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Leishmania persistence and host cell interactions

Michael Mandell
Washington University in St. Louis

WASHINGTON UNIVERSITY IN SAINT LOUIS

Division of Biology and Biomedical Sciences

Program in Molecular Microbiology and Microbial Pathogenesis

Dissertation Examination Committee:
Stephen M. Beverley, Chair
Keril J. Blight
Mark J. Miller
L. David Sibley
Herbert W. Virgin
Joseph P. Vogel

LEISHMANIA PERSISTENCE AND HOST-CELL INTERACTIONS

by

Michael Aaron Mandell

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ABSTRACT OF THE DISSERTATION

Leishmania parasites are the causative agent of leishmaniasis, a neglected tropical disease. An important aspect of *Leishmania* biology is asymptomatic parasite persistence, which typically occurs after clinical cure. Persistent parasites remain enigmatic despite their importance as reservoirs for transmission, having roles in maintaining protective immunity, and posing the risk of reactivation. I developed methods for assessing parasite replication by BrdU labeling and showed that persistently infected mice harbor two sub-populations of *L. major*, one labeling similarly to acute-phase parasites, with the other showing much less labeling. That persistent parasite replication occurs without a commensurate increase in parasite number implies parasite killing. Continual parasite replication and destruction within antigen presenting cells provides an attractive model explaining the role of persistent parasites in maintaining immunity, namely through constant presentation of antigens derived from dead parasites and subsequent immune boost. While many of the persistent parasites are within host cells expressing high levels of iNOS, there is no apparent correlation between this and the parasite's survival/replication status. Attenuated *lpg2*- *L. major*, a proposed model of parasite persistence, resemble WT persistent parasites for most parameters tested. However, more *lpg2*- parasites are associated with host cells expressing elevated levels of arginase 1, which further studies implicate as a negative correlate of immunity. While persistent parasites immunize their hosts against pathology from subsequent infection, experiments using marked parasites showed that persistently infected mice could be super-infected. This has implications for the generation of parasite phenotypic diversity, as genetically distinct parasites could be simultaneously transmitted to sand flies, the site of parasite

sexual recombination. In addition to my studies of *Leishmania* persistence, I also identified markers that differentiate amastigote- from metacyclic-stage parasites, and used them to assay parasite differentiation within different host cell types *in vitro*. Although the markers were induced in the same sequence in all host cell types, the parasites in bone marrow-derived macrophages and dendritic cells were slower to lose LPG expression and resume replication. These data show that invading *L. major* can retain virulence factors, potentially playing a role in situations where parasites are transferred from one host cell to another.

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Introduction

This chapter was written entirely by M. Mandell.

Leishmaniasis, a neglected tropical disease

'Neglected diseases' are defined as diseases that are not major focuses of the pharmaceutical industry despite great global demand for improved treatments and/or vaccines (1). The spectrum of diseases caused by infection with protozoan parasites of the genus *Leishmania*, collectively referred to as leishmaniasis, is a prime example of such a disease. Leishmaniasis is common throughout tropical, sub-tropical, and temperate regions of the Americas, Europe, Asia, and Africa, with an estimated 12 million people infected and more than 350 million people at risk (2). However, as the overwhelming majority of cases are amongst the very poor in the developing world, there is little profit motive for the development of new cures or preventative measures.

The clinical manifestations of leishmaniasis are quite diverse, with different species of *Leishmania* or even by different isolates of the same *Leishmania* species causing drastically different pathologies (2). In general, there are three classifications of disease manifestations. The first is cutaneous leishmaniasis (CL), which is defined by non-life threatening skin lesions of varying severity. One species which causes CL is *Leishmania major*, the organism that is the focus of my work. The second major type of leishmaniasis is muco-cutaneous disease (MCL), which is the prevalent form of the disease in some parts of Latin America. MCL patients present first with a skin lesion at the site of *Leishmania* infection, but eventually develop lesions of the mucus membranes of the face that are very severe and disfiguring despite the fact that very few parasites are found in those lesions (2). The third disease manifestation is visceral leishmaniasis, in which parasites of *L. donovani* or *L. infantum* disseminate to the spleen and liver of infected patients and there replicate to high titers, resulting in disease that is fatal if untreated (2).

Perhaps a fourth classification of leishmaniasis could be "asymptomatic leishmaniasis" as most human infections with *Leishmania* result in asymptomatic persistent infections that last for the rest of the patient's life (2). Although such infections may actually be beneficial under normal circumstances by conferring protective immunity against subsequent *Leishmania* infections (3), they pose a substantial risk of reactivation resulting in severe disease in the event that the patient becomes immunosuppressed (4). Reactivation has become a serious problem as the prevalence of HIV/AIDS has increased in regions in which leishmaniasis is common (5).

Vaccines and treatments for leishmaniasis

There is currently no vaccine to prevent leishmaniasis caused by any *Leishmania* species (2). However, there is reason to hope that the development of such a vaccine might be possible, as CL patients with healed lesions are protected against pathology from subsequent infections (3). This knowledge is the basis for the centuries-old practice of leishmanization, in which live virulent parasites are intentionally infected into an inconspicuous site on the body such as the buttocks to protect against pathology in a more visible site. To date, many different vaccination approaches (e.g. live-attenuated parasites, recombinant parasite proteins, etc.) have been tried in a laboratory setting with varying success (6, 7).

Several drugs are available to treat leishmaniasis, but all have serious drawbacks. The first drugs used against leishmaniasis are based on pentavalent antimony, and have been in use since the early 20th century. While reasonably inexpensive, antimony-based treatments are associated with serious side effects including death, and have lost efficacy

as the parasites in several parts of the world, especially India, have developed resistance (8). Other drugs available include miltefosine and liposomal amphotericin B. Miltefosine is associated with severe gastrointestinal side effects and teratogenicity and its efficacy is highly dependent on the *Leishmania* species/strain infecting the patient (2). Liposomal amphotericin B is a highly effective anti-parasitic agent, but is associated with serious side-effects and its cost is beyond the reach of most of the patients who are likely to need it (2). Because of these challenges, there remains great need for new treatment options.

Overview of the Leishmania *life cycle*

Leishmania parasites alternately infect sand fly and vertebrate hosts with transmission taking place as a result of the sand fly taking a blood-meal (Figure 1-1). Once in the sand fly, the parasites differentiate into the procyclic promastigote stage, which have relatively long flagella and express the multifunctional virulence factor lipophosphoglycan (LPG) on their surface. LPG mediates attachment of these parasites to the sand fly midgut, preventing their excretion (9). Over the course of the next 10-14 days, the parasites replicate, undergo several developmental changes, detach from the midgut, and finally enter a G1 cell cycle arrest, becoming metacyclic promastigotes (10). Metacyclic-stage parasites are considered the transmissible form of the life-cycle, and consistent with this role, these parasites express virulence factors important for the establishment phase of vertebrate infection and are found within the mouth parts of the sand fly awaiting the next blood-meal (10). These parasites are then taken up by phagocytic immune cells and are eventually found within fusogenic phagolysosomes of macrophages, in which they replicate as amastigote-stage parasites. Amastigotes differ from metacyclics in several

ways, both in terms of their gene expression and morphology. Relative to metacyclics and other promastigotes, amastigotes are rounder and have much shorter flagella.

In many "textbook" versions of the *Leishmania* life cycle, metacyclic-stage parasites are depicted as interacting exclusively with macrophages, and as such, most studies of the roles of *Leishmania* virulence factors have focused on macrophages. In reality, however, this is likely an over-simplification and parasites deposited into a host encounter several different host cell types in addition to macrophages, thus complicating the typical reductionist *in vitro* studies and raising questions as to their relevance to biology. In particular, neutrophils (PMNs) and dendritic cells (DCs) have been shown by two-photon microscopy studies to infiltrate the site of parasite inoculation within a few hours after infection, where they engulf the parasites (11-13). Interactions between metacyclics and these cell types may in fact predominate over metacyclic-macrophage interactions, necessitating a re-evaluation of the textbook description of the parasite life cycle and new studies of the virulence factors required for survival within and/or parasitism of PMNs and DCs.

Although many or most parasites are found within PMNs and DCs shortly after inoculation, by 48 hours after infection most parasites at the inoculation site are found within macrophages (12). There are a number of models that could explain how this transfer occurs. One possibility is that the parasites within PMNs and DCs die and are cleared, leaving only those parasites that infected macrophages at 48 hours after infection. However, substantial evidence suggests that this is not the case as several *in vitro* studies have demonstrated parasite survival within PMNs and DCs, and the transfer of parasite-loaded PMNs into a naïve mouse yields a lesion comparable to the inoculation

of an equal number of free parasites (12, 14-17). Another possibility that is probably more relevant for DCs than for PMNs is that infected DCs could leave the inoculation site and migrate to the regional lymph node, resulting in infection of that tissue and leaving infected PMNs or macrophages at the infection site (11).

A third model is that the parasites infect PMNs/DCs first and are subsequently transferred from these cells to macrophages. Under this scenario, the parasites could enter macrophages in one of two ways. The first involves the release of the parasite from an infected DC/PMN, and then the free parasite is engulfed by a macrophage. Consistent with this, two-photon microscopy studies identified instances in which infected PMNs appeared to die, releasing an intact parasite that appeared to then be taken up by another cell (12). While this sounds similar to what likely happens with amastigote-infected macrophages in which after several rounds of parasite replication the macrophage bursts releasing amastigotes that can then infect new macrophages, it is important to note that at 48 hours after infection the parasites are unlikely to have had sufficient time to undergo substantial replication.

The other way in which sequential infection could take place is by the macrophage engulfing the infected PMN/DC in what is referred to as the “Trojan horse” model of infection (16, 18). This model primarily deals with PMNs, which are very short-lived cells and that typically die by apoptosis. In this model, metacyclic-stage parasites enter PMNs and survive, even though their host cells ultimately undergo apoptosis. Macrophages are then recruited to the site of infection to clear the apoptotic PMNs, many of which contain viable parasites. Phosphatidyl serine on the surface of the infected apoptotic PMNs induces the macrophages to adopt a deactivated phenotype, thus

allowing safe entry for the parasite into its "preferred" host cell (18). Despite much effort, *L. major* entry into macrophages within PMN "Trojan horses" has not been observed *in vivo* (12, 13). However, macrophages have been observed to phagocytose apoptotic PMN that contain apparently intact *Leishmania in vitro* (14, 16).

Regardless of the manner of parasite transfer from the first cell type infected to macrophages, one important question is whether the parasite undergoes differentiation within the host cell types that it encounters first and what stage of the parasite life cycle (metacyclic or amastigote) is encountered by the macrophage. This question has relevance for a number of temporally regulated virulence factors, including LPG, GP63 and GP46, which are known to be important in the establishment-phase of mouse infections but are not expressed on amastigotes (19-23). If most parasites enter DCs/PMNs and differentiate into amastigotes prior to encountering macrophages, then macrophages would rarely encounter these virulence factors *in vivo*, and it is likely that they have evolved for interactions with other cell types. Alternatively, the parasites may retain the expression of these virulence factors as they transit through these cells allowing for the virulence factors to subsequently impact their interactions with macrophages.

To date, the only data regarding the ability of the parasites to differentiate into the amastigote stage within non-macrophage host cells comes from studies of infected neutrophils, in which at least some of the parasites appear to remain in the metacyclic stage for up to 42 hours post-infection as assessed by retaining long, motile flagella (16). This study, however, made no mention of the percent of parasites with long flagella nor addressed other changes associated with differentiation such as the loss of promastigote-specific virulence factors. In Chapter 2, I address whether *L. major* metacyclic-stage

parasites differentiate within dendritic cells, one of the other first cell types to encounter invading parasites, and for how long they retain the expression of the early virulence factor LPG. As part of these studies, I characterized five markers that are differentially expressed between amastigotes and metacyclics. Two of these were amastigote-specific antigens that are recognized by monoclonal antisera that were first generated by Charles Jaffe (24). The localization pattern of both of those antisera was particularly interesting, and is detailed in Appendix I.

Leishmania major *infections in mice*

The typical course of *L. major* infection in susceptible and resistant mice strains is shown in Figure 1-2. Following a either sand fly vector-mediated infection or needle inoculation of mice, *L. major* parasites replicate rapidly for several weeks in the absence of overt pathology in what is referred to as the 'silent phase' of the infection (25, 26). During this phase of the infection, the leishmaniasis-promoting IL-4 is the predominant cytokine produced by infection site-derived cells (25). In 'susceptible' BALB/c mice, IL-4 production is maintained throughout the infection, resulting in Th2 polarization and, ultimately, fatal leishmaniasis (27). In contrast, in 'resistant' strains of mice such as C57BL/6, cells derived from the infection site begin to express the Th1 cytokines IL-12 and interferon-γ co-incident with the inception of lesion pathology (4-5 weeks post-infection) (25, 27). Ultimately, Th1-cytokine producing cells overwhelm IL-4 producing cells, leading to infected macrophages adopting a "classically activated" phenotype, which involves the expression of iNOS and subsequent generation of nitric oxide, a molecule that is essential for the control of *Leishmania* infection in mice (28-32). As a result, parasite number declines and the lesions eventually resolve ~12 weeks post-

infection (26). Healed mice are then protected against pathology from subsequent *L. major* infections (33).

Interestingly, despite the absence of pathology and the presence of this strong protective immune response, healed hosts continue to harbor a small, roughly constant number of viable parasites for the remainder of their life (34). These persistent parasites are important for several reasons. First, despite their limited numbers (~100 - 1000 in mice) (25, 35, 36) persistent parasites can still be transmitted to sand fly vectors, and as such, are a reservoir for the pathogen (26, 37). Second, as is the case with infected people, the parasites in persistently infected mice can "reactivate" in the event of immunosuppression, leading to severe disease (4, 29). Finally, they may be beneficial to hosts with intact immune systems because they help maintain protective immunity against pathology from subsequent *Leishmania* infections (38). In fact, treatment of persistently infected mice to achieve a sterile cure renders those mice susceptible to new infections (36). As described below, the focus of Chapters 3 and 4 is to better understand the biology of these persistent parasites.

The biology of L. major *persistence*

Asymptomatic persistence of *Leishmania* in their hosts, either in experimentally infected mice or in human patients, is a far more common result than sterile cure (34). Such infections are characterized by low parasite titers, the lack of pathology, and protective immunity against subsequent the pathology from infections. In fact, it is possible that the entry of the parasites into an asymptomatic persistent state may in fact be advantageous to the parasites, as they can still be transmitted from persistently infected hosts to new

sand flies (26, 39). Supporting this, when *L. tropica* parasites are transmitted by sand flies to their natural rodent hosts, these parasites directly enter a persistence-like state without inducing pathology (39).

Despite their importance, both medically and in terms parasite biology, very little is known about persistent *Leishmania*, perhaps resulting from the fact that no *in vitro* model exists, and from the challenges associated with studying persistence *in vivo* (e.g. long infection times and low parasite numbers). On important unknown is whether or not persistent *L. major* replicate, as the number of persistent *L. major* remains relatively constant for the lifespan of its host (35, 40). Two models could explain this constant parasite population. One possibility is that the persistent parasites could be in a quiescent state that resists killing by the host. Alternatively, the parasites could replicate as in the acute phase, but their numbers do not increase as any replication is offset by parasite destruction. This second model is particularly attractive as it may help explain the requirement of persistent parasites to maintain immunity: constant presentation of antigens from killed parasites would serve to maintain the effector T cell population necessary for anti-*Leishmanial* immunity (41). Thus, a crucial question that I address in chapter 3 is whether or not persistent parasites replicate.

It is clear from both clinical and laboratory studies that the host's immune system is responsible for maintaining the parasites in an asymptomatic persistent state. Similar to what is seen when infected persons become immunosuppressed as a result of HIV/AIDS, persistent infections 'reactivate' in mice treated with inhibitors of iNOS or interferon-γ signaling, or in which $CD4^+$ T cells have been depleted (29).

Although the host's immune response clearly is involved in preventing the reactivation of persistent parasites, the parasites appear to be able to prevent it from completely clearing the infection. Two non-exclusive models have been proposed to explain how why sterile cure is not achieved. The first model to explain how *Leishmania* persist despite their host's protective immunity against disease pathology is based on the finding that the immunomodulatory cytokine IL-10, along with immunosuppressive $CD4^+CD25^+$ regulatory T cells (Treg), are important for maintaining persistent infections, and that the blockade of IL-10 signaling or Treg depletion results in sterile cure of *L. major*-infected mice (36, 42, 43). IL-10 and Tregs are thought to facilitate parasite persistence by preventing host cell activation and iNOS expression, thus providing a safe niche in which the parasites can survive (36).

The second model is referred to as the "safe-cell" model (44). This model is based on the observation that the majority of *L. major*-infected iNOS-negative cells in persistently infected lymph nodes express fibroblast, but not macrophage, markers. In contrast, almost all infected macrophages from the same persistently infected tissue expressed iNOS (44). As the nitric oxide produced in iNOS-positive cells is lethal to *Leishmania* (29), it was proposed that these cells are sites of parasite destruction, while the fibroblasts are "safe cells" in which the parasites survive (44). Another candidate "safe cell" is the alternatively activated macrophage. These cells have been shown to support enhanced replication of *L. major in vitro* and *in vivo* as a result of their expression of arginase-I, which simultaneously blocks the formation of leishmanicidal NO while generating polyamines that the parasites can use for growth (45-47). Since first being proposed by Bogdan et al. in 2000 (44), the safe cell model has been cited 87 times, but neither the

study's findings nor the proposed model have been independently tested. Thus one of the aims of chapter 3 is to test the safe cell model. One assumption of the model is that parasites should be found in safe cells such as fibroblasts or alternatively activated macrophages at all sites of persistence (i.e. the inoculation site and the draining lymph node). As Bogdan's study focused exclusively on parasites within lymph node tissue, I sought to determine what types of host cells were infected at the footpad inoculation site to see if infection of proposed safe cells occurred at that site as well. Another assumption of the safe cell model is that iNOS-expressing macrophages are an unsafe cell in which persistent parasites would be killed. I therefore asked whether this assumption was correct or whether persistent parasites could survive within iNOS-expressing host cells.

As mentioned above, persistent parasites are difficult to study, at least in part due to the long infection periods required to establish a persistent infection in mice. Interestingly, several parasite lines (including parasites lacking fructose-1,6-bisphosphatase or the nucleotide sugar transporter LPG2) have been generated that appear to directly enter a persistent-like state without first inducing pathology, suggesting that the parasite genes required for persistence differ from those required for virulence (35, 48). The *LPG2* gene encodes a protein Golgi-localized GDP-mannose transporter which is involved in the synthesis of parasite virulence factors including phosphoglycans. Despite their long-term persistence in mice, these parasites appear incapable of replicating to high numbers and causing disease, as they are asymptomatic even in immunodeficient mice or in mice lacking iNOS (35). Nevertheless, they resemble WT persistent parasites in the number of viable parasites recoverable from infected mice and in the fact that *lpg2*- parasites are capable of generating and maintaining protective immunity against subsequent *L. major*

infections in some strains of mice (35, 49, 50). For these reasons, *lpg2*- and other "persist without pathology" parasites may be valuable both as models of *Leishmania* persistence and have potential as live-attenuated vaccine candidates if they resemble persistent wild type parasites (35). A final aim of the data presented in chapter 3 is to compare the replication and localization of *lpg2*- parasites in mice with what is seen with persistent wild type parasites.

Leishmania, concomitant immunity, and evolution

Because infected hosts are usually capable of developing an appropriate immune response, infection with *Leishmania major* is rarely fatal (51). Instead, mammalian hosts typically control the infection and heal. At the same time, while still harboring persistent parasites, they become protected against pathology from subsequent *Leishmania* infections: a condition known as concomitant immunity or premunition (52-54). Although the benefits to the host resulting from an effective anti-*Leishmania* immune response and subsequent immunity are obvious, there are likely also benefits to the parasite. One such benefit comes as a consequence of parasite persistence within a healthy host: the longer the host survives, the better the chances that the parasite will be transmitted (55-57).

The term "concomitant immunity" is most commonly used in reference to infections with parasitic worms such as *Schistostoma mansoni*, where excrement from adult worms contains antigens that promote protective immunity against juvenile worms, which are the infectious forms of the worm's life cycle. The adult worms are thought to benefit

from vaccinating their host against super-infection by eliminating intraspecific competition (52, 58).

Another potential benefit to the pathogen comes if the host's immune response is sufficiently strong to prevent super-infection, thus gaining for the first pathogen to infect the host exclusive transmission rights for its genome. A pathogen capable of such exclusive infection would have a strong selective advantage over those that could not, and the ability to generate exclusivity would likely spread quickly through a population, as has been proposed for lysogenic bacteriophage (59).

In Chapter 4, I address whether the immunity maintained by persistent *L. major* is capable of generating 'exclusivity', or in other words, if the immunity maintained by persistent *L. major* is strong enough to prevent super-infecting parasites to establish their own persistent infections. In addition to being an important evolutionary question, this question has relevance for understanding the development and maintenance of *Leishmania* phenotypic diversity, as sterilizing immunity to re-infection would greatly diminish the chances of an infected host to pass on a mixed infection to a sand fly where parasite sexual recombination could take place (60).

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Figure legends

Figure 1-1. The *Leishmania* life cycle. A. Sand flies are infected after taking a blood-meal from an infected host. The parasites differentiate into procyclic promastigotes, which are retained in the sand fly's midgut via interactions between parasite LPG and receptors on the sand fly's intestinal epithelium. B. Parasites eventually detach from the midgut and develop into infectious metacyclic-stage parasites, which are found in the mouthparts of the sand fly awaiting the next blood-meal. C. Metacyclic-stage parasites are deposited into the dermis of their mammalian host, where they interact with several different types of host cells including macrophage, dendritic cells, and neutrophils. D. Ultimately, parasites are found as amastigote forms in acidified phagolysosomes of macrophages. E. Parasites replicate within phagolysosomes eventually resulting in the lysis of the macrophage and the release of amastigotes which can either go on to infect new macrophages or be ingested by a biting sand fly, completing the cycle.

