mixture including 1 part 96% H_2SO_4, 3 parts 85% H_3PO_4 and 7 parts water. α-isonitrosopropiophenone reacts with urea and produces a pink colour that we used as a measurable output of the amount of urea in the sample. Forty microlitres of 9% α-isonitrosopropiophenone (in ethanol) was added and the solution left to incubate for 30 minat 95°C. Optical density was measured at a wavelength of 540 nm. One unit of enzyme activity is defined as the amount of enzyme that converts L-arginine to 1 µM urea/min.

For *in vivo* arginase activity assay, infected feet were rinsed in chlorhexiderm and ethanol and homogenized by hand in PBS containing 2% Pen/Strep. The homogenate was first centrifuged at low speed (500 rpm for 5 min) to remove large tissue debris and the supernatant was then centrifuged again at high speed (3000 rpm for 15 min) to pellet the cells and amastigotes. Both pellets were incubated for 30 min in 2 ml lysis buffer (0.1 M Tris-HCl, 300 µM NaCl, 1 µM phenylmethanesulfonyl fluoride, 1% TritonX) and the lysate was assayed for arginase as previously described [257].

Prostaglandin Assay

Prostaglandin E2 was measured in lymph cell culture supernatant using a competitive ELISA kit from Cayman Chemicals (Ann Arbor, MI) by following the manufacturer's directions. Briefly, 96-well plates pre-coated with polyclonal goat anti-mouse IgG are incubated for 18 hours with equal volumes of tracer molecule, PGE_2-linked acetylcholinesterase (AChE), the sample, and PGE_2 monoclonal antibody at 4°C. The tracer and free PGE_2 from the sample compete for binding to the monoclonal antibody, which binds to the goat anti-mouse coating. The plates are washed to remove unbound reagent and 200 µl of Ellman's reagent is added as a source of substrate for AChE before the plate is developed. The product of this reaction, 5-thio-2-Nitrobenzoic acid, has a yellow colour the intensity of which is determined spectrophotometrically at a wavelength of 412 nm. The intensity is proportional to the amount of tracer bound to the well and inversely proportional to the amount of PGE_2 in the sample. The sensitivity of the assay is 15 pg/ml.

Carboxyfluorescein succinimidyl ester (CFSE) Labeling

Five ml of single cell suspensions were prepared at 8 x 10^6 cells/ml. An equal volume of CFSE (2.5µM) was added to the cells and mixed in the dark for 3 to 5 minutes. To quench the CFSE, 30% of FBS was added before cells were centrifuged at 1200 rpm for 5 min. Complete DMEM was then added to the cells before counting with a haemocytometer. Finally, cells were cultured for 5 days in the presence of 50µg/ml SLA and then analyzed by flow cytometry with or without stimulation with PMA (50 ng/ml), ionomycin (500 ng/ml) and BFA (10 µg/ml) for 4-6 hr.

Statistical Analysis

A two-tailed Student's t-test or analysis of variance (ANOVA) was used to compare means of lesion sizes, parasite burden, and cytokine production from different groups of mice within a given timepoint. If the results were statistically significant ($p<0.05$), a post-hoc Tukey's test was conducted. Results were not significantly different unless otherwise indicated by an *. Correlation statistics were analyzed by Pearson's correlation at the 95% confidence interval. Significance was considered if $p < 0.05$.

4. Results

4.1 The Role of Parasite-Derived Arginase in Primary Infection with *Leishmania major*

i. Lack of parasite-derived arginase compromises *L. major* proliferation *in vitro*

Previous studies using Δarg null mutants in *L. mexicana* have shown that these mutants are impaired in axenic cultures in the absence of exogenous polyamines [237]. In addition, experimental administration of NOHA to infected mice or cells has also shown the dependence of *Leishmania* on host-derived arginase [45-47, 138]. To evaluate the importance of parasite-derived arginase *in vitro* in our system, we infected bone marrow derived and peritoneal macrophages with wild-type (WT), Δarg null mutants *arg*$^-$ (KO) or complemented *(arg*$^-$/+ARG, AB) *L. major* and monitored parasite proliferation by microscopy over the course of three days (Figure 1 A and B, BMDM; C and D, peritoneal). Initial infection of bone marrow derived or peritoneal macrophages was not affected by the gene deletion, such that at 6 hours post infection the numbers of parasites per cell and percentage of infected cells was the same between all groups. Clearly, lack of endogenous arginase does not impair the ability of *L. major* to invade the cell, *in vitro*. However, parasite-derived arginase does play a role in maintenance of infection because by 48 (in bone marrow derived macrophages) and 72 hours post infection (in peritoneal macrophages) significant reductions in infectivity and intracellular parasite proliferation were evident. By 72 hours post infection, there was as much as a 50% and 40% reduction in infectivity and number of parasites per infected cell respectively. In summary, these results show that the absence of parasite-derived arginase significantly impaired but did not completely abolish *in vitro* macrophage infection by *L. major*.

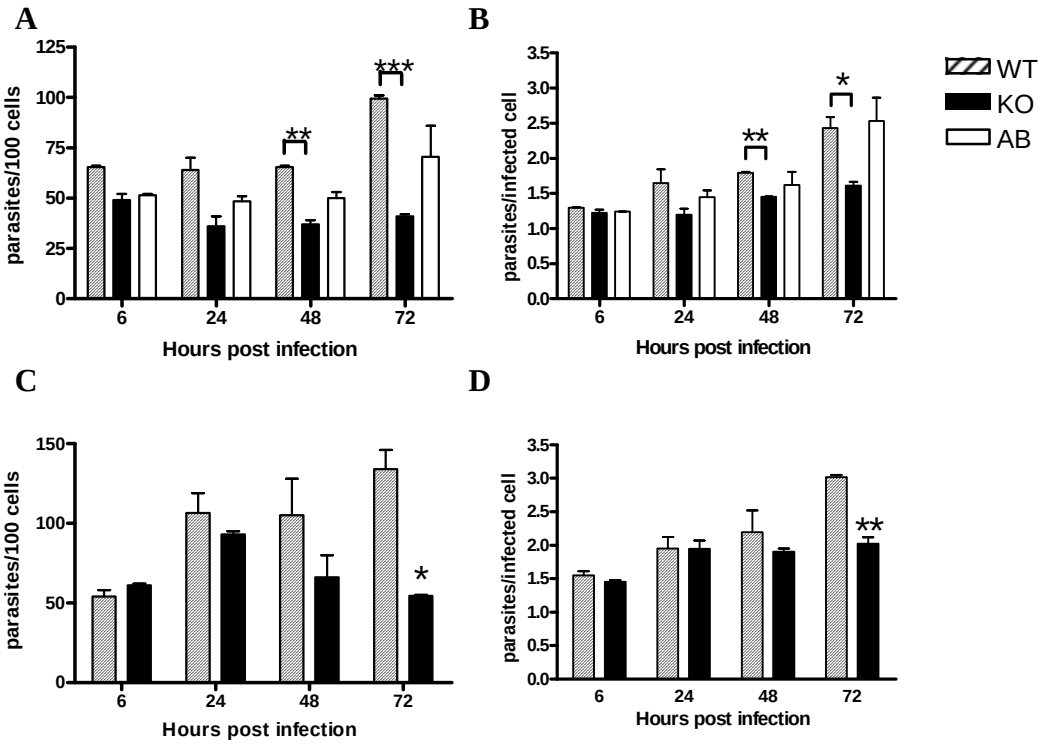

Figure 4. Parasite-derived arginase is necessary for *in vitro* parasite proliferation.
Bone marrow derived macrophages (A and B) and non-elicited peritoneal macrophages (C and D) were infected in polypropylene tubes with wild type (WT), arginase deficient (*arg*$^-$), or *arg*$^-$/+ARG (AB, add-back) *L. major* promastigotes (MOI 1:10; macrophage to parasite ratio). After 6 hr, free parasites were washed away and the cells were resuspended in 500 µl of fresh medium and incubated at 37^{o}C. At various times post-infection, slides were prepared by cytospin and stained with Giemsa. Slides were examined under a microscope at 1000X magnification to estimate infection levels. Each point is the mean ± SE of 3 slides. Data are presented as mean ± SE and is a representative of 3 different experiments with similar results. * $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ii. *arg*$^-$*-L. major* -infected BMDM show decreased arginase activity *in vitro*

Previous work has shown that increasing cellular host arginase activity, for example by IL-4 administration, promotes intracellular parasite proliferation [45-47, 138]. Given that parasite proliferation was impaired *in vitro*, we hypothesized that parasite-derived arginase would increase total arginase activity in infected host cells thereby enhancing polyamine availability for the parasites. Thus, we measured arginase activity in infected macrophages over the course of infection. Figure 5A shows the changes in arginase activity in BMDMs up to 144 hours post-infection with WT and *arg*$^-$-*L. major*. Consistent with our findings of impaired proliferation *in vitro*, arginase activity between groups remained the same until after 72 hours post infection. By 96 and 144 hours post infection, there was significant induction of arginase activity above background levels – as much as five times higher than cells alone in WT *L. major* infection. In *arg*$^-$-infected cells however, arginase activity was significantly lower than WT-infected controls. It is important to note that these differences are not solely additive since arginase activities measured in parasites alone (Figure 5B) are negligible compared to those measured in cells.

It is widely believed that T cell-derived IL-4 increases arginase activity in murine macrophages [219, 242, 258-260]. We next wanted to see how addition of exogenous IL-4 would impact the impaired arginase activity of *arg*$^-$-infected cells. To test this we added IL-4 (20ng/ml) to bone marrow derived macrophage cell cultures at the time of infection and monitored infection over 144 hours post infection. Figure 5C and D show the parasites per cell and parasites per 100 cells respectively in this IL-4-added infection. Notably, the number of parasites was increased when IL-4 was added to infected cells but

not to WT levels. This trend was mirrored in arginase activity at 96 hours post infection (Figure 5E). While addition of IL-4 into the cultures increased arginase levels in all treatment groups, this IL-4-induced increase in arginase levels was not sufficient to rescue the impaired cellular arginase activity in the absence of parasite-derived arginase. The total arginase levels in cells infected with *arg*$^{-}$*-L. major* + IL-4 were significantly ($p < 0.001$) lower than those infected with WT *L. major* + IL-4 (Figure 5E). These experiments suggest that while IL-4 can increase arginase activity in infected macrophages, the effect of the cytokine alone is not sufficient to overcome the parasite's deficit.

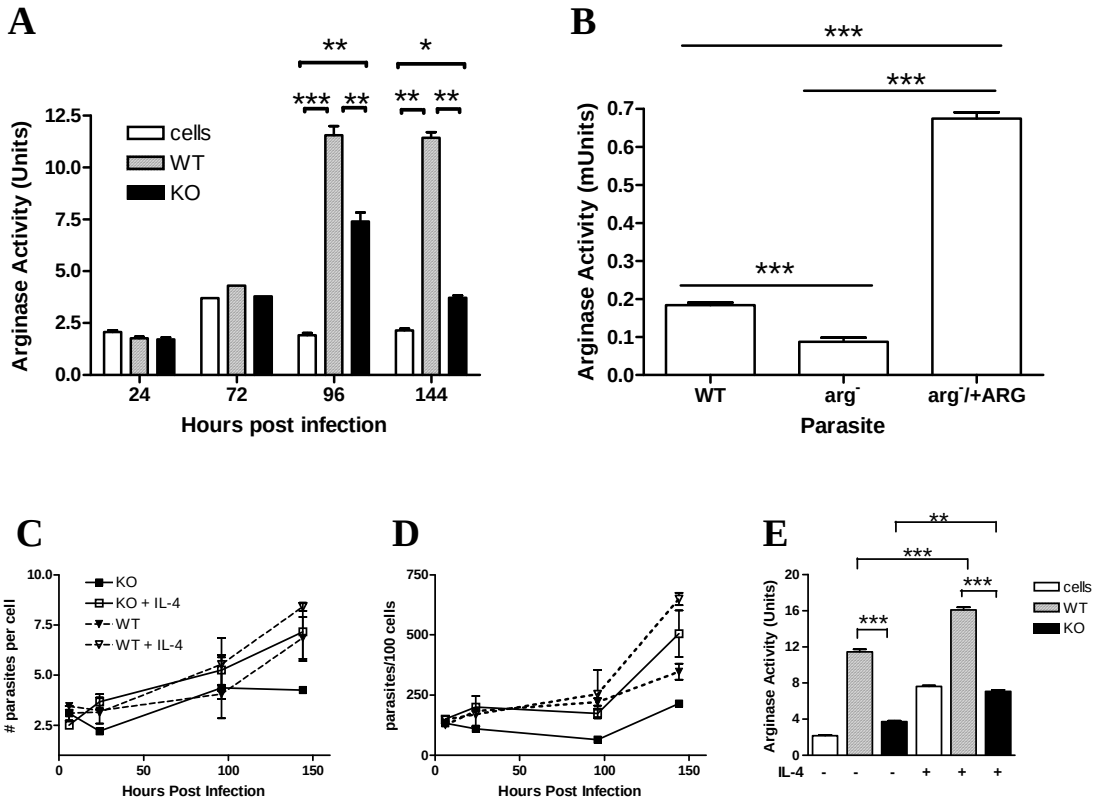

Figure 5. Reduced host cellular arginase during infection with *arg*$^-$*-L. major* is not rescued by addition of IL-4.

