THE ROLE OF PARASITE – DERIVED ARGINASE IN THE INFECTION OF *LEISHMANIA MAJOR*

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II Abstract

Leishmaniasis is a disease caused by an intracellular parasitic protozoan belonging to the genus *Leishmania*. *Leishmania* species contain a gene that encodes for the enzyme arginase, which is important for polyamine synthesis and subsequently parasite proliferation. Arginase has a role in both polyamine synthesis and the Th II immune-response pathway. In this study, it was investigated if the presence of this enzyme shifts the Th I/Th II immune response in the parasite's favor. The species *Leishmania major* was used to test *in vitro* infection of macrophages, as well as nitric oxide production from the infected cell, using an arginase knockout version of the parasite. Knockout parasites were unable to proliferate inside the host cells and showed a significant difference in the number of parasites per infected macrophage ($p < 0.005$) in comparison to the wild type parasites. However, there were no significant differences in nitric oxide production between the macrophage cells infected with either type of parasite. This, along with further testing of cytokine production in the infected cells, leads to the conclusion that the presence of arginase does not alter the immune system of the host, but is present solely to provide the required polyamines for proliferation of the parasites.

III Acknowledgments

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1 Introduction

Leishmania spp are unicellular, parasitic protozoa that typically cause the vector-borne zoonotic disease called leishmaniasis. The disease is primarily transmitted by various species of phlebotomine sand flies that are indigenous to the five continents leishmaniasis is currently prevalent in, namely Africa, Asia, Europe, North America and South America. According to the World Health Organization (WHO), over 12 million people are currently infected with the disease with a population of over 350 million at risk (Reiner and Locksley 1995).

Leishmania infections can produce diverse symptoms in the mammalian host, depending on the species of parasites, and the host genetic makeup. *L. donovani* and *L. infantum* cause visceral leishmaniasis, the most severe form of the disease. *Leishmania braziliensis* causes mucocutaneous leishmaniasis, infecting the mucous membranes of the host. Lastly, *L. tropica* and *L. major* cause simple cutaneous leishmaniasis, producing skin ulcers, and is the most common type of the disease. Again depending on the host genetics, the cutaneous leishmaniasis are characterized by localized skin ulcers on the exposed parts of the body, (simple cutaneous leishmaniasis), or serious widespread lesions and ulcers all over the body (diffuse cutaneous leishmaniasis). While the simple cutaneous form is self-limiting (healing with strong immunity to re-infection), the diffuse cutaneous form never heals and tends to relapse after treatment.

The parasites are transmitted from animal to human, from human to human, and plausibly from human to animal as well. Leishmania species are digenetic, existing in two different life forms (Figure 1).

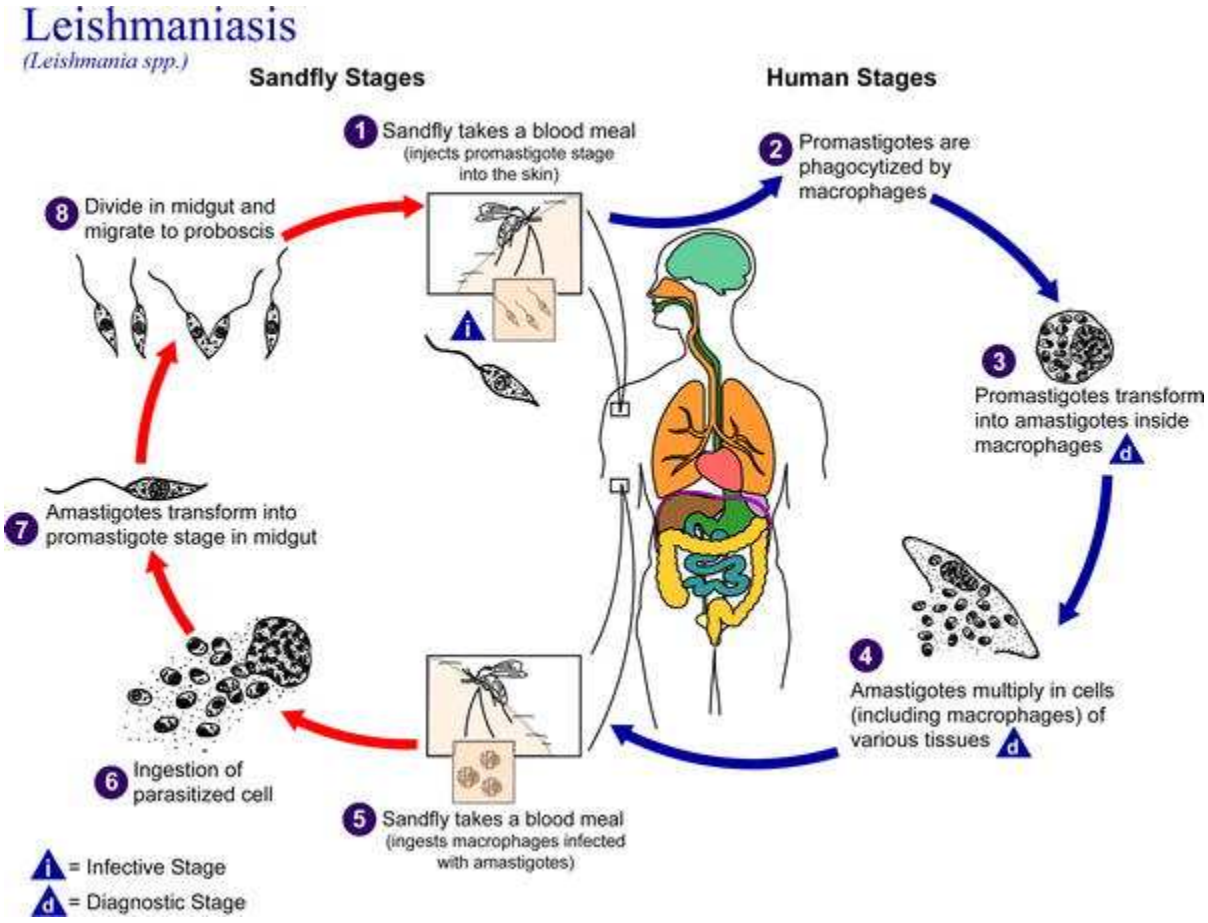


Figure 1: Life cycle of *Leishmania* species. (CDC: http://www.dpd.cdc.gov/dpdx)

In the infective stage, the promastigotes are spindle-shaped motile cells, which exist extracellularly inside the digestive tract of the sand fly (the insect vector). When the sand fly bites a mammalian host, the promastigotes are injected into the skin of the human, rodent or domestic animal. Once inside the host, the promastigotes quickly infect the monocytes, macrophages and dendritic cells, where they rapidly differentiate into amastigotes. Amastigotes are non-motile and oval or round-shaped, and undergo extensive proliferation inside infected cells, such as macrophages, leading to their lysis. This lysis of infected cells results in release of the parasites, which go on to infect new cells. The infected cells are introduced back into the sand fly when the sand fly acquires its blood meal. Once back inside the gut of the sand fly, the amastigotes differentiate into the promastigotes again.

Due to the varied and extremely hostile environments that they reside in during their lifecycle, the parasites have adapted innovative methods of survival. For example, the host cell (macrophage) that the parasites inhabit in the mammalian host is also important in the protection of the body against infection and other noxious substances. Because of their intracellular life style inside the mammalian host, antibodies made against the parasites are ineffective in controlling the infection, and might actually be exploited by the parasite for evasion of host killing mechanisms (Padigel *et al*. 2003). Complete understanding of the *Leishmania spp* survival in the human host has not yet been accomplished.