Figure 1-2. The course of *L. major* infection in susceptible and resistant mouse strains. Both graphs. Black lines represent parasite number, grey lines represent lesion pathology. Parasite numbers increase dramatically during the first ~4 weeks after infection in the absence of pathology in what is termed the 'silent phase' of infection. Top graph. Infection of 'susceptible' BALB/c mice results in Th2 skewing associated with uncontrolled parasite growth and eventually fatal leishmaniasis. Bottom graph. Infection of 'resistant' C57BL/6 mice results in Th1 skewing, associated with lesion healing and a decrease in parasite number starting 6-8 weeks post infection. Lesions typically heal 12-15 weeks after infection. Healed mice are immune to subsequent *L. major* infections and continue to harbor ~1000 parasites for the remainder of their lives (persistent phase).

Figure 1-1: The *Leishmania* life-cycle

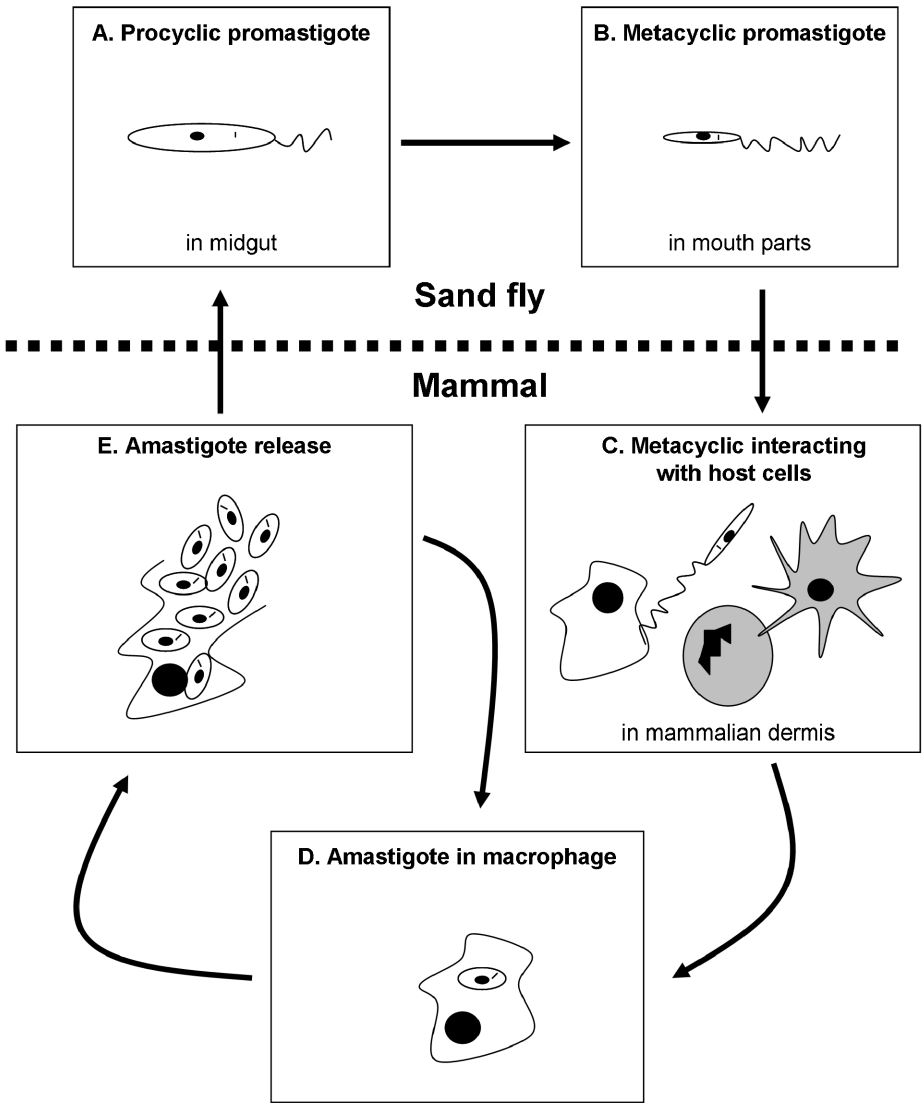
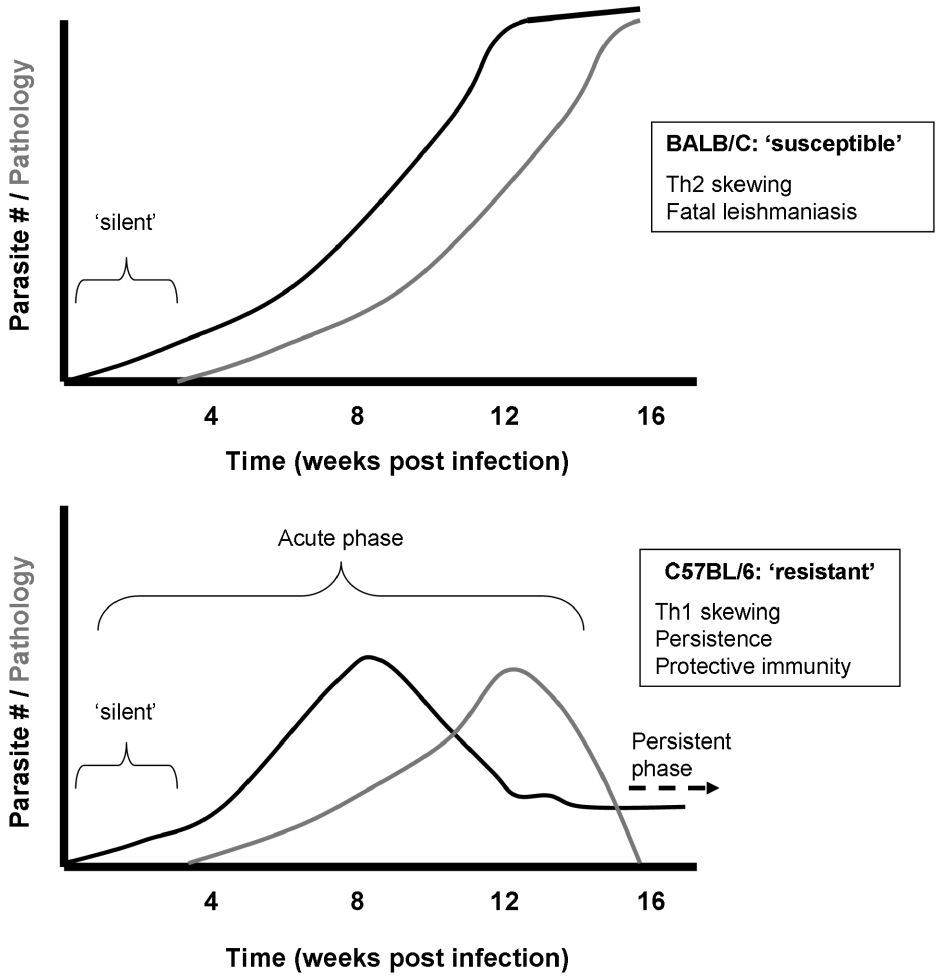


Figure 1-2: *L. major* infections of susceptible and resistant mice



Chapter 2

Running title: *L. major* stage differentiation and LPG retention in DCs

Quantitative profiling of *Leishmania* differentiation markers establishes that Lipophosphoglycan (LPG) is at high levels for extended periods of time in a large fraction of bone marrow derived macrophages and dendritic cells

Michael A. Mandell[1], Stephen A. McCartney[2], Marco Colonna[2], and Stephen M. Beverley[1,3]

[1]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis MO 63110 USA.

[2]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis MO 63110 USA.

[3]Corresponding author; Dept. of Molecular Microbiology, Washington University School of Medicine, Campus Box 8230, 660 S. Euclid Ave., St. Louis MO 63110; Tel: 314-747-2630; e-mail beverley@borcim.wustl.edu.

Abbreviations used: DC – dendritic cell; BrdU - 5-bromo-2-deoxyuridine;

Author contributions

M.A.M. performed experiments.

S.A.M and M.C. provided bone marrow-derived cells.

M.A.M and S.M.B. wrote the text.

Abstract

Many workers have proposed that neutrophils and dendritic cells (DCs) are the predominant cell types first engulfing *Leishmania* following the transmission of metacyclic parasites by a sand fly bite. Following entry into such 'transit' cells, parasites are ultimately transferred to the macrophage, although some parasites likely enter macrophages directly as well. This raises the question as to whether parasite virulence determinants whose functional roles are restricted to the earliest phases of establishment of *Leishmania* infection persist long enough in transit to play significant roles in the macrophage. One of such early-acting virulence factors include the most abundant parasite surface molecule, lipophosphoglycan (LPG). We first identified a set of experimental 'landmarks' that clearly differentiate infective metacyclic promastigotes from replicating macrophage amastigotes; these markers show an orderly transition post-infection, allowing the tempo and properties of parasite differentiation to be visualized microscopically. In peritoneal macrophages, loss of promastigote flagella and expression of amastigote differentiation markers occurs within 12 hr, followed by entry into the cell cycle as assessed by BrdU incorporation after 24 hr. LPG levels declined such that by 48 hr it was undetectable on most parasites. Bone marrow derived macrophages or DCs maintained a similar sequence of marker transitions, but with different kinetics for some but not all markers, and with fewer parasites showing evidence of replication even by 72 hr, suggesting overall a slower rate of differentiation to amastigotes. Notably LPG was lost much less rapidly in both DC and BMM, maintaining levels as high as that seen in metacyclics in 37% percent of the parasites after 72 hr, well beyond estimates of the 'transit' time to macrophages. While these LPG^{+} parasites have not re-entered the cell,

they otherwise resemble amastigotes and therefore present an intermediate phenotype. These data suggest that LPG and potentially other ‘early’ virulence factors would have the opportunity to impact macrophage function through either the transit or direct route, potentially facilitating parasite establishment.

Introduction

Leishmaniasis, caused by protozoan parasites of the genus *Leishmania*, is considered a neglected tropical disease because there is a lack of good treatment options despite many millions of people at risk of infection (1). *Leishmania* parasites are transmitted to their mammalian hosts by the bite of an infected sand fly which deposits non-replicating, flagellated metacyclic-stage parasites into the dermis while taking a blood meal. Once in the dermis, the parasites interact with phagocytic cells of the immune system, and are eventually found within lyososome-like vacuoles in macrophages where they replicate intracellularly as non-motile amastigotes.

Although many *in vitro* studies have focused on interactions of infectious parasites with macrophages, recent *in vivo* data suggest that other cell types, in addition to macrophages, also encounter and engulf invading metacyclic-stage parasites. These include neutrophils and dendritic cells (DCs), which are rapidly recruited to the infection site and collectively may predominate over macrophages as the first host cells for *Leishmania* (2, 3). However, by 24-48 hours after infection, the percentage of *L. major* within these cell types declines, such that at later time points macrophages are the predominant cell type infected (2-4). How this transfer of the parasite from the first cell type infected to macrophages is under debate, but presumably parasites are either released from the infected cell and subsequently taken up by a macrophage or, alternatively, the infected cell itself is engulfed by a macrophage (5).

Regardless of the manner of parasite transfer, an important question is whether the parasite undergoes differentiation within its cells of first contact and what stage of the parasite life cycle (metacyclic or amastigote) is encountered by the 'destination'

macrophage. The answers to these questions could have profound consequences to our understanding of the role of parasite virulence factors, especially those known to function early in the initial stages of establishment of intracellular parasitism. A number of parasite virulence factors such as lipophosphoglycan (LPG), GP63, and GP46 are temporally regulated and not expressed by amastigotes despite having important roles in the establishment of infection by metacyclic-stage parasites (6-10). Thus, invading metacyclic-stage parasites could be taken up by 'transit' host cells such as neutrophils and/or DCs, undergo differentiation and cease expression of these 'early' virulence factors prior to their transfer into macrophages. Under this scenario, macrophages would not encounter 'early' virulence factors in natural infections. Alternatively, the parasites could retain expression of the 'early' virulence factors throughout the transit period until they reach a macrophage, thus retaining the armamentarium of virulence factors for deployment within the macrophage. Virulence factor retention could be mediated by a variety of mechanisms, for example specific control of early virulence factor expression or more global mechanisms such as delayed or even arrested differentiation in the transit PMN/DC.

As an example of an 'early' virulence factor, we focused on the surface promastigote glycoconjugate, lipophosphoglycan (LPG). LPG is the most abundant molecule on the surface of invading metacyclic-stage parasites (6). Experiments with genetically-modified *Leishmania* lacking genes essential for LPG synthesis have demonstrated that LPG plays key roles in parasite survival upon infection of macrophages, including prevention of lysis by complement, protection against oxidants, and transiently inhibiting phagolysosomal fusion (7-10). In addition, LPG has been shown to be important in

protecting *L. donovani* parasites from killing by neutrophil extracellular traps (11), and in establishing a safe niche for these parasites facilitating their survival within neutrophils (12).

To address the question of whether *L. major* metacyclics differentiate into amastigotes within the cells types of 'first contact' and how long the expression of LPG is retained as the parasites transit through these cells, we identified a set of markers suitable for immunofluorescence microscopy that would allow an assessment of the differentiation status of *Leishmania* following host cell uptake. These included markers expressed by metacyclic promastigotes but not amastigotes such as the flagellum, employing paraflagellar rod proteins (13), and markers expressed by amastigotes but not metacyclic promastigotes (unknown antigens recognized by antisera T17 or T18) (14), activation of amastigote gene expression (SSU:YFP), and replication (BrdU incorporation). We compared the expression of these markers in peritoneal and bone marrow macrophages (BMMs) first, in order to establish a baseline for macrophage differentiation. For these studies, peritoneal macrophages (PEMs) serve as our gold-standard host cell because parasites are known to enter these cells and become replicating amastigotes by 72 hours post-infection (7, 15, 16). We then studied the expression of the stage-specific markers in dendritic cells, one of the first cell types to be infected *in vivo* (2). In all three cell types, we found that the markers of 'amastigotigenesis' were induced in the same order, with amastigote marker induction occurring prior to the onset of parasite DNA synthesis. We then used similar approaches to assess LPG level, and showed that even after 72 hr within DCs a high fraction of intracellular parasites retained high levels of LPG. As this is well beyond the estimated transit time of *Leishmania* through DCs in the literature (~24 hr),

these data establish that the destination macrophages will encounter a significant number of *Leishmania* retaining LPG at biologically relevant levels able to mediate virulence functions characterized previously in macrophages.

Materials and Methods

Parasite strains and culture

L. major Friedlin V1 strain (MHOM/IL/80/Friedlin; abbreviated as LmjF) parasites were grown at 26˚C in M199 medium (US Biologicals) supplemented with 40 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) pH 7.4, 50 μM adenosine, 1 μg ml^{-1} biotin, 5 μg ml^{-1} hemin, 2 μg ml^{-1} biopterin and 10% (v/v) heat-inactivated fetal calf serum (17), in some cases containing selective drugs. LmjF parasites expressing YFP (yellow fluorescent protein; *SSU:IR1PHLEO-YFP*) were described elsewhere (18). *L. major* LV39cl5 *lpg1-* were described previously and (19) were cultured in the above media supplemented with 2 mM L-glutamine, 9 μg ml^{-1} folate and RPMI Vitamin Mix (Sigma). Infective metacyclic-stage parasites were recovered using the density gradient centrifugation method (20). Prior to infection of host cells, purified metacyclic-stage parasites were opsonized with serum from C5-deficient mice.

Mammalian host cells

Cells were isolated from female C57Bl/6J mice (6-10 weeks old; Jackson Labs). Peritoneal macrophages (PEMs) were elicited by a peritoneal injection of potato starch and harvested and maintained in DMEM (Invitrogen) containing 10% FCS and 2 mM L-glutamine as described (21). Bone marrow-derived macrophages (BMMs) and dendritic cells (DCs) were harvested as describe previously (22). Briefly, bone marrow was flushed from the femurs of mice and cultured in dendritic cell or macrophage growth media at 37˚ for 6 days. DCs were cultured in RPMI media without L-glutamine (Gibco) supplemented with 10% fetal calf serum (Hyclone), Glutamax (Gibco), Na pyruvate, non-essential amino acids, and kanamycin (DC media) with the addition of 2% GM-CSF.

BMMs were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum, 5% horse serum, Glutamax (Gibco), Na pyruvate, non-essential amino acids, and kanamycin (as described above for the macrophage media) with the addition of 30% L-cell media as the source of M-CSF. For infections, cells were cultured in DC or macrophage media without growth factors. Prior to infection, PEMs, DCs, and BMMs were adhered to sterile glass coverslips in 24 well dishes overnight.

Infection of host cells

Parasites were added to host cells at a ratio of 5:1. Typically, extracellular parasites were removed by extensive washing 2 hours after parasites were added to host cells. Infected cells were maintained in the media described above, which was changed daily for the duration of the experiments. For DNA labeling studies, containing 0.1 mM 5-bromo-2'-deoxyuridine (BrdU; Sigma) or 0.1 mM 5-ethynyl-2'-deoxyuridine (EdU; Life Technologies) was added for the duration of time indicated in the text.

Antibodies

L. major nuclei were detected with a pool of rabbit antibodies raised against *L. major* histones H_2A, $H_2A_{variant}$, H_2B, H_3, and H_4 (Wong and Beverley, in preparation). Were pooled at a ratio of 3:2:3:3:1 by titer as determined by Western blot and used at a dilution of 1:750 (Wong and Beverley, in preparation). BrdU was detected with a rat monoclonal antibody (Abcam) used at 10 μg ml^{-1}. For dual-labeling experiments involving YFP, Alexafluor488-conjugated rabbit anti-GFP antisera (Invitrogen) was used at a concentration of 8 μg ml^{-1}. The amastigote-specific mouse monoclonal antisera T17 and T18 were a gift from Charles Jaffe (Hebrew University) and were diluted 1:400 (14). Paraflagellar rod (PFR) was detected with the mouse monoclonal antibody L8C4

(provided by Keith Gull), and was used at a dilution of 1:50 (13). Lipophoshoglycan (LPG) was detected using two different antisera. For most experiments, Gal-substituted LPG was detected with the mouse monoclonal antibody WIC79.3 (23), which was used at a 1:250 dilution. Where specified, "metacyclic LPG", in which most of the galactose side chains are capped with arabinose, was detected with the mouse monoclonal antibody 3F12 (23), used at a 1:100 dilution.

The following fluorescent secondary antibodies were used: Alexafluor488 goat anti-rabbit, Alexafluor555 goat anti-rabbit, Alexafluor633 goat anti-rabbit, Alexafluor488 goat anti-mouse, Alexafluor594 goat anti-mouse, and Alexafluor488 goat anti-rat (Invitrogen, all used at a concentration of 2 μg ml^{-1}).

Immunofluorescence staining and confocal microscopy

At the designated time points, samples were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes. Samples were washed in PBS, and then blocked and permeabalized in PBS containing 5% (v/v) normal goat sera (Vector labs) and 0.1% (v/v) Triton-X-100 for 30 min. The samples were then stained with various combinations of primary antibodies (as described in the text) for 1 h. Unbound antibody was then washed off in PBS and primary antibodies were detected with combinations of fluorescent secondary antibodies (as described in the text) for 40 min, followed by a second wash in PBS. In experiments involving BrdU, fixed samples were washed with distilled water prior to a 40 minute incubation in 2 N HCl. Samples were then extensively washed in PBS prior to blocking and permeabilization as described above. Samples were incubated in anti-BrdU antisera for 2 hours. For experiments involving EdU, we labeled

the EdU according to the manufacturer's protocol (Life Technologies) prior to antibody labeling.

Following staining, all samples were mounted in ProLong Gold (Invitrogen). All microscopy was performed on a Zeiss 510 META confocal laser scanning microscope. Cutoffs for saturation and background levels were adjusted with Photoshop software (Adobe).

Quantitation of LPG abundance

Samples were stained to detect parasite histones and PGs and confocal microscopy performed as described above. 3-dimensional confocal image stacks were then compressed into a single 2-dimensional image which was then used for subsequent analysis. For samples harvested less than 24 hours post infection, at which time the parasite's outline could be visualized with WIC79.3 staining, Volocity software (Improvision) was used to trace the outline of the parasites and then measure the sum of the WIC79.3 (red) intensity within the traced area. For samples harvested 24 hours or later after infection, this method was unusable because the outline of the parasites was increasingly invisible. Thus, we measured the sum of WIC79.3 intensities within a 44.8 μm^2 circle centered at the parasite nucleus. We did not use this method with parasites at time points prior to 24 hours because a circle is a poor approximation of the elongated shape of metacyclic-stage parasites.

Statistics

Unless stated otherwise, the data reported throughout the paper is the mean of at least three independent experiments in which >400 parasites were scored per experiment. Data

are presented as the mean ± the standard deviation. *P* values were determined either by a Student's t-test or a Chi-square test.

Results

Characterization of differentiation markers and their expression following L. major *infection of peritoneal macrophages*

First we sought markers that clearly distinguished *L. major* metacyclic parasites from authentic amastigotes, obtained after 120 hr infection of peritoneal macrophages *in vitro*, or visualized in sections taken from mice infected for several weeks. While a number of genes showing quantitative differences in promastigote or amastigote expression are known from microarray or proteomic studies, few show qualitatively on/off properties suitable for use in characterization of the tempo of amastigote differentiation on a cellular level (24-29). We explored a number of candidates, and ultimately identified five suitable for use. As shown in Fig. 1, these clearly distinguish between metacyclic-stage parasites and parasites 72 hours after infection of peritoneal macrophages (PEMs), a time at which many of the parasites in these cells are replicating amastigotes (below).

As a marker 'on' in promastigotes and 'off' in amastigotes, we chose the expression of the paraflagellar rod protein PFR1, which accompanies the loss of the flagellum during differentiation (Figure 1A) (30). As markers 'off' in promastigotes and 'on' in amastigotes, we followed the expression of the amastigote-specific antigens recognized by the monoclonal antibodies T17 and T18 (14). The epitopes recognized by the two antisera clearly differed in their cellular localization within amastigotes, suggesting they recognize different amastigote molecules. A third such marker consisted of a YFP transgene inserted into the ribosomal SSU locus, where YFP is "off" in metacyclic promastigotes but "on" in other stages (Figure 1D). While the mechanism controlling the YFP transgene expression have not been definitively established, preliminary studies

suggest that the reduction in YFP fluorescence and protein results from a decrease in YFP mRNA abundance (data not shown). Lastly, we monitored parasite DNA synthesis following metabolic labeling with BrdU or EdU (31, 32), to detect the transition between non-replicating metacyclics and replicating amastigotes.