(A) Bone marrow derived macrophages were infected with WT or *arg*$^-$*-L. major* as in Figure 4 above and free parasites were washed away after 6 hrs. At the indicated time, the cells were lysed with Triton X and the total arginase activity was determined as described in section 3. (**B)** 1 x 10^8 Parasites were harvested from *in vitro* culture, washed in PBS and lysed according to the arginase activity protocol described in section 3. **(C and D)** Bone marrow derived macrophages were infected in polypropylene tubes with wild type (WT) or arginase deficient (KO) *L. major* promastigotes (MOI 1:10; macrophage to parasite ratio). After 6 hr, free parasites were washed away and the cells were resuspended in 500 µl of fresh medium, ± 20ng/ml IL-4, and incubated at 37^{o}C. At various times post-infection, slides were prepared by cytospin and stained with Giemsa. Slides were examined under a microscope at 1000X magnification to estimate infection levels. Each point is the mean ± SE of 3 slides. **(E)** 96 hr after infection, cells were lysed with Triton X and the total arginase activity was determined as described in section 3. Data are presented as mean ± SE. * $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

iii. The decreased arginase activity in *arg*$^-$-*L. major* -infected macrophages is unrelated to increased NO production

INOS and arginase are competitively regulated because they use the same substrate: L-arginine. In addition, some of the intermediate products of the enzymatic pathways of each inhibit the other directly and indirectly [48, 151, 233, 242] . Due to this reciprocal regulation, it is possible that the reduced arginase activities in *arg*$^-$-infected cells (due in part to the absence of parasite-derived arginase) confers an advantage to iNOS by increasing substrate availability. This would imply that one function of parasite-derived arginase is to limit L-arginine availability to iNOS thereby reducing intracellular NO. To test this hypothesis, we measured nitrite production (as a function of iNOS activity) by Griess assay in WT and *arg*$^-$-*L. major* -infected macrophages over 96 hours post infection. To ensure a measurable amount of nitrite production, we stimulated the cells with a number of stimuli including CpG ODN, αCD40 antibodies, IFN-γ, LPS, or LPS in combination with IFN-γ. Surprisingly, despite the observed differences in arginase activity (Figure 5*A*), there was no significant difference in nitrite levels between cells infected with WT or *arg*$^-$-*L. major* at all times tested and under all stimulations (Figure 6). Stimulation of infected cells with CpG ODN or anti-CD40 did not lead to any detectable nitrite production. Stimulation with IFN-γ resulted in measurable nitrites only at 72 and 96 hours post infection. These results indicate that, contrary to the theory of reciprocal regulation, depressed total arginase activities in cells infected with *arg*$^-$-*L. major* is neither due to nor results in increased iNOS activities. They further suggest that parasite-derived arginase is not a virulence factor responsible for the suppression of nitric oxide production in *L. major*-infected cells.

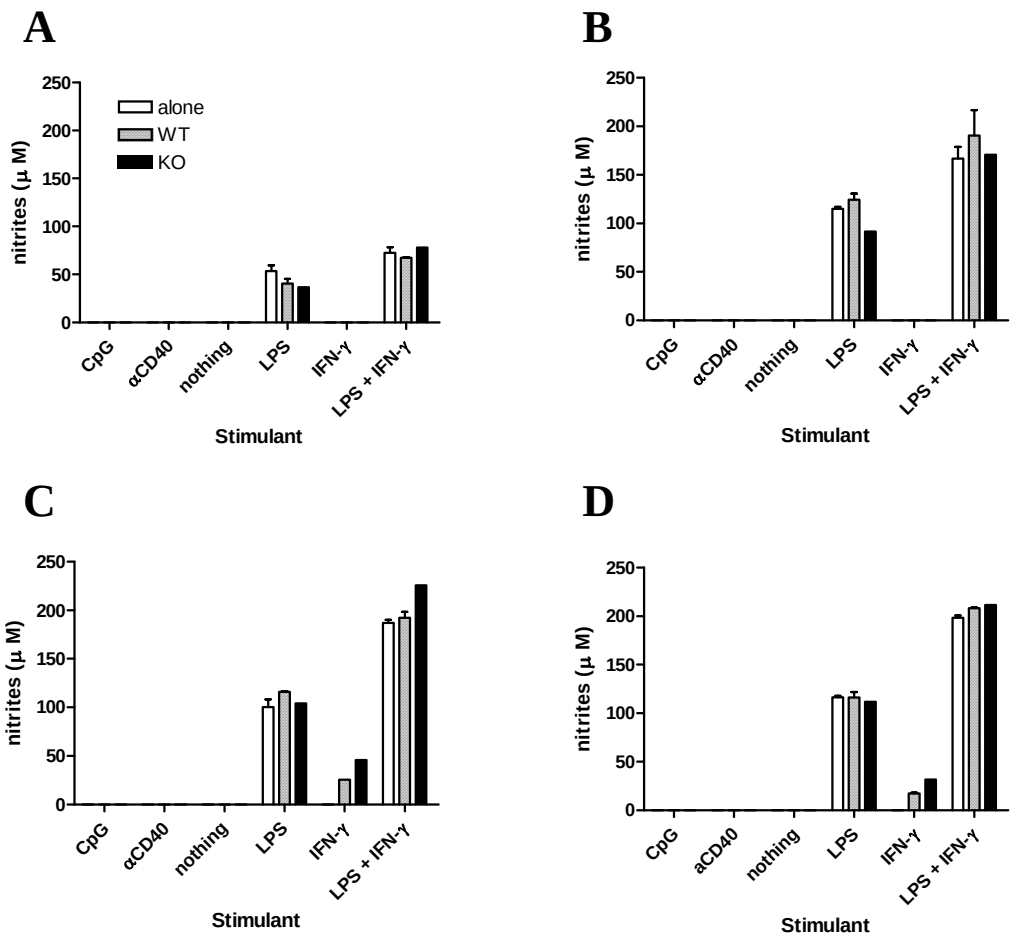

Figure 6. *in vitro* infection with *arg*$^-$*-L. major* does not increase NO production more than WT infection.

Bone marrow derived macrophages were infected with WT or *arg*$^-$*-L. major* as in Figure 4 above and the free parasites were washed away after 6 hr. Some infected cells were stimulated with CpG ODN (10μg/ml), aCD40 antibodies (10μg/ml), IFN-γ (100 U/ml), LPS (10 μg/ml) , or LPS + IFN-γ (100 U/ml) and nitrite levels in culture supernatant fluids were determined using the Griess assay method at (A) 24, (B) 48, (C) 72 and (D) 96 hours post-infection. Data are presented as mean ± SE.

iv. Lack of parasite-derived arginase compromises parasite proliferation *in vivo*

We next wanted to examine if the impaired proliferation of *arg*$^{-}$*-L. major in vitro* was also obtainable in the *in vivo* infection. We infected BALB/c mice with WT, *arg*$^{-}$ and add-back (*arg*$^{-}$ */+ARG*) *L. major* (stationary phase metacyclic promastigotes) and monitored the onset and progression of lesion development over time (Figure 7). *arg*$^{-}$ parasites are highly attenuated *in vivo*. First, visible lesions took up to two weeks longer to develop in these mice, and the pathology did not reach the same level of severity as seen in WT or *arg*$^{-}$*/+ARG L. major* infection (Figure 7*A*). It is important to note that even without parasite-derived arginase, *Leishmania* can still induce pathology upon infection. However, due to severe pathology at seven weeks post infection in mice infected with WT *L. major*, we had to euthanize WT-infected mice and as such the long-term pathology associated with *arg*$^{-}$*-L. major* infection in BALB/c mice remains undefined. To get a better picture of the disease process, we also quantified parasite burden in the infected footpad at different times post-infection (Figure 7*B*). By 2, 4 and 7 weeks post infection, parasite burden in WT and *arg*$^{-}$*/+ARG L. major*-infected footpads was 2 to 3-logs higher than in *arg*$^{-}$*-L. major* -infected mice, indicating that without endogenous arginase *L. major* cannot replicate successfully *in vivo*. There were no significant differences between WT and *arg*$^{-}$*/+ARG L. major*-infected mice in either of these parameters

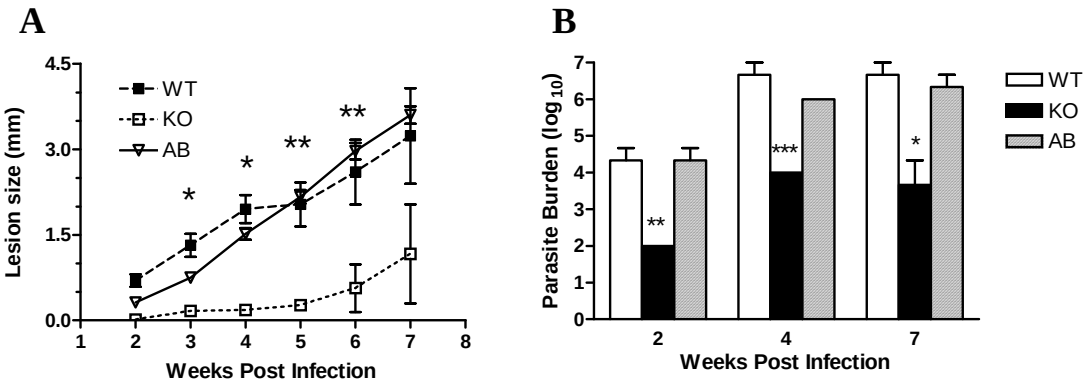

Figure 7. *arg*$^-$*-L. major* parasites are highly attenuated *in vivo*.
(A) BALB/c mice were infected in the right footpad with 2 million (stationary phase promastigotes) WT, *arg*$^-$ (KO) and *arg*$^-$ /+ARG (AB, add-back) and lesion sizes were monitored weekly with calipers. Asterisks indicate significant difference between WT and KO. **(B)** At different times after infection (ending at 7 wk due to extensive ulceration in WT-infected controls), mice were sacrificed and parasite burden at the infected footpad was determined by limiting dilution assay. Data are presented as means ± SE and are representative of 4 independent experiments with similar results. * $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

v. Arginase deficient and WT *L. major* induce comparable immune responses *in vivo*

At the single cell level, Th1/Th2 cytokine responses regulate macrophage activation status and this mediates the outcome of infection with *L. major*. Because of this link between cytokines and arginase, we wanted to quantify cytokine balance in our system. Furthermore, in other knockout constructs of *L. major*, e.g., *lpg2*$^-$and *dhfr-ts*$^-$, attenuated pathology and lower parasite burden are associated with low or altered cytokine responses *in vivo* [169, 261]. We hypothesized the same would hold true for our *arg*$^-$ parasites.

At different times post-infection, we sacrificed WT, *arg*$^-$*/+ARG* and *arg*$^-$-infected mice and used sandwich ELISA to determine the production of cytokines (IL-10, IL-13, IFN-γ and IL-4) by cells from lymph nodes draining the infection site (dLN) following their stimulation for three days with soluble leishmanial antigen (SLA). Figure 8A-D shows the ELISA results for IL-4, IFN-γ, IL-10 and IL-13 respectively from the culture supernatant fluids at different times post infection. Although there were some significant differences in IFN-γ at 4 weeks post infection (significantly lower in *arg*$^-$-infected mice) and IL-4 at seven weeks post infection (significantly higher in *arg*$^-$-infected mice) an overall Th1- or Th2- skewed response could not be found in the *arg*$^-$ infection.

We also sought to determine whether differences in cytokine responses in WT- and *arg*$^-$-infected mice could be observed by intracellular staining of cells from infected mice. Figure 9A shows the expressions of IFN-γ, IL-4 and IL-10 in CD4+ T cells. In some experiments, IL-4 and IFN-γ expression was higher in cells from *arg*$^-$-infected mice than cells from WT-infected mice (Figure 9A), but when the results from all experiments were compiled (Figure 9B), the differences were less evident. Overall, there was no

significant difference in cytokine expression between cells from WT or *arg*$^{-}$-infected mice. The results disprove our hypothesis for, even though pathology and parasite burden are low in mice infected with *arg*$^{-}$-*L. major*, these parasites still induce robust cytokine immune responses.

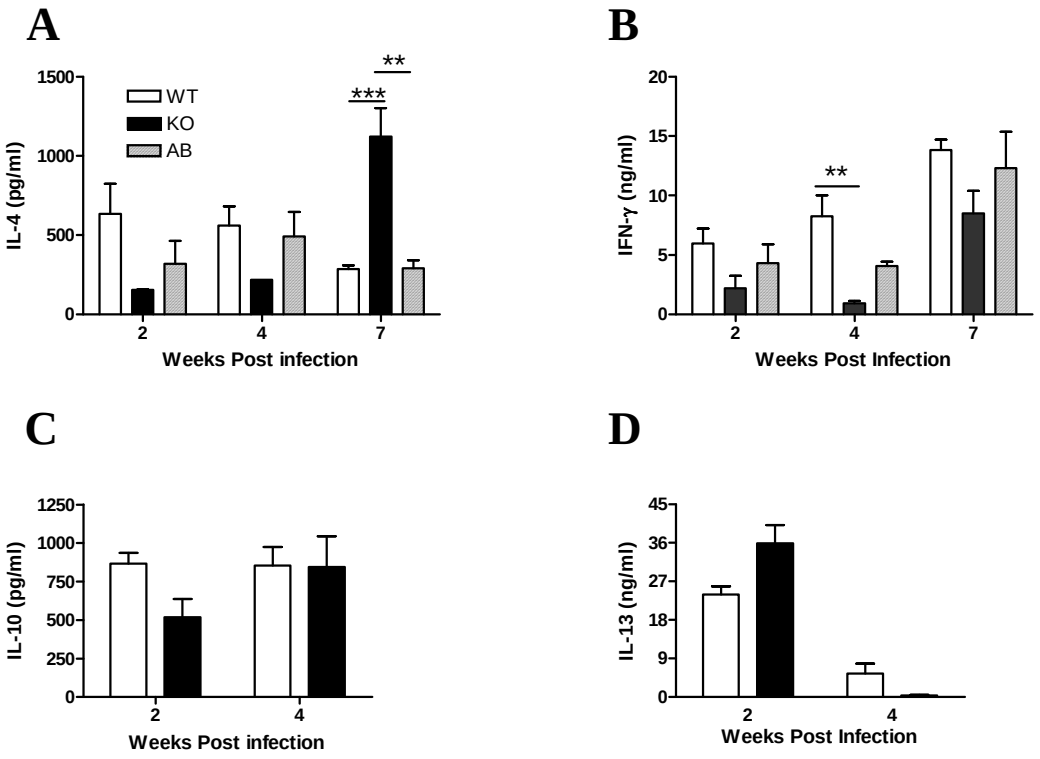

Figure 8. *arg*$^-$ and WT *Leishmania major* induce comparable immune responses *in vivo*.
BALB/c mice were infected in the right footpad with 2 million (stationary phase promastigotes) WT, *arg*$^-$ (KO) and *arg*$^-$ /+ARG (AB, add-back). At various times after infection, mice were sacrificed and draining lymph nodes were removed, made into single cell suspensions and cultured for 3 days in the presence of soluble *Leishmania* antigen (SLA, 50 μg/ml). After 72 hr, the culture supernatant fluids were collected and assayed for **(A)** IL-4, **(B)** IFN-γ, **(C)** IL-10 and **(D)** IL-13 by ELISA. Data are presented as means ± SE. * $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