Macrophages are white blood cells, more specifically phagocytes, acting in the nonspecific defense as well as the specific defense system of humans and other vertebrate animals. Their role is to phagocytize cellular debris and pathogens, either as stationary or

mobile cells. The attraction of wandering macrophages to a damaged site occurs through chemotaxis, triggered by a number of things. Primarily, damaged cells and pathogens release chemical substances, which macrophages are attracted to and activated by. Activation of macrophages is an important step for the killing of intracellular pathogens, including *Leishmania*. Activated macrophage cells produce two key enzymes that regulate the killing ability of macrophages, inducible nitric oxide synthase (iNOS) and arginase (Iniesta *et al*. 2002). Both enzymes metabolize arginine but with different results.

Arginine is oxidized by iNOS to produce nitric oxide (NO) and citrulline. The NO is then used as an important effector molecule in cell-mediated immune reactions for killing and/or inhibiting the proliferation of intracellular pathogens including leishmania (Reiner and Locksley 1995). The cellular and molecular mechanism in which nitric oxide applies its cytotoxic activity is not well understood; however, recent studies have found numerous targets of NO in *Leishmania* parasites (Holzmuller *et al*. 2002). These targets include metabolic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase, aconitase and cysteine proteinase, as well as extensive nuclear DNA fragmentation (Holzmuller *et al*. 2002). The overall toxic effect of NO in *Leishmania* parasites is the induction of necrosis as well as apoptosis.

Arginase metabolizes arginine by two, separately encoded, distinct isoforms of the enzyme in mammalian hosts (Tapiero *et al*. 2002). These isoforms differ in their location and tissue expression, as well as their function. Type one arginase (arginase I) is usually found in the cytosol, and is expressed in the liver as a component of the urea cycle. Type-two arginase (arginase II) is a mitochondrial enzyme that is wide spread in

numerous tissues of the body. It is involved in biosynthetic functions, such as the synthesis of ornithine. Both isoforms of arginase hydrolyze arginine to produce urea, a waste product, and ornithine, which is the substrate for the key enzyme in polyamine synthesis (Bansal and Ochoa 2003). Because polyamines are essential to the growth of leishmania, this pathway is responsible for the survival of the parasites, and the promotion of their growth (Roberts *et al*. 2004). It has been found that activated macrophages up-regulate arginase I, thus making this enzyme important in the pathogenesis of leishmaniasis (Munder *et al*. 1998).

The activities of arginase and iNOS *in vivo* can be profoundly regulated by the cytokines produced by CD4+ T cells (T helper cells). Murine T helper cells can be classified into Th1 or Th2 subsets based on their cytokine patterns. Th1 cells secrete interleukin-2 (IL-2) and interferon-γ (IFN-γ) and mediate cell-mediated immunity, while Th2 cells produce IL-4, IL-5 and IL-10 and predominantly favor the production of antibodies (Liew *et al*. 1997; Modolell *et al*. 1995). Healing in resistant mice infected with *L. major* is associated with the development of Th1 cells producing IFN-γ, which activates macrophages to produce nitric oxide, an effector molecule for killing intracellular *Leishmania* parasites (Iniesta *et al*. 2001; Munder *et al*. 1999; Munder *et al*. 1998; Reiner and Locksley 1995). In contrast, susceptible mice produce IL-4 early, which promotes the development and expansion of Th2 cells that produce IL-4 and IL-10. These are cytokines that deactivate macrophages and inhibit intracellular parasite killing (Iniesta *et al*. 2001; Munder *et al*. 1998; Reiner and Locksley 1995). Activation of macrophages to produce NO (via the iNOS pathway) is mediated by cytokines IFN-γ and TNF-α produced by Th1 cells. In contrast, IL-4 and IL-10 produced by Th2 cells enhance

arginase activities and downregulate the iNOS pathway. Conversely, while cytokines produced by Th2 cells enhance the activity of arginase, the Th1 cytokines downregulates arginase (Liew *et al*. 1997). Thus, the balance between Th1 and Th2 immune responses *in vivo* directly or indirectly regulate the preferential activation of macrophages via the arginase or iNOS pathways with distinct and opposing results (Munder *et al*. 1998; Kropf *et al*. 2005).

It has been shown that the limiting factor that regulates the activities of arginase and iNOS is the availability of their substrate, arginine (Tapiero *et al.* 2002). The competitive regulation between the two pathways is enhanced by the limited coupling of iNOS. This particular dimer enzyme is only able to dimerize in the presence of arginine. In its absence, the enzyme is inactive. As well, inhibitors of each pathway can be found in the alternative pathway as a product or intermediate. For example, N^{w}-Hydroxy-L-Arginine (NOHA), an intermediate product in the conversion of arginine to nitric oxide by iNOS, is the most effective physiological inhibitor of arginase, while the polyamines produced by the arginase pathway can act as inhibitors of NO production (Bansal and Ochoa 2003).

Several studies have been carried out to investigate the role of these alternative pathways of arginase metabolism in the pathogenesis of *Leishmania major* in mice. When infected with *L. major*, the iNOS protein is apparent in the cells earlier and in higher quantity in clinically resistant mice (C57BL/6) in comparison to the non-healing BALB/C mice (Stenger *et al*. 1994). In addition, fewer parasites were found in sections taken from the infection site from the resistant C57BL/6 mice and this was directly correlated with high *in situ* expression of iNOS protein. This suggests that there is a

correlation between the amount of iNOS in the host cell and the susceptibility of the host to the parasites.

In addition, mice having a disrupted gene encoding for the iNOS enzyme, and hence unable to produce the iNOS protein, are significantly more vulnerable to infection from the parasites than wild type controls. This is in spite of having high levels of the Th1 cytokines. This is not surprising because the iNOS gene activation that leads to resistance is downstream of Th1 response in the cascade of events (i.e. it is activated by IFN-γ).

In contrast, treating *L. major*-infected mice with the Th2 cytokines, IL-4 and IL-10, activates arginase, leading to a significant increase in parasite burden (Iniesta *et al.* 2002). Thus, the activity of arginase I promotes pathology and uncontrolled proliferation of amastigotes (Kropf *et al*. 2005). Inhibiting the activity of this enzyme causes a reduced pathology and a control of parasite proliferation. Because the Th2 response is not altered in these mice, the results indicate that arginase regulates parasite growth by affecting polyamine synthesis. Consistent with this, it has been found that addition of large amounts of ornithine or smaller amounts of putrescine promotes parasite proliferation (Iniesta *et al*. 2002).

Like mammals, *Leishmania* have a gene that encodes for an arginase enzyme. The reason why *Leishmania* species have conserved this gene is not known but it is thought to relate to their intracellular survival. The presence of additional arginase activity (parasite-derived) in the host cell could lead to an overall increase in the production of polyamines, which can then be used by the parasites for nutritional requirements needed to survive. In addition, parasite-derived arginase may also be used by the parasite to influence the

competitive outcome between arginase and iNOS. Additional arginase enzymes, and hence increased activities, will further deplete the available arginine in the cell, making the competition for arginine greater for iNOS. The aim of this project was to investigate the role of *Leishmania*-derived arginase on survival and immunopathogenesis of *Leishmaniasis in mice*.

Hypothesis:

Leishmania-derived arginase is vital for disease pathogenesis and it influences the host immune response.