Sequence and timing of metacyclic/amastigote differentiation marker expression.

We compared the sequence and timing of amastigote marker induction in PEMs. These results are summarized in Fig. 1E. The first marker change was the loss of reactivity with anti-PFR1 antisera. By 4 hours after infection (the earliest time point attempted), most parasites had lost anti-PFR staining (89 ± 6%), and by 24 hours essentially no parasites (0.2 ± 0.6%) were recognized with this antibody. Interestingly, loss of PFR1-reactivity did not completely coincide with the disappearance of long flagella, as staining with anti-phosphoglycan antisera or mAb T17 (see below) revealed that some parasites retained long flagella even at 24 hours post-infection (data not shown).

Next we observed that reactivity with mAbs T17 and T18 rapidly appeared. At four hours after infection, a small percentage of parasites (<5%) were weakly reactive with either MAb, but by 8 hours post-infection 37 ± 7% and 41 ± 12% were positive for T17 or T18 reactivity, respectively (Fig. 1E). By 11 hours after infection, >90% of the parasites were recognized by these antisera, and at 24 hours post infection, 94 ± 4% of the parasites are $T17^+$ and 92 ± 8% are positive for T18 reactivity.

SSU:YFP transgene expression first became weakly detectable 8 hours after infection, with 18 ± 3% of the parasites YFP^+ at 12 hours post-infection. By 24 hours, the percentage of YFP^+ parasites had increased to 51 ± 13%. Interestingly, while the percent of parasites displaying YFP fluorescence continued to increase reaching 70 ± 17% at 72

hours post-infection, it never became 100%. Similarly, only ~70% of parasites within infected mouse footpads were YFP^+ 2 weeks following infection, a time of vigorous parasite replication and expansion. Currently we do not understand why 100% YFP expression is not attained.

The last marker transition was the commencement of DNA replication. While all other markers had achieved 'full' expression by 24 hr (PFR off, T17, T18, and SSU:YFP on), only 8 ± 1% of the parasites showed labeling with anti-BrdU antisera at this time. The percentage of BrdU-positive parasites increased such that at 72 hours post-infection, 72 ± 13% of the parasites were $BrdU^+$. Together, these data show that the amastigote markers are induced in an orderly sequence with promastigote-specific PFR1 gene expression turning off by 4 hr post-infection, T17 and T18 expression turning on by 8-11 hr, SSU:YFP expression turning on by 11-24 hr, and finally replication commencing around 24 hr (Fig 1F). While we did not seek to rigorously establish whether various markers appeared homogeneously in sequence, the quantitative aspects suggest that this is likely. This supposition was supported by limited preliminary tests examining co-expression (not shown).

These data establish a useful developmental sequence of marker expression for analysis of *Leishmania* differentiation. Whether these transitions are functionally connected or interdependent, or occur independently of one another, has not been investigated.

Amastigote marker induction in bone marrow-derived macrophages and dendritic cells

We then examined the 'differentiation sequence' markers above in *L. major* metacyclic infections in two other cell types, bone marrow-derived macrophages (BMM) or dendritic cells (DCs). These data are plotted for each marker separately for the three cell types in

Fig. 2, or by cell type in Fig. 3 (BMM) and Fig. 4 (DC). Overall, the relative order of developmental marker expression was conserved amongst the three cell lines, albeit with a few differences amongst specific markers and in the overall rate.

Loss of anti-PFR1 reactivity in BMM and DCs was similar to what is seen in PEMs at either 4 or 24 hours post-infection ($P > 0.17$ by Student's t-test; N = 3 experiments) with 35 ± 25% and 0.5 ± 0.6% of parasites in BMMs and 15 ± 13% and 0.4 ± 4% of parasites in DCs labeling at these time points (Figure 2A). The induction of T18 reactivity and SSU:YFP fluorescence also showed similar time courses in the three cell types ($P > 0.16$ for T18 and $P > 0.2$ for YFP by Student's t-test; N = 3 experiments; Figs. 2B and 2C). Induction of T17 reactivity appeared to be slower in BMMs than in PEMs in that a lower percentage of the parasites at 8 hours post-infection were $T17^+$ (10 ± 8%, $P < 0.01$ by Student's t-test; N = 3 experiments). This difference disappeared by 24 hours, with 89 ± 10% of the parasites within BMMs showing T17-positivity. With the T17 marker, parasites within DCs were intermediate between the profile seen for PEMs and BMMs, but were not significantly different from the PEM results ($P > 0.08$ by Student's t-test; N = 3 experiments; Figure 2D). Lastly, parasites within BMMs and DCs also did not initiate DNA synthesis until after 24 h, as measured by BrdU-incorporation, as only 8 ± 2% of parasites within DCs and 5 ± 4% of parasites within BMMs were positive at 24 h. At 72 hours post infection significantly fewer parasites within DCs (46 ± 9%; $P = 0.02$ by Student's t-test; N = 3 experiments) and BMMs (35 ± 15%; $P = 0.01$ by Student's t-test; N = 3 experiments) are $BrdU^+$ than is seen in PEMs (72 ± 13%; Figure 2E).

When plotted by cell type, it is evident that the parasites show a similar progression in developmental marker expression in all three cell types (Fig. 1E, 3, 4). For the most part

the timing of marker expression was also conserved, other than reduced numbers of parasites entering the cell cycle in BMM and DCs relative to PECs (Fig 2E).

Quantitation of LPG expression.

LPG expression was assessed at different time points after infection of PEMs by its reactivity with the phosphoglycan (PG) specific monoclonal antibody WIC79.3, which recognizes galactose modifications of LPG and proteophosphoglycan (PPG) (23). While these modifications are often capped by arabinose in "metacyclic" LPG in *L. major*, we have shown previously that >90% of metacyclic parasites enriched through the gradient centrifugation protocol performed here retain some level of non-capped Gal-residues (20, 23), and thus are detected by this antibody. While PPGs also react with WIC79.3, they are expressed at much lower levels than LPG (<1%) (19).

First LPG expression was assessed qualitatively, scoring parasites as either "LPG-positive" or "LPG-negative". By this assay, 99 ± 1% of metacyclic stage parasites were LPG^{+}. By 24 hours after infection of PEMs, this number had declined to 81 ± 2%, and by 72 hours post infection, only a few (5 ± 3%) of the parasites had detectable LPG. Preliminary experiments monitoring the presence of arabinose-capped metacyclic-specific LPG using the monoclonal antibody 3F12 (23), yielded comparable results with a modest increase in the percentage of 3F12-negative parasites by 24 hours after infection and almost complete loss of $3F12^{+}$ parasites by 72 hours post infection (Supplemental figure S1).

The intensity of WIC79.3 reactivity was then used to quantitate LPG expression per cell (Figure 5A). To establish the background we used the LPG-deficient *lpg1-* mutant described previously (19). By this assay, all metacyclic parasites show LPG expression,

with a mean labeling intensity of 45,900 ± 23,300 arbitrary units (see the Methods for the procedure used for delineating parasite boundaries and the intensity of binding). Interestingly, at 20 minutes post infection the mean per-cell WIC79.3-reactivity increases to 66,100 ± 24,500 units. Thereafter, LPG expression gradually declined (albeit with some heterogeneity) such that at 48 and 72 hours post infection, the parasites reach background levels of WIC-reactivity (below 20,000 units). Consistent with the results from the qualitative assay a few (5-10%) of the parasites at 48 and 72 hours post infection retained detectable WIC79.3-reactivity, which in some cases was as high as seen in metacyclics. Thus, LPG is no longer present at significant levels on most parasites by 48 hours post infection of PEMs.

High levels of LPG retained on parasites in BMMs and DCs

We then examined LPG expression following infection of *L. major* metacyclics of BMM and DCs (Figs. 4B-D). At 24 hours post-infection, 19 ± 2% of parasites within PEMs have lost LPG expression by the qualitative assay described above. In contrast, fewer parasites in BMMs (8 ± 5%; $P < 0.05$ by Student's t-test; N = 3 experiments) lost LPG expression at this time point, with the parasites in DCs yielding a result intermediate between the two types of macrophages (12 ± 6% LPG-negative). The most striking difference was seen at 72 hr; while 95 ± 3% of parasites within PEMs are LPG^- at 72 hours after infection, many parasites in both BMMs and DCs retained LPG expression at this time point, with 65 ± 5% of parasites in BMMs ($P < 10^{-4}$ by Student's t-test; N = 3 experiments) and 37 ± 14% ($P < 0.02$) of parasites in DCs retaining high levels of LPG (Fig. 5B and C).

We then measured LPG expression per cell on all three types at 24 and 72 hr (Fig. 5D). Consistent with the results from the qualitative assay described above, parasites within BMMs and DCs retained LPG expression for a longer period of time. Whereas the mean per-cell WIC79.3 intensity of parasites within PEMs 24 hours after infection was reduced by ~30% relative to that of metacyclic stage parasites ($P < 0.001$ by Student's t-test; N = 3 experiments), the mean LPG levels in parasites within BMMs and DCs had not declined significantly, remaining above 41,300 units. By 72 hours, however, this value did decline for parasites within DCs and BMMs, which had mean LPG expression of 24,900 ± 15,400 and 28,600 ± 19,200 units, respectively. At 72 hr however many individual parasites within both BMMs and DCs showed little LPG expression, similar to that of the *lpg1*- LPG negative parasites. Nevertheless, the mean WIC79.3 intensity of parasites within PEMs at 72 hours after infection was 17,600 ± 8,400 units, significantly less than that for parasites within either BMMs or DCs ($P < 10^{-5}$ by Student's t-test; N = 3 experiments). Importantly, even at 72 hours post-infection, many of the parasites that were classified as "LPG^{+}" showed LPG expression comparable to metacyclic-stage parasites. This suggests that a population of parasites within BMMs and DCs retained biologically relevant amounts of LPG for at least 72 hr in BMM and DCs, but not PECs.

LPG-retaining parasites within bone-marrow dendritic cells are non-dividing

We asked whether the sub-population of cells retaining high levels of LPG after 72 hr following infection of BMM or DCs had initiated DNA synthesis or not. For these experiments, we made use of the Click-iT EdU system (Life Technologies, to facilitate simultaneous visualization of LPG and the incorporation of thymidine analogue (EdU) into parasite DNA. DCs were infected with *L. major* metacyclics and then cultured in the

presence of EdU for 72 hours, after which the samples were fixed and stained to simultaneously detect *L. major* histones, LPG, and EdU. For these studies, the results obtained for the percent of parasites that were labeled with EdU were in the ballpark of the results described above with BrdU. We imaged a total of 1174 parasites, 38% of which were EdU^+ and 21% of which were LPG^+ (Figure 4A). Assuming that there is no relationship between a parasite's LPG-positivity and DNA synthesis, as a null hypothesis we would expect that 38% of the LPG^+ parasites should be EdU^+. Instead, while 46% of LPG^- parasites are EdU^+, only 7% of the LPG^+ parasites are EdU^+, significantly less than what would be expected ($p < 10^{-30}$ by a Chi-square analysis, N = 2 experiments, 1174 parasites; Figure 4B).

Thus, the parasites within DCs retaining high levels of LPG expression have not yet entered the cell cycle. This finding raises the question as to whether the loss of LPG and the induction of DNA synthesis is an ordered process. To address this, we performed the same experiment as above, only this time on PEMs 24 hours post-infection, a time point at which most parasites retain LPG expression and in which some parasites have become BrdU-positive (see Figure 1F). Unlike in DCs at 72 hours post infection, in PEMs at 24 hours post-infection a similar percentage of LPG^+ and LPG^- parasites are EdU^+, suggesting that LPG loss and DNA synthesis are not ordered in PEM infections ($p = 0.41$ by a Chi-square analysis; N = 2 experiments, 1255 parasites; Figure 4B).

Discussion

Amastigote development in peritoneal macrophages

Although the primary aim of our study was to address parasite differentiation and LPG loss within DCs, we first had to characterize the differentiation process within macrophages, cells in which the parasites are known to differentiate. These studies were carried out within starch-elicited peritoneal macrophages, which represent our gold-standard host cell type because they are permissive to all of the stages of parasitism (entry, differentiation, and parasite replication). Some of the developmental changes associated with amastigogenesis, such as the loss of PFR expression, happened very rapidly upon parasite infection of these cells, with more than 90% of the parasites displaying a PFR^- phenotype by 4 hours post-infection. Other developmental changes as assessed by amastigote markers occurred later, with T17 and T18 reactivity being the next "markers" to be induced, followed by the induction of YFP fluorescence. The findings that the induction of amastigote markers appears to occur in an ordered manner is consistent with the ordered progression of gene expression changes seen by microarray studies as *L. donovani* differentiates in axenic culture (27). These changes preceded re-entry into the cell cycle, as they had largely gone to completion by 24 hours post infection, a time point at which only 8% of the parasites were $BrdU^+$.

All of the amastigote markers are induced on parasites within DCs

Having established the timing and sequence of amastigote marker induction within PEMs, we then asked whether *L. major* metacyclic-stage parasites behave similarly within DCs, one of the host cell types with which parasites interact first upon infection *in vivo* and which may be important either as hosts for or killers of parasites and have

important roles in the establishment of either protective or pathological immune responses (2, 33-35). As a control for these studies, we also assessed the differentiation of the parasites within bone marrow-derived macrophages (BMMs) in order to better understand the range of parasite differentiation phenotypes in different types of macrophages. Parasites within BMMs and DCs clearly did not remain metacyclics, as they became PFR^-, $T17^+$, $T18^+$, and YFP^+ in a manner similar to that of parasites within PEMs. In addition, by 24 hours after infection, a comparable percentage of the parasites within BMMs and DCs were $BrdU^+$. Thus, at this time point each amastigote marker is present on at least a few parasites, consistent with at least partial if not complete differentiation within DCs.

Fewer parasites in BMMs and DCs undergo DNA synthesis or lose LPG expression

Although all amastigote markers were present on parasites within DCs and BMMs, significantly fewer of the parasites within these cells became $BrdU^+$ or LPG^- by 72 hours post-infection than what is seen in PEMs. Importantly, these LPG^+ parasites likely have biologically relevant levels of LPG, as quantitation demonstrated that they express comparable amounts of LPG to metacyclic-stage parasites on a per-cell basis. Dual-labeling experiments showed that the LPG^+ parasites in DCs at this time point tended to be preferentially $BrdU^-$, suggesting that the parasites with residual LPG were not replicating. Nevertheless, we argue that the LPG^+ parasites are viable for several reasons. First, while killed intracellular *Leishmania* are degraded very quickly by host cells (36), the LPG staining of the parasite's surface revealed that the LPG^+ parasites appeared intact and morphologically normal. In addition, in BMMs where 65% of the parasites are LPG^+, we saw that 66% of total parasites showed bright YFP fluorescence, implying that

at least some of the LPG^+ parasites were positive for endogenous YFP fluorescence. Such fluorescence would be lost in killed parasites arguing that LPG^+ parasites are alive (37).

Taken together, these results demonstrate that in bone marrow-derived DCs and macrophages, a population of parasites exist up to 72 hours after infection which have a phenotype that is intermediate between metacyclics and amastigotes. Similar to amastigotes, they are PFR^-, $T17^+$, $T18^+$, with some being YFP^+. However, they express levels of LPG similar to what is seen on metacyclic stage parasites and have not resumed replication. Such intermediate phenotypes could arise either because amastigogenesis is a slower process within BM-derived cells or alternatively the parasites could instead be arrested in some intermediate stage in development. Further studies will be required to distinguish between these two models. Regardless, either process results in the existence of parasites which retain the expression of LPG and potentially other 'early' virulence factors. The retention of these virulence factors may have important effects on the infected hosts. LPG has demonstrated roles in parasite survival in macrophages and neutrophils, protection against complement-mediated lysis, oxidant avoidance, and the inhibition of phagolysosomal fusion (7, 8, 12). LPG retention by the parasites may serve to prolong the effects of this virulence factor as the parasites transit through cell types of 'first contact'.

What type of parasite could be transferred from DCs to macrophages?

Invading metacyclic-stage parasites are thought to interact predominantly with cell types other than macrophages *in vivo*, yet by 48 hours after infection most parasites are found within macrophages (38). If we assume that all parasites enter macrophages by way of DCs, then the parasite phenotype encountered by macrophages would be entirely

dependent on the parasite phenotype(s) present at the time of transfer. We evaluated the phenotype of parasites within DCs at 24 and 72 hours post-infection, time points book-ending the time during which parasite transfer from DCs to macrophages likely takes place. At 24 hours post infection, almost all parasites are positive for the amastigote markers PFR, T17, and T18, yet retain LPG and cell cycle arrest. By 72 hours after infection roughly a third of the parasites retain LPG, cell cycle arrest, and potentially other 'metacyclic' characteristics. Thus, should parasite transfer occur at any time between 24 and 72 hours post infection, macrophages would be expected to encounter a mix of parasite phenotypes including both LPG^- and LPG^+ parasites, with the LPG potentially exerting effects not just on the DC but also on the macrophage upon subsequent transfer.

In reality, DCs are not the only cell type with which metacyclic-stage *L. major* interact, and the parasites are thus transferred to macrophages from these other cell types as well. In order to understand the parasite phenotype(s) that may be transferred from these cells to macrophages, the ability of the parasites to differentiate within these cells must be determined. Although it may be tempting to assume that the timing of parasite differentiation within different host cells may be similar to what is seen by one of the three cell types assessed here, our data suggest that amastigogenesis is strongly dependent on conditions, and such assumptions may not be correct. Some of the most profound differences seen between cell types in terms of parasite differentiation were between different sources of macrophages (PEMs versus BMMs). These two types of macrophages could differ in several ways that are potentially relevant to the parasite, including in their lysosomal pH or contents or the fusogenicity of *Leishmania*-containing

endosomes, with such differences being either positive or negative regulators of amastigogenesis. Thus, the ability of *L. major* to undergo amastigogenesis within other host cell types must be addressed experimentally. In fact, our results in DCs appear to contrast with what is seen in human peripheral blood-derived neutrophils, in which at least some *L. major* parasites retain the long, highly motile flagella that is characteristic of promastigotes up to 42 hours post-infection (39).

Potential uses of amastigote markers

In order to address the ability of *L. major* to differentiate within DCs, we first had to develop amastigote markers, as such tools are limited with *Leishmania* in general and *L. major* in particular. As the induction of some of the markers (PFR loss and induction of T17 and T18 antigens) appear to be ordered, these markers can be used to determine the extent to which a parasite has undergone differentiation, with a parasite that is positive for all markers being considered fully differentiated. These markers potentially have numerous applications in addition to those for which they were used here. Such uses might include the evaluation of putative *L. major* axenic amastigote lines, with the goal of finding conditions in which parasites are "positive" for all amastigote markers. In addition, these markers could be used to identify environmental signals that positively or negatively affect amastigogenesis *in vitro*, as a higher percentage of parasites would be expected to be positive for the amastigote markers as differentiation conditions improve. Ultimately, the identification of amastigote markers lays the groundwork for genetic studies of amastigogenesis so as to better understand parasite developmental biology.

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Figure legends

Figure 1. Characterization of amastigote markers in peritoneal macrophages (PEMs). (A-D) Comparison of marker expression between metacyclic parasites (left) or parasites 72 hours post infection of PEMs (i.e. amastigotes, right). (A) Parasite nuclei are detected with antisera against *L. major* histone proteins (green), and PFR is shown in red. (B) Parasite histones, red. mAB T17, green. (C) Parasite histones, red. mAB T18, green. (D) Endogenous YFP fluorescence (yellow) overlaid onto DIC image. Scale bar represents 5 μm. (E) Timing of marker induction. The percentage of parasites showing an "amastigote-like" phenotype for the various markers is plotted as a function of time after infection. N = 3 experiments, error bars represent standard deviation. (F) Summary of the data in (E) showing the relative timing of amastigote marker induction in PEMs.

Figure 2. Parasites within bone marrow-derived macrophages (BMMs) and dendritic cells (DCs) induce PFR, T17, T18, and YFP markers similarly to in PEMs. For all graphs, PEM data is represented with black diamonds, BMM data is represented with grey squares, and DC data is represented with open triangles. The data shown is the average of three experiments, and error bars represent the standard deviation. (A) PFR loss. (B) Induction of T18 reactivity. (C) Induction of YFP fluorescence. (D) Induction of T18 reactivity. Data shown is the mean of three independent experiments, error bars represent standard deviation. (E) BrdU-incorporation by parasites in PEMs, BMMs, and DCs. Infected host cells were cultured in media containing BrdU for 24 or 72 hours after infection. Black bars, PEMs. Grey bars, BMMs. White bars, DCs. ** denotes $P < 0.05$ by Student's t-test.

Figure 3. The percentage of parasites within BMM showing an "amastigote-like" phenotype for the various markers is plotted as a function of time after infection. N = 3 experiments, error bars show standard deviation.

Figure 4. The percentage of parasites within DCs showing an "amastigote-like" phenotype for the various markers is plotted as a function of time after infection. N = 3 experiments, error bars show standard deviation.