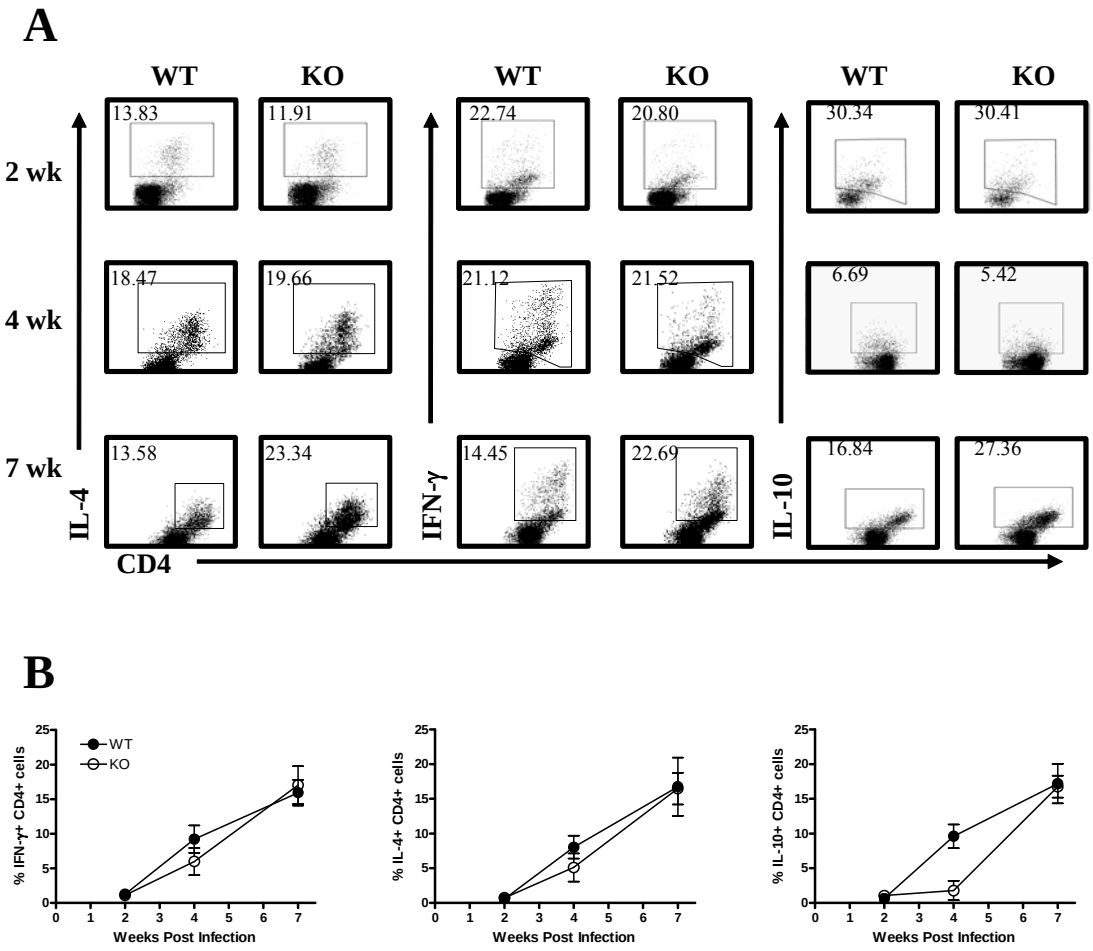

Figure 9. *arg*$^-$ and WT *Leishmania major* induce comparable immune responses *in vivo*.
BALB/c mice were infected in the right footpad with 2 million (stationary phase promastigotes) WT, *arg*$^-$ (KO) and *arg*$^-$ /+ARG (AB, add-back). At various times after infection, mice were sacrificed and draining lymph nodes were removed, made into single cell suspensions and cultured for 3 days in the presence of soluble *Leishmania* antigen (SLA, 50 μg/ml). After 72 hr, the cultures were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 hr and Brefeldin A (BFA, 10 μg/ml) was added in the last 2 hr to enhance intracellular protein accumulation. The cells were surface stained for CD4 and intracellularly for IFN-γ, IL-4, and IL-10. **(A)** Dot plot from one experiment representative of 4 independent experiments with similar results. **(B)** Pooled data from 4 experiments, each point is the mean ± SE.

vi. Parasite number in the infected footpad correlates with arginase activity *in vivo*

Previous studies have demonstrated that *in vivo* arginase activity, regulated by IL-4, increased over the course of *L. major* infection [45, 46, 48, 138, 262] . Given that the IL-4, IL-13 and IL-10 responses in WT and *arg*$^-$-*L. major* infected mice were similar (Figures 8 and 9), we decided to measure arginase activity in footpad homogenates of infected mice. Figure 10 shows arginase activity over 7 weeks of infection. At two weeks post infection, there were no significant differences in arginase activity between the groups. In contrast, arginase activity was significantly lower in the *arg*$^-$-infected mice (by as much as 90%) than WT infected mice at four weeks post-infection, corresponding to the onset of significant differences in cutaneous lesions (Figure 7). By seven weeks post infection however, arginase activity in *arg*$^-$-infected mice had reached equivalent levels to WT infection although arginase activity in footpads from *arg*$^-$*/+ARG L. major*-infected was still significantly higher than in *arg*$^-$-infected mice and not different from WT controls.

Unexpectedly, and in contrast to the central dogma of Th1/Th2 cytokines regulating iNOS/arginase activities, the results of *in vivo* arginase activity did not mirror our Th1/Th2 cytokine results. To investigate this, we conducted Pearson's correlation analysis and compared IL-4 ELISA results with *in vivo* arginase activity results. Using pooled results from all groups (Figure 11A), there was no significant correlation between *in vivo* arginase activity and IL-4 ($r^2=0.01164$,$p=0.6158$). This finding was consistent when the results were broken down by group and no correlation was found between these parameters in WT, *arg*$^-$*/+ARG* or *arg*$^-$-infected mice (WT, $r^2=0.2494$, $p=0.0983$; AB, $r^2=0.02247$, $p=0.7231$; KO, $r^2=0.09257$, $p=0.6957$, Figure 11B). Therefore, we

hypothesized that *in vivo* arginase activity may have more to do with pathology than cytokine response and therefore we investigated a correlation between parasite titer and arginase activity. Pearson correlation analysis of pooled results, showed a significant positive correlation between parasite titer and arginase levels ($r^2=0.6744$, $p<0.0001$; Figure 11C). When this result was examined by group (Figure 11D, we found the correlation remained significant in WT and *arg*$^-$*/+ARG L. major*-infected mice (WT, $r^2=0.8773$, $p=0.0002$; AB, $r^2=0.5566$, $p=0.0053$) but not in *arg*$^-$*-L. major* -infected mice ($r^2=0.2598$, $p=0.3017$) showing that the correlation is not valid at very low arginase activities. Thus, contrary to current views, host arginase activities in mice infected with *L. major* may be less controlled by levels of the Th2 cytokine IL-4 and correspond more closely to other cytokines, parasite-derived factors or other inflammatory mediators.

To investigate further the role of parasite numbers in regulation of host arginase activities, we compared arginase activity levels in mice infected with WT and *lpg2*$^-$*-L. major*. *lpg2*$^-$*-L. major* has an intact arginase gene, but lacks the surface glycoconjugate molecules that are essential for *in vitro* and *in vivo* virulence [253, 263]. As a result, infection with *lpg2*$^-$*-L. major* does not result in lesion development or any measurable cytokine response even though low levels of parasite persist at infected sites for years [4, 254]. As shown in Figure 12 and similar to *arg*$^-$*-L. major*, *lpg2*$^-$ parasites were also unable to induce the significant levels of *in vivo* arginase activity seen in the WT infected mice (over 70% less than WT parasites). Importantly and consistent with previous reports, parasite burden was low in *lpg2*$^-$-infected mice (Figure 12). Collectively, these results suggest that host cytokine response in mice infected with *L. major* may not be tightly linked to *in vivo* arginase activity and arginase levels. Together with our *in vitro*

results, they strongly suggest that parasite numbers or perhaps inflammatory mediators also direct arginase enzyme activity directly or indirectly and therefore can significantly influence the outcome of *L. major* infection.

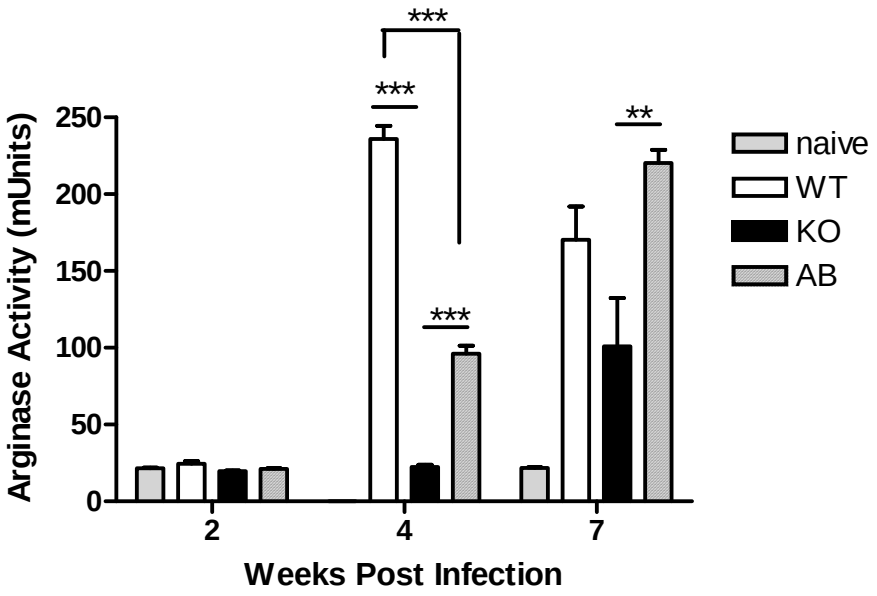

Figure 10. Delayed induction of host arginase following *in vivo* infection with *arg*$^-$*-L. major*. BALB/c mice were infected in hind footpad with 2 x 10^6 WT and ***arg*$^-$*-L. major*** and at various times (2, 4 and 7 weeks post-infection), mice were sacrificed and the infected footpads were homogenized in buffer as described in section 3 and the total arginase activity was determined. Data are presented as means ± SE and are representative of 4 independent experiments with similar results. **, $p < 0.01$; ***, $p < 0.001$.

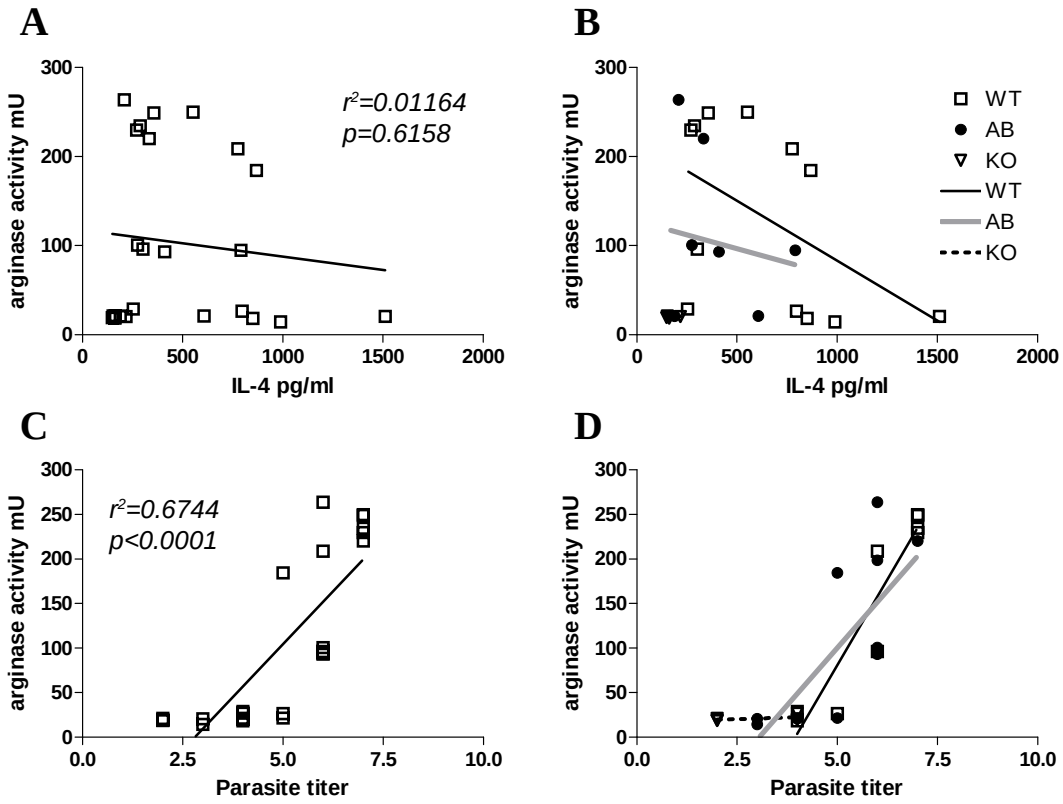

Figure 11. *in vivo* arginase activity correlates with parasite burden but not IL-4.
BALB/c mice were infected with 2 x 10^6 WT and ***arg*⁻*-L. major*** and at various times post-infection mice were sacrificed, the infected footpads homogenized in buffer as described in section 3 and the total arginase activity determined. At sacrifice, draining lymph nodes were removed, made into single cell suspensions and cultured for 3 days in the presence of soluble antigen (SLA, 50 μg/ml). After 72 hr, the culture supernatant fluids were collected and assayed for IL-4 by sandwich ELISA. Mice were tracked individually through the assays allowing comparison of the parasite burden and IL-4 with *in vivo* arginase activity using Pearson correlation test. **(A and C)** Results from the three groups were pooled. **(B and D)** Assay results were separated based on the genotype of the infecting parasite. **(B)** WT, $r^2=0.2494$, $p=0.0983$; AB, $r^2=0.02247$, $p=0.7231$; KO, $r^2=0.09257$, $p=0.695$. **(D)** WT, $r^2=0.8773$, $p=0.0002$; AB, $r^2=0.5566$, $p=0.0053$; KO, $r^2=0.2598$, $p=0.3017$. Each data point represents one mouse.