Objectives:

1. To determine the role of parasite-derived arginase in infectivity (*in vitro* and *in vivo*) of *L. major*;
2. To determine the influence of parasite-derived arginase in production of nitric oxide by infected macrophages; and
3. To determine the influence of parasite-derived arginase on CD4+ T helper cell response *in vivo*.

2 Materials and Methods

2.1. *Mice*

Female BALB/c mice (6-8 weeks old) were purchased from the GMC mouse colony of the Central Animal Care Services (CACS), University of Manitoba. Mice were maintained in the Level 2 facilities of the CACS according to the standards recommended by the Canadian Council for Animal Care.

2.2 Parasites and infections

Leishmania major strain LV39 clone 5 promastigotes (Rho/SU/59/P; [WT]) were grown in M199 medium supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, 2 µg/mL of biopterin, 5 µg/mL of hemin, 1.0 µg/mL of biotin, 0.1mM of adenine, and 40 mM of HEPES (pH 7.4; complete M199 medium). The homozygous *arg*- (arginase knockout) mutant and parasites complemented with Arginase, termed *arg*-/+ (arginase add back), all derived from the WT clone, were kindly provided by Dr. Steve Beverley (Washington University, St Louis MO). The parasites were also maintained in complete M199 medium supplemented with putrescine (*arg*-). For infection of mice, parasites were washed three times in phosphate-buffered saline (PBS) and 50 µL containing 5 x 10^6 parasites were injected into the right hind footpad. Infected mice were monitored for development of footpad lesion, which was measured with calipers.

Parasite quantification

Parasite burden in the infected footpad was quantified by limiting dilution. Briefly,

infected feet were cut just above the ankle joint, rinsed in 70% alcohol and homogenized with a tissue grinder. The homogenates were plated in 10-fold serial dilutions in Grace's insect medium starting at a 1/100 dilution. Each sample was plated in quadruplicates, and the mean of the negative log parasite titer was calculated after 7 days. Results were presented as means $\pm$ SEM for 3-5 mice per group.

Cell culture and Cytokine production

At different times after infection, groups of mice were sacrificed and single cell suspensions from draining lymph nodes of infected mice were made. Cells (4×10^6/mL) were plated in 24- or 96-well plates (1 mL or 200 µL, respectively) in complete tissue culture medium (DMEM supplemented with 10% FBS, 5×10^{-5} µM 2-Methoxyethanol (2-ME), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin) and stimulated with 50 µg/mL soluble leishmanial antigen (SLA), freezed-thawed antigen (FTAg) or 1 µg/mL anti-CD3 and anti-CD28 monoclonal antibody. Cells were incubated at 37^{o}C for 72 hr and supernatants assayed for cytokines by ELISA.

Proliferation assays by [^{3}H] thymidine incorporation

Lymph node cells, obtained from sacrificed mice as above, were cultured in complete medium and stimulated with soluble anti-CD3 (1 µg/mL) and anti-CD28 (1 µg/mL) antibodies or SLA (50 µg/mL). After 72 hr, the cultures were pulsed with [^{3}H] thymidine (0.5 µCi) overnight and incorporation of thymidine assessed by a scintillation counter.

Bone marrow derived macrophages

Bone marrow cells were obtained by flushing out the bone marrow of murine femur bones. Bone marrow derived macrophages (BMDM) were derived from bone marrow cells by growing them on Petri dishes in complete tissue culture medium supplemented with 30% L929 cell-conditioned medium. After 7 days of incubation at 37°C, adherent cells were harvested from the plates using disposable cell scrapers, washed twice, and suspended at a concentration of 1 x 10^6 cells/mL in 5-mL polypropylene tubes. Some tubes were primed with 10 U/mL IFN-γ and 100 ng/mL LPS (L6143; Sigma-Aldrich, St. Louis, MO) for 4 hr and infected in suspension cultures with stationary phase *L. major* promastigotes at a 4:1 parasite:cell ratio. After 3 hr, the cells were washed twice to remove excess parasites and incubated at 34°C. At the indicated times, the percentage of infected cells and number of parasites per 100 macrophages were enumerated from staining cytospin preparations with Wright Giemsa. The supernatant fluid was collected and assayed for the presence of cytokines by ELISA (see below) and nitric oxide (NO) by the Greiss assay.

Peritoneal macrophage cells

Peritoneal macrophage cells were obtained by injecting about 10 mL of RPMI medium into the peritoneal cavity using a 21-gauge needle. The cell suspension was washed, counted and re-suspended in medium for use in experiments.

2.3 Cytokine Determination

Cytokines (IL-4, IL-10 and IFNγ) in the supernatants of the tissue culture experiments were determined by ELISA using paired antibodies according to the manufacturers' suggested protocols (Groovy Blue Genes Biotech Ltd, Vineland, ON). The measuring range of the ELISA tests for all three cytokines was 31.25 pg/mL to 2000 pg/mL.

Intracellular cytokine staining was also performed to detect the presence of IL-4, IL-10 and IFNγ by flow cytometry, using a fluorescence-activated cell scanner (FACs machine) and Cellquest pro program. After incubation, the tissue culture cells were harvested and washed in FACS buffer (PBS with 0.1% bovine serum albumin (BSA) and 0.1% Na azide). The cells were incubated for five minutes on ice with Fc Block (1μg/test tube) then surface stained (CD4-PE at 1.25 μL/20μL). After a 25 minute incubation period on ice, the cells were washed and fixed with 0.5 mL of four percent pariformaldehyde in PBS. They were incubated on ice for 15 mins, washed, and then 1 mL of saponin buffer (FACS buffer containing 0.1% saponin) was added. After another ten minute incubation period, the cells were spun down and the labeled anti-cytokine antibodies (Ab) at pre-determined concentrations were added at 20 μL/test tube. The Ab were diluted with saponin buffer at the following concentrations: IL4-APC at 1.25 μL/tube, IFNγ-FITC at 0.5 μL/tube, and in a separate tube, IL10-APC at 1.25 μL/tube. The cells were incubated on ice with the antibodies for 30 mins, and then washed with saponin buffer. The cells were subsequently washed with 2 mL of FACs buffer, and re-suspended in 0.3 mL of FACs buffer for acquisition.

2.4 Measurement of nitric oxide production

Nitric oxide rapidly reacts with oxygen to form nitrite and nitrate which are relatively stable *in vitro* and thus can be used for quantification of NO (Moncada *et al*. 1991). Nitrite concentration in the culture supernatant was measured by the Griess reaction according to a previously described microassay (Ding *et al*. 1988). Briefly, 50 µL of the supernatants from the cultures was added to a fresh 96 well plate, and a standard curve was prepared with a $NaNO_2$ stock solution, starting at 125 µM concentration. The testing solution was made immediately before addition and was comprised of a one to one ratio of sulfanilamide (1%) in 2.5% H_3PO_4 (Sigma) and napthylethylenediamine dihydrochloride (0.1%) in 2.5% H_3PO_4. One hundred µL of this testing solution was added to each well in the 96-well plate. The reaction was allowed to proceed for a minimum of ten minutes before reading at 538nm on an ELISA plate reader. The sensitivity of this assay was 2 µM.