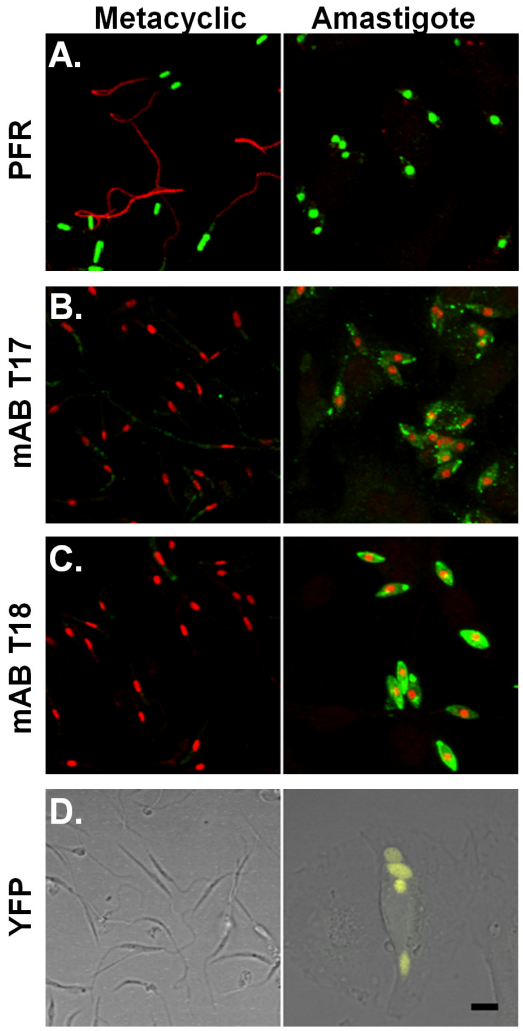
Figure 5. Loss of LPG expression following infection of PEMs, BMMs, and DCs. (A) Anti-LPG fluorescence intensity on a per-parasite basis. Anti-LPG intensity of WT metacyclic-stage parasites as well as at various time points after infection of PEMs was measured as described in Methods. As a negative control, the anti-LPG intensity of *L. major lpg1-* (open circles) was measured 0.3 hr after infection of PEMs. The grey line shows the mean anti-LPG intensity of "LPG-negative" WT parasites as determined by the qualitative assay plus two standard deviations, and any parasite with anti-LPG values below that line would be considered LPG-negative. Data shown is pooled from at least two independent experiments. Black bars represent geometric mean of the data for each sample. (B) Representative images comparing the LPG-positivity of purified metacyclics and parasites within PEMs, DCs, and BMMs at 72 hours post-infection. Parasite nuclei are shown in green, LPG is shown in red. Scale bar represents 5 μm. (C) Percent of parasites within the three host cell types that are LPG^+ as determined by the qualitative assay. PEM data is represented with black diamonds, BMM data is represented with grey squares, and DC data is represented with open triangles. N = 3 experiments, error bars show standard deviation. (D) Quantitation of LPG on the surface of parasites within PEMs, BMMs, and DCs at 24 and 72 hours post infection. For comparison, anti-LPG

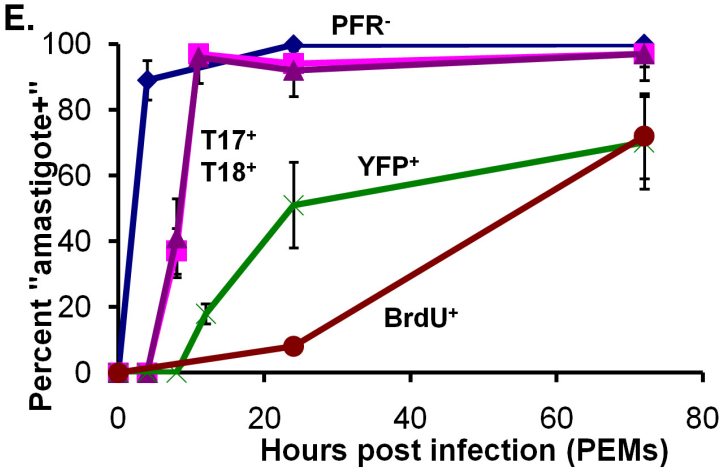
intensity data for metacyclic-stage parasites is also shown (open circles). The grey line shows the cut-off for LPG-positivity. Data shown is pooled from three independent experiments. Black bars represent geometric mean of the data for each sample, and P values were calculated by a Student's t-test.

Figure 6. LPG-retaining parasites within DCs 72 hours post-infection have not undergone DNA synthesis. Parasites were cultured in the presence of thymadine analogue (EdU) for 72 hours following infection of DCs or PEMs. Cells were stained to detect EdU-incorporation (green), LPG (red) and parasite nuclei (blue). (A) Representative image of parasites within DCs stained as above. Scale bar represents 5 μm. (B) Percent of parasites within DCs 72 hours post-infection (white bars) or PEMs 24 hours post-infection (grey bars) that are positive for EdU-incorporation. Data shown include the percent of total parasites that are EdU^+, as well as the EdU-positivity of parasites that are either LPG-negative or LPG-positive. In DCs at 72 hours post-infection, almost all EdU^+ parasites are LPG-negative. This is not the case in PEMs 24 hours post-infection, in which a similar percentage of parasites within the LPG^+ and LPG-negative pools are EdU^+. N = 2 experiments. * denotes statistical significance as determined by a χ^2 test described in the text.

Supplemental figure S1. Loss of arabinose-capped "metacyclic" LPG by parasites within PEMs. PEMs were infected with *L. major* and harvested 2, 24, and 72 hours after infection. (Top) Ara-capped LPG is detected with mAB 3F12 (green), and parasite histones are shown in red. Scale bar represents 5 μm. (Bottom) Percent of parasites that were $3F12^+$ at indicated time points.

Figure 1





F.

PFR OFF (4 h)

↓

T17/T18 ON (8-11 h)

↓

SSU::YFP ON (16-24h)

↓

REPLICATION (>24 h)

Figure 2

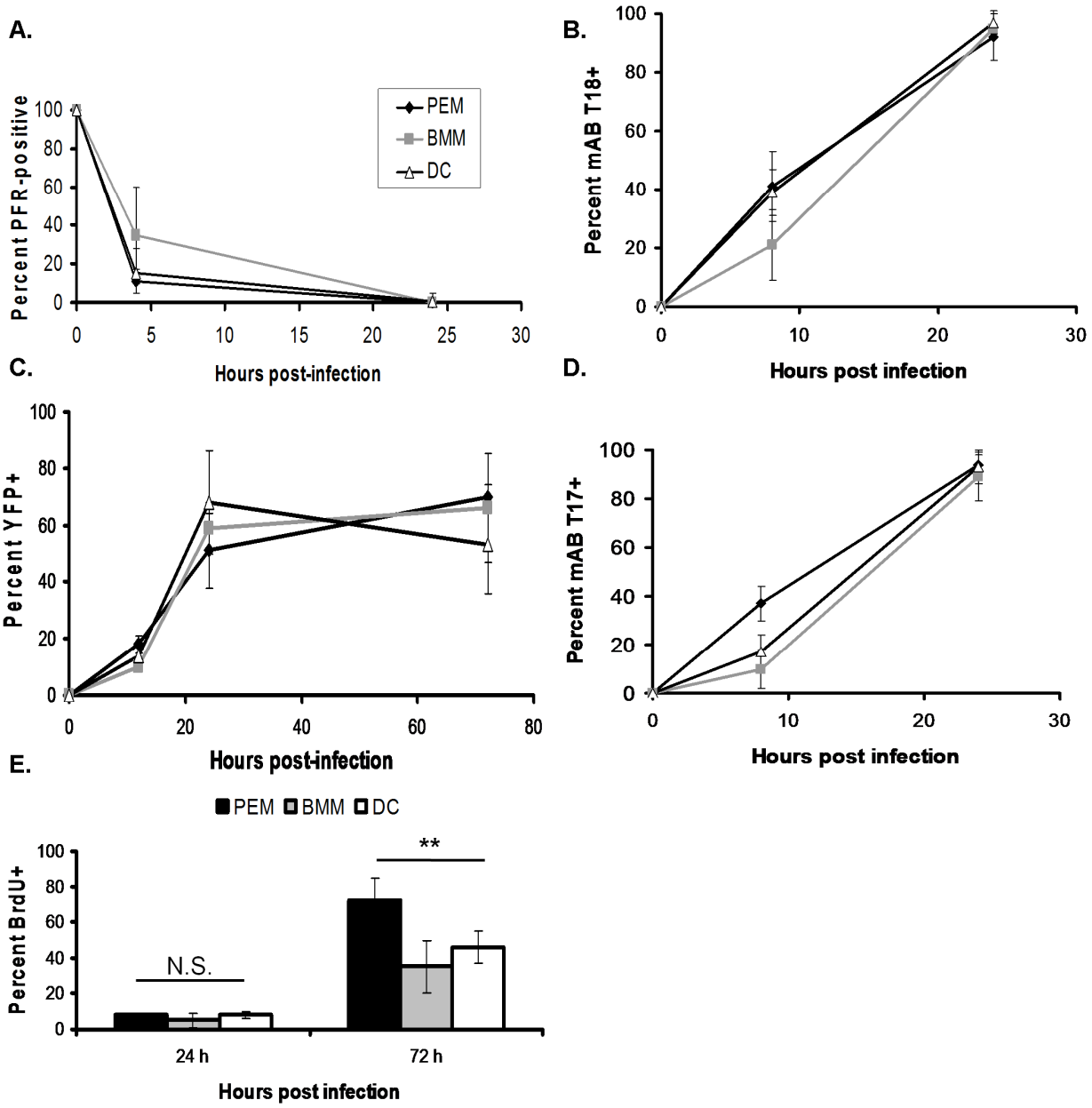

A.
Percent PFR-positive
PEM
BMM
DC
Hours post-infection
B.
Percent mAB T18+
Hours post infection
C.
Percent YFP+
Hours post-infection
D.
Percent mAB T17+
Hours post infection
E.
PEM BMM DC
Percent BrdU+
N.S.
**
24 h
72 h
Hours post infection

Figure 3

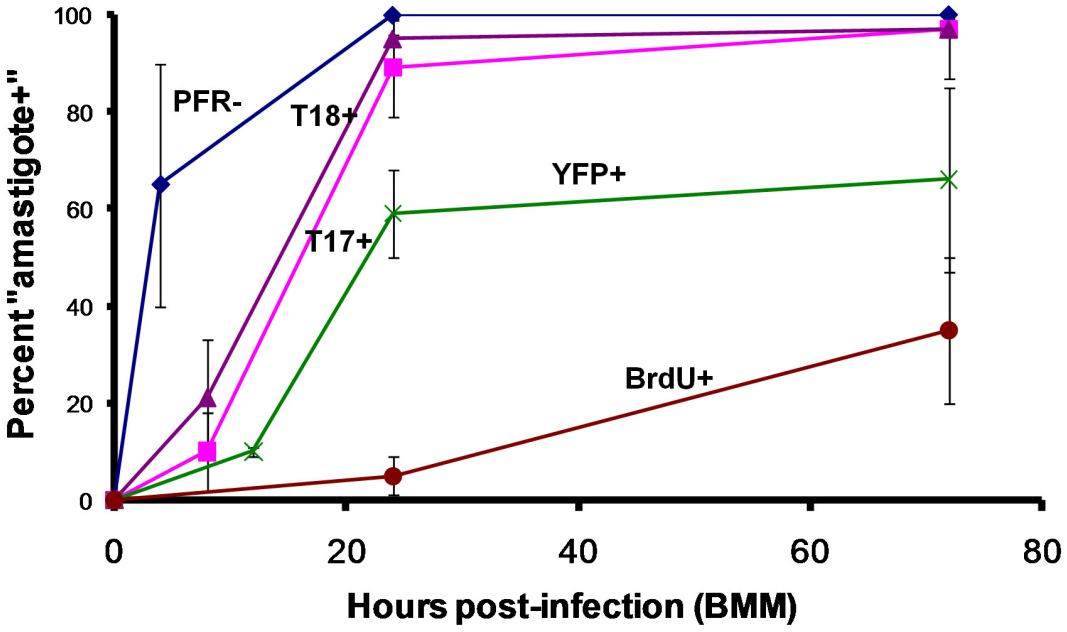

Percent"amastigote+"
100
80
60
40
20
0
PFR-
T18+
T17+
YFP+
BrdU+
0
20
40
60
80
Hours post-infection (BMM)

Figure 4

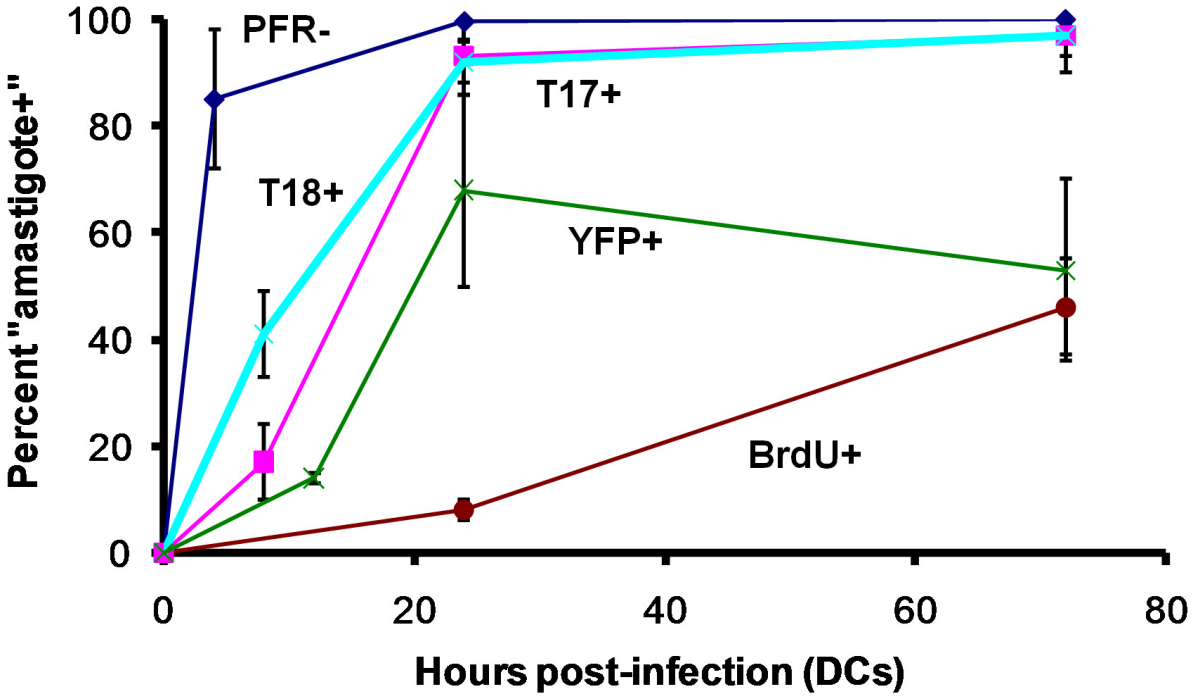

Percent "amastigote+"
100
80
60
40
20
0
PFR-
T17+
T18+
YFP+
BrdU+
0
20
40
60
80
Hours post-infection (DCs)

Figure 5

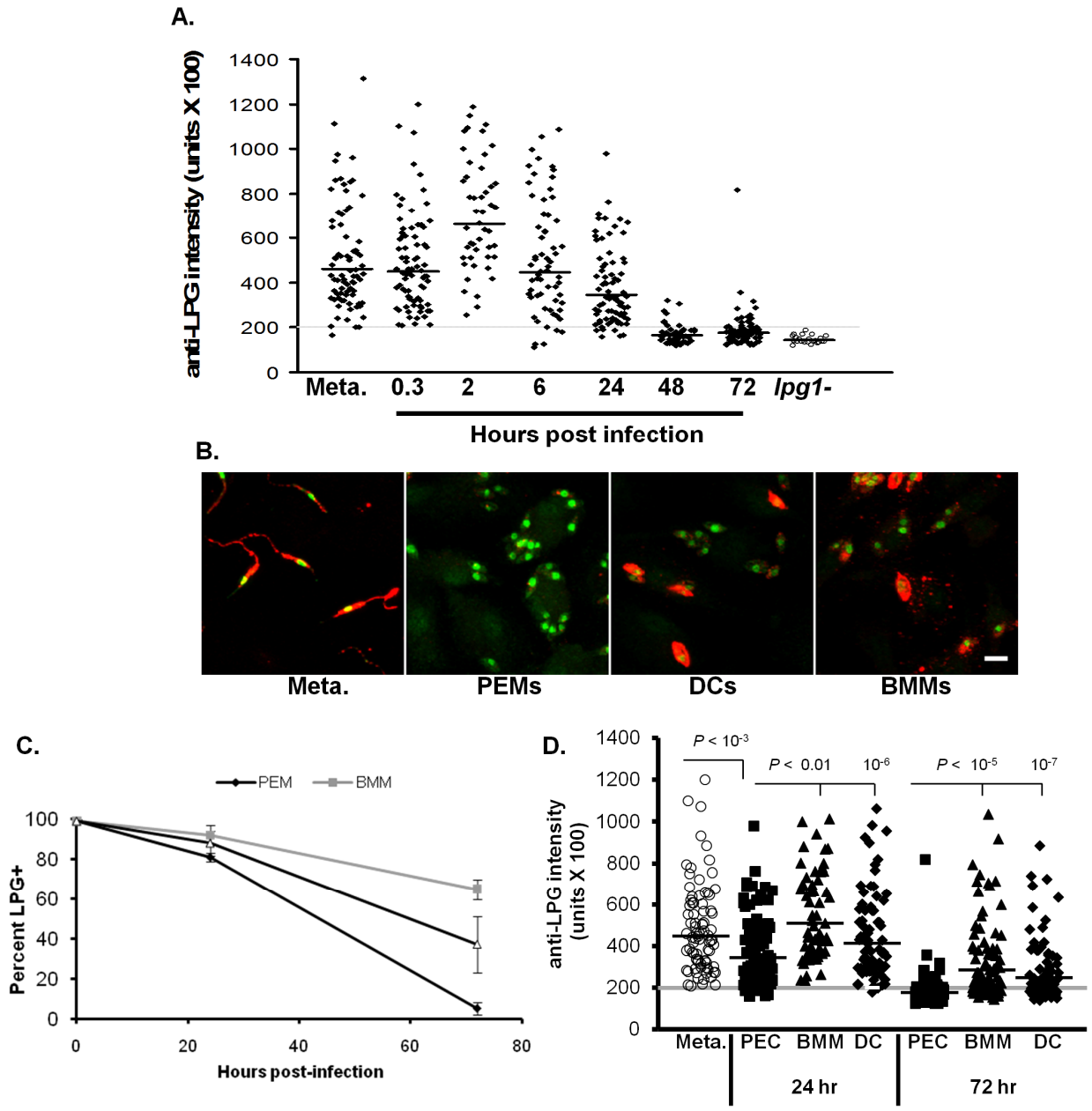
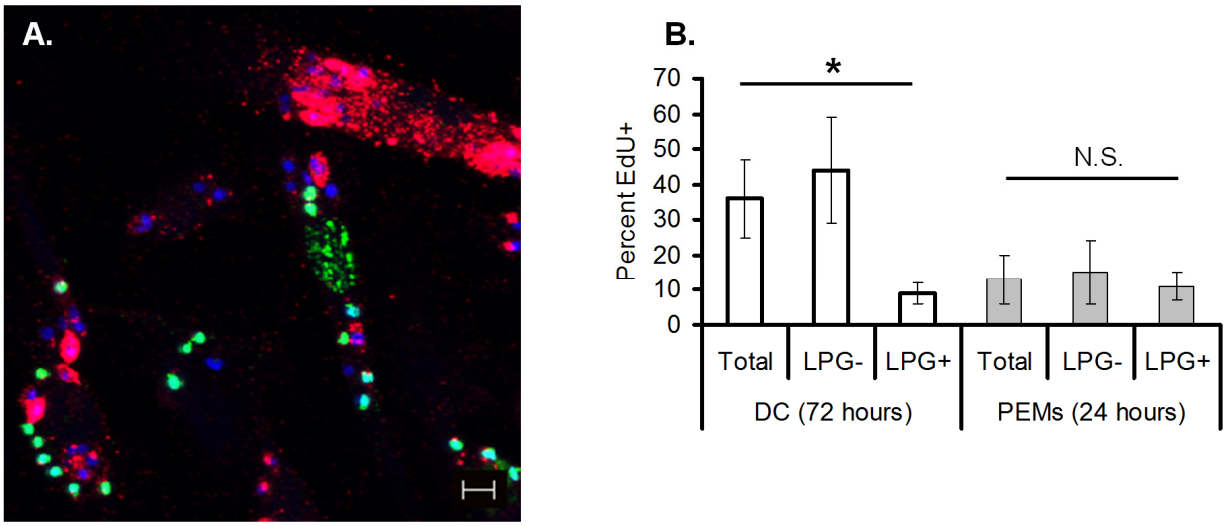
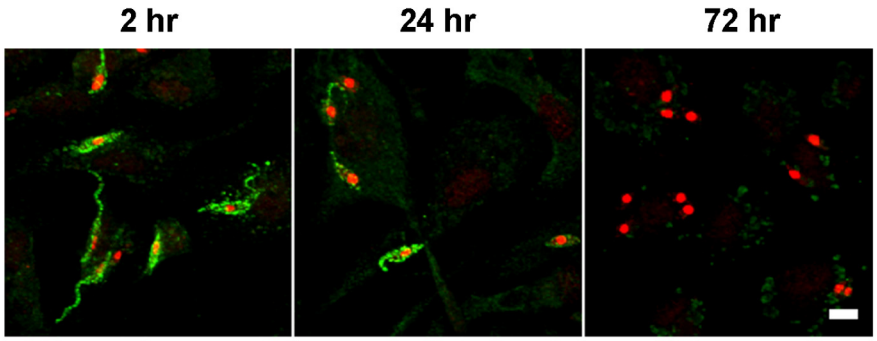

A.
anti-LPG intensity (units X 100)
1400
1200
1000
800
600
400
200
0
Meta.
0.3
2
6
24
48
72
lpg1-
Hours post infection
B.
Meta.
PEMs
DCs
BMMs
C.
PEM
BMM
Percent LPG+
100
80
60
40
20
0
0
20
40
60
80
Hours post-infection
D.
anti-LPG intensity (units X 100)
P < 10-3
P < 0.01
10-6
P < 10-5
10-7
Meta.
PEC
BMM
DC
PEC
BMM
DC
24 hr
72 hr

Figure 6


A.
B.
*
N.S.
Percent EdU+
70
60
50
40
30
20
10
0
Total
LPG-
LPG+
Total
LPG-
LPG+
DC (72 hours)
PEMs (24 hours)

Supplementary figure S1



% 3F12^{+}	**71% (N =163)**	**61% (N = 109)**	**3% (N = 239)**

Chapter 3

Replicating and quiescent sub-populations of persistent *L. major* suggest a "stem immunogen" model of concomitant immunity

Author contributions

All experiments were performed by Mike Mandell.