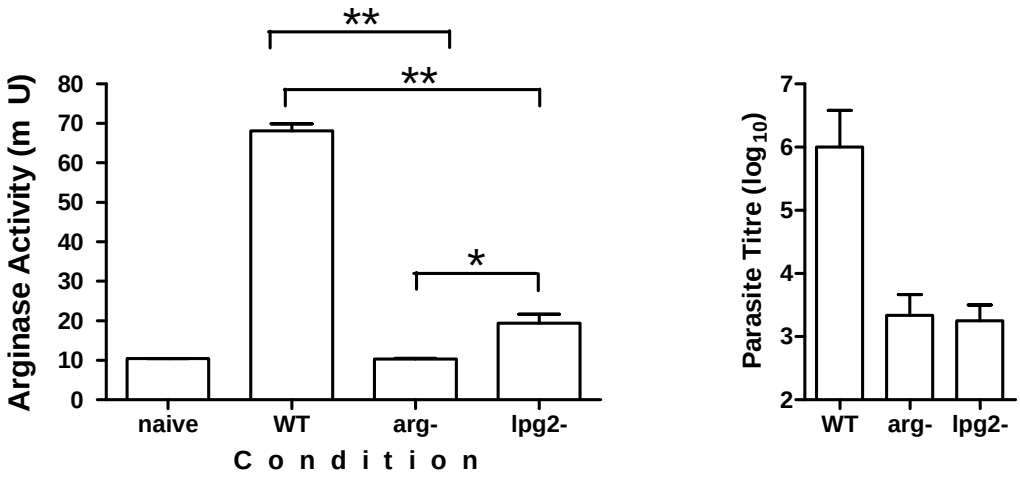

Figure 12. *in vivo* infection with *lpg2*$^{-}$*-L. major* induces less host arginase activity than WT infection. BALB/c mice were infected in the hind footpad with 2x 10^6 wild-type (WT), *arg*$^{-}$, or lipophosphoglycan deficient (*lpg2*$^{-}$) stationary phase *L. major* promastigotes. Four weeks post infection, mice were sacrificed and infected footpads homogenized in buffer as described in section 2.x. Total arginase activity was determined. At the same time point, parasite burden was determined by limiting dilution. Data presented as mean ± SE of one experiment representative of two similar experiments. *, $p < 0.05$; **,$p < 0.01$.

vii. Lack of parasite-derived arginase does not impact host nitric oxide production.

Due to reciprocal regulation between arginase and iNOS, increased arginase arginase activity in cells may impair function of iNOS by substrate depletion and the mechanisms outlined in section 1.3 part four. *L. major* may exploit reciprocal regulation by expressing its own arginase and so increase intracellular arginase and decrease harmful NO. If so, we would expect to find higher nitric oxide production in cells from mice infected with *arg*$^-$ parasites. To examine this possibility, we used the Griess assay to measure nitrites produced by dLN cells from infected mice, as a measure of *in vivo* nitric oxide production (Figure 13). Consistent with our *in vitro* findings in Figure 6, there were no significant differences in NO production from draining lymph node cells at any time tested. These results intimate that parasite-derived arginase is not a virulence factor and functions solely as a nutritional support for *L. major*.

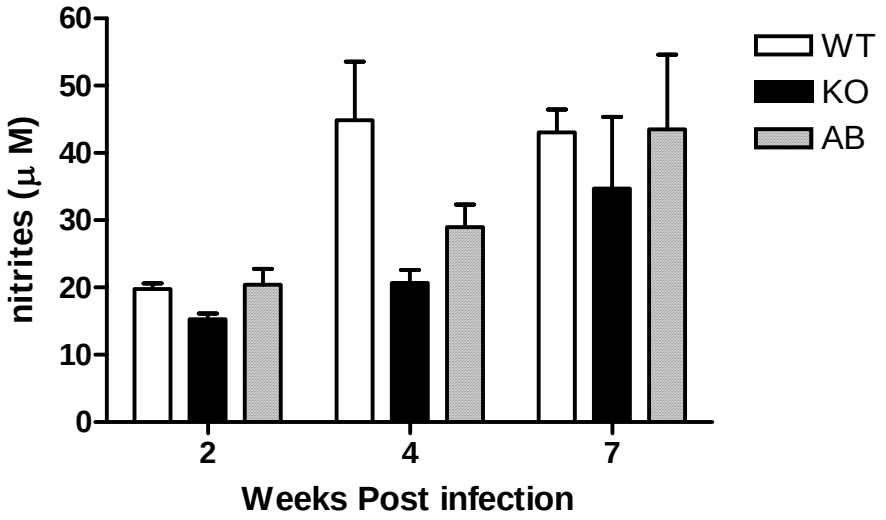

Figure 13. *in vivo* infection with *arg*$^-$*-L. major* does not increase NO production more than WT infection.
BALB/c mice were infected in the right footpad with 2 million (stationary phase promastigotes) WT, *arg*$^-$ (KO) and *arg*$^-$ /+ARG (AB, add-back). At various times post-infection, mice were sacrificed and draining lymph nodes removed. Single cell suspensions were made and cultured for three days with SLA **(**50 μg/ml). After 72 hours, cell culture supernatants were assayed for nitrite production by Griess assay as a measure of nitric oxide production. Data presented are mean ± SE.

viii. *arg*$^-$*-L. major* elicits decreased prostaglandin E_2 production *in vivo*.

In addition to cytokines, the inflammatory mediators prostaglandins, particularly prostaglandin E2 (PGE_2), have also been found to increase arginase activity [219, 264]. In the local tissue microenvironment, prostaglandin may increase host arginase activity by a mechanism independent of the adaptive immune response. This "inflammation-mediated" induction of arginase is physiologically relevant because arginase plays an important role in wound healing where it promotes proliferation of new cells, fibrosis, and collagen deposition via the production of proline [190]. Thus, during lesion pathology following *L. major* infection, PGE_2 may attempt to bring about lesion resolution but have the unfortunate effect of promoting parasite proliferation by increasing polyamine production in macrophages. Furthermore, PGE_2 is induced during infection by a number of *Leishmania* species including *L. major* [120, 264, 265]. Therefore, we investigated PGE_2 production in cell culture supernatant from the popliteal lymph nodes draining the infection site. At 3 days and 2 weeks post-infection, there was no significant difference in PGE_2 production. By 4 weeks post infection, PGE_2 levels in WT-infected mice were significantly different than in *arg*$^-$*-L. major* -infected mice ($p < 0.05$, Figure 14). Since PGE_2 has been shown to influence inflammatory responses [266, 267], this result is not surprising given the significantly lower inflammation in *arg*$^-$*-L. major*-infected mice.

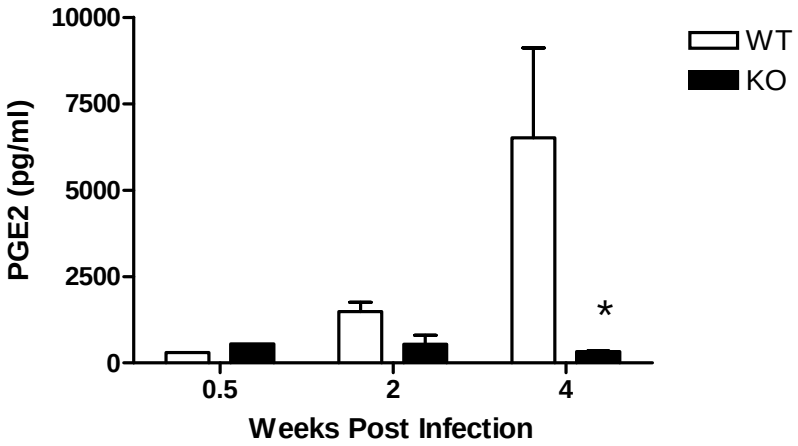

Figure 14. Infection with *arg*$^-$-*L. major* does not induce prostaglandin E2 production *in vivo*.
BALB/c mice were infected in the right footpad with 2 million (stationary phase promastigotes) WT, *arg*$^-$ (KO) and *arg*$^-$/+ARG (AB, add-back). At various times post-infection, mice were sacrificed and draining lymph nodes removed. Single cell suspensions were made and cultured for three days with SLA (50μg/ml). After 72 hours, cell culture supernatants were assayed for PGE_2 by bench top assay as described in section 3. Data presented are mean ± SE of one experiment representative of two experiments with similar results. *, $p < 0.05$.

4.2 Influence of parasite-derived arginase on the quality of memory anti-*Leishmania* immunity following secondary challenge

i. Infection of C57BL/6 mice with *arg*$^-$-*L. major* results in chronic infection but induces comparable immune response to WT infection.

Naturally occurring or experimentally generated avirulent pathogen strains are attractive vaccine candidates because they contain the same antigens required for generating a successful immune response without the pathology of the full-blown disease. Null mutant constructs of *L. major* are no exception and considerable research has been done to create such attenuated strains for clinical use [169, 253, 263, 268] . With this in mind, we began to examine the quality of the memory immune response generated after primary infection with arginase-deficient *L. major* since memory is an important attribute and correlate of protective immunity. C57BL/6 mice infected with WT *L. major* can heal the infection and generate life-long immunity concomitant with the presence of persistent parasites and for this reason B6 mice were used in this part of the work.

To begin these experiments, we infected B6 mice in the hind footpad with WT, *arg*$^-$*/+ARG* and *arg*$^-$*-L. major* and monitored lesion development for 14 weeks post-infection. As expected, mice infected with WT *L. major* were able to control the infection and resolve their lesions (Figure 15). As in BALB/c mice, lesions in arg *arg*$^-$-infected animals were slow to develop but, surprisingly, *arg*$^-$-infected mice developed an uncontrolled chronic infection, which, while never reaching the severity of the WT infection, remained uncontrolled until the time of sacrifice. The ability to clear infection was restored when mice were infected with *arg*$^-$*/+ARG L. major.* Consistent with lesion sizes, parasite burden four days post infection was significantly lower in *arg*$^-$-infected mice than WT infected mice (Figure 15B) but surprisingly, this trend did not hold over

the course of infection because by 3 and 7 weeks there were no significant differences (Figure 15C).

We next examined the immune response in C57BL/6 mice following infection with WT and *arg*$^-$*-L. major*. Draining lymph node cells were cultured with soluble leishmanial antigen for 72 hours and cytokines in cell culture supernatant were measured by ELISA. Consistent with our findings in BALB/c mice, there was no trend toward a Th2 response in *arg*$^-$-infected mice (Figure 16). At four days post infection, no measurable IL-4 could be found (data not shown). IFN-γ was significantly higher in *arg*$^-$-infected mice which is consistent with the significantly lower parasite burden at this time point (Figure 16A). At three and seven weeks post infection, there was no significant difference in IL-4 production (Figure 16B). IFN-γ levels were similar between the groups at three weeks but significantly lower in *arg*$^-$-infected mice than WT-infected mice at 7 weeks post infection (Figure 16C). The results show that the defect in *arg*$^-$*-L. major* that prevents them from proliferating in susceptible BALB/c mice, somehow enables them to remain undetected in resistant B6 mice relative to WT and *arg*$^-$*/+ARG* controls and so develop into a chronic infection. In keeping with our findings in BALB/c mice, despite differences in lesion size and parasite burden, the cytokine profiles between *arg*$^-$, *arg*$^-$ /+ARG and WT *L. major* infections were not skewed to a Th1 or Th2 response.

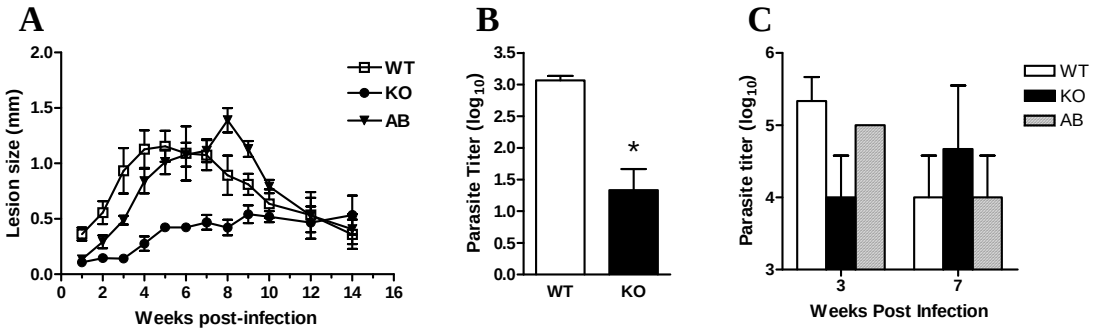

Figure 15. *arg*$^-$*-L. major* causes chronic infection in resistant C57BL/6 mice.
C57BL/6 mice were infected in the hind footpad with 2 x 10^6 stationary phase promastigote WT, *arg*- or *arg*-/+ARG *L. major* parasites. A) As a measure of lesion size, footpad thickness was measured weekly with digital calipers. At 4 days (B) and 3 and 7 weeks C) post infection, mice were sacrificed and footpad parasite burden quantified by limiting dilution assay as outlined in section 3. Data presented are mean ± SE. *, $p < 0.05$.

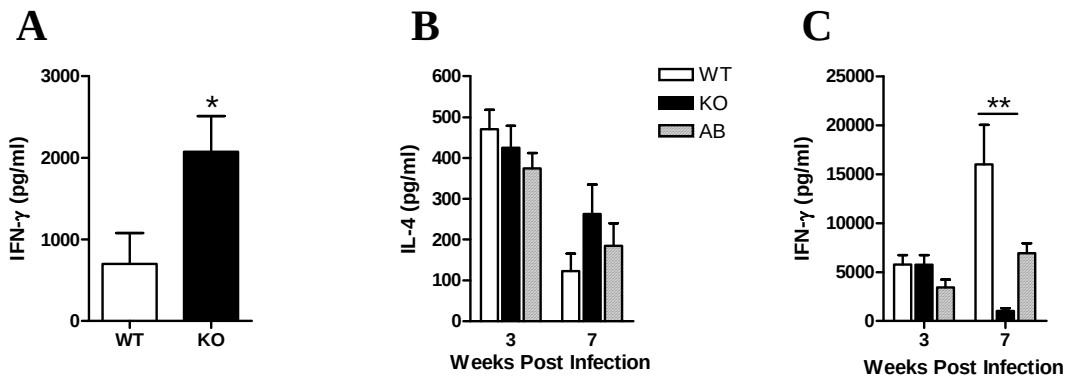

Figure 16. Infection with *arg*⁻ or WT *L. major* induce comparable immune responses *in vivo* in resistant mice.

C57BL/6 mice were infected in hind footpad with 2 x 10^6 stationary phase promastigote WT, *arg*- or *arg*-/+ARG *L. major* parasites. **A – C)** At various times post infection, mice were sacrificed and single cell suspensions made from draining lymph nodes. Cells were cultured with SLA for 72 hours and cytokine levels in culture supernatant was measured by ELISA. Four days post infection is shown in **A)**. Data presented are mean ± SE. Data are presented as mean ± SE. * $p < 0.05$; **, $p < 0.01$.

ii. Primary infection with *arg*$^-$-*L. major* does not protect host against secondary challenge infection

We next wanted to examine if the chronic infection in B6 mice affected their ability to develop infection-induced immunity. Considerable literature in viral systems, has theorized that resolution of infection is necessary to develop effective memory responses [269, 270]. In *L. major* infection, the case is slightly different because immunity cannot be maintained without persistent parasites [3, 5, 6]. At 16 weeks post infection we sacrificed mice infected with WT *L. major* (that had completely healed their lesions) and mice infected with *arg*$^-$-*L. major* (that remained chronically infected) and measured parasite burden in infected footpads (Figure 17A). Parasite burden was not significantly different between the three groups, WT, *arg*$^-$ and *arg*$^-$ /+ARG. At this time, we also examined the populations of memory CD4+ T cells in the spleens of healed animals (Figure 17B). Though both WT and *arg*$^-$-infected mice had higher T_{cm} and T_{eff} populations than naïve mice, there was surprisingly no significant difference in cell numbers between WT and *arg*$^-$-infected mice.