2.5 Statistical Evaluation

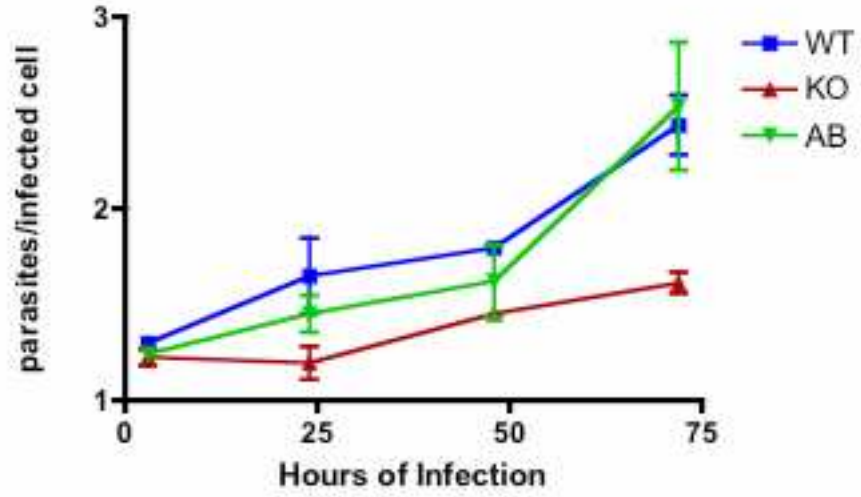
Results were analyzed by analysis of variance (ANOVA) tests, and Student's paired t-test at the stated confidence levels.

3 Results

3.1 Infection of bone marrow-derived macrophages with wild type, arginase knockout and arginase add back *Leishmania major* parasites.

Three independent experiments were performed *in vitro* with bone marrow-derived macrophages to investigate the effect of deletion of the gene encoding arginase enzyme on infectivity and proliferation of *Leishmania major*. Stationary phase promastigotes of wild type, arginase gene deficient (*arg*-) and arg- complemented with arginase gene (add back) were used to infected bone marrow-derived macrophages and the numbers of intracellular amastigotes were counted at 24, 48 and 72 hours. The results show both the wt and arg- parasites can infect macrophages with initial similar efficiency (Figure 2), suggesting that deletion of this gene has no effect in the entry of parasites into macrophages. While the number of parasites inside infected macrophages increased over time in cells infected with wt, indicative of proliferation, the number of parasites remained fairly stable in cells infected with arg- parasites. The number of parasites inside infected cells was significantly lower in cells infected with arg- than in those infected with wt at 24, 48, and 72 hr. As expected, the behavior of add back parasites was similar to that with wt, suggesting that the inability of the arg- to proliferate was due to specific deletion of this gene and not as a result of non-specific effects resulting from the genetic manipulation process. These results suggest that parasite-derived arginase plays an important role in survival of leishmania inside infected macrophages.

A)



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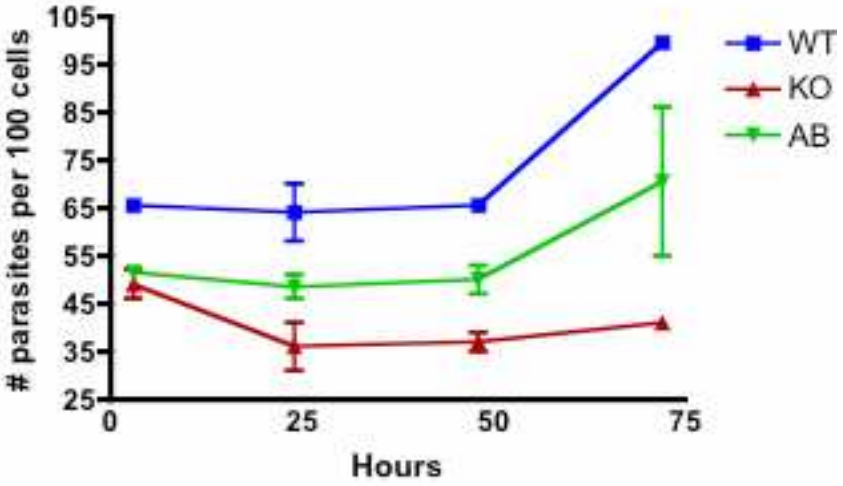
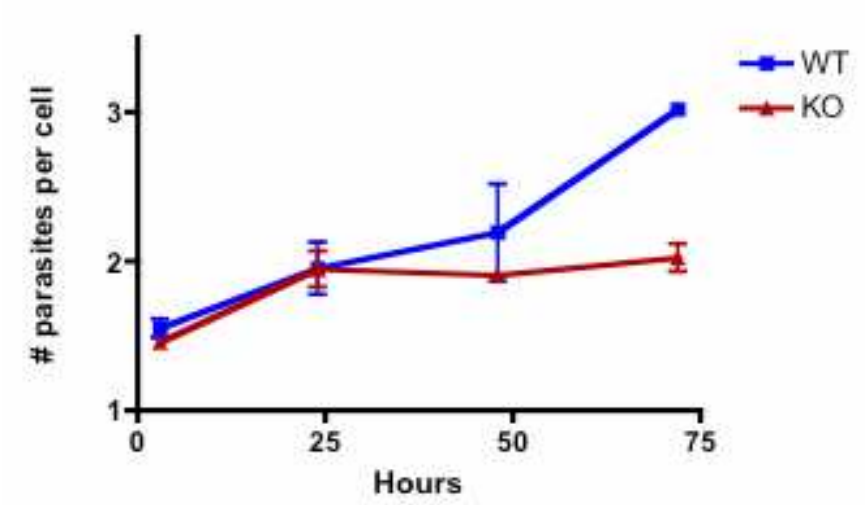


Figure 2: Infectivity and growth (proliferation) pattern of wild type (WT), arginase knockout (KO), and arginase add-back (AB) *Leishmania major* parasites in bone marrow-derive macrophages (BMDM). BMDM were infected with parasites (see materials and methods) and the proliferation of parasites was determined at indicated times by staining cytospin preparations with Giemsa stain. A) The number of parasites present for each observed infected macrophage. B) The total amount of parasites present per 100 (non-infected and infected) observed macrophages.

3.2 Infection of peritoneal macrophages with wild type, arginase knockout and arginase add back *Leishmania major* parasites.

The previous experiment was performed using BMDM differentiated *in vitro* from bone marrow cells with L929 supernatant fluid. To investigate whether the pattern of infectivity observed with *in vitro* differentiated cells will occur in naturally differentiated macrophages, the experiment was repeated using peritoneal macrophages. These macrophages were obtained by flushing the peritoneum with normal (RPMI) medium. As with BMDMs, there was no difference in the initial infection of these cells by wt and arg- parasites (Figure 3). However, consistent with the BMDM data, the number of parasites inside each infected macrophage (Figure 3A), as well as the total number of parasites per 100 cells were significantly higher in cells infected with wt than with arg- parasites (Figure 3B). Furthermore, while the percentage of macrophages that infected with wt parasites increased significantly as with time, cells infected with arg- parasite showed only a slight increase in percentage, followed by a continuing decrease over time (Figure 3C). After 72 hours of incubation, there was a significant difference ($p < 0.005$) in the total number of parasites per infected cell, with the arg- parasites having a much lower number.