Text was written by Mike Mandell with contributions by Stephen Beverley.

Abstract

Following *Leishmania major* infection, small numbers of parasites persist indefinitely in the host in the absence of pathology. Persistent parasites play important roles in concomitant immunity and act as reservoirs for transmission and/or reactivation. We sought to examine the replication and localization of persistent *L. major* in mice. We used an *in vivo* BrdU-labeling assay to show that persistent parasites replicate, albeit ~50% as much as acute-phase parasites resulting from the existence of a poorly-replicating sub-population of parasites unique to persistent infections. Persistent parasite replication occurs within macrophages and dendritic cells, ~80% of which synthesized high levels of iNOS protein, an enzyme implicated in parasite killing. However, the parasites within $iNOS^+$ cells appeared morphologically normal and showed comparable BrdU labeling to parasites within $iNOS^-$ cells suggesting at least transient survival within $iNOS^+$ cells. Since parasitemia remains roughly constant over time, persistent parasite replication implies that parasites must also be destroyed. These data shed new light on the persistent parasite paradigm, invoking a 'stem immunogen' model for concomitant immunity in which a generally quiescent reservoir periodically undergoes replication, thus maintaining itself while targeting many progeny parasites for destruction within professional antigen presenting cells and consequent maintenance of immunity. Attenuated *lpg2- L. major*, a proposed model of WT persistence capable of vaccinating susceptible BALB/c mice against virulent challenge, closely resembled persistent WT in most respects (e.g. replication and localization within iNOS-expressing phagocytes), but differed in its association with host arginase I expression in C57BL/6, but not BALB/c,

mice. As *lpg2*- fails to vaccinate C57BL/6 mice, elevated arginase I expression may be a negative correlate of anti-*Leishmania* immunity.

Introduction

As long-term infection of a host can increase a pathogen's chances of transmission, an array of viral, bacterial, and eukaryotic pathogens have evolved the ability to prolong their relationship with their hosts. A subset of pathogens, including *Mycobacterium tuberculosis*, *Toxoplasma gondii*, and *Leishmania major* remain indefinitely within their hosts in small numbers without causing obvious illness: a condition which we refer to as a "persistent" infection. Despite the absence of overt pathology, such persistent infections are of great medical importance as they serve as reservoirs for transmission, reactivate to cause disease (1), or protect against subsequent infections either by their own species or by different pathogens (2), a process known as concomitant immunity.

Persistence is a significant but under-studied aspect of the biology of parasites of the genus *Leishmania*, which are the causative agents of leishmaniasis. These parasites are transmitted as metacyclic-stage promastigotes to humans by the bite of an infected sand fly. They are then engulfed by phagocytes such as macrophages, where they differentiate into the amastigote stage and begin to replicate. In the case of *Leishmania major*, this may produce an ulcerating skin lesion. In most human cases, as well as in experimental infections of "resistant" mouse strains, the infection is eventually controlled by a Th1 type immune response, an important component of which is the induction of iNOS. It is generally thought that iNOS-derived nitric oxide (NO) is responsible for killing intracellular *L. major,* since iNOS knock-out mice fail to control infection (3, 4). Following the development of this protective response, the number of parasites in infected tissue declines dramatically, the lesion heals, and the host becomes immune to subsequent *L. major* infection. However, a small, roughly constant population of viable

parasites remains at the site of infection and in the lymph node draining that site for the rest of the host's life (5).

These persistent parasites are important for several reasons. First, despite their limited numbers (~1000), persistent parasites can still be transmitted to sand fly vectors, and as such, are a reservoir for the pathogen (6, 7). Second, they pose a substantial risk to infected people in the event of immunosuppression, as the persistent parasites can "reactivate" leading to severe disease (8). Finally, they may be beneficial to hosts with intact immune systems, as they help maintain protective immunity against subsequent *Leishmania* infections (9). Indeed, healed *Leishmania* infections are the gold-standard in anti-*Leishmania* immunity, and to date no other vaccination approaches have been successful in humans (10). Importantly, treatment of persistently infected mice to achieve a sterile cure renders those mice susceptible to new infections (11), suggesting that the persistent parasites are actively contributing in some way towards their host's anti-*Leishmania* immunity.

Because of the strong protective immunity conferred by persistent parasites, an attenuated parasite line that could persist indefinitely without causing pathology would be a promising candidate for development into a vaccine against leishmaniasis (12). Several mutant lines of *L. major*, (including parasites lacking fructose-1,6-bisphosphatase or the nucleotide sugar transporter *LPG2*) have these properties (13). Of these, *lpg2*- (which lacks a GDP-mannose transporter required for the synthesis of parasite virulence factors such as phosphoglycans), can vaccinate susceptible mice against virulent challenge (14). In fact, because the number of viable *lpg2*- in an infected mouse is comparable to that of WT persistent parasites, *lpg2*- has been proposed as a model for *L. major* persistence.

One advantage of this model would be that it would allow the generation of a persistent infection only a few weeks after infection with *lpg2*-, as opposed to months following infection with WT (12).

Considerable evidence suggests that the host's immune response is important to simultaneously prevent reactivation and clearance of persistent parasites. For instance, treatment of persistently infected mice with immunosuppressive drugs, iNOS inhibitors, or the blockade of interferon-γ signaling rapidly results in increased parasite numbers and the reappearance of disease symptoms (4). In contrast, depletion of $CD4^+CD25^+$ regulatory T cells or the blockade of IL-10 signaling both result in sterile cure in mice (11, 15). It remains unknown, however, how persistent parasites modulate the host's immunostimulatory and immunosuppressive responses, or what role persistent parasites (or attenuated lines such as *lpg2*-) have in maintaining protective immunity. In addition to immunological studies, studies of persistent parasites themselves are crucial to help us address these questions.

As the number of persistent parasites is roughly constant, we sought to determine if the parasites at this stage of the infection replicate or if they are quiescent. To this end, we developed a BrdU-incorporation assay and used it to show that acute-phase levels of replication was taking place by a sub-population of parasites, while another sub-population replicated poorly if at all. Taken together, persistent parasites replicate about half as much as acute-phase parasites, in which a poorly-replicating sub-population is not detected. We found that parasite replication takes place within macrophages and dendritic cells in both sites of persistence (the footpad infection inoculation site and the draining lymph node). Other cell types, including ER-$TR7^+$ reticular fibroblasts, harbored 10% or

less of persistent parasites. Constant parasite numbers despite constant replication implies that parasite killing is also taking place. We hypothesized that iNOS-expressing cells were the sites of parasite killing and found that ~70% of persistent parasites were within such cells. Although the parasites observed within these cells appeared to be healthy and replicating, we presume that at least some of the parasites within these cells are killed. Continual parasite destruction and repopulation suggest a model by which persistent *L. major* maintain protective immunity, namely by continual presentation of antigens from killed parasites. This paradigm of constant immune stimulation could also explain the concomitant immunity generated by other pathogens including herpesviruses and *Toxoplasma*. We also assessed the replication and localization of *lpg2*- parasites. For most parameters tested, WT persistent parasites and *lpg2*- were indistinguishable, further supporting the use of *lpg2*- as a model of WT persistence. However, we found small differences in the phenotype of the host cells infected by *lpg2*- and persistent WT, one of which (an increased association within host arginase 1 expression) negatively correlates with the ability of *lpg2*- to vaccinate different strains of mice.

Materials and Methods

Parasite strains and culture

For most experiments, *L. major* strains LV39c5 (Rho/SU/59/P), Friedlin V1 (MHOM/IL/80/Friedlin), and *lpg2- L. major* (Δ*lpg2::HYG*/ Δ*lpg2::HYG*) (12) were grown at 26^{O}C in M199 M199 medium (US Biologicals) supplemented with 40 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) pH 7.4, 50 μM adenosine, 1 μg ml^{-1} biotin, 5 μg ml^{-1} hemin, 2 μg ml^{-1} biopterin and 10% (v/v) heat-inactivated fetal calf serum (16). "Slow-growing" promastigote cultures were grown in RPMI 1640 + L-glutamine (Invitrogen) supplemented with 37 mM HEPES pH 7.4, 47 μM adenosine, .93 μg ml^{-1} biotin, 4.7 μg ml^{-1} hemin, 1.9 μg ml^{-1} biopterin and 0.9% (v/v) heat-inactivated fetal calf serum(17). The WT LV39c5 parasites used here expressed GFP from the ribosomal locus (*SSU::IR1SAT-GFP*) and were generated by transfecting SwaI-cut plasmid B3538 into WT LV39c5 as described (16) and selecting for resistance to 100 μM nourseothricin and bright green fluorescence. The clone used in this study exhibited virulence similar to WT in BALB/c mice (data not shown). Parasites deficient in the arginase gene *arg*- (Δ*arg::HYG*/Δ*arg::PAC*) (18) were cultured in the above media supplemented with 50 mM putrescine. Infective metacyclic-stage parasites were recovered using the density gradient centrifugation method (19). Propidium iodide staining of promastigotes was performed as described (20).

Mouse infections

Female C57Bl/6J mice (6-10 weeks old; Jackson Labs) were injected subcutaneously in the left hind footpad with either 10^5 metacyclic WT or 10^6 metacyclic *lpg2*- parasites.

Following infection with WT parasites, the mice developed lesions that resolved, as determined by the absence of footpad swelling relative to the uninfected foot, ~4 months after infection. For the purposes of this study, "persistent" infections were defined as any time >1 month following the resolution of footpad swelling. Most studies with *lpg2*- were performed between 1-2 months following infection. Where indicated, some studies with *lpg2*- were performed 5 months after infection. Because *lpg2*- parasites can revert to amastigote virulence (21), it was important to eliminate possible revertants from our analysis. To do this, we did not include data from *lpg2*- -infected mice in which we found > 150 parasites in a single section. We chose this cutoff because we calculate that such a mouse would likely have ~10000 parasites in the footpad (based on the fact that a normal footpad can yield ~60 sections and our observation that the number of parasites within a section remains roughly constant within one mouse.)

Antibodies used

L. major nuclei were detected with a pool of rabbit antibodies raised against *L. major* histones H2A, $H2A_{variant}$, H2B, H3, and H4 (pooled at a ratio of 3:2:3:3:1 by titer) (Wong and Beverley, in preparation). For some experiments, this pool was used at a dilution of 1:750. For others, this pool of antibodies was directly conjugated to Alexafluor488 monoclonal antibody labeling kit according to the manufacture's protocol (Invitrogen) and the directly conjugated antibody used at a final concentration of 0.15 mg ml^{-1}. GFP was detected with a chicken anti-GFP antibody (AbCam) at a final concentration of 0.02 mg ml^{-1}. F4/80 was detected with a rat monoclonal antibody (clone A3-1, AbD Serotec) diluted to 1:250. CD11c was detected with a hamster monoclonal antibody (clone N418, eBioscience) diluted to 1:250. ER-TR7 was detected with a rat monoclonal antibody

(BMA Biomedicals) used at a final concentration of 0.01 mg ml^{-1}. iNOS was detected with a rabbit anti-iNOS (BD Transduction Labs) used at 1 μg ml^{-1}. Relmα was detected with a rabbit polyclonal antibody (Abcam) used at 0.8 μg ml^{-1}. BrdU was detected with a rat monoclonal antibody (Abcam) used at 10 μg ml^{-1}. Goat anti-Arg1 (Santa Cruz) and goat anti-Arg2 (Santa Cruz) were used at 2 μg ml^{-1}. Rat anti-LY-6C mAb (clone RB6-8C5; kindly provided by L. D. Sibley) was used at a 1:250 dilution. Rabbit anti-*Leishmania* arginase (kindly provided by B. Ullman) was used at a 1:1000 dilution. The following antisera were screened for reactivity to nitrotyrosine in interferon-γ/LPS stimulated macrophages: rabbit anti-nitrotyrosine (Santa Cruz Biotechnology, sc-55256; Millipore, #06-284; Abcam, ab50185), mouse anti-nitrotyrosine (Santa Cruz Biotechnology, sc-32757), and rat anti-nitrotyrosine (Abcam, ab6479))

The following fluorescent secondary antibodies were used: Alexafluor555 goat anti-rabbit, Alexafluor633 goat anti-rabbit, Alexafluor488 goat anti-rat, Alexafluor555 goat anti-rat, Alexafluor633 goat anti-rat, Alexafluor568 goat anti-hamster, Alexafluor488 goat anti-chicken, Alexaflour555 donkey anti-goat, and Alexafluor647 donkey anti-rabbit (Invitrogen, all used at 2 μg ml^{-1} concentrations).

Tissue preparation and histological staining

After euthanasia, infected draining popliteal lymph nodes or feet were harvested. The infected tissue was then fixed for 1 h at room temperature in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). After fixation, tissues were incubated at $4^{O}C$ for in 10%, 20%, and then 30% (w/v) sucrose in PBS. After an overnight incubation in 30%

sucrose, the tissues were embedded in O.C.T. compound (Ted Pella, Inc.), cut into 6 μm thick sections using a cryostat, and mounted onto microscope slides.

Unless otherwise indicated, tissue sections were stained as follows. Slides were washed in PBS, and tissue were then blocked and permeabolized in PBS containing 5% (v/v) normal goat sera (Vector labs) and 0.1% (v/v) Triton-X-100 for 30 min. The sections were then stained with various combinations of primary antibodies (as described in the text) for 1 h. Unbound antibody was then washed off in PBS and primary antibodies were detected with combinations of fluorescent secondary antibodies (as described in the text) for 40 min, followed by a second wash in PBS. For some experiments, we needed to simultaneously stain tissue sections with different antibodies that were both generated in rabbits. To do this, the tissue was stained with an unlabeled rabbit primary antibody and a fluorescently-labeled secondary antibody as described above. Next, the tissue was blocked for 30 min with a buffer containing 5% (v/v) normal rabbit sera (Sigma Aldrich), and then the second fluorescently conjugated primary antibody was used.

For TUNEL staining of the tissue sections, after all primary and secondary antibody staining was finished, the sections were stained with the *In Situ* Cell Death Detection Kit, TMR Red (Roche), according to the manufacturer's protocol. All sections were mounted in ProLong Gold reagent (Invitrogen).

BrdU staining and in vitro experiments

For all BrdU experiments, paraformaldehyde-fixed samples were permeabilized with 0.1% (v/v) Triton-X-100 in PBS for 15 min, washed in distilled water, and then immersed in 2 M HCl for 40 min to denature the DNA. After extensive washing with

PBS, the samples were incubated in a blocking buffer containing PBS and 5% (v/v) normal goat sera and 0.1% (v/v) Triton-X-100, and were then stained as described in the text in the methods above. All BrdU stains used a 2 h incubation with the anti-BrdU antibody.

To test BrdU in promastigotes in culture, either log- or stationary-phase *L. major* promastigotes were cultured in M199 media that contained 0.1 mM BrdU (Sigma) for the indicated time, after which they were fixed in 4% (w/v) paraformaldehyde in PBS for 10 min and stained as described above. To test BrdU in infected macrophages *in vitro*, starch-elicited peritoneal macrophages (PEMs) were harvested and infected as described with stationary-phase parasites (22). Two hours after parasites were added, the macrophages were washed to remove extracellular parasites and placed in media containing 0.1 mM BrdU. The infected macrophages were maintained in BrdU-containing media for the remainder of the five-day experiment. At two hours, 1 day, 2 days, and 5 days post-infection, samples were fixed and stained as described above.

BrdU incorporation assay in vivo

Several different methods were attempted to administer BrdU to the infected mice. In the preferred method, infected mice were injected every 3 h hours for 18 h with 200 µl of PBS containing 4 mg ml^{-1} BrdU into the peritoneal cavity and 50 µl of this solution directly into the infected footpad, yielding a total dose of 6 mg BrdU. We also tried administering BrdU in the drinking water (1 mg ml^{-1}), via infusion using osmotic pumps (Alzet #2001D, 7.2 mg total dose), and single intraperitoneal injections of 200 µl of PBS containing 4 mg ml^{-1} BrdU. 24 h after the first dose, the mice were euthanized and

footpad tissue prepared and stained as described above. Volocity image analysis software (Improvision) was used to assist counting.

Generation of alternatively activated macrophages

PEMs were harvested and plated on glass coverslips. 24 h after isolation, the media was replaced with fresh media containing 100 U ml^{-1} each of recombinant IL-4 (BD Pharmingen) and IL-13 (BD Pharmingen) for 48 hours.

Comparison of iNOS staining intensity

PEMs were isolated and allowed to attach to glass coverslips. 24 h after isolation, the media was replaced with fresh media containing 100 U ml^{-1} recombinant interferon-γ (Chemicon) and 100 ng ml^{-1} LPS (Sigma). 24 h later, the cells were fixed in 4% (v/v) paraformaldehyde and stained in parallel with footpad tissue sections from *lpg2-* -infected mice with antibodies against *L. major* histones and iNOS, and nuclei were stained with TOPRO-3 (Invitrogen). To determine the fluorescence intensity of iNOS per cross-sectional area, the outline of each cell from a confocal stack was traced in Volocity software (Improvision) and the sum intensity of all "red" pixels (iNOS) in the selected area was divided by the total number of pixels in that area, yielding a mean pixel intensity for the cross section.

Comparison of arginase staining intensity between persistent parasites and promastigotes

Footpad tissue sections infected with persistent WT or log-phase WT promastigotes were labeled to detect parasite arginase with a rabbit anti-*Leishmania* arginase antibody (a gift

from B. Ullman, diluted to 1:1000) and parasite histones (with the fluorescently-conjugated anti-histone antibody). Confocal images were acquired using identical settings, and then Volocity image analysis software was used to determine the total arginase fluorescence intensity on a per-cell basis. To do this, confocal stacks were compressed into a single plane, and then the total arginase fluorescence intensity was determined within a 2.28 μm radius circle centered on parasite nuclei.

Macrophage infections

PEMs were harvested and plated on glass coverslips in 24 well dishes. The following day, some cells were stimulated with 100 U ml^{-1} recombinant interferon-γ (Chemicon) and 100 ng ml^{-1} LPS (Sigma) with or without the iNOS-inhibitor L-NIL (Cayman Chemical) at a concentration of 10 μM. Four hours later, metacyclic stage WT *L. major* strain Friedlin V1 (MHOM/IL/80/Friedlin) that had been opsonized in C5-deficient mouse sera were added to the wells with a parasite to PEM ratio of ~10:1. Two hours later, extracellular parasites were removed by extensive washing, and some coverslips were removed and stained with the DNA stain Hoechst to determine initial parasite titers. 24 hours after infection, nitrite production was determined by the Greiss assay (Sigma) and samples were removed to determine parasite titers. The remaining samples were left alone until 72 hours after infection, at which time some samples were removed to determine parasite titers. Media containing various combinations of interferon-γ + LPS and L-NIL was added at this time point. At 96 hours after infection, nitrite production was determined by the Greiss assay, and all samples were stained to detect parasite nuclei and DNA. For these experiments, 'percent survival' is defined as the ratio of the number

of parasites per 100 PEMs at either 24 or 94 hours after infection with the number of parasites per 100 PEMs 24 hours earlier.

Microscopy

All microscopy of was performed on a Zeiss 510 META confocal laser scanning microscope. Cutoffs for saturation and background levels were adjusted with Photoshop software (Adobe).

Statistics

Throughout the manuscript, data are presented as the geometric mean ± the standard deviation. *P* values are calculated by the Student's t-test method.

Results

Development of a BrdU incorporation assay for Leishmania

Preliminary studies of the *L. major* cell cycle suggest that the parasites extend the duration of G_1 phase, but not S- or G_2 phase, under conditions of slow growth *in vitro* (Supplementary figure S1). This suggests that a BrdU-incorporation assay (23) would allow us to determine if persistent parasites replicate in their hosts and, if so, to compare the doubling time of persistent parasites with that of acute-phase parasites. To develop this assay, we first examined parasites growing in logarithmic phase *in vitro*, labeled with 0.1 mM BrdU for various periods followed by fixation and staining with a pool of antibodies specific to *L. major* histone proteins to label parasite nuclei and anti-BrdU antisera (24). As expected, BrdU was incorporated into both the kinetoplast (mitochondrial) DNA network and the nucleus. Under conditions where parasites replicated with a doubling time of approximately 8 hr, the percent of $BrdU^+$ cells rose from 0 to 90% over a period of 9 hr (Figure 1A). Other studies employing longer labeling times failed to increase the percentage to higher values, suggesting that this is the technical limit of this experimental protocol. As expected, increasing parasite doubling time results in a lower fraction of $BrdU^+$ parasites (Supplementary figure S1) and addition of BrdU for 12 hr to cultures that had been in stationary phase for 24 hours yielded no $BrdU^+$ cells (data not shown). Importantly, culture of *L. major*-infected macrophages *in vitro* in the presence of BrdU for 72 hr resulted in up to 90% BrdU-labeling of intracellularly replicating amastigotes, indicating that BrdU can enter the phagolysosome.

We next tested the ability of BrdU to label replicating parasites in acute mouse infections (2-3 weeks post-infection). We tried several different methods to deliver BrdU to the mice including in the drinking water, by intraperitoneal injection, through subcutaneous infusion from surgically implanted osmotic pumps, and via injection of BrdU into the peritoneum combined with direct injection of BrdU into the infected footpad. Mice were sacrificed 24 hr after the beginning of the dosing period and the strategy that yielded the highest percentage of $BrdU^+$ parasite nuclei was determined.

Although all of the above strategies resulted in $BrdU^+$ host cell nuclei within our acutely-infected tissue sections, $BrdU^+$ parasites were only detected in mice that received the combined intraperitoneal and subcutaneous footpad BrdU injections. We found that the percentage of parasites that labeled in a 24 h period increased linearly with the number of doses (Figure 1B). We adopted a dosing regimen of 6 doses given every 3 h as a 'standard' protocol as it presented the maximum dosing schedule tolerable to both the mouse and experimenter. Assuming a 24 hr labeling period and a parasite doubling time of 60 h (as determined by the use of luciferase-expressing parasites; Hickerson et al, unpublished data), we calculate that 40% of acute-phase parasites should be $BrdU^+$. Using the above protocol, 44 ± 6% of acute-phase *L. major* were $BrdU^+$ 24 h after the first dose of BrdU, a value which is consistent with our calculations and which we use as representative of "maximal" replication. Throughout the remainder of this manuscript, we will use the following nomenclature to summarize our summary statistics: N = # of experiments (E) / total # of mice (M) / total # of parasites (P). Thus, for the experiment described above, N = 2E / 5M / 5289P.