To measure the ability of immune cells to recall a protective response to parasite antigen, spleen and draining lymph node cells from *arg*$^-$ and *arg*$^-$ /+ARG infected animals were cultured with soluble leishmanial antigen for 72 hours and IFN-γ measured by ELISA from cell culture supernatant. Spleen cells from *arg*$^-$*/+ARG* infected mice responded to *in vitro* challenge with significantly more IFN-γ than cells from *arg*$^-$-infected mice (Figure 17C). In dLN cells this difference was not significant but followed the same pattern (Figure 17D). These results suggested that infection with *arg*$^-$-*L. major* may not induce the same level of protection as WT infection because after healing immune cells were less able to respond with a robust Th1 response.

To test this possibility, we challenged mice infected with WT, *arg⁻/+ARG,* and *arg⁻-L. major* at 16 weeks post-infection with 2 x 10^6 parasites in the contra lateral footpad. Three days after challenge, footpad thickness was measured as an indicator of the delayed type hypersensitivity (DTH). As shown in Figure 18A, mice that healed from WT and *arg⁻/+ARG* infections mounted considerable DTH responses to challenge infection that were significantly different from naïve mice and *arg⁻*-infected mice but not significantly different from each other. Consistent with this, parasite burden at 3 weeks post challenge was significantly greater in naïve mice and *arg⁻*-infected mice than in mice that healed WT and *arg⁻/+ARG L. major* infections and there was no significant difference between WT and *arg⁻/+ARG* healed mice (Figure 18B). Thus, the resulting chronic infection generated by *arg⁻-L. major* is not sufficient to stimulate protective immune responses in mice upon re-challenge.

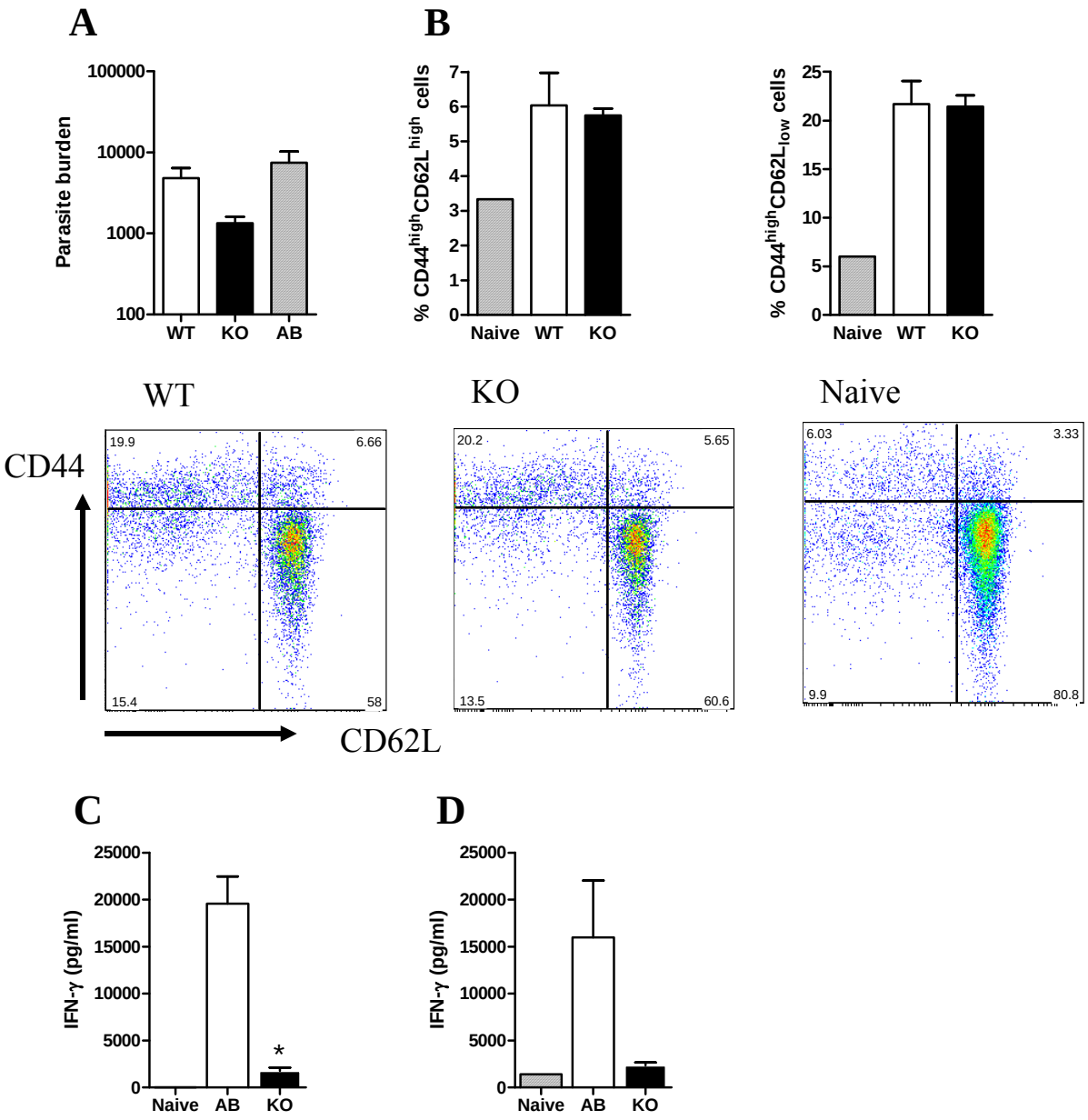

Figure 17. Despite normal memory populations, primary infection with *arg*⁻*-L. major* results in impaired *in vitro* IFN-γ recall response.
Sixteen weeks after infection, mice previously infected with WT and *arg*⁻*-L. major* were sacrificed. **A)** Footpads were homogenized and parasite burden was estimated by limiting dilution. **B)** Single cell suspensions from spleens were surface stained with flurochrome-conjugated antibodies against CD4, CD62L and CD44. Cells were gated on CD4+ populations and levels of CD62L and CD44 quantified by flow cytometry as a measure of T_{cm} and T_{eff} populations. Shown is a representative dot plot and graphs from one experiment with 2 mice per group. Single cell suspensions from spleen, **C),** and dLN, **D),** were stimulated for 72 hours *in vitro* with SLA and the production of IFN-γ was measured by ELISA. Bars represent mean ± SE. * $p < 0.05$.

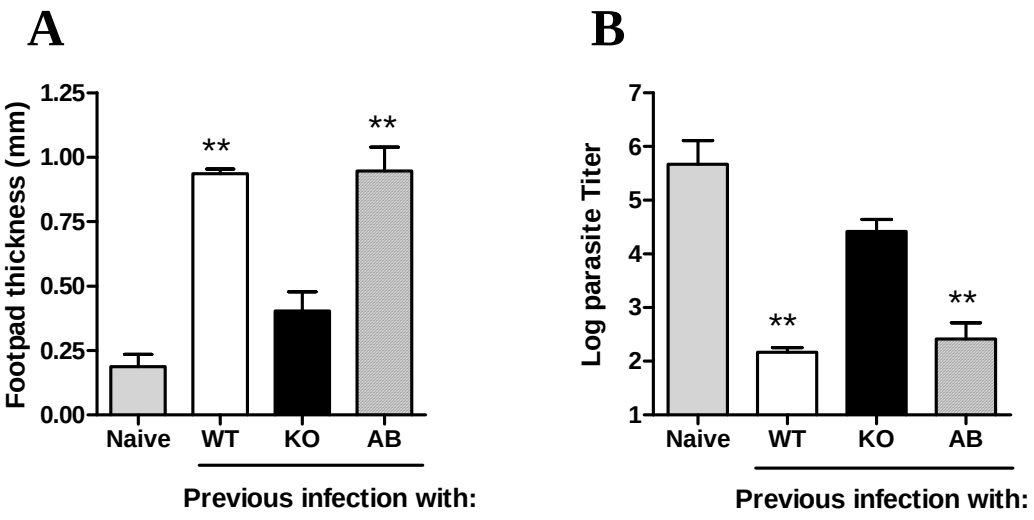

Figure 18. Primary infection with *arg*$^-$-*L. major* does not protect host against secondary challenge. Sixteen weeks after infection, naïve mice and mice previously infected with WT, *arg*-/+ARG (AB), and *arg*- (KO) *L. major* were challenged in the contralateral footpad with 2 x 10^6 WT (LV39) *L. major* stationary phase promastigotes. **A)** 72 hours post infection, footpad thickness was quantified with digital calipers as a measure of delayed type hypersensitivity. **B)** Three weeks post infection, mice were sacrificed and infected footpads were homogenized. Parasite burden was estimated by limiting dilution. Data presented are mean ± SE. ** $p < 0.01$.

iii. Cells from *arg*$^-$-infected mice do not protect naïve mice upon adoptive transfer

Primary infection with *arg*$^-$-*L. major* caused a chronic infection that was insufficient in generating protective immunity when infected mice were subsequently challenged (Figure 18). To assess the protective ability of cells from *arg*$^-$-infected mice, we adoptively transferred whole spleen cells from WT and *arg*$^-$-infected mice into naïve C57BL/6 mice, challenged these recipient mice 24 hours later with WT *L. major*, and determined parasite burden after 3 weeks post infection. Firstly, lesion size in recipients of cells from WT-infected mice was significantly lower than in mice that received cells from *arg*$^-$-infected or naïve mice (Figure 19A). Secondly, parasite burden was up to two logs higher in mice that received cells from *arg*$^-$-infected or naïve mice (Figure 19B). Collectively these results show that infection with *arg*$^-$-*L. major* does not generate enough memory T cells capable of transferring protection to a naïve recipient host.

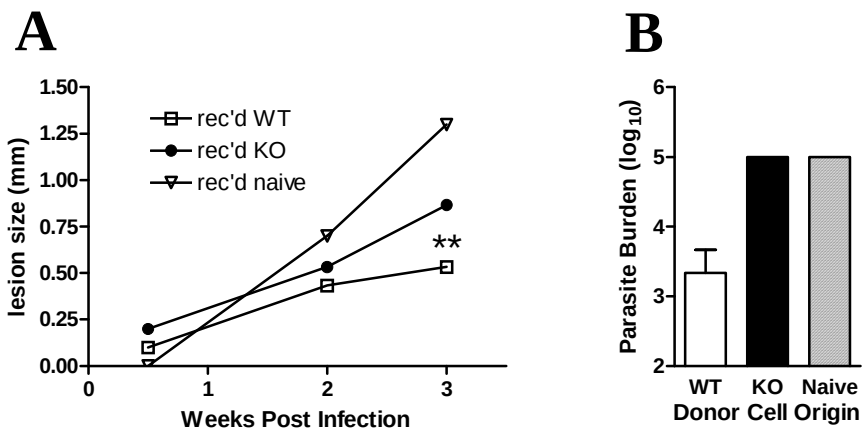

Figure 19. Adoptive transfer of cells from *arg*$^-$-infected mice does not protect naive recipients against virulent challenge.

Forty million spleen cells from naïve, WT or *arg*$^-$*-L. major* infected mice were adoptively transferred by tail vein injection into naïve recipients. Twenty-four hours after transfer, mice were challenged in hind footpad with 2 x 10^6 LV39 *L. major* Footpads were measured weekly with digital calipers **(A)**. Asterisks indicated WT is significantly different from KO and naive. Three weeks post infection mice were sacrificed. Footpads were homogenized and parasite burden quantified by limiting dilution **(B)**. Data represent mean ± SE. ** $p < 0.01$.

iv. Immune cells from WT or *arg*$^-$-infected mice behave similarly in recipient mice upon challenge

In the previous experiments, we adoptively transferred cells into naïve hosts, challenged the recipient mice with *L. major*, and found that similar to challenge experiments in primary hosts, these naïve recipients of cells from *arg*$^-$-infected mice were not protected upon infection. We next wanted to observe the behaviour of "memory" cells purified from WT- and *arg*$^-$-infected mice upon challenge. We hypothesized that cells from *arg*$^-$-infected mice would be defective in IFN-γ production and/or proliferation in recipient mice and this may be the cause of their inability to protect. In order to monitor the behaviour of donor cells during the challenge infection, we employed congenic Thy1.1 mice as recipients in an adoptive transfer of immune cells from healed mice. Thy 1.1 and 1.2 are two allelic forms of the T cell surface marker also known as CD90 that is involved in T cell adhesion and signal transduction. By using Thy 1.1 mice as recipients and Thy 1.2 mice as donors, we were able to selectively monitor the donor cells by flow cytometry using a fluorescent-labeled antibody against Thy 1.2. In addition, we labeled donor cells with CFSE, which allowed us to track donor cell proliferation in response to the new infection.

Surprisingly, there were few significant differences in the behaviour of cells from WT-healed or *arg*$^-$-infected mice when they were transferred into naïve recipients and challenged. As shown in Figure 20, there were few significant differences between cells from WT or *arg*$^-$-infected mice in any of the parameters tested regardless of whether those cells were captured from the spleen or dLN of recipient mice. Only the overall proliferation of *arg*$^-$ donor cells was significantly different from 5 to 14 days post-challenge in the spleen of recipient mice (Figure 20). Despite the lack of significant

differences, when comparing the proliferation results in donor cells captured from recipient spleens there is a clear pattern in cells from *arg*$^{-}$-infected mice . These cells consistently had higher proliferation levels at 5 days than at 14 days post-challenge. Proliferation in cells from WT-healed mice remained relatively constant from 5 to 14 days post-challenge. The same pattern of higher levels at 5 days than 14 days post-challenge was found in donor T_{eff} ($CD44^{high}CD62L^{low}$) cells captured from spleens of recipient mice (Figure 21), but otherwise there were no differences in T_{cm} or T_{eff} cell proportions.