A)



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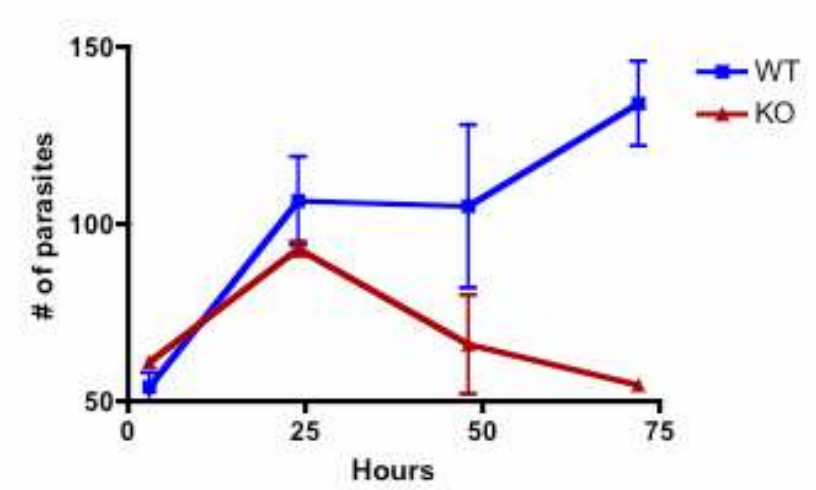


C)

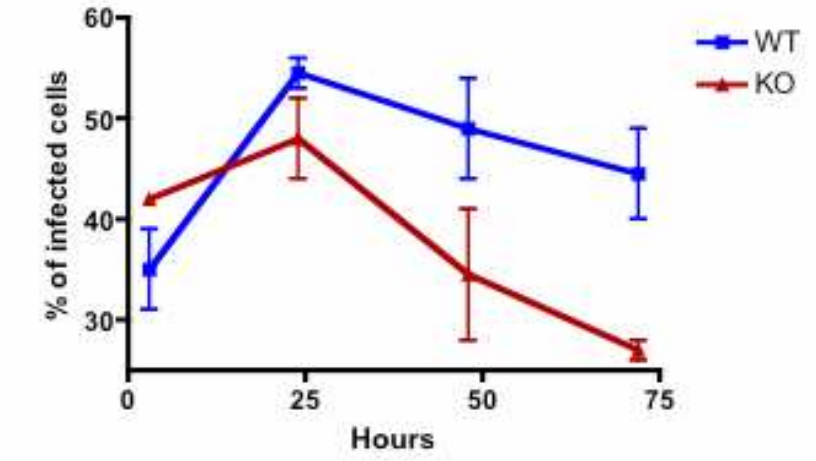


Figure 3: Infectivity and growth (proliferation) pattern of WT and arginase KO *Leishmania major* parasites in non-elicited peritoneal (primary) macrophages from BALB/c mice. Peritoneal macrophages obtained by flushing the peritoneum of mice with medium were infected with WT and KO parasites (see materials and methods) and the proliferation of parasites was determined at indicated times by staining cytospin preparations with Giemsa stain. A) Average number of parasites per infected cells. B) The total amount of parasites present per 100 (non-infected and infected). C) Total percentage of infected cells at the indicated times.

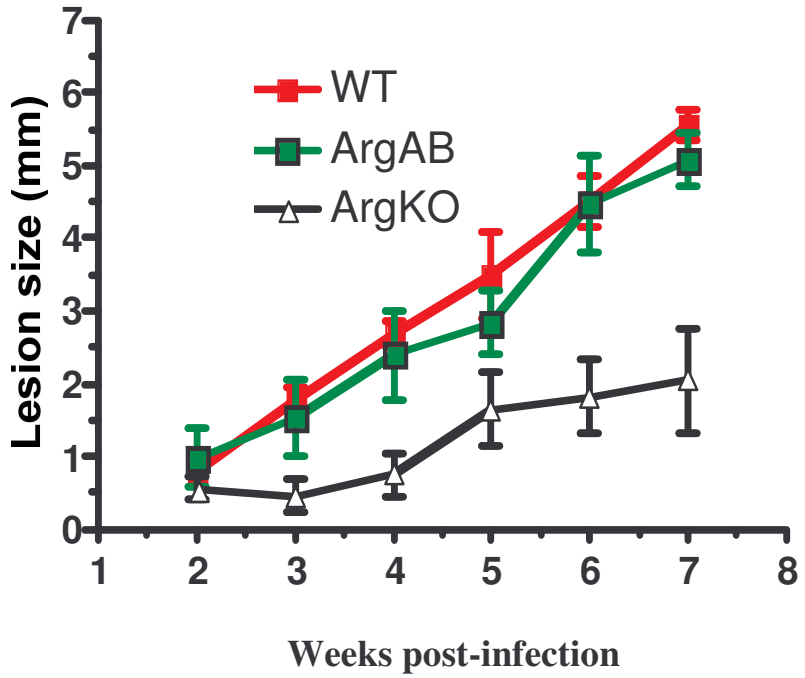
3.3 *In vivo* studies with wild type and arginase knockout parasites

BALB/c mice were infected with 5 million stationary phase promastigotes of wt, arg- or add back, and the lesion sizes were monitored weekly. As shown in Figure 4A, mice infected with wt parasites developed progressive lesions that began to ulcerate around 8 weeks and hence mice were euthanized. This development of progressive lesion was associated with massive parasite proliferation in the infected footpad (Figure 4B). In contrast, mice infected with arg- parasites showed delayed lesion development that was non-progressive (Figure 4A). These mice also had significantly lower parasites in their footpad (Figure 4B). The lesion progression and parasite numbers in mice infected with add back parasites were similar to those obtained with wt parasites. These results indicate that deletion of arginase gene in *L. major* is associated with reduced pathology *in vivo* in mice.

3.4 *In vivo* immune response of Balb/c mice infected with wild type and arginase knockout parasites.

To investigate the influence of parasite-derived arginase on the host early immune response, infected mice were sacrificed at different times after infection. The draining popliteal lymph nodes were collected, made into single cell suspension and cultured *in vitro* in the presence or absence of SLA (antigen). After 72 hr, the cells were pulsed with ^{3}H-thymidine to measure proliferation. Infection with *L. major* caused a marked increase in the number of cells in the draining popliteal lymph node three days after infection

A)



B)

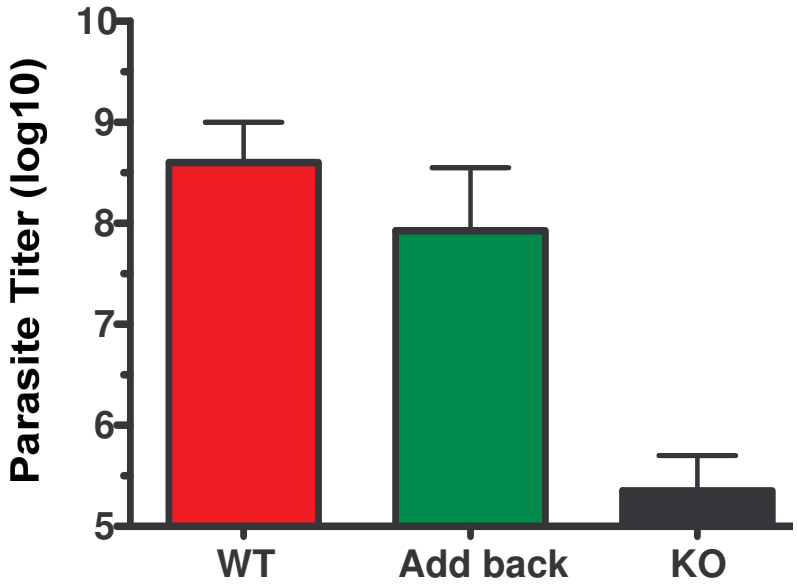


Figure 4: Kinetics of footpad lesion progression (A) and parasite burden (B) in BALB/c mice infected with WT, KO and AB L. major. BALB/c mice (6 mice per group) were infected in the right hind feet with 5 million stationary phase promastigotes of each parasite and footpad swelling was measured weekly with calipers. Eight weeks after infection, mice were sacrificed and parasite burden in the infected feet was determined by limiting dilution.