Persistent parasites replicate in vivo

We then tested whether persistent *L. major* incorporate BrdU into their DNA. Here, we define the asymptotic "persistent" phase as >1 month following the resolution of footpad swelling at the inoculation site. When persistently infected mice were treated with BrdU as described above, 19 ± 6% of persistent parasites had $BrdU^+$ nuclei (N = 2E / 6M / 578P; Figure 1C and D). These data establish for the first time that persistent parasites replicate *in vivo*.

As a second sign of parasite replication, we developed an indirect assay based on the distribution of parasites within the tissue. This assay is based on the assumption that in the persistently-infected state, host cells are initially infected by a single parasite, as the low number of parasites present in the tissue makes multiple independent infections of the same host cell unlikely. Thus, host cells containing two or more parasites can be viewed as sites of intracellular replication. Using this indirect assay, we regularly found instances in which host cells contained between 2-20 *L. major* cells, which we refer to as "parasite clusters". Roughly half of all infected cells contain 2 or more parasites, and ~80% of all parasites are found in "clusters" (N = 3E/8M/888P; 386 infected cells; Figure 2A and B).

Interestingly, the percentage of $BrdU^+$ parasites was about 50% as much as that of acute-phase parasites ($P < 0.0001$ by Student's t-test). Assuming that persistent parasites replicate as a homogeneous population, the percent labeling observed here suggests about 2-fold slower replication, or a doubling time of ~120 hr. Alternatively, these data would fit a model where about ½ of the parasites replicate at rates comparable to exponentially growing parasites (44% labeling), while the others are either dead or in some kind of arrest. We sought to differentiate between these models by plotting the percent of parasite

clusters as a function of the percent BrdU-labeling within those clusters. Assuming that the parasites are replicating homogeneously, we would anticipate a distribution of percent labeling centered at the mean. Acute-phase parasites fit this model, with 61% of all clusters showing between 21% and 70% labeling. In contrast, persistent parasites do not show a single distribution centered at 19% labeling, but rather appear to have two peaks representative of two different types of clusters (Figure 2C). The first type, which accounts for ~60% of the total clusters, is not labeled with BrdU during the duration of the experiment. The other population had a mean labeling frequency of 57 ± 21% (N = 2E/5M/170P/50 clusters). This second population resemble acute phase parasites (mean labeling frequency of 46 ± 29%; N = 2E/5M/585P/77 clusters). While this analysis strongly suggests the existence of a poorly-replicating sub-population, it excludes data from parasites not in 'clusters', which account for ~20% of all persistent parasites. We thus re-analyzed the above data, plotting percent BrdU labeling as a function of the number of parasites per infected cell. Assuming homogeneous parasite replication, roughly 44% of acute-phase and 19% of persistent parasites should be $BrdU^+$, regardless of the number of parasites per host cell. The data from acute-phase parasites very closely matches this prediction, with 40-50% BrdU-labeling independent of the number of intracellular parasites (N = 2E/5M/976P/176 infected cells). However, once again the persistent parasite data do not fit a homogeneous replication model. ~12% of the parasites in host cells containing 1 to 3 parasites are BrdU+, while ~46% of the parasites within 'clusters' containing 4 or more parasites are $BrdU^+$ (Figure 2D; N = 2E/6M/379P/167 infected cells). These results suggest that a population of host cells contain persistent parasites that replicate similarly to acute phase parasites, whereas another population of

host cells contains parasites that replicate poorly. This second population is not apparent in acutely infected mice.

Persistent parasites predominantly reside in macrophages and dendritic cells

We also sought to determine the localization of persistent parasites. Footpad and lymph node sections were stained to detect *L. major* nuclei and host cell-type specific markers. At least three cell types have been proposed as hosts for persistent parasites: fibroblasts, macrophages, and dendritic cells (25-27). Here, we define fibroblasts as cells that are recognized by anti-ER-TR7 antisera, dendritic cells (DCs) as cells expressing CD11c (either $F4/80^+$ or $F4/80^-$), and macrophages as F4/80-positive, CD11c-negative cells. For this and other experiments, confocal microscopy allowed us to ensure that persistent parasites were actually 'within' the host cells, as we could visualize the host cell plasma membrane or cytoplasm (depending on the antibody used) in the X, Y, and Z planes. Only 2 ± 3% of persistent parasites in footpad tissue and 10 ± 7% of persistent parasites in lymph nodes were within are within ER-$TR7^+$ host cells (footpad N = 3E / 6M / 528P; lymph node N = 2E / 4M / 191P), despite the presence of numerous ER-$TR7^+$ cells in each field from both sites. In contrast, 80 ± 6% of footpad persistent parasites and 87 ± 12% of lymph node persistent parasites were found within $F4/80^+$ cells in tissue (footpad N = 3E / 8M / 983P; lymph node N = 2E / 4M / 208P). 13 ± 2% of persistent footpad parasites and 61 ± 19% of lymph node parasites were within $CD11c^+$ cells (footpad N = 3E / 8M / 1074P; lymph node N = 2E / 4M / 208P; Figure 3A and B). By dual-staining sections to simultaneously detect *L. major* histone proteins, CD11c, and F4/80, we found that 78 ± 9% of footpad persistent parasites were within $F4/80^+CD11c^-$ macrophages, and 16 ± 6% were within $F4/80^+CD11c^+$ DCs in footpad tissue (N = 2E / 4M / 266P). In

lymph nodes, 30 ± 18% of persistent parasites were within F4/80$^+$CD11c$^-$ cells, and 61 ± 19% were within F4/80$^+$CD11c$^+$ DCs. Importantly, essentially all footpad persistent parasites (97 ± 3% in footpads and 91 ± 5% in lymph nodes) imaged in this experiment were labeled with one or both markers, showing that almost all persistent parasites are within macrophages and dendritic cells at both sites (with ~10% of lymph node parasites in ER-TR7$^+$ fibroblasts) and that a major host cell type was not missed (Figure 3C).

The distribution of persistent parasites suggests intracellular replication in both macrophages and dendritic cells

We used the "parasite cluster" analysis described above to address where parasite replication takes place. Similar to the distribution of total persistent parasites within host cell types in footpads, 74 ± 5% of parasite clusters occurred within host cells expressing F4/80, while 17 ± 5% were within CD11c$^+$ cells. These data suggest that the intracellular replication takes place within both macrophages and dendritic cells as the percentage of parasite clusters within F4/80$^+$ cells (which include CD11c$^+$ cells) greatly exceeds the percentage of clusters within CD11c$^+$ cells (Table 2). We plotted the percent of infected cells expressing CD11c as a function of the number of parasites per cell to determine if dendritic cells preferentially harbor the 'static' sub-population which tends to be within host cells containing <3 parasites but did not see any obvious correlation (Supplementary figure 2A; N = 3E/8M/283P/124 infected cells.)

Macrophages infected with persistent L. major in footpad tissue do not express markers of alternative activation.

We hypothesized that some F4/80$^+$ cells containing persistent *L. major* may be alternatively activated macrophages and that these cells may provide a favorable environment for parasite survival. To test this, we stained footpad sections from persistently infected mice to detect parasite histones, F4/80, and RELMα (a marker of alternatively activated macrophages) (28, 29). As above, the majority of persistent *L. major* were within F4/80$^+$ cells (96 ± 2%), but none of these infected cells expressed RELMα (N = 2E/3M/284P). Positive controls included the visualizing of F4/80$^+$RELMα$^+$ cells elsewhere in the same tissue sections and the ability of the anti-RELMα antibody to yield 70-80% positive cells in alternatively activated macrophages obtained by IL-4 and IL-13 treatment of peritoneal macrophages (Supplementary figure S1). These data suggest that persistent parasites do not favor alternatively activated macrophages as a site of replication.

Most persistent L. major are found within iNOS$^+$ macrophages and dendritic cells

The finding that persistent parasites replicate suggests that they must also be destroyed, or else parasites number would increase. Nitric oxide (NO), generated from L-arginine via iNOS, is essential for the control of *L. major in vivo*, and as such, it has been assumed that *L. major* is killed within iNOS-expressing host cells (3, 4, 30). Thus we asked whether some fraction of persistent parasites was found within iNOS$^+$ host cells and whether it is within these cells that the parasites are destroyed. 59 ± 15% of persistent parasites in footpads were found within iNOS-expressing cells (N = 3 E / 8M / 2535P). In addition, 80 ± 19% percent of persistent lymph node parasites are within iNOS$^+$ cells (N = 2E / 4M / 477P), in agreement with previously published data (30).

Both of the major persistent parasite-harboring host cell types (macrophages and dendritic cells) were found to express iNOS when infected with persistent *L. major*. 10 ± 6% of total persistent parasites were found within CD11c/iNOS double positive cells (Figure 4A; N = 3E / 8M / 1074P). As our instrument can capture at most 3-color images, we were unable to simultaneously visualize parasites with F4/80, CD11c, and iNOS. However, the fact that the percentage of parasites within $F4/80^{+}iNOS^{+}$ cells (42 ± 14%; N = 3 E / 8M / 983P) is more than 3 times the total fraction of parasites within $CD11c^{+}$ cells argues that many of the $F4/80^{+}iNOS^{+}$ cells were macrophages (Figure 4B).

Persistent L. major survive and replicate within iNOS-expressing cells

We asked next whether the persistent parasites seen in $iNOS^{+}$ cells showed evidence of destruction. First, we evaluated whether the parasites in iNOS-expressing host cells were morphologically intact. The parasites used in these experiments express GFP, allowing the parasite cytoplasm within host cells to be clearly delineated using a chicken anti-GFP antibody while iNOS expression is simultaneously visualized using a rabbit anti-iNOS antibody. In these studies, parasites within $iNOS^{+}$ host cells were morphologically normal and indistinguishable from persistent parasites within $iNOS^{-}$ host cells, suggesting that the parasites in iNOS-expressing cells are intact (Figure 4C). In addition to overall cellular morphology, the integrity of the parasite's nuclear genome is a good marker for parasite viability (31). Of the 80 parasite nuclei visualized in these experiments with the anti-histone antisera, 79 were within $iNOS^{+}$ cells. However, none of these parasite nuclei had $TUNEL^{+}$ nuclei regardless of their localization within $iNOS^{+}$ or $iNOS^{-}$ cells (Figure 4D; N = 2E/3M/80P). In contrast to the result for nuclear DNA integrity, a population (26 ± 15%) of persistent parasites had $TUNEL^{+}$ kinetoplast DNA. As kinetoplast replication

involves the transient formation of double-stranded breaks that are recognized by the TUNEL reaction (32), this may be expected of healthy cells.

To determine if parasite replication was taking place in $iNOS^+$ cells, we determined if parasites within iNOS expressing cells incorporate BrdU. We stained tissue sections from persistently-infected mice that had been injected with BrdU (as described above) to simultaneously detect parasite histones, BrdU, and iNOS. In these sections, 82 ± 10% of the parasites were within $iNOS^+$ cells, slightly higher than that seen in other experiments ($P = 0.03$ by the Student's t-test). 85 ± 13% of the $BrdU^+$ parasites were within $iNOS^+$ cells, suggesting that persistent parasite replication does not preferentially occur within $iNOS^-$ host cells (Figure 4E and Table 1, N = 2E / 3M / 254P)

To further examine if persistent parasites replicate within iNOS-expressing cells *in vivo*, we used the indirect assay of replication looking for "parasite clusters" within iNOS expressing cells and found that 61 ± 9% of persistent parasites "clusters" (Table 1, N = 3E / 8M / 329 clusters) occurred within cells expressing iNOS. The percent of parasites within $iNOS^+$ cells does not obviously correlate with the number of parasites per infected cells (Supplementary figure 2B; N = 3E/8M/305P/137 infected cells), suggesting that neither the fast-replicating nor poorly-replicating sub-population of parasites are preferentially found within these cells. Taken together, these data show that infected iNOS-expressing cells may be at least transiently permissive host cells for persistent parasites.

Persistently infected cells express high levels of iNOS

One explanation for the apparent survival of persistent parasites within iNOS$^+$ cells might be that the level of iNOS expression by persistently infected cells is lower than that of cells which generate lethal levels of NO. To test this, we compared the iNOS staining intensity between persistently infected iNOS$^+$ cells with that of interferon-γ/LPS-activated PEMs, which kill *L. major* in an iNOS-dependent manner (33). Quantitation of iNOS staining intensity per cell area showed that *in vivo* persistently infected host cells actually expressed higher relative iNOS levels than did the activated PEMs *in vitro* (Figure 5; 1.6 fold higher average fluorescence intensity; $P < 0.001$ by Student's t-test). Thus reduced iNOS expression cannot account for the survival of persistent parasites in iNOS$^+$ host cells, suggesting that the cells containing persistent parasites *in vivo* express sufficient iNOS to generate leishmanicidal levels of NO.

Neither host nor parasite arginase is up-regulated in association with infected iNOS-expressing host cells

iNOS mediated NO synthesis requires arginine, and depletion of arginine through the action of arginase in infected or neighboring cells could serve to limit NO production despite high levels of iNOS (34). Mice synthesize two isoforms of arginase which differ in their sub-cellular localization: Arg1 which is cytoplasmic, and Arg2, which is mitochondrial (35). Parasite infected host cells were visualized using anti-histone antisera and scored for iNOS$^+$ and arginase(1 or 2)$^+$; additionally, we scored whether adjacent cells (defined as in physical contact with the parasite-infected cell) were arginase$^+$. We chose to look at cells 'adjacent' to the infected cell because some arginase-expressing cells are thought to deplete arginine in their immediate vicinity (36, 37). In these experiments 83 ± 14% of persistent parasites were within iNOS expressing cells,

however only 3 ± 3% were within cells that simultaneously expressed iNOS and Arg1, and only 12 ± 11% of WT persistent parasites within $iNOS^+$ cells were adjacent to an $Arg1^+$ host cell (Figure 6A). Pooling these data we find that 15 ± 14% of persistent parasites within iNOS-expressing cells are either within or adjacent to an $Arg1^+$ cell (N = 3E / 5M / 317P). We did not detect any persistent footpad parasites within $iNOS^+$ cells that were also within or adjacent to $Arg2^+$ host cells (N = 2E / 2M / 175P) despite seeing regions of intense Arg2 staining elsewhere in the tissue (all of which were > 75 microns from parasites) indicative of proper Arg2 reactivity (data not shown).

While *L. major* also have an arginase gene, promastigotes do not appear to express sufficient arginase to affect NO production by activated macrophages *in vitro* (18, 38). However, persistent parasites may express higher levels of arginase. We stained in parallel *L. major* promastigotes and footpad tissue sections from persistently-infected mice to detect parasite histones and parasite arginase and compared their relative arginase staining intensity on a per-cell basis (Figure 6B and C). We find that most, if not all, persistent parasites express some level of arginase (N = 2E / 2M / 65 P). However, the mean relative arginase fluorescence intensity of promastigotes was 2-fold higher than that of persistent parasites when differences in background fluorescence are taken into account ($P = 10^{-6}$ by the Student's t-test), suggesting that *L. major* do not up-regulate arginase during the persistent phase of the infection (Figure 6D).

Amastigotes are more resistant to NO than metacyclics within activated macrophages in vitro

Our findings that persistent parasites within iNOS$^+$ host cells appear viable was surprising and suggested that persistent parasites may be more tolerant of NO than promastigotes. As a surrogate for persistent parasites, we sought to compare the NO tolerance of amastigotes with that of metacyclic promastigotes *in vitro*. As expected, metacyclic-stage parasites were rapidly killed in an within peritoneal macrophages (PEMs) that had been pre-treated with interferon-γ and LPS, with 10-fold fewer parasites surviving 24 hours after infection than parasites added to untreated PEMs (Figure 7A; $P < 0.05$). Treatment of activated PEMs with the specific iNOS inhibitor L-NIL (39) reduced the parasite mortality showing that parasite killing is iNOS-dependent (Figure 7A). In contrast, parasite killing by activated macrophages was greatly reduced when metacyclic-stage *L. major* were allowed to infect PEMs for 72 hours (in which time they differentiate into amastigotes and begin to replicate) prior to the addition of interferon-γ and LPS. Under these conditions, 94 ± 17% of the parasites remain 24 hours after host cell stimulation despite the production of substantial quantities of NO by the stimulated macrophages. Relative to unstimulated controls or cells stimulated in the presence of L-NIL (in which the parasite number continues to increase) there is a 1.5-fold reduction in parasite titers in the stimulated PECs (Figure 7B). These data suggest that amastigotes are substantially more tolerant of NO than are promastigotes (94% versus 6% survival; $P < 0.001$ by the Student's t-test).

*Attenuated lpg2- L. major resembles WT persistent parasites in most respe*cts

L. major parasites lacking the *LPG2* gene (*lpg2-)* are a proposed model of *Leishmania* persistence (12). We sought to determine the cell types infected by *lpg2-* in footpad tissue and whether these parasites replicate *in vivo*. Because *lpg2-* appears to enter a

persistence-like state almost immediately, we performed most experiments 1 month after infection (versus 5 or more months post infection with WT parasites). At this time point, *lpg2*- resembled WT persistent parasites for most of the parameters tested above (Table 2). Among these WT persistent parasites and *lpg2*- were indistinguishable from each other in terms of the percent of parasites labeling with BrdU, the percent of parasites within F4/80$^+$ cells, and the percent of parasites found within iNOS$^+$ host cells. Like persistent WT parasites, *lpg2*- parasites were not found within cells labeled with the markers ER-TR7, RELMα, nor cells that express high levels of Arg2. In addition *lpg2*-, like WT persistent parasites, replicate within iNOS-expressing cells by both BrdU and cluster analysis.

More lpg2- parasites are within dendritic cells or associated with Arg1$^+$ cells than persistent WT

We found two differences between the phenotype of cells infected with *lpg2*- versus those infected with persistent WT parasites (Table 2). First, the frequency of *lpg2*- within CD11c$^+$ cells was elevated 3-fold relative to WT persistent parasites (46 ± 29%, $P <$ 0.006; N = 3E / 10M / 1004P). Among the *lpg2*- -infected mice, there appears to be a bimodal distribution of parasites within CD11c$^+$ cells (in one group, <40% of the parasites are within CD11c$^+$ cells, while in the other, >60% of the parasites are within these cells). Both of these groups were present in all experiments. We also looked to see if either group was associated with higher expression of iNOS relative to the other group, but found no obvious correlation.

There was also a clear difference between WT and *lpg2*- parasites in terms of their association with Arg1. 37 ± 41% of *lpg2*- parasites were found in $iNOS^+/Arg1^+$ host cells with another 35 ± 32% found in $iNOS^+$ host cells adjacent to an $Arg1^+$ host cell. In total, 72 ± 26% of persistent footpad *lpg2*- parasites were associated with Arg1 expression which is roughly 5-fold higher than WT ($P = 0.002$, N = 3E / 5M / 477P).

Differences between the cell association of persistent WT and lpg2- parasites are not due to the duration of infection

The observed differences between the two parasite lines could result from differences in the duration of infection (e.g. one month for *lpg2*- versus >5 months for persistent WT). Thus, we compared the association of *lpg2*- parasites one month after infection with that of *lpg2*- 5 months after infection focusing on their association with CD11c and Arg1. 78 ± 13% of *lpg2*- parasites are within $CD11c^+$ DCs 5 months after infection (Figure 8A; N = 2E / 5M / 520P). This is 6-fold higher than what is observed for persistent WT ($P < 10^{-6}$) and 1.7-fold higher than *lpg2*- at one month ($P = 0.03$). In addition, 85 ± 12% of *lpg2*- parasites within footpad tissue 5 months after infection are either within or directly adjacent to a cell expressing Arg1 (Figure 8B; N = 2E / 5M / 901P). Although this was indistinguishable from *lpg2*- at one month post-infection (68 ± 27%), it is 5-fold higher than persistent WT ($P < 10^{-3}$). Taken together, these data suggest that the differences observed between persistent WT and *lpg2*- are not the result of differences in the duration of the infection but are more likely due to differences in the interactions between the two parasite lines and the host.

Association with Arg1 negatively correlates with the ability of lpg2- to vaccinate mice

Unlike WT persistent parasites, *lpg2*- does not vaccinate C57BL/6 mice without the use of CpG DNA as an adjuvant (40). We asked whether the observed differences between *lpg2*- and persistent WT correlate with the relative inability of *lpg2*- to vaccinate mice of this strain. To address this, we investigated the association of *lpg2*- parasites and host cells expressing these proteins in BALB/c mice, which become highly immune to *L. major* infections following vaccination with *lpg2*- (14). The percent of *lpg2*- within $CD11c^+$ cells in BALB/c mice is indistinguishable from that of *lpg2*- in C57BL/6 mice (44 ± 21% versus 46 ± 29%; N = 2E / 10M / 745P), and significantly higher than what is seen for WT persistent parasites (Figure 8A; $P = 0.001$). Thus, the presence of *lpg2*- parasites within $CD11c^+$ dendritic cells does not correlate with the ability of the parasite to vaccinate its hosts.

On the other hand, the association of *lpg2*- with host Arg1 expression does change depending on its ability to vaccinate mice. Unlike *lpg2*- within C57BL/6 mice, in which a relatively high percentage of the parasites (72%) are either within or adjacent to an Arg1-expressing cell, only 16 ± 25% of *lpg2*- in BALB/c mice is associated with Arg1 (Figure 8B; $P < 0.005$; N = 2E / 9M / 1105P), and are thus indistinguishable from WT persistent parasites in a C57BL/6 mouse. These data suggest that Arg1 expression may be a negative correlate of *L. major* immunity.