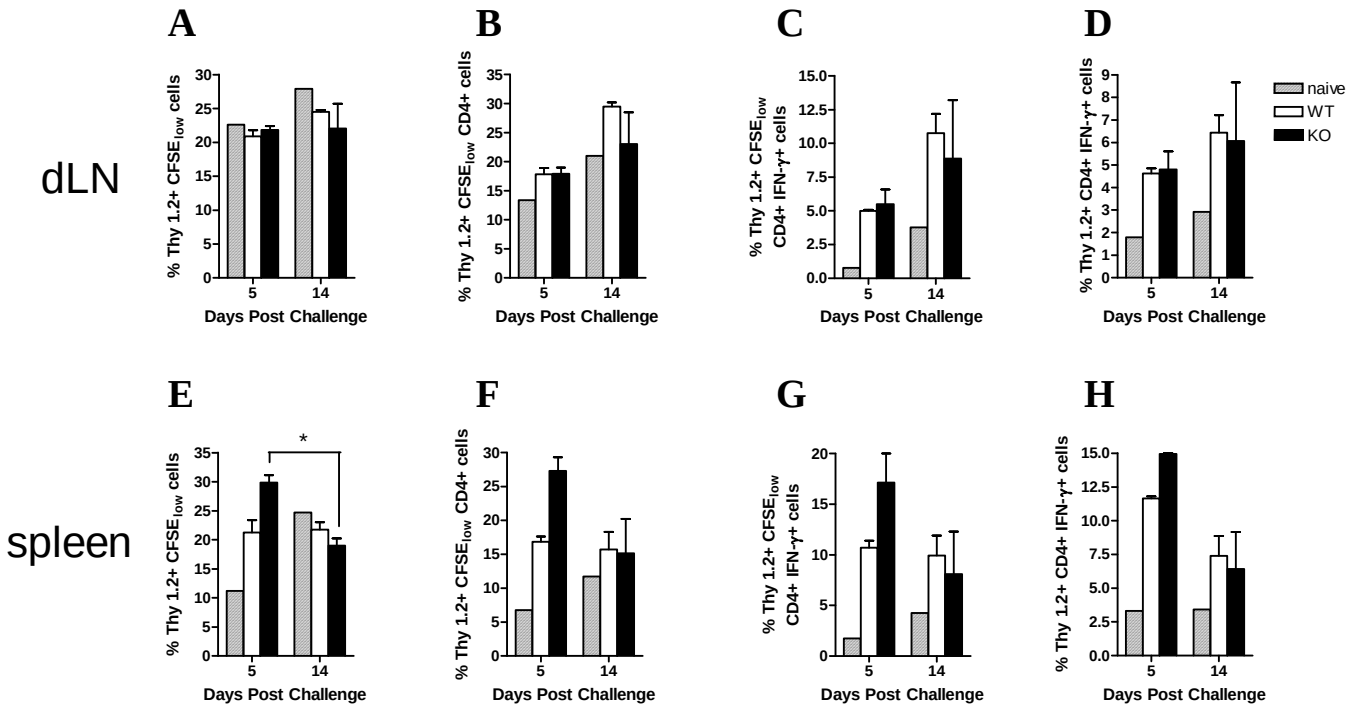

Figure 20. Donor cells from WT- or *arg*$^-$-infected mice behave similarly in recipient mice upon challenge.

Thy 1.2 naïve, healed and chronically infected mice were sacrificed and spleen cells labeled with CFSE. 4 x 10^7 labeled spleen cells were adoptively transferred by tail vein injection into naïve Thy 1.1 recipients. 24 hours after transfer, mice were challenged in hind footpad with 2 x 10^6 LV39 *L. major* stationary phase promastigotes. Mice were sacrificed 5 and 14 days post challenge and single cell suspensions made from dLN (A-D) and spleen (E-H). Cells were then surface stained with fluorochrome-conjugated antibodies against CD4 and proliferation of donor Thy 1.2+ cells assayed by flow cytometry (A, B, E, F). Some cells were cultured *in vitro* with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 hr and Brefeldin A (BFA, 10 μg/ml) in the last 2 hr to enhance intracellular protein accumulation. These cultures were perforated with 0.1% saponin and intracellularly stained with flurochrome-conjugated antibodies against IFN-γ (C, D, G, H). Data represent mean ± SE of the percentages of positive cells. * $p < 0.05$.

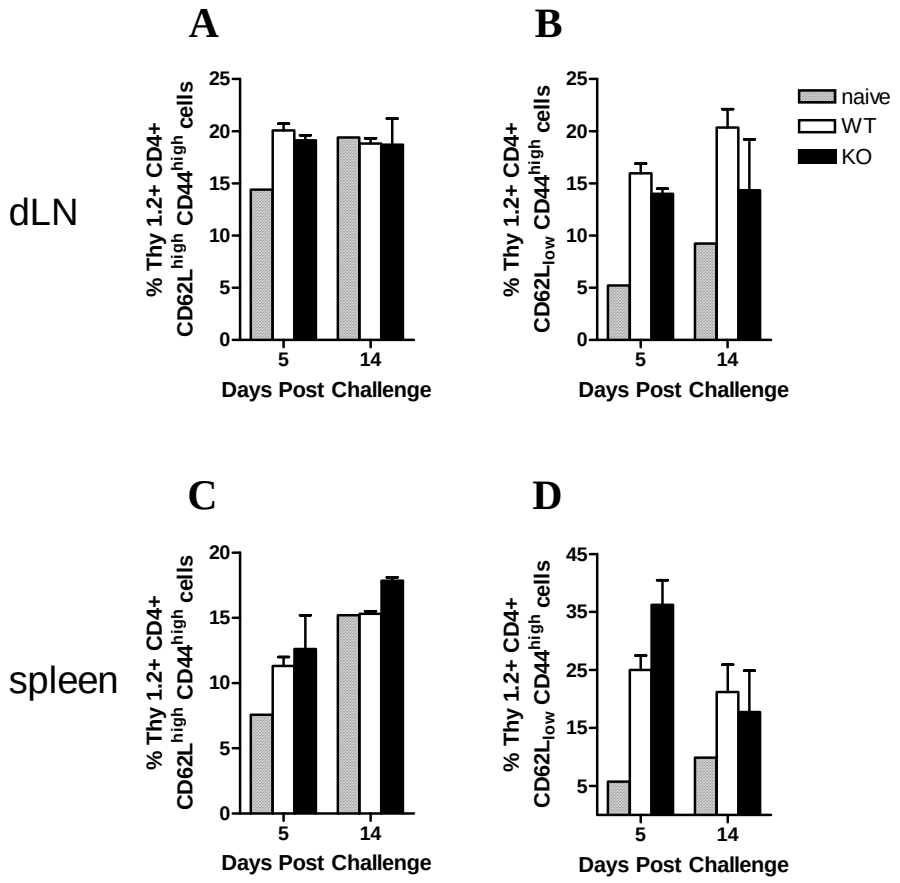

Figure 21. Similar proportions of T_{eff} and T_{cm} populations from WT- and *arg*$^-$-infected mice colonize the dLN and spleen of recipient mice upon challenge.
Thy 1.2 naïve, healed, and chronically infected mice were sacrificed and spleen cells labeled with CFSE. 4 x 10^7 labeled spleen cells were adoptively transferred by tail vein injection into naïve Thy 1.1 recipients. 24 hours after transfer, mice were challenged in hind footpad with 2 x 10^6 LV39 *L. major* stationary phase promastigotes. Mice were sacrificed 5 and 14 days post challenge and single cell suspensions made from dLN (A-B) and spleen (C-D). Cells were then surface stained with flurochrome-conjugated antibodies against CD4, CD44 and CD62L and the populations of donor Thy 1.2+ CD4+ cells assayed by flow cytometry. T_{cm} (A,C) T_{eff} (B,D). Data represent mean ± SE of the percentages of positive cells.

5. Discussion

5.1 The Role of Parasite-Derived Arginase in Primary Infection with *Leishmania major*

Experimental evidence has shown that parasite-derived arginase is an essential enzyme in murine *L. major* from a nutritional perspective. In this work, we set out to examine if parasite-derived arginase is also a virulence or immune evasion factor because of its reciprocal regulation with iNOS. We hypothesized that parasite derived arginase influences parasite survival and host immune response to *L. major*. Using an arginase null mutant in *in vitro* and *in vivo* infections, we found that parasite-derived arginase enhances infectivity *in vitro* and disease pathogenesis *in vivo*. However, parasite arginase does not modulate host NO production and may not be solely regulated by Th2 cytokines like IL-4. Our results suggest that parasite arginase may be of most importance in early colonization of the host. Possibly, once infection is well established *Leishmania major i*s able to induce host arginase to sufficient levels that host polyamines are sufficient for continued intracellular success.

i. Parasite-derived arginase is necessary for optimal intracellular proliferation *in vitro* and *in vivo*

In the *in vitro* infection, parasite proliferation was significantly reduced in the absence of endogenous parasite-derived arginase but the initial ability to invade macrophages remained unaffected (Figure 4). Furthermore, arginase deficient parasites were unable to induce overall increases in macrophage arginase levels as well as wild-type parasites (Figure 5A). Arginase activity measured in infected BMDM was not an additive result of host and parasite-derived arginase combined because arginase activity measured from parasites alone is a minuscule fraction of BMDM derived arginase

activity (Figure 5B). Therefore, factors inherent in the parasite or the process of cell entry induce host-arginase activity and, arguably, AAMϕ. The extent of this induction depends on the strain of mouse as shown in [46] where infected BMDM from C57BL/6 mice displayed significantly less arginase activity than infected BMDM from BALB/c mice even without the influence of Th2 cytokines. Thus, resistance or susceptibility to *L. major* may be the result of the capacity of host macrophages to inherently respond to intracellular amastigotes by CAMϕ even before cytokine help.

Consistent with the *in vitro* results, *in vivo* pathology and parasite number were significantly reduced in *arg*$^-$-infected mice. (Figure 7), again highlighting that *arg*$^-$-*L. major* is impaired *in vivo* but retains the ability to infect. In fact, in all experiments conducted, pathology in *arg*$^-$-infected mice developed between 2 and 4 weeks later than in WT-infected mice, suggesting that parasite-derived arginase may be of key importance early in host colonization. Also consistent with *in vitro* results, at four weeks post infection, arginase activity as measured from infected footpads was significantly lower in *arg*$^-$-infected mice than WT or *arg*$^-$ /+ARG infected mice (Figure 10). This result did not hold true by seven weeks post infection when arginase activity was similar between *arg*$^-$ and WT infected mice. The reason for this increase in footpad arginase activity likely has more to do with lesion pathology than immune response. Arginase is an important enzyme in the physiology of wound healing [190, 271] where the abilities of polyamines to promote cell proliferation and collagen deposition are best put to use. In man, arginase is present and active in wounds [191] and its activity is further increased when those wounds are infected [272]. Futhermore, research in BALB/c mice has shown that individual macrophages can sequentially change their activation status during wound

healing from an anti-microbial phenotype early after activation to a proliferative phenotype in the late phase [273]. In our study, the increase in footpad arginase activity took place at the same time point as lesion establishment in *arg*$^-$-infected mice. Iniesta et al [138] found that arginase I expression in infected footpads increased with inflammation and cellular infiltration of the lesion. In resistant mice, arginase-positive staining peaked at four weeks post infection concurrent with peak footpad swelling [138].

ii. Parasite-derived arginase is not a virulence factor in *L. major* infection.

Because of their intracellular lifestyle, the nature of macrophage activation significantly impacts on the outcome of *L. major* infection [149]. As discussed in sections 1.3 and 1.4, a key factor that regulates the outcome of macrophage activation is the product of L-arginine metabolism, a process that is regulated by the expression of two key enzymes: iNOS and arginase. iNOS and arginase are reciprocally regulated by Th1/Th2 cytokines, respectively, by their shared dependence on intracellular l-arginine, and also by their metabolic pathways and by-products. Reciprocal regulation of iNOS and arginase has been shown experimentally in a number of studies and models [48, 115, 191, 219, 236] . Interestingly, despite the marked increase in arginase activity induced by wild-type *L. major* infection *in vitro* (Figure 5), there was no significant difference in nitric oxide production either between *arg*$^-$ and WT *L. major* infected cells or between uninfected and infected cells (Figure 6). Other studies examining reciprocal regulation in macrophages [48] and in *L. major* [274] have found that measurable nitrites decreased following arginase induction (i.e., after IL-4 stimulation). Because of our contradictory results, we stimulated the cells with additional stimuli including, CpG ODN and anti-CD40 antibodies. We chose these stimulants because of their activating potential in

macrophages. However, in our hands, these stimuli were unable to activate macrophages to produce nitric oxide. We found similar responses *in vivo* where significant differences in footpad arginase activity at 4 weeks post infection were not mirrored in nitrite production from dLN cells (Figures 10 and 13).

Initially, we hypothesized that parasite-derived arginase was serving as an immune evasion mechanism and lack thereof would provide a competitive advantage to host iNOS. This may have occurred primarily because of increased substrate availability but also, possibly through the feedback mechanisms found in the arginase and iNOS metabolic pathways e.g. dependence of iNOS on L-arginine for enzyme stability [234]. These results suggest that in infected cells, the additional parasite-derived arginase plays an insignificant role in influencing the competition for L-arginine between arginase and iNOS. Thus, we speculate that impaired parasite growth in cells infected with *arg*$^{-}$*-L. major* may be directly related to decreased polyamine availability (due to decreased arginase activity) and is unrelated to compensatory iNOS overactivity and NO production. Similar effects have been observed in mice treated with nor-NOHA, a synthetic analog of arg-OH, which blocks arginase activity *in vitro* and *in vivo* [45-47, 274].

iii. Cytokine response is not the sole determinant of host arginase activity.

Previous studies suggest that in BALB/c mice infected with *Leishmania*, the overproduction of IL-4, IL-10 and IL-13 leads to enhanced arginase activities, production of polyamines, excessive parasite proliferation and uncontrolled disease pathology [46, 47, 138, 258, 264]. This increased arginase activity in macrophages is believed to deplete L-arginine availability and competitively inhibit host iNOS activity and nitric oxide production. In the

present study, although addition of rIL-4 significantly enhanced the proliferation of *arg*$^-$ parasites in infected macrophages, this was not enough to rescue them to the level seen in cultures containing WT and rIL-4 (Figure 5C and D). Also the addition of exogenous IL-4 *in vitro* was not sufficient to change the pattern of *arg*$^-$ parasites inducing significantly less arginase activity than wild-type parasites (Figure 5E), a first indication that some other factor(s) (distinct from cytokines) may also play a role in regulating host cellular arginase activity.