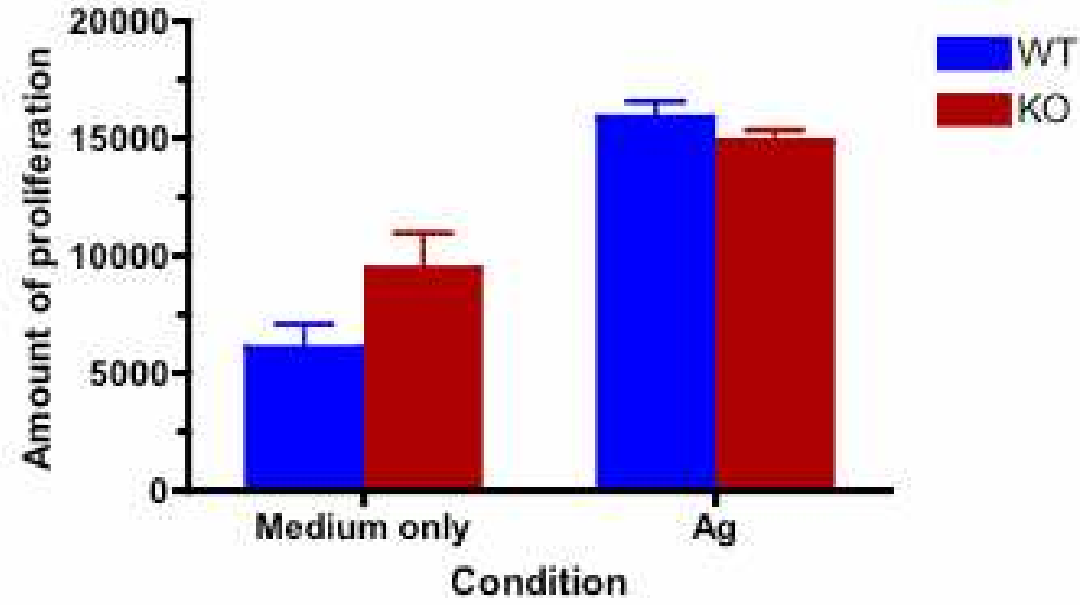


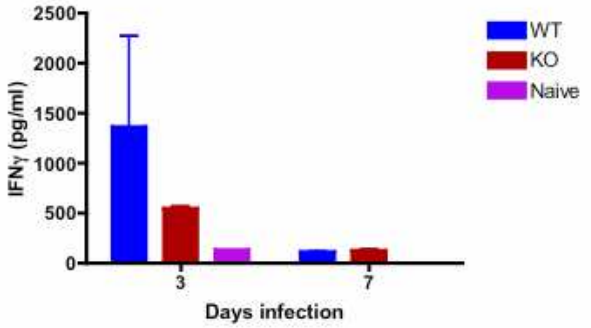
Figure 5: Antigen-specific proliferation of cells from the draining lymph node (dLN) of mice infected with wild type (WT) and knockout (KO) parasites. BALB/c mice were infected with 5 million stationary phase promastigotes from either type of parasite and sacrificed 3 days later. The dLN cells were stimulated with freeze-thawed Leishmania antigen (Ag) for 4 days then pulsed with ^{3}[H] thymidine. Proliferation was measured by measuring the number of cells using a scintillation counter.

(Figure 5). However, there was no significant difference in cell numbers in lymph nodes (LNs) from mice infected with wt and arg- parasites, suggesting that the absence of parasite-derived arginase does not influence the early inflammatory response against *L. major*. Similarly, there was no difference in antigen-specific proliferative response of cells taken from mice infected with wt and arg- parasites.

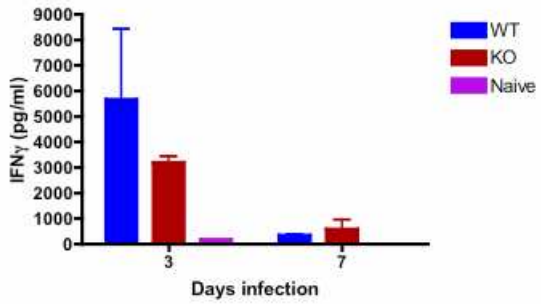
3.5 Cytokine (IL-10, IL-4 and IFN-γ) responses of mice infected with wt and arg- *L. major* parasites.

The level of arginase activity has been shown to influence the nature of CD4+ T cell response *in vivo*. We speculated that parasite-encoded arginase could raise the overall level of arginase in infected cells and hence influence the cytokine response. Therefore the levels of cytokines were measured in the supernatant fluids of cells from mice infected with wt or arg- parasites. As shown in Figure 6, there was a strong induction of antigen-specific IL-4, IL-10 and IFN-g responses following infection with *L. major*, compared with uninfected, or naïve, controls. Surprisingly, there was no significant difference in the production of these cytokines by cells from mice infected with wt and arg- parasites at all points analyzed. Similar results were obtained following polyclonal stimulation of the cells with anti-CD3/anti-CD28. Paradoxically, this trend was maintained even at 4 weeks despite the fact that mice infected with arg- parasites had several fold lower number of parasites than those infected with wt or add back parasites (Figure 7). These results suggest that parasite-derived arginase has no significant influence on the host cellular immune response. However, they suggest that reduction in parasite numbers observed in arg- may be related to alternative mechanism(s) that

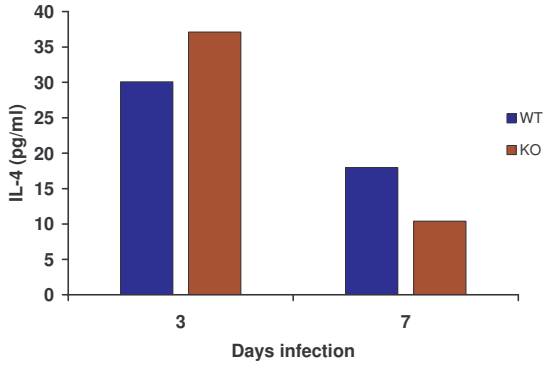
A) IFNγ detection in cells in medium



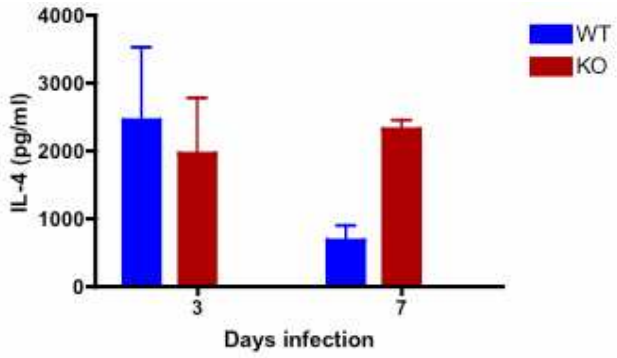
B) IFNγ detection in cells stimulated with SLA