Discussion

Replication and localization of persistent L. major

In this study, we examined the localization of persistent *L. major* in the footpad inoculation site and the draining lymph node. At both sites, parasites were overwhelmingly found within either F4/80^{+}CD11c^{-} macrophages or F4/80^{+}CD11c^{+} dendritic cells with ~10% of lymph node persistent parasites within ER-TR7^{+} reticular fibroblasts. A high percentage of both infected macrophages and dendritic cells expressed iNOS in lymph node and footpad tissue.

Because the number of persistent parasites remains roughly constant over time, it has previously been difficult to determine whether these parasites are replicating or not, as constant populations could result from either a long-lived, non-replicating form of the parasite or continual parasite replication and destruction. Traditional methods for determining parasite replication *in vivo* (such as limiting dilution assays) cannot distinguish between these two scenarios. We therefore developed a BrdU-incorporation assay to detect parasite replication *in vivo*. Using this assay, we found for the first time that persistent parasites do indeed replicate in footpad tissue.

Identification of fast-replicating and poorly-replicating subpopulations

Interestingly, persistent parasites showed about 2-fold less BrdU incorporation than acute-phase parasites after a 24 hour labeling period. Further analysis of our data identified two populations of host cells. The first population is characterized by a low number of intracellular parasites (less than 3) and a low percentage of BrdU-labeling

(~12%) while the other population had a larger number of intracellular parasites and BrdU-labeling comparable to acute-phase parasites (46%). These data suggests that there are either two populations of parasites based on their relative replication or two populations of host cells based on their relative ability to permit parasite replication. As the poorly-replicating population of parasites tends to be within host cells containing 1-3 parasites, we looked to see if cells expressing CD11c or iNOS were correlated with relatively low intracellular parasite burdens, but we found no obvious association. Another question is whether or not the poorly replicating parasites are alive and in some sort of stasis or are dead/dying. While current technologies do not allow us to definitively answer this question, we favor the model that the poorly replicating parasites are alive because we were unable to detect any parasites that were morphologically abnormal or that had degraded DNA. Further suggesting that these parasites are alive, parasites killed intracellularly are digested by the macrophage and disappear rapidly (31) and therefore would be difficult to find.

Our finding that a large percentage of persistent *L. major* is in a non-replicating state fits in well with data from other organisms which cause life-long asymptomatic persistent infections. Herpes viruses, which express only a few select transcripts during latent infections (41), are probably the best example of a pathogen that has adopted a strategy of quiescence to facilitate persistence. In addition, *Toxoplasma gondii* persist within their host in the poorly replicating bradyzoite stage (42), and a population of persistent *Mycobacterium tuberculosis* are proposed to be in a quiescent 'persister' state (43). Presumably, quiescence helps these pathogens avoid the immune response and increase

their antimicrobial tolerance. *Leishmania* may be benefited in the same ways, which may explain the difficulty of achieving a sterile cure of persistent infections (44).

With the exception of persistent *Leishmania*, the quiescent forms of the pathogens listed above are known to have distinct gene expression profiles from the actively replicating forms (45-47). It is not yet known whether or how the quiescent persistent parasites differ from the replicating persistent parasites or if persistent parasites in general differ from acute-phase amastigotes. Future studies comparing the gene expression profiles of persistent parasites with acute phase parasites, as well as quiescent persistent parasites (enriched in small clusters) versus replicating persistent parasites (enriched in large clusters) will be crucial to see if *Leishmania* also has a stage of its life cycle that is specifically devoted to persistence.

Persistent parasites within iNOS-expressing cells appear healthy

As NO-synthase activity is an essential component of *L. major* killing by activated macrophages *in vitro* (33) and in the control of experimental leishmaniasis by resistant mice (3, 48) we postulated that iNOS-expressing cells were sites of parasite destruction. Surprisingly, however, the parasites that we observed within these cells were not 'corpses' but rather were morphologically normal and had intact nuclear genomes as determined by TUNEL staining. Furthermore, these parasites appeared to be replicating both by the 'parasite cluster' criteria and by BrdU-incorporation data. Taken together, these data suggest the parasites we observed within iNOS-expressing cells were at least transiently viable.

Our data therefore raise the question of whether these infected iNOS-expressing cells generate lethal amounts of NO. The level of iNOS protein within persistently infected cells is comparable to that of interferon-γ/LPS-treated peritoneal macrophages, which produce lethal amounts of NO, suggesting that the capacity of persistently infected cells to generate NO is not limited by insufficient iNOS expression. Although we cannot measure the levels of reactive nitrogen within infected iNOS-expressing cells *in vivo*, we can look at known pathways that may attenuate iNOS activity; one of which being the arginase pathway. This pathway could potentially compete with iNOS for its substrate (L-arginine), resulting in less nitric oxide production. As mentioned above, very few WT persistent parasites within iNOS-expressing cells are associated with elevated levels of Arg1 expression and we see no association of persistent parasites with elevated levels of Arg2. As such, it is unlikely that either isoform of host arginase attenuates the ability of iNOS-expressing cells to generate NO. In addition to Arg1 and Arg2, *L. major* express their own arginase. However, the arginase activity within *L. major* is extremely low *in vitro* and does not appear to impact the amount of NO produced by classically activated macrophages infected with promastigote-stage parasites (18, 38) . Using antibodies specific to parasite arginase, we show that persistent parasites (most of which were within iNOS-expressing cells) do not increase arginase expression relative to promastigotes, suggesting that parasite arginase is not an anti-iNOS defense mechanism. Furthermore, preliminary data show that *arg- L. major* are capable of persistence within resistant mice following healing (J. Uzonna, personal communication). Based on these data, it seems unlikely that parasite-derived arginase affects the capacity of host cells to generate NO. While there may be other mechanisms, such as aggresome formation or iNOS miss-

localization, by which high levels of iNOS are expressed without the parasites experiencing high levels of NO (49-51), we saw no evidence of such mechanisms in our samples and, as such, we suspect that at least some if not all iNOS-expressing cells are capable of generating large quantities of NO. Survival of persistent parasites within such cells would suggest that persistent parasites are more resistant to the ill effects of NO than are promastigotes. Indeed, our *in vitro* studies show that amastigotes are substantially more resistant to NO than are promastigotes.

Comparison of WT persistent and lpg2- identified arginase 1 as a negative correlate of immunity

One of the aims of this work was to compare the candidate vaccine line *lpg2-* with WT persistent parasites: the gold standard in anti-*Leishmania* vaccines. Previous work has shown that *lpg2- L. major* differs from WT parasites in terms of its ability to survive within macrophages *in vitro* and to cause pathology *in vivo*. However, it resembles WT persistent parasites since it is found in mice in numbers comparable to WT persistent parasites (12) and can vaccinate susceptible (BALB/c) mice against virulent challenge (14). Here, we found that lpg2- was indistinguishable from WT persistent parasites in most respects. Like persistent WT, *lpg2-* parasites are not found within ER-TR7^{+} cells but do infect macrophages and DCs, some of which express iNOS. In addition, our preliminary data also suggests that *lpg2-* recruits Foxp3^{+} cells to the site of infection in BALB/c mice (data not shown), a phenomenon that is also reported for persistent WT parasites (11). Finally, *lpg2-* and WT persistent parasites are indistinguishable in terms of replication, most of which takes place within iNOS expressing cells. Taken together,

these data continue to support the use of *lpg2*- parasites as a model of *Leishmania* persistence.

However, our work has also demonstrated clear differences between WT persistent parasites and *lpg2*-. In particular, significantly more *lpg2*- parasites were within DCs and are associated with Arg1 expression in the footpads of C57BL/6 mice than is the case with WT persistent parasites. We ruled out the possibility that these differences are the result of differences in the duration of the infection as the association of *lpg2*- parasites with CD11c and Arg1 in mice infected for more than 5 months more closely resembles that of *lpg2*- at one month post-infection than WT persistent parasites. Instead, these differences are likely related to differences in the mouse's immune response to the two parasite lines, and may correlate with the inability of *lpg2*- to vaccinate resistant (C57BL/6) mice without the addition of CpG DNA as an adjuvant (40). Indeed, we found that the association of *lpg2*- and host Arg1 correlate inversely with the ability of *lpg2*- to vaccinate its host, as *lpg2*- parasites within BALB/c mice (which are protected by vaccination with *lpg2*-) more closely resemble persistent WT than *lpg2*- in a C57BL/6 mouse with regards to Arg1 association. Whether the increased association of parasites with Arg1 results from or is causative of conditions leading to vaccine failure remains unknown. However, Arg1, along with iNOS, can lead to arginine-depleted microenvironments which have strong inhibitory effects on both T-cell proliferation and function and induce regulatory T-cell differentiation (36, 52). In fact, arginine depletion has been shown to impair *Leishmania*-specific T-cell responses (53). In addition, products of Arg1 activity such as urea and polyamines may also have immunosuppressive effects (54-56). Thus, the up-regulation of Arg1 in *lpg2*- -infected C57BL/6 mice

provides a viable explanation for why the immunity generated by this attenuated parasite line is weak in these mice.

Models of L. major *persistence*

It has long been assumed that persistent *L. major* required "safe cells" to evade lethal nitric oxide that would otherwise result in parasite clearance. Two cell types, ER-TR7^{+} reticular fibroblasts and alternatively activated macrophages have been implicated as such "safe cells" as they do not express high levels of iNOS (25, 26). However, as mentioned above, the persistent *L. major* found within iNOS^{+} cells are apparently healthy by all criteria tested. As such, a cell's capacity to express high levels of iNOS protein may not be a good indicator of how "safe" that cell would be for persistent parasites.

In addition, if the parasites do indeed require safe cells, then such cells should be found at all sites of parasite persistence (i.e. the site of inoculation and the lymph node draining that site). Although our data is in agreement with the published literature about the presence of a population of persistent parasites within ER-TR7^{+} fibroblasts in lymph nodes, we find little, if any, association of persistent *L. major* with this marker at the site of inoculation (the footpad). Instead, we find that the vast majority of the parasites in both footpad and lymph node tissue are within macrophages and dendritic cells. Further investigation revealed that infected macrophages do not express RELMα, a marker of alternative activation, and instead expressed iNOS, a marker of classical activation that is repressed in alternatively activated macrophages (29). While these data do not exclude the possibility that there is a "safe cell type" that serves as a reservoir for persistent *L.*

major, they demonstrate that neither reticular fibroblasts nor alternatively activated macrophages are major reservoir host cell types for parasite persistence.

Our data suggest an alternative model of *L. major* persistence (Figure 9). We have shown that a population of persistent parasites replicate comparably to acute-phase parasites. However, despite substantial replication by these parasites, the parasite number remains roughly constant, thus implying that parasite replication is offset by parasite killing. In this model, the offspring of replicating parasites infect new host cells where they have three potential fates. They may either continue active replication, become 'static' or be destroyed. How the eventual fate of the parasites is decided is not known and may be either stochastic or depend on the phenotype of the host cell infected.

A stem immunogen model of concomitant immunity

The constant replication and destruction of persistent *Leishmania* may help explain the concomitant immunity induced by either persistent WT or *lpg2*- parasites. While replicating or static parasites likely have roles in perpetuating the infection, killed parasites might be a good source of antigen that could be presented to the immune system, maintaining a robust anti-*Leishmania* response. This assumption is reasonable, as antigens from dead parasites can be presented to the immune system whereas live parasites have been shown to inhibit antigen presentation by their host cell (57-59). As parasite replication and destruction would be a continual process in a persistently infected host, such constant boosting would result in the life-long immunity observed in healed *Leishmania* patients. Thus, from the host's perspective, persistent parasites serve as a continually self-renewing vaccine.

In some respects, the model we propose here is comparable to stem cell biology (Figure 9). Like stem cells, persistent *L. major* are capable of distinct fates: either self-regeneration or 'differentiation' into a cell that has functional consequences which in this case is death and immune stimulation. Here, the replicating parasites represent a continually self-renewing "stem" with some of the progeny parasites surviving either as replicating or quiescent forms. However, many of the progeny parasites become 'terminally differentiated' within antigen presenting cells, which could result in immune stimulation. Modulations of the host's immune response would affect the flux of parasites going down one pathway versus the other, resulting in reactivation or sterile cure as extremes (4, 11).

The stem immunogen model relies on the assumption that immune stimulation results from the presentation of antigens derived from killed persistent parasites, a point that has not been established. In fact, although *in vitro* data suggest that parasite killing may be a prerequisite for the presentation of antigens found within the parasite's cytoplasm (60) and it is well established that live *Leishmania* inhibit antigen presentation by their host cells (57-59), it is possible that live parasites, rather than those that are being destroyed, are responsible for the persistent parasite-dependent immune stimulation and maintenance of immunity. Thus, an important future question is what host cells present *Leishmania* antigens: those that contain viable parasites or those that have previously killed parasites. In this study, we were unable to find the latter class of host cells, and so new approaches will be required to properly address this question.

If supported by further studies with persistent *Leishmania*, the stem immunogen model of concomitant immunity could also be proposed for other persistent pathogens such as

Toxoplasma gondii and herpesviruses. Both persist indefinitely while conferring life-long protective immunity against pathology associated with re-infection. In addition, there is evidence of at least sporadic sub-clinical reactivation from the persistent/latent stage of the infection which would result in the production of antigens for immune stimulation (61, 62). Currently, it remains unknown whether these sub-clinical reactivations have a role in maintaining immunity or even whether persistent *Toxoplasma* or latent herpes virus infections are required for immunity. However, there is some evidence that at least partial reactivation is required to elicit protective immunity against herpesvirus challenge. Whereas a replication-deficient γHV68 (ORF50STOP) with a block in immediate early gene expression established latency but failed to protect mice from a challenge from WT virus (63), a latent attenuated virus (ORF31STOP) capable of partial reactivation involving immediate early and early gene expression stimulated robust cellular immunity to WT challenge (64), potentially by continually boosting the host's immune response.

The fact that live attenuated vaccine lines that can persist and at least partially replicate (e.g. *L. major lpg2*- or γHV68-ORF31STOP) can effectively vaccinate their hosts while those that do not partially replicate (e.g. *L. major dhfr-ts* or γHV68-ORF50STOP) are much less effective suggests that the best vaccines may be those consisting of an attenuated pathogen capable of long term persistence and replication/reactivation but incapable of causing pathology. Such a vaccine may have the added benefit of conferring protection to other pathogens as well (2).

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Figure legends

Figure 1. BrdU-incorporation assay with *Leishmania*. A. Graph comparing parasite growth as promastigotes in culture media containing BrdU with BrdU-incorporation. Data shown is one of three experiments. B. The percentage of BrdU-positive acute-phase parasite nuclei after 1, 3, or 6 doses of BrdU in a 24 hour period. Data shown is from one experiment, with three mice per treatment. Error bars represent standard deviation. C. Representative image of six persistent parasite nuclei (red), two of which are $BrdU^+$ (green). Nuclei are stained blue. Scale bar indicates 5μm. D. The percentage of $BrdU^+$ parasite nuclei for acute and persistent *L. major*. Horizontal bars represent the mean for all mice. *P* value was determined by the Student's t-test.

Figure 2. Distribution of persistent parasites within host cells. A. The number of infected host cells is plotted as a function of the number of parasites per host cell. ~83% of cells infected with persistent parasites contain 3 or less parasites. B. The number of persistent *L. major* plotted as a function of the number of parasites per infected host cell. 78% of parasites are within host cells containing 2 or more parasites ('clusters'). C. Percent of acute- or persistent-phase 'clusters' (host cells containing 2+ parasites) as a function of the percent BrdU-positivity within each cluster. D. Percent BrdU-positivity of acute- or persistent-phase parasites as a function of the number of parasites per infected cell. Numbers within bars represent the number of infected cells scored for each category.

Figure 3. Association of persistent parasites with the markers ER-TR7, F4/80, and CD11c in footpads and lymph nodes. A. Representative pictures of persistent parasites from the footpad (top) or lymph node (bottom) with the markers ER-TR7, F4/80, CD11c

individually and F4/80 and CD11c in combination (far right). Parasites are detected with a pool of antibodies raised against *L. major* histone proteins (green). Cell-type markers are depicted in red, except for F4/80 in the far-right panel, which is depicted in blue. Scale bar indicates 5μm. B. The percentage of persistent parasites within cells expressing each marker individually in footpads (FP) or lymph nodes (LN). Each data point represents one mouse, and horizontal bars represent the mean for all mice. C. The percentage of persistent parasites within macrophages ($CD11c^{-}F4/80^{+}$) and dendritic cells ($CD11c^{+}F4/80^{+/-}$) in footpads and lymph nodes.

Figure 4. Persistent *L. major* survive and replicate within iNOS-expressing cells. A and B. Persistent parasites nuclei (green) within an iNOS-expressing (blue), $CD11c^{+}$ (red, A) or $F4/80^{+}$ (red, B) host cell. C. A persistent GFP-expressing parasite (green) within an iNOS-expressing host cell (red). Nuclei are stained blue. The parasite appears intact. D. Persistent parasites within an iNOS-expressing cell have intact nuclear genomes. Parasite nuclei were detected with antibodies against histone proteins (green), degraded DNA is detected by TUNEL-staining (red), and iNOS is stained blue. Absence of co-localization of nuclei and TUNEL-staining shows that parasite nuclear genomes are intact. Some parasites (white arrows) had $TUNEL^{+}$ kinetoplasts which would be expected even in healthy parasites. Scale bar indicates 5 μm. E. Persistent parasites incorporate BrdU in iNOS expressing cells. Parasite histone proteins are stained green, BrdU is stained red, and iNOS is stained blue. All three parasites are within an $iNOS^{+}$ cell and are $BrdU^{+}$. Scale bar indicates 5 μm.

Figure 5. Comparison of iNOS expression levels by macrophages activated *in vitro* and cells infected with *lpg2*- parasites *in vivo*. A. Representative image of starch elicited

peritoneal macrophages that were cultured in the presence of interferon-γ and LPS for 24 h and then stained to detect parasite histones (green) iNOS (red) and nuclei (blue). These cells were not infected with *L. major*. B. Representative image of an infected cell in footpad tissue stained identically to the cells in 'A'. Images of activated macrophages *in vitro* or iNOS-expressing infected cells from footpad tissue were captured by confocal microscopy using identical settings. Scale bar indicates 5 μm. C. Comparison of average red (iNOS) fluorescence intensity per μm^2 within *in vitro* activated peritoneal macrophages (PEM) or iNOS-expressing infected cells from footpad tissue. Each data point represents one cell. Black horizontal bars represent mean for all cells. The average background intensity is represented by the grey horizontal line. $P < 0.001$ by the Student's t-test.

Figure 6. Association of persistent parasites with Arg1 expression. A. Representative image of footpad tissue from mice infected with persistent *L. major* showing an infected $iNOS^+$ cell (blue) adjacent to an $Arg1^+$ (red) cell. Parasite nuclei are stained green. B. Representative image of log-phase promastigotes stained to detect histones (green) and arginase (red). C. Representative image of persistently-infected footpad tissue stained identically as the parasites in (B). Scale bar equals 5 microns. D. Analysis of images showing relative fluorescence intensity of persistent parasites and promastigotes. This value was determined by measuring the sum "red" (arginase) intensity within a circle with a 2.28 micron radius centered on the parasite nucleus on confocal images such as those in (A) and (B) in which the confocal stack had been compressed into a single plane. Grey horizontal bars represent the average background, black bars represent the mean

arginase reactivity for all cells. Each data point represents one parasite. *** represents *P* < 10-6 by the Student's t-test.

Figure 7. Amastigotes are more resistant to NO than are metacyclic-stage parasites. A. Nitrite production (left side) and percent parasite survival (right side) 24 hours after infection of control peritoneal macrophages, macrophages activated with interferon-γ and LPS, or activated macrophages treated with the iNOS inhibitor L-NIL. B. Nitrite production and percent parasite survival measured at 96 hours post-infection and 24 hours after the addition of interferon-γ and LPS with or without iNOS inhibitor. For all plots, the data shown is the average of three independent experiments, and error bars represent standard deviation.

Figure 8. Comparison of the association with CD11c and Arg1 between WT persistent parasites, *lpg2*- 1 month after infection in C57Bl/6 mice, *lpg2*- >5 months after infection in C57Bl/6 mice, and *lpg2*- 1 month after infection in BALB/c mice. For both plots, each data point represents one mouse and the horizontal bars represents the mean for all mice. *P* values were calculated by the Student's t-test. A. The percentage of parasites within $CD11c^+$ cells. For CD11c, the elevated percentage of *lpg2*- parasites within $CD11c^+$ cells does not depend on the duration of the experiment (1 versus 5 months after infection), and is also present in BALB/c mice. B. The percentage of parasites associated (either within or adjacent to) an Arg1-expressing host cell. In terms of Arg1 association, *lpg2*- within BALB/c mice more closely resembles WT persistent parasites in C57BL/6 mice than it does *lpg2*- in C57BL/6 mice. Thus, the elevated association with Arg1 correlates with vaccine failure. Horizontal bars represent the mean for all mice.

Figure 9. Stem immunogen model of concomitant immunity. Top: acute-phase *L. major* cell (green oval) within host cell (HC) replicates, with progeny parasites going on to infect new host cells where they too will replicate. Middle: quiescent *L. major* cell resides within macrophage or DC (HC). Some quiescent parasites re-enter cell cycle and begin to replicate similarly to acute-phase parasites. While some of the progeny parasites successfully infect new cells and maintain the infection, most are killed, resulting in antigen presentation and maintenance of immunity. Bottom: cartoon of stem cell biology in which a quiescent stem cell re-enters the cell cycle in response to some signal and divides. Daughter cells can then either remain stem cells or can differentiate, depending on a variety of signals. What cues signal quiescent *L. major* to re-enter the cell cycle or how the fate of progeny parasites is determined is unknown, but alterations of the host's immune status would affect the proportions of progeny parasites destined for survival or destruction.

Supplementary figure S1. Slow-growing *L. major* increase duration of G_1 phase. A. Cell density of parasites cultured in M199-based (grey) versus RPMI-based (black) media as a function of time post inoculation. Parasites in M199 media double every 9.0 hours, whereas parasites cultured in the RPMI-based media double every 29.6 hours. B. DNA content of cells grown in M199 (grey) and RPMI (black) as assessed by propidium iodide (PI) staining intensity. C. Percent of parasites in M199 (grey bars) or RPMI (black bars) that are in G_1, S, or M/G_2 phase in the cell cycle (corresponding to M1, M2, and M3 in panel B, respectively). Also shown is the percentage of parasites from each growth condition that are $BrdU^+$ 2 hr after the addition of BrdU to the culture media. For the

BrdU experiment, error bars represent standard deviation, N = 3 coverslips; > 227 parasites scored per condition. *** represent $p < 3X10^{-5}$ by the Student's t-test.