Furthermore, despite the reduced arginase activities *in vivo*, the production of both Th1 and Th2 cytokines by cells from the draining lymph nodes of mice infected with *arg*$^-$*-L. major* was not consistently lower than their WT counterparts across all time points (Figure 8 and 9). In support of this, correlation analysis showed no relationship between arginase levels at the site of infection and IL-4 production from draining lymph node cells. Parasite titre however, directly correlated with arginase levels *in vivo* (Figure 11 C and D). This correlation was significant when the three groups were pooled but not in *arg*$^-$-infected mice alone. In these mice, arginase activity is low for most of the time course investigated but some of the parasite burdens in WT and *arg*$^-$ /+ARG infected mice (see Figure 11D) are equivalent to those in *arg*$^-$-infected mice. Therefore, the correlation does not hold at very low arginase activities. Furthermore, infection of mice with *lpg2*$^-$ parasites did not induce WT levels of host arginase despite the expression of an intact parasite-derived arginase gene (Figure 12). In these experiments, parasite titer again mirrored arginase activity. This is not the first study to find a positive correlation between parasite burden and footpad arginase activity. Kropf et al [45] also reported this correlation in infected BALB/c mice and in resistant CBA mice where host arginase activity mirrored the pattern of parasite load. As mentioned above, Iniesta et al [138] also found arginase-positive

staining in infected footpads localized to inflammatory sites highlighting the recruitment of arginase to wounds.

iv. Prostaglandin E2 (PGE_2) may modulate host arginase activity in *L. major* infection.

Prostaglandin E2 (PGE_2) is an important mediator of inflammation produced by a variety of immune and non-immune cells during tissue damage [266, 267]. In response to inflammatory stimuli, arachidonic acid is released from the cell membrane and converted to the PGE_2 precursor prostaglandin H2 by the cyclooxygenases (COX) [275]. This pathway takes place in a wide variety of cell types including endothelial cells, fibroblasts and macrophages [265, 276, 277] . In addition to its many roles in inflammation, PGE_2 can also inhibit production of Th1 cytokines in part at least by its inhibition of IL-12 signaling but has no effect on Th2 cytokines [119, 278].

Induction of PGE_2 has been observed during infection with a number of *Leishmania* species [265]. However, the cells where parasite-driven induction PGE_2 takes place differs from species to species. For example, *L. donovani* induces significant PGE_2 in infected macrophages but *L. major* infection decreases macrophage PGE_2 production [120, 265]. The molecular pathways by which parasite-driven PGE_2 induction occur are unclear but may involve protein tyrosine kinase and protein kinase C [279]. In *L. major*, *L. tropica* and *L. donovani*, a population of immunosuppressive spleen cells has been identified that mediate their effect by production of PGE_2 [120, 280, 281]. The identity of these cells is again strain-specific as they are likely macrophages in *L. donovani* but not macrophages in *L. major* [120, 280]. Inhibition of PGE_2 by the drug indomethacin highlights the importance of this mediator in *Leishmania* infection.

Although indomethacin treatment of BALB/c mice cannot render them resistant, it does decrease lesion pathology and parasite burden, skew the immune response from Th2 to Th1 and result in more NO production [282]. This result was further potentiated when indomethacin was used in combination with IL-12 [283].

As outlined in section 1.3 and 4.1, PGE_2 increases arginase activity in macrophages [194, 219, 264] . From a physiological standpoint, this inflammation-mediated induction of arginase may serve to regulate the tissue damage caused during inflammation. While PGE_2 mediates increased vascular permeability and the infiltration of powerful immune cells, it also induces arginase activity thereby promoting extracellular matrix regeneration. In our study, we found that prostaglandin levels increased substantially over the course of WT infection but remained relatively unchanged in *arg*$^-$ infection (Figure 14). This result is logical given the increasing parasite titer over the course of WT infection and the fact that the parasites themselves are capable of inducing PGE_2. We hypothesize that in addition to parasite-induced PGE_2, the pathology associated with increasing parasite proliferation induces PGE_2 in lesions and these combined effects increase host arginase activity. In the *arg*$^-$ infection, parasite burden and lesion size remain significantly lower than in WT infection over the course of the study. We believe that during the *arg*$^-$ infection low levels of PGE_2 are insufficient to induce host arginase levels similar to those observed in WT infections. These hypotheses can be tested using the PGE_2 inhibitor indomethacin with which we would expect to see a decrease in total arginase levels in WT infection. It is conceivable that additional PGE_2 may not rescue the proliferation defect of *arg*$^-$ infection just as additional polyamines did not rescue parasite proliferation *in vitro*.

Matlashewski et al [265] propose at least two roles for Cox-2 and PGE_2 in *Leishmania*, i) promotion of Th2 responses and ii) where it is induced in infected macrophages, contributing to lymph node escape and eventual visceralization [280, 284]. We propose a third role for PGE_2 in *Leishmania* in increasing local arginase expression and providing a larger polyamine pool, enhancing parasite proliferation.

v. An argument against contradictory findings

In 2007, researchers from the University of Iowa and colleagues [247] characterized an *L. mexicana* arginase null mutant *in vitro* and *in vivo*. The inability of *arg⁻-L. mexicana* to proliferate in macrophages was attributed to overall lower arginase activity in host cells, which is in part contributed by the absence of parasite derived arginase resulting in more substrate availability for NO production by the enzyme iNOS [247]. This study reported increased NO production in *arg⁻*-infected macrophages over those produced by WT-infected cells at 48 hours post-infection. Since this study also found no differences in total arginase levels in WT and *arg⁻*-infected cells at 48 hr post-infection, it is unlikely that the increased nitric oxide production observed at this time in *arg⁻*-infected cells was due to enhanced iNOS activity resulting from decreased arginase activity [247]. Furthermore, we did not observe any increase in NO production (over that induced by WT parasites) in the absence of parasite-derived arginase at any time during *in vitro* and *in vivo* infections (Figures 6 and 13). Thus, our data are consistent with our conclusion that arginase activity in infected cells or tissues does not influence iNOS activity and the impaired proliferation of *arg⁻-L. major* is unrelated to increased NO production. Gaur et al. also found that *arg⁻-L. mexicana* infection induces a type one

immune response based on their finding of higher IFN-γ levels and lower IL-4 levels in culture supernatant of cells from *arg*$^-$-infected mice at 4 weeks post-infection compared to WT infection [247]. In contrast, we show that the cytokine response in WT and *arg*$^-$-*L. major* -infected mice show lack of predictable dominance of type one over type two immune response or vice versa over a period of 7 weeks post-infection (Figures 8 and 9). The *L. mexicana arg*$^-$ study investigated the adaptive immune response only at one time point (at 4 weeks) post-infection providing only a snapshot of the response whereas we undertook a systematic kinetic examination of immune response over 7 weeks.

In addition to differences in experimental design, differences in the biology of New and Old world parasites may also be important. For instance, there are marked differences in the nature of interaction of *L. major* and *L. mexicana* with macrophages, particularly in the formation of parasitophorous vacuoles where in *L. major* they are small and generally contain only one amastigote, whereas in *L. mexicana* they contain many amastigotes [285]. Furthermore, lesion progression also differs between these two strains being slower in *L. mexicana* and inducing a different immune response [286]. Importantly, recent reports show that the functional consequences, such as virulence, of genetic ablation of key conserved genes, such as *LPG2,* are different in *L. major* and *L. mexicana* [253, 287, 288] .

vi. Characterization of *arg*$^-$*-L. major*

Recently, Beverley et al. [218] characterized a clone of the *arg*$^-$ mutant used in this study. Since Gaur et al [247] characterized an *arg*$^-$ mutant of *L. mexicana*, Beverley wanted to compare results in *L. major*. Similar to our findings, lesions in *arg*$^-$-infected

mice took 2 weeks longer to develop, but in contrast, they found that after ten weeks *arg*$^-$-infected mice developed larger lesions than WT-infected mice, though the difference was not statistically significant. Furthermore, the difference in parasite burden was statistically insignificant between WT and *arg*$^-$ infection at seven weeks post-infection. Similar results were found in mice infected with recovered amastigotes. In our study, lesion pathology in WT mice forced us to sacrifice mice at 7 weeks post infection. It is possible that had we extended our experiments to ten weeks post infection we would find a similar result. However, it is important to note that we infected mice with 2 x 10^6 parasites while Beverley et al. infected mice with 1 x 10^5 promastigotes or 1 x 10^4 amastigotes. Two million parasites is several orders of magnitude higher than in a real infection but is a common amount for studies of this kind. In nature, a sandfly injects only 100 to 200 parasites, for components of sandfly saliva modulate the local immune response to favour the establishment of infection [31, 289-291] . In the work by Beverley et al., lesions became evident in WT infected mice between five and seven weeks post-infection. In our study, lesions in WT controls became evident between 2 and 3 weeks post infection. Because BALB/c mice can control infection with less than 1000 parasites, it seems that even a ten-fold reduction in parasite dose has a measurable outcome in lesion pathology. In their *in vitro* experiments, Beverley et al. report that *arg*$^-$ /+ARG parasites demonstrated a 30-fold increase in arginase activity compared to WT controls. In our study, we found a 4-fold increase in these arginase complemented mutants because we used a different clone of parasite. Surprisingly, despite higher arginase activity, intracellular L-ornithine levels were 20% lower in *arg*$^-$ /+ARG parasites than in WT parasites. The authors hypothesize that ornithine is either being excreted from the cell or

that there is increased flux through ODC and the downstream pathway. Since *arg*$^-$ /+ARG is ten-fold more resistant to the ODC inhibitor DFMO, the second hypothesis is more likely. Not surprisingly, *arg*$^-$ /+ARG is completely insensitive to NOHA. Finally, the authors concluded, as this thesis has shown, that inhibition of parasite-derived arginase is not a suitable therapeutic aim because mutant parasites can still develop *in vivo*. Two features of *Leishmania* allow them to scavenge polyamines from the host. Firstly, several species express arginine and polyamine transporters and secondly, the acidic nature of the parasitophorous vacuole can encourage accumulation of basic nutrients like the polyamines L-ornithine and L-arginine [218].

vii. The role of parasite-derived arginase in primary infection: proposed model

With respect to the exact role of *L. major*-derived arginase in *de novo* polyamine synthesis, we suggest a "take rations for the invasion" hypothesis. The intracellular environment of macrophages is extremely hostile. During the early phase of infection, the newly transformed amastigotes require survival factors to proliferate successfully inside the cell. Parasite-derived arginase may function to enhance the initial survival and proliferation of *L. major* inside macrophages by increasing local polyamine concentration around the parasitophorous vacuoles thereby enhancing their availability to the parasites. In the absence of parasite-derived arginase, early proliferation and establishment of *L. major* parasites may be impaired to such an extent that the addition of exogenous polyamines, and the parasite's ability to scavenging from the host, cannot rescue their proliferation to WT levels. A testable prediction from this hypothesis is that increasing polyamine concentration in macrophage cultures will not be effective in rescuing the

growth defects of *arg*$^-$*-L. major in vitro*. Indeed, Gaur et al., [247] found that although additional putrescine and ornithine can increase intracellular parasite proliferation, they were unable to rescue the defect of *L. mexicana arg*$^-$ infection. Thus, it appears that there is distinct compartmentalization of responses between what influences the cell as a whole and what influences the intracellular amastigote microenvironment. Hence, the ability to synthesize polyamines *de novo* directly after infection gives *L. major* a survival advantage during this difficult time. Once a significant number of amastigotes are present in the host, parasite-derived products or exoantigens would be produced in sufficient quantity to sustain the infection. We hypothesize that one or more of these products is a molecule for upregulating host arginase.

viii. Induction of host arginase by *L. major*: a colonization mechanism?

The metabolism of polyamines (putrescine, spermine, and spermidine) is an integral part of cellular function in all living cells [242]. In mammals, the cellular polyamine content correlates with the rate of cell division, for example, polyamine levels are increased in cancer cells compared to normal cells [242, 262] . As in mammals, polyamines are essential for the growth of all *Trypanosomatidae* yet, arginine metabolism in these organisms differs slightly from those of mammals and even among species. For instance, African trypanosomes (including *T. brucei* and *T. congolense*) encode most of the mammalian enzymes, or their homologs, necessary for the biosynthesis of polyamines [242]. In contrast, *Trypanosoma cruzi*, which is closely related to *Leishmania*, lacks the enzyme ornithine decarboxylase (ODC) and therefore relies heavily on the uptake and interconversion of putrescine and spermidine from their environment. This process is

aided by an enzyme, cruzipain, which is encoded by the parasite [242, 292, 293]. Similar to IL-4, cruzipain can increase cellular arginase levels and hence trigger alternative activation of macrophages and production of polyamines thereby promoting intracellular proliferation of *T. cruzi* [292]. Importantly, cruzipain also down-regulates iNOS expression and nitric oxide production in macrophages, ensuring the maintenance of an alternatively activated state in these cells. The discovery of cruzipain and its ability to induce arginase expression suggests that other members of the *Trypanosomatidae* may also possess such molecules. Indeed, parasites from other families have been found to possess such arginase effectors; for example *Fasciola hepatica* secretes the antioxidant thioredoxin peroxidase (TPx) which is hypothesized to induce alternative activation and subsequent arginase upregulation in peritoneal macrophages [294]. The possession of a protein capable of increasing host arginase activity is advantageous for the parasites as it provides a larger polyamine pool for optimal parasite proliferation. In both this work and a study by Kropf et al [45] there is a positive correlation between parasite titer and arginase activity (Figure 11) supporting the hypothesis that *Leishmania major* may also produce such a molecule which, combined with parasite-derived arginase, ensures optimal availability of polyamines thereby facilitating efficient intracellular survival.

ix. Conclusion

Clearly parasite-derived arginase is of important nutritional benefit to parasite proliferation in the host. We conclude that it does not play a role as a virulence factor by reducing substrate availability to iNOS. In our kinetic study, we show that adaptive immune response may not predict *in vivo* host arginase activity or vice versa as parasite-

derived product(s) or inflammatory mediators like PGE_2 also modulate host arginase activities *in vivo*. From the parasite's perspective, if individual *L. major* organisms are the soldiers in the battle of leishmaniasis, parasite-derived arginase is the food carried in their packs. *L. major*'s use of this enzyme in infection exemplifies *Leishmania*'s prowess as a pathogen because not only do they subvert host responses but by creating lesions they use the natural pathway of host healing, arginase, to their advantage.