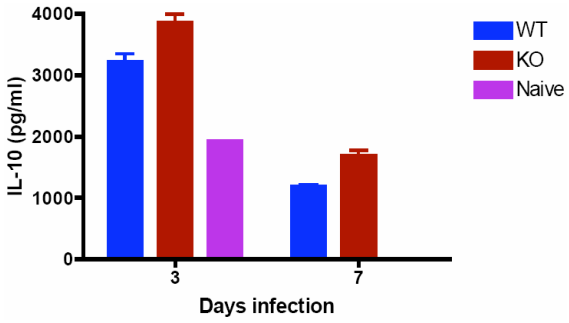
C) IL-4 detection in cells in medium



D) IL-4 detection in cells stimulated with SLA



E) IL-10 detection in cells in medium



F) IL-10 detection in cells stimulated with SLA

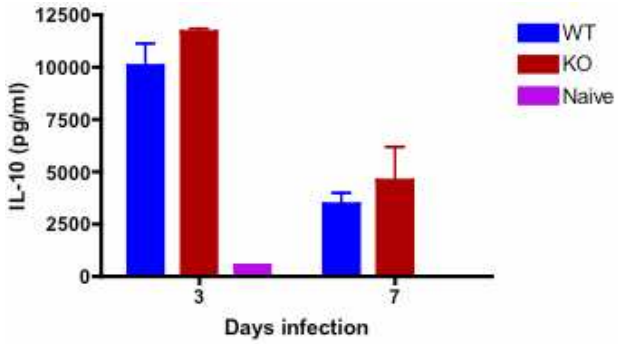


Figure 6: In vivo early cytokine responses in mice infected with WT and arginase KO parasites. Cytokine detection in Balb/c infected mice with wild type and arginase knock out parasites using ELISA detection method. A) Cells were not stimulated with anything, but left in their medium and analyzed after three and seven days of infection for the presence of IFNγ. B) Cells were stimulated with SLA and analyzed after three and seven days of infection for the presence of IFNγ. C) Cells were not stimulated with anything, but left in their medium and analyzed after three and seven days of infection for the presence of IL-4. D) Cells were stimulated with SLA and analyzed after three and seven days of infection for the presence of IL-4. E) Cells were not stimulated with anything, but left in their medium and analyzed after three and seven days of infection for the presence of IL-10. F) Cells were stimulated with SLA and analyzed after three and seven days of infection for the presence of IL-10.

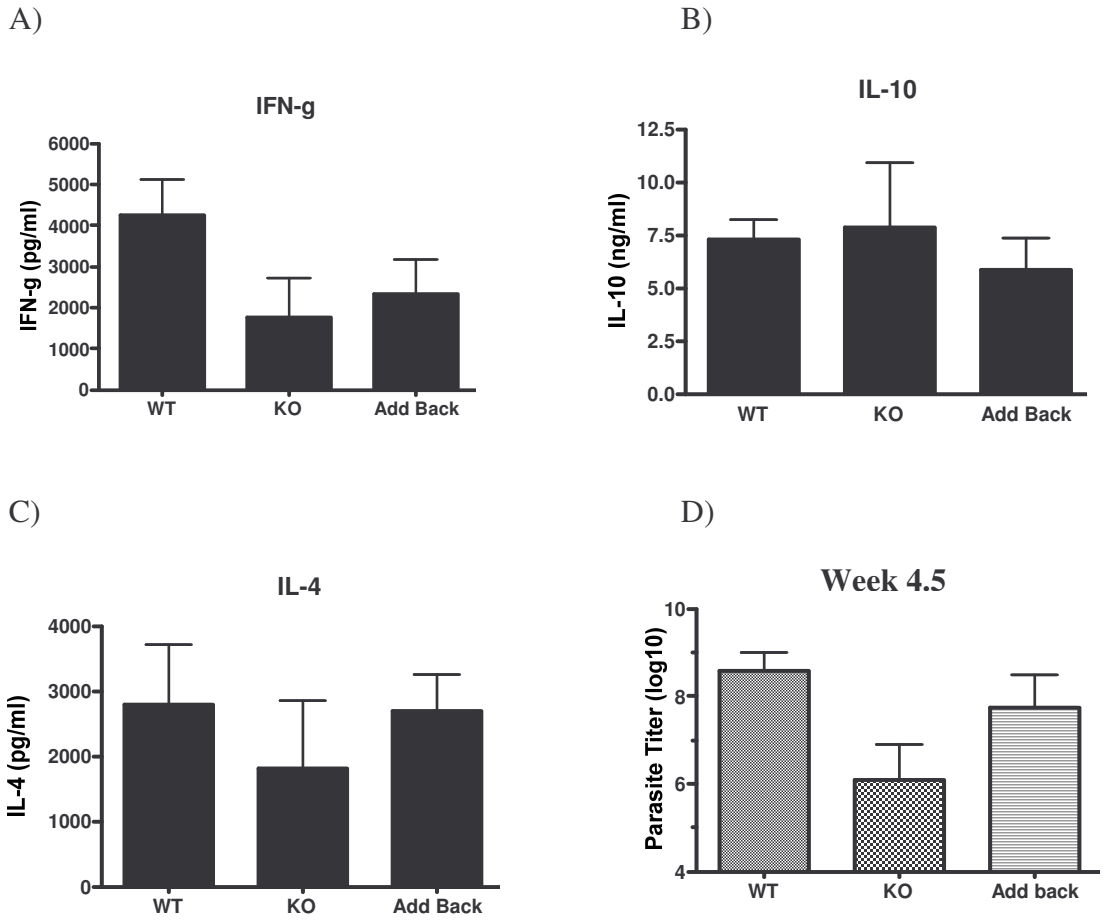


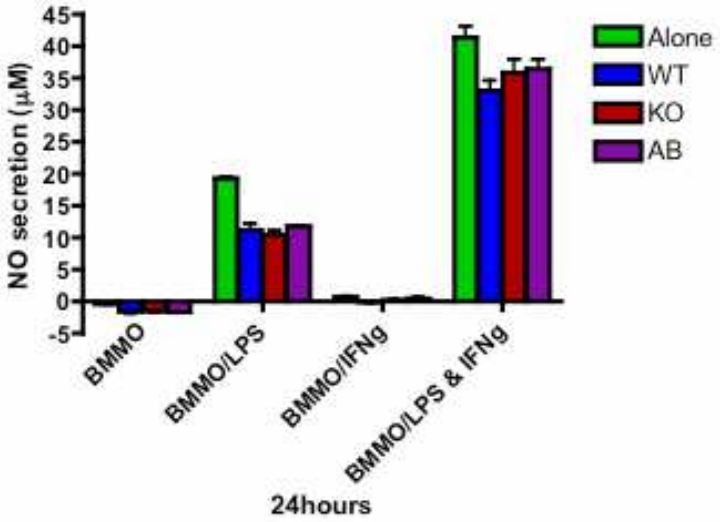
Figure 7: Analysis of cytokine production and parasite load in mice infected with either the wild type, arginase knock out or arginase add back parasites, after 4 weeks of infection. Cells from dLNs were cultured for 3 days in the presence of 50 μg/ml soluble Leishmania antigen (SLA). The supernatant fluids were then harvested and assayed for IFN-γ (A), IL-10 (B) and IL-4 (C) by ELISA. At the time of sacrifice, parasite burden in the infected feet was also determined by limiting dilution (D).

regulate parasite proliferation that is independent of the classical Th1/Th2 cytokine regulation of immunity to *L. major*.

3.6 Nitric oxide production in the supernatants of tissue cultures infected with the wild type, knockout, or add back parasites, then stimulated with either LPS, IFNγ, or both.