5.2 Influence of parasite-derived arginase on the quality of memory anti-*Leishmania* immunity following secondary challenge

In resistant mice, resolution of primary infection with *L. major* results in life-long immunity to re-infection. With the objective of testing the efficacy of the arginase null mutant as an avirulent vaccine candidate, we set out to examine the quality of memory anti-*Leishmania* immunity following secondary challenge. Curiously, infection of normally resistant C57BL/6 mice with *arg*$^-$-*L. major* resulted in a chronic infection. This primary infection was unable to induce immunity as *arg*$^-$-infected mice had significantly higher parasite burden than their WT-infected counterparts upon secondary challenge and adoptive transfer of cells from *arg*$^-$-infected mice did not protect naïve mice against challenge. Oddly, we were unable to find any differences in immune memory parameters between cells from *arg*$^-$-infected mice and those from WT-infected mice after the adoptive transfer. We therefore cannot recommend *arg*$^-$ null *L. major* as a vaccine candidate but this result presents an interesting question in what parasite factors determine a successful immune response capable of controlling or promoting *L. major* infection.

i. Normally resistant C57BL/6 mice develop a chronic infection in response to *arg*$^-$-*L. major* infection.

In normal conditions, C57BL/6 mice are resistant to *Leishmania major* - the mice resolve lesions and control parasite burden. We have found that the same mice infected with *arg*$^-$-*L. major* develop a chronic infection that, though slow to develop, persists at least up to 16 weeks post infection (Figure 15A). Four days after infection, parasite burden is significantly lower in *arg*$^-$-infected compared to WT-infected mice (Figure 15B). Therefore, consistent with our findings in BALB/c mice, parasite proliferation was

impaired without parasite-derived arginase. *L. major* uses many mechanisms to subvert the host immune response [36, 40-42]. Logically the success of these mechanisms depends in part on the number of parasites present. Therefore, *arg*$^{-}$*-L. major,* having impaired *in vivo* proliferation, can be expected to have a reduced ability to thwart host responses compared to WT *L. major.* As such, early after infection with the already weak arginase null mutants innate immunity may be sufficient to impair parasite proliferation. We hypothesize that this early control is the beginning of interplay between the immune system and the parasites. At later time points, parasite burden is the same between groups (Figure 15C) even until the WT-infected mice are considered healed (Figure 17A). IFN-γ follows a corresponding pattern being significantly higher (Figure 16A) in *arg*$^{-}$-infected compared to WT-infected mice at four days post-infection and IFN-γ significantly less in the *arg*$^{-}$-infected mice (Figure 16C) at 7 weeks post-infection.

In summary, *arg*$^{-}$*-L. major* is impaired because they do not have arginase to help colonize the host during the most sensitive initial infection. Due to this deficiency, they are unable to overcome the early immune response and the infection does not expand to WT levels but continues in a chronic form. In WT *L. major*, parasites have the full nutritional component to begin a strong infection that can withstand the early host response. This requires the host to mount an even stronger protective response that eventually controls parasite proliferation (clonal expansion, causing significantly higher IFN-γ at seven weeks post infection Figure 16C). We hypothesize that *arg*$^{-}$*-L. major* is more susceptible to the host's early immune response than WT *L. major.* Deficiency in arginase does not abolish *L. major*'s ability to persist - after all, in resistant mice and in

human cutaneous leishmaniasis, the host never succeeds in clearing parasites. The persistent nature of this pathogen is likely the key to *arg*$^-$-*L. major* chronicity.

ii. Infection with *arg*$^-$-*L. major* does not result in protective immunity

Although the chronic nature of *arg*$^-$-*L. major* infection makes it useless as an avirulent vaccine, as scientists we were still interested in the memory response generated by these parasites. At the time when WT-infected mice can be considered healed, *arg*$^-$-infected mice have the same level of parasite burden (Figure 17A). Since, this number of parasites is equivalent to the persistent parasites of the WT infection will they provide protection?

IFN-γ is the key cytokine for control of *L. major* [99] so we measured this cytokine in infected mice to see how well they were controlling infection. Sixteen weeks after infection, when WT-infected mice are considered healed, antigen-specific IFN-γ production by WT spleen cells was significantly greater than that produced by cells from *arg*$^-$-infected mice (Figure 17C). The same pattern was evident in dLN cells. This result was our first clue that *arg*$^-$-*L. major* cannot protect mice against secondary infection. Therefore it was not surprising that when we challenged WT- and *arg*$^-$-infected mice (Figure 18) or adoptively transferred their cells into naïve mice and challenged the recipients (Figure 19) neither the *arg*$^-$-infected nor the recipients of cells from *arg*$^-$-infected mice were protected.

What was surprising was that cells from WT-infected and *arg*$^-$-infected mice appeared to behave similarly when adoptively transferred into naïve recipients even though the recipients of cells from *arg*$^-$-infected mice were not protected (Figure 20 and

21). Overall, there was significantly more proliferation of cells from *arg*$^-$-infected mice recovered from recipient spleens at 5 days post infection than at 14 days post infection (Figure 20E). Although this difference was not statistically significant in CD4+ cells or those CD4+ cells producing IFN-γ, the trend remained consistent (Figure 20E-G) and could also be found in donor T_{eff} cells recovered from spleen (Figure 21D). Since *arg*$^-$-infected mice were in the midst of a chronic infection when cells were taken for adoptive transfer, it is logical that they would have such a high level of proliferation. In future experiments, we could examine CFSE proliferation of cells from WT- and *arg*$^-$-infected mice 16 weeks after infection in response to SLA. By fourteen days post infection it appears that proliferation decreases to baseline (WT) levels.

We found no differences in the parameters chosen to examine the behavior of cells from *arg*$^-$-infected mice during secondary infection; however, there are other possibilities. Current theories on memory T cells suggest that the primary infection must include sufficient clonal expansion and contraction for generation of effective memory responses [270]. At first, typical *L. major* infection may seem contrary to this because infection-induced immunity depends on the presence of persistent parasites [2, 3, 5]. Classical activation of macrophages is the key mechanism by which *L. major* is controlled [47, 204, 205, 213, 221]. The cells adoptively transferred from *arg*$^-$-infected mice in our experiments seem equivalent to those transferred from WT-infected mice but may be substandard in their ability to classically activate macrophages because, as outlined in section 1.2 part three, classical activation of macrophages requires more than just IFN-γ. Signaling though costimulatory molecules like CD40L may be substandard. Furthermore, although we examined T cell responses in spleen and dLN, we did not look

for these cells at the site of infection. Possibly improper generation of memory T cells impairs their ability to migrate to the infected footpad. Finally, as mentioned in section 1.2 part four, CD62L and CD44 are also expressed by naïve T cells upon activation and this makes their use to identify memory T cells slightly problematic. Usually the application of CD62L and CD44 to identify memory T cells is reasonable because they are being identified in healed mice where one would not expect to find significant T cell activation. However, in *arg*$^-$-infected mice we have observed a chronic infection so the cells adoptively transferred may not be bonafide memory cells. If this is the case, the lack of protection may be due to exhaustion of this pool of cells such that though they can proliferate and make IFN-γ the same as WT elicited memory T cells (Figures 20 and 21) they may still be inefficient at activating macrophages.

iii. Conclusion

As in BALB/c mice, proliferation of *arg*$^-$*-L. major* is impaired in C57BL/6 mice however in this normally resistant strain, the early immune response is able to counter the infection. Due to the persistent nature of *Leishmania* in general, this results in a chronic, low-level infection. Infection with *arg*$^-$*-L. major* is unable to induce protection against challenge infection either in the primary host or in recipients of cells from *arg*$^-$-infected mice. We believe that the chronic infection caused by the arginase null mutants exhausts T cells and impairs their ability to classically activate macrophages. In conclusion, *arg*$^-$*-L. major* is not a useful vaccine candidate because not only does it result in a chronic infection but also it is unable to provide protection upon challenge. This null mutant does provide an interesting model for the study of the generation of immune memory.

6. Future Directions

This work has shown that parasite derived arginase influences parasite survival but not host immune response to *L. major*. In both BALB/c and C57BL/6 mice and *in vitro*, infection with *arg*$^-$*-L. major* altered the pathogenesis of infection. However, infection with this mutant did not have a strong influence on cytokine profiles or NO production. Furthermore, infection with *arg*$^-$*-L. major* does not provide protection against challenge infection.

Our work found that host arginase activity is less related to cytokine response (specifically IL-4) than previously believed (Figure 11) and may be largely regulated by parasite titer (Figure 11 and 12) or inflammatory mediators (Figure 14). The caveat to these *in vivo* findings is that arginase activity was measured in the footpad while nitric oxide production, cytokine responses and PGE_2 were measured in the culture supernatant of dLN cells. Although cells from the dLN travel to the site of infection and individual parasites can be found in cultures of dLN cells, different results may be found if these parameters were measured in the same tissue. The conclusion that inflammatory mediators may be of greater importance than cytokines in regulating host arginase would be stronger if these were measured in the same tissue. Recently, an arginase specific ELISA was developed for recognition of human arginase I in culture supernatant [295-297]. If this system was developed for murine arginase, then nitric oxide production, cytokine responses, PGE_2 and arginase could all be measured in dLN cell culture supernatant. Alternatively, tissue specific PGE_2 assay or cytokine immunohistochemistry would achieve the same end.

Future experiments into the nature of memory responses generated or not in response to primary infection with *arg*$^-$*-L. major* may investigate the proliferative responses of T cells in the first few days after infection with this null mutant. Is there really a difference in clonal expansion of T cells between WT and *arg*$^-$*-L. major* and what does this mean for T cell priming? To answer this question, we can CFSE label T cells isolated a few days post infection, stimulate them *in vitro* with SLA and monitor proliferation. Our results suggest that there is some level of early control of *arg*$^-$*-L. major* in C57BL/6 mice. We could investigate this by daily kinetic measurement of IFN-γ production by these mice and/or parasite burden and compare these results to WT infection. Immunofluororescent labeling of footpad slices may also show us if macrophages are taking up more parasites in *arg*$^-$-infected footpads than in WT-infected footpads at early times in the infection. One possibility for the lack of protection of *arg*$^-$-infected mice against secondary challenge is that the T cells are exhausted. This can be tested by examining CFSE proliferation of cells from WT- and *arg*$^-$-infected mice 16 weeks after infection in response to SLA. Furthermore, if T cells are isolated from WT- and *arg*$^-$-infected mice at this timepoint, pulsed with SLA and co-cultured with infected BMDM we could use the ability of the macrophages to kill parasites as readout of the protective ability of these cells.

Because of the level of pathology in our WT-infected mice, we had to abort our experiments at 7 weeks post infection. Reguera et al. [218] showed that when BALB/c mice were infected with 1×10^5 promastigotes or 1×10^4 amastigotes, lesion size caught up to that of WT infected mice by seven weeks post infection and parasite burden was not statistically different at this time. Possibly, if we examined lesion size beyond seven

weeks post infection we would find the same in our system highlighting the importance of parasite-derived arginase mostly in the early stage of host colonization and further confirming our model.

7. References

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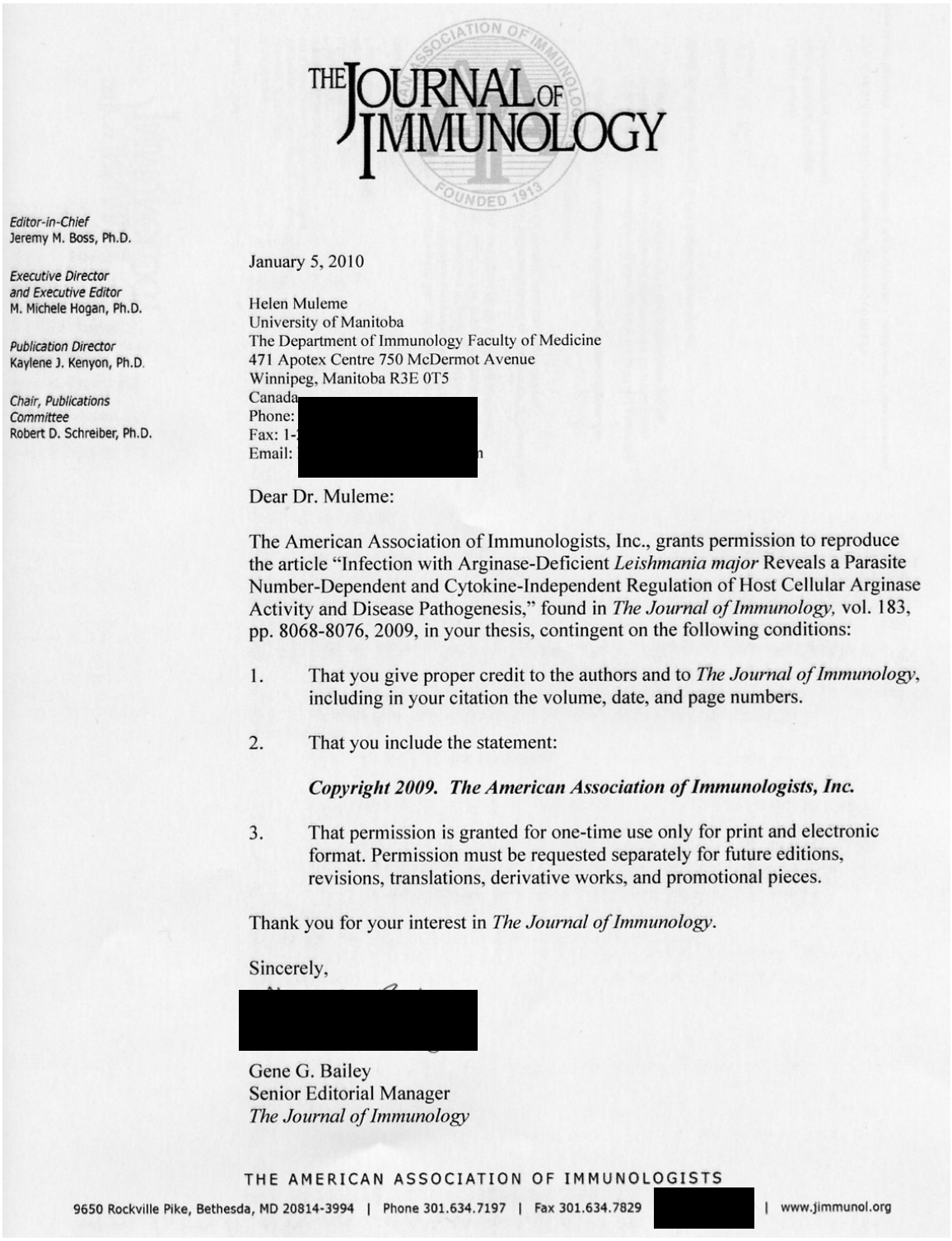
Appendix



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January 5, 2010

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