Parasite-derived arginase may also be used to influence the competitive outcome between arginase and iNOS. Additional arginase enzymes, and hence increased activities, could deplete the available arginine in the cell, making the competition for arginine greater for iNOS. Hence we speculated that in the absence of parasite-derived arginase, the activities of iNOS may be enhanced. Bone marrow derived macrophages were infected with wt or arg- *L. major* and stimulated with IFN-g, LPS or both (to enhance iNOS activity) and the production of NO (nitrite) was measured by Griess assay. The cell cultures that were not stimulated, or were only stimulated with IFNγ, did not have any measurable nitric oxide production after 24, 48 or 72 hours of infection (Figure 8A, 8B, 8C, respectively). In contrast, cells stimulated with LPS or LPS + IFN-g produced significant amounts of NO. There seem to be a trend that infection with *L. major* decreases the amount of NO produced by macrophages. However, there were no differences in NO production between cells infected with wt and arg- parasites at any point tested.

A)



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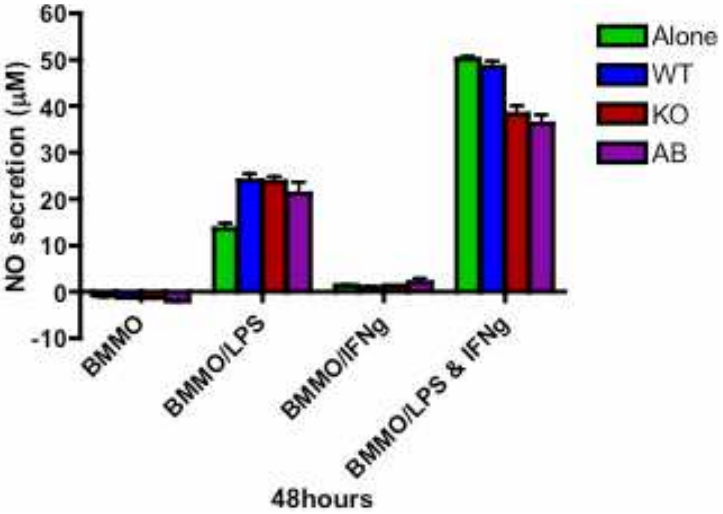

C)

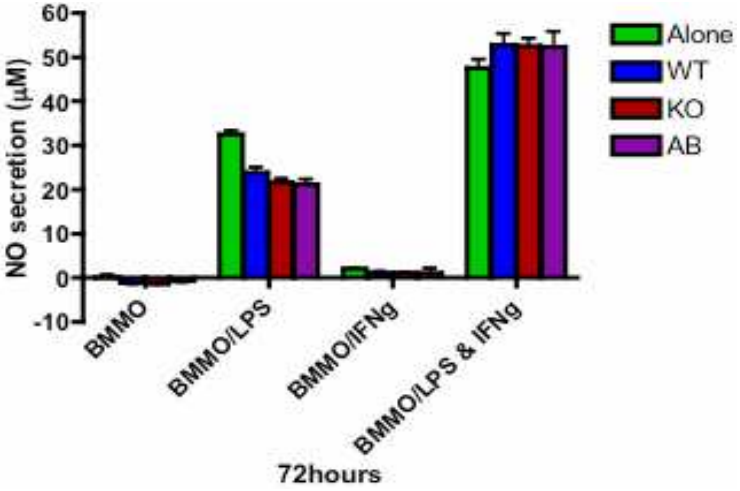

Figure 8: Nitric oxide detection in bone marrow derived macrophage cell cultures not infected (alone), or infected with wild type (WT), arginase knockout (KO), and arginase add back (AB) parasites using the Greiss assay method. Cultures were stimulated with LPS, IFNγ or both. A) Cells were incubated for 24 hours after simultaneous infection and stimulation. B) Cells were incubated for 48 hours after simultaneous infection and stimulation. C) Cells were incubated for 72 hours after simultaneous infection and stimulation.

4 Discussion

Killing of *L. major* parasites inside infected cells is mediated by nitric oxide, enhanced by IFN-g produced by activated CD4+ Th1 cells. The production of nitric oxide in macrophages is regulated by several factors including the availability of the substrate, arginine, and competition by arginase, another enzyme whose substrate is arginine. This study focuses on determining the effect of parasite-derived arginase on the host immune response leading to control of parasite proliferation. The effects of arginase enzyme on *Leishmania major* growth have been addressed by a number of previous studies. The synthesis of polyamines from ornithine following the degradation of arginine by arginase is essential for *Leishmania* proliferation (Iniesta *et al*. 2001). Thus, the availability of arginase in the host cell is crucial for parasite viability. *Leishmania* organisms have a gene that encodes arginase. Interestingly, parasite-derived arginase has been shown to metabolize host arginine to provide polyamine precursors (Roberts *et al*. 2004). Alternatively, parasite-derived arginase may also be used to suppress Th1 response and favor the production of Th2 cytokines, thereby allowing the parasite to evade the host's immune response (Iniesta *et al*. 2001). Here, it was shown that parasite-encoded arginase is vital for optimum parasite survival inside infected host cells. Interestingly, infected host cells contained detectable numbers of parasites at all times investigated suggesting that there may be a parasite encoded independent pathway to salvage polyamines. Similar results were obtained, when mice were infected with arg- parasites, where there was a persistence of parasites at infected sites with minimal pathology. The mechanisms by which arg- parasites salvage polyamines were not tested in this study.

The expression level of arginase in host cells is regulated by the balance between Th1 and Th2 cytokines produced by activated CD4+ T helper cells. It was found that in spite of lacking arginase activity, arg- induced early inflammatory and cytokine responses comparable to wt parasites. Furthermore, there was no difference in the induction of key CD4+ T cell cytokines ((IL-4, IL-10 and IFN-g) following infection with wt and arg- parasites. Because it was previously shown that arginase levels could also influence Th1 and Th2 response, it was speculated that parasite-derived arginase could raise the overall cellular concentration and activity of this enzyme and therefore regulate the cytokine responses *in vivo*. However, it was shown that this does not seem to be the case. Furthermore, we found no significant differences in NO production by macrophages infected with wt or arg- parasites. These results refute the hypothesis that additional arginase enzyme shifts the immune response in the favor of the arginase pathway.

Although the proliferation of arg- parasites in macrophages was severely impaired, these parasites were not completely destroyed. The levels of the parasites inside infected cells were found to plateau with time. These results would suggest that lack of proliferation is possibly related to diminished polyamine synthesis, rather than enhanced NO-mediated killing. This would be the main reason for the observed reduction in proliferation of arg- compared to wt parasites. The implication of the lingering parasites in macrophages *in vitro* and in infected mice *in vivo* supports the view that the additional arginase activity (parasite-derived) does not enhance parasite infection by helping to evade the host's immune system, but rather does so by allowing the required optimal amounts of nutrients for optimum parasite proliferation to be produced. It will therefore be highly interesting to investigate the growth and disease pathology of arg- parasites in

iNOS deficient mice, which are incapable of metabolizing arginine via the iNOS pathway.

5 Conclusion

1. The arginase knock out parasites were found to be unable to proliferate inside the infected macrophage, indicating that the parasite-derived arginase has a role in parasitic survival.
2. There was no significant difference in NO production between macrophage cells infected with either the wild type, knock out, or add back parasites, indicating that the immune pathway of the cell was not altered by the presence of the parasite-derived arginase.
3. Though the knock out parasites were not able to proliferate inside the infected macrophage, they were also not completely destroyed, further indicating that the parasite derived arginase does not alter the immune response of the cell, but is solely present to provide nutrients for the parasite.

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