Supplementary figure S2. Neither CD11c- (A) nor iNOS-expression (B) correlate with the number of persistent parasites per infected host cell. The numbers within the bars represent the number of infected cells scored for each category.

Supplementary figure S3. Positive controls for RELMα staining. A and B. Starch-elicited peritoneal exudate cells were either cultured in media without cytokines (A) or cultured in the presence of IL-4 and IL-13 (100 U ml^{-1}) for 48 h (B) and then stained to detect RELMα (green) or nuclei (blue). Cytokine treatment induced RELMα which was easily visualized with anti-RELMα antisera. Scale bar equals 5μm. C. Representative image of a footpad tissue section from a foot persistently-infected with WT stained to detect parasite histones (green, no parasites visible in this field), RELMα (red) and F4/80 (blue). Scale bar equals 5μm.

Table 1. Persistent *L. major* replicate comparably within $iNOS^+$ and $iNOS^-$ host cells. The percentage of both 'clusters' and BrdU-positive parasites within $iNOS^+$ host cells is the same as the percentage of total parasites within $iNOS^+$ cells.

Table 2. Comparison of persistent WT and *lpg2*- parasites in footpad tissue. For the summary statistics presented here, E = # of experiments, M = total # of mice, and P = total # of parasites. A. N = 2E/7M/1003P. B. N = 3E/10M/616P. C. N = 3E/10M/712P. D. N = 3E/10M/1004P. E. N = 3E/3M/346P. F. N = 3E/8M/2535P. G. N = 2E/9M/1105P. H. N = 1E/2M/123P. I. N = 3E/5M/477P. J. N = 3E/10M/175 parasite clusters. K. N = 3E/10M/512 parasite clusters. L. N = 3E/10M/213 parasite clusters.

Figure 1

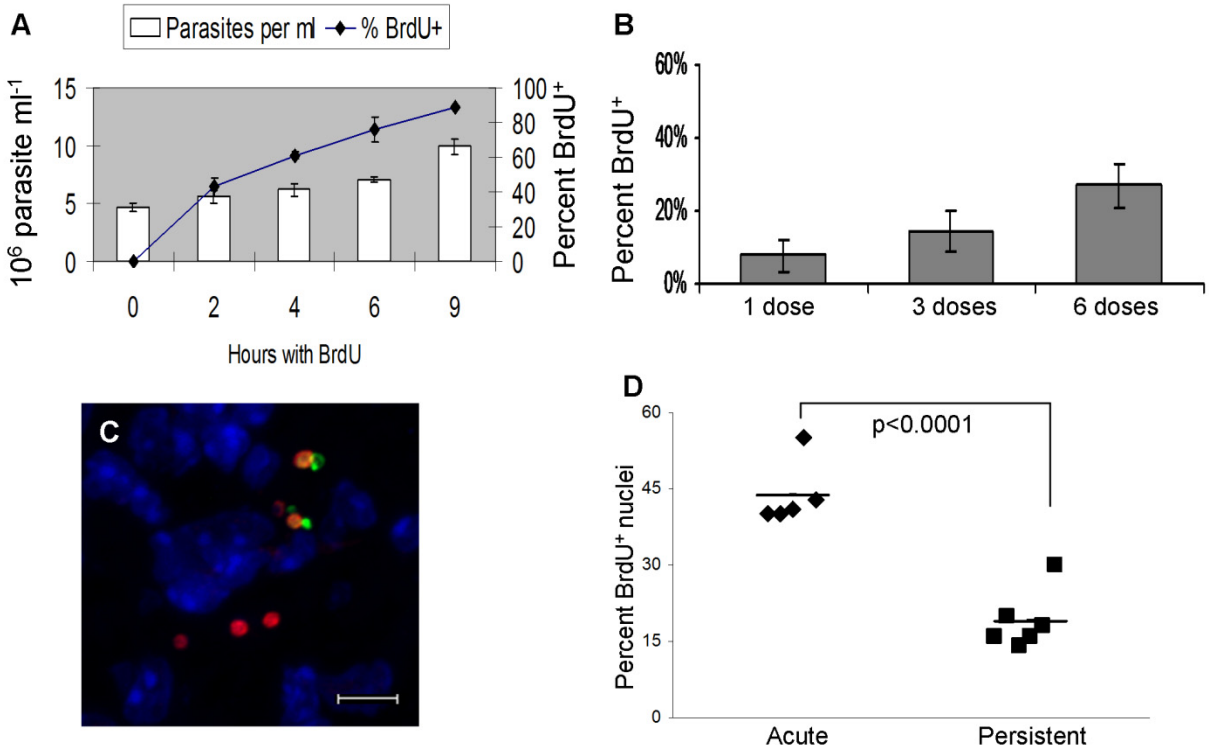

A
Parasites per ml
% BrdU+
10^6 parasite ml^{-1}
Percent BrdU+
Hours with BrdU
B
Percent BrdU+
1 dose
3 doses
6 doses
C
D
Percent BrdU+ nuclei
p<0.0001
Acute
Persistent

Figure 2

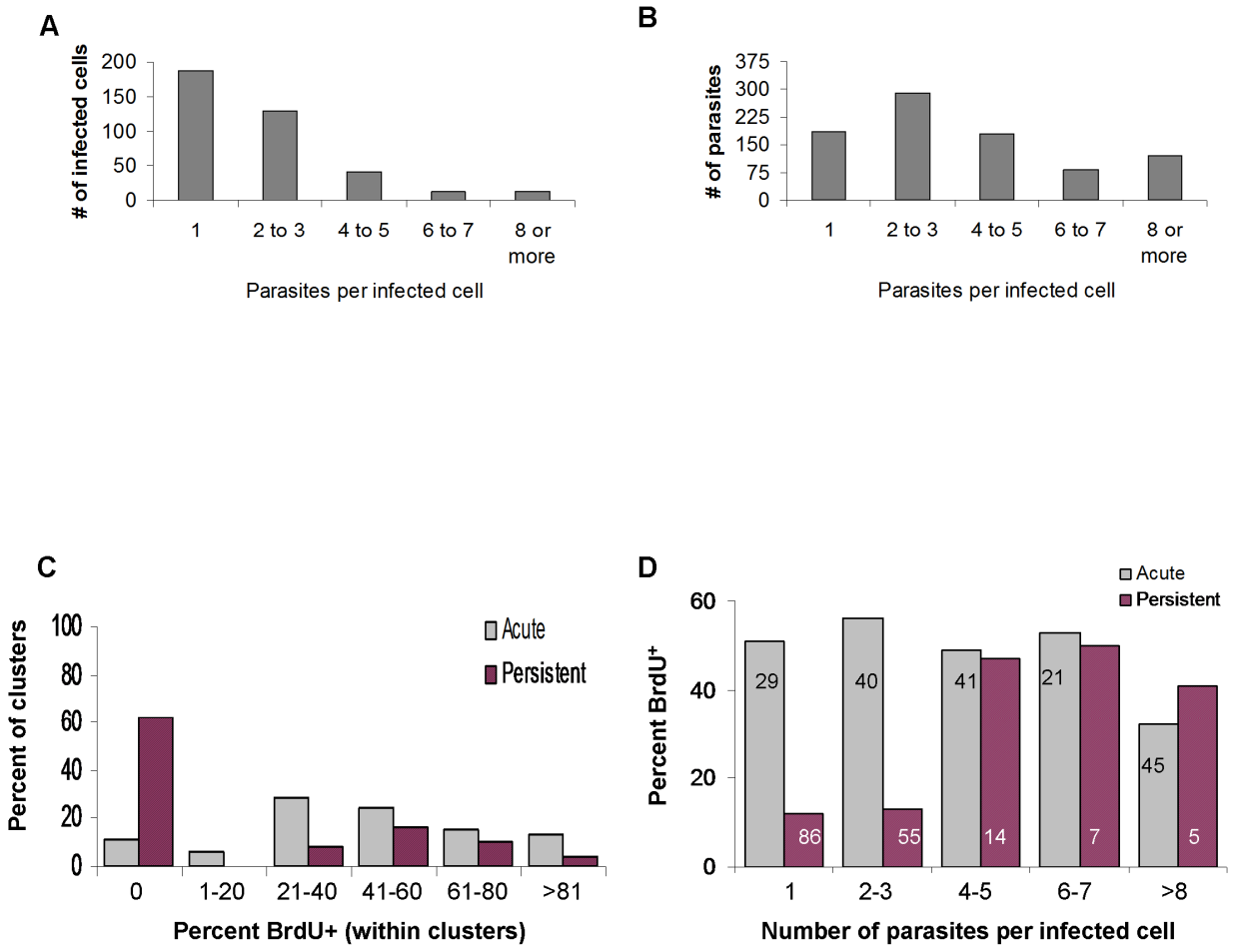

A
# of infected cells
200
150
100
50
0
1
2 to 3
4 to 5
6 to 7
8 or more
Parasites per infected cell
B
# of parasites
375
300
225
150
75
0
1
2 to 3
4 to 5
6 to 7
8 or more
Parasites per infected cell
C
Percent of clusters
100
80
60
40
20
0
Acute
Persistent
0
1-20
21-40
41-60
61-80
>81
Percent BrdU+ (within clusters)
D
Percent BrdU+
60
40
20
0
Acute
Persistent
29
86
40
55
41
14
21
7
45
5
1
2-3
4-5
6-7
>8
Number of parasites per infected cell

Figure 3

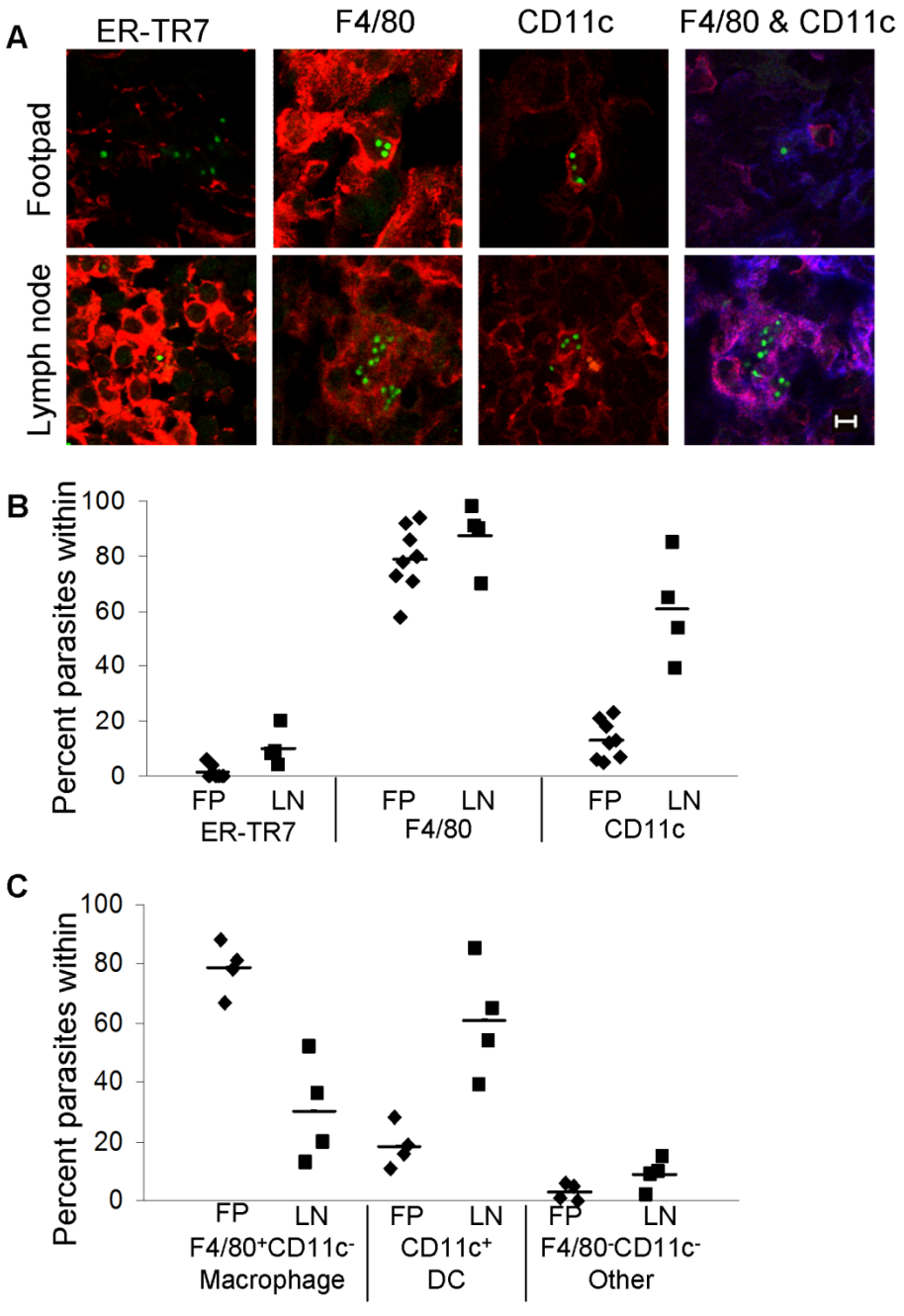

A
ER-TR7
F4/80
CD11c
F4/80 & CD11c
Footpad
Lymph node
B
Percent parasites within
100
80
60
40
20
0
FP
LN
ER-TR7
F4/80
CD11c
C
F4/80+CD11c-
Macrophage
CD11c+
DC
F4/80-CD11c-
Other

Figure 4

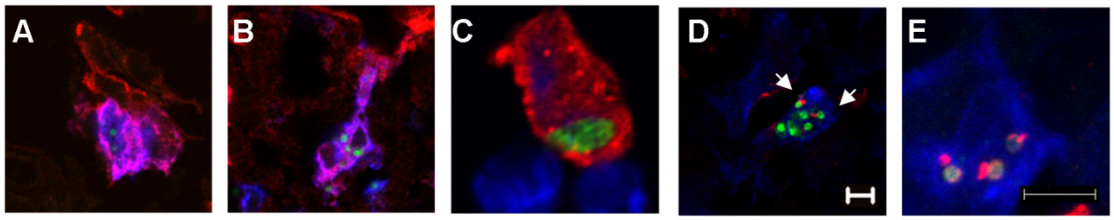


Figure 5

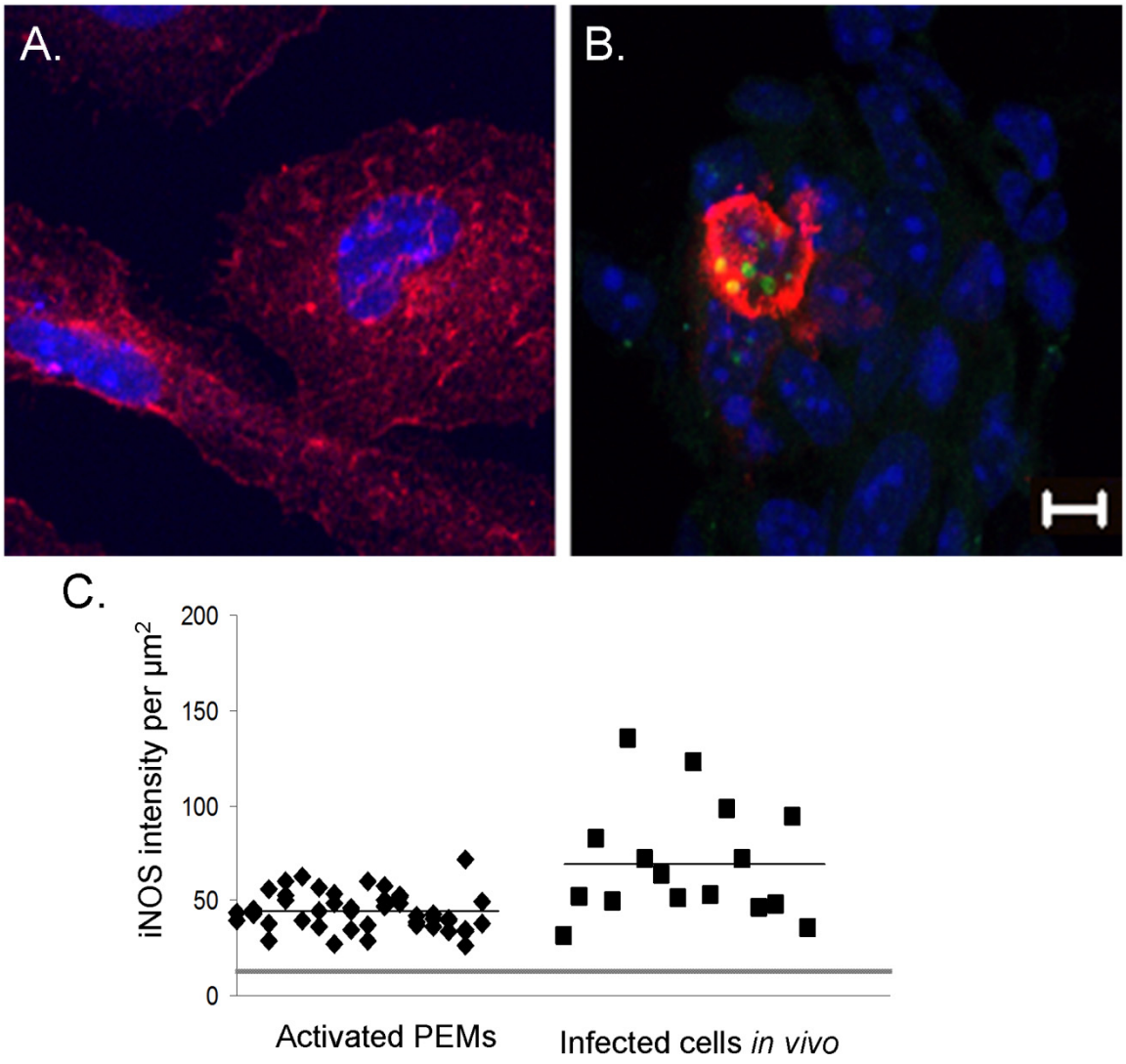

A.
B.
C.
iNOS intensity per µm2
200
150
100
50
0
Activated PEMs
Infected cells in vivo

Figure 6

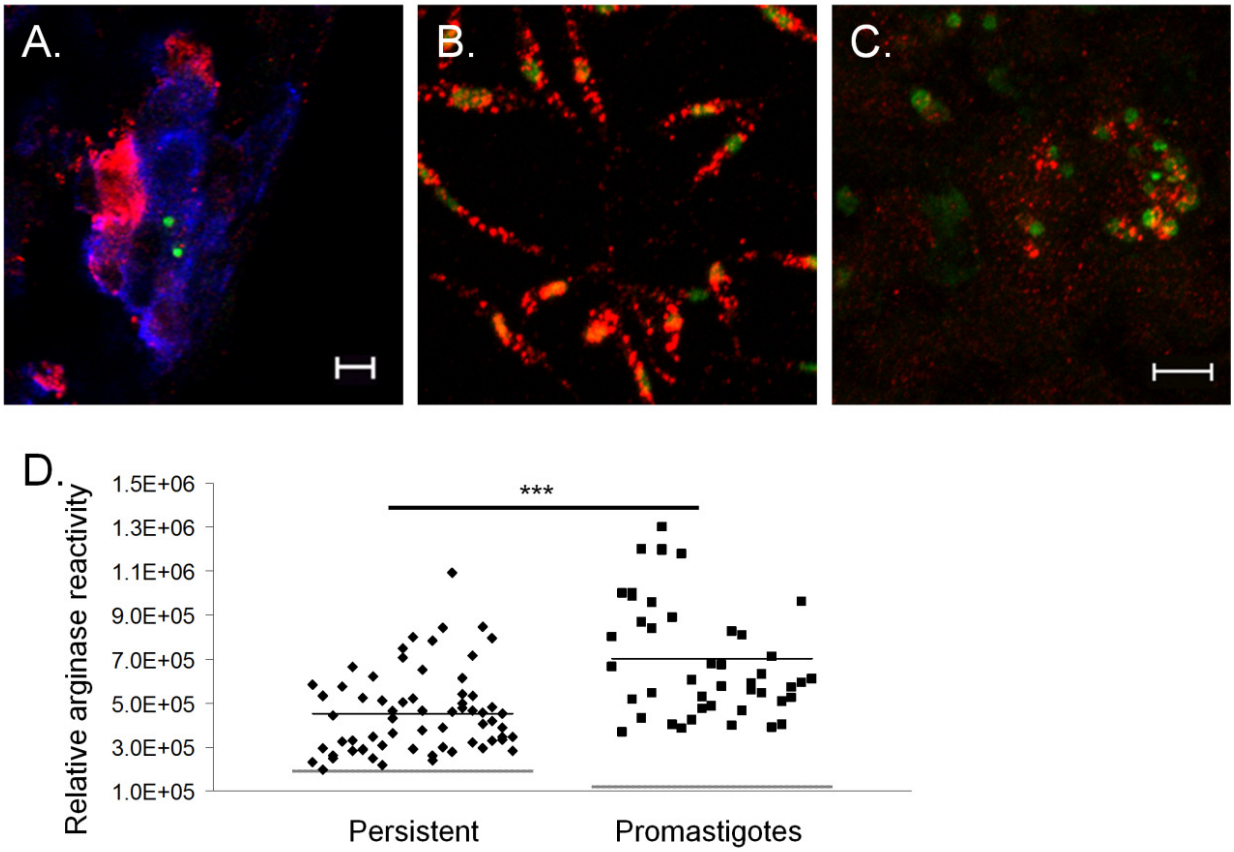

A.
B.
C.
D.
Relative arginase reactivity
1.5E+06
1.3E+06
1.1E+06
9.0E+05
7.0E+05
5.0E+05
3.0E+05
1.0E+05
***
Persistent
Promastigotes

Figure 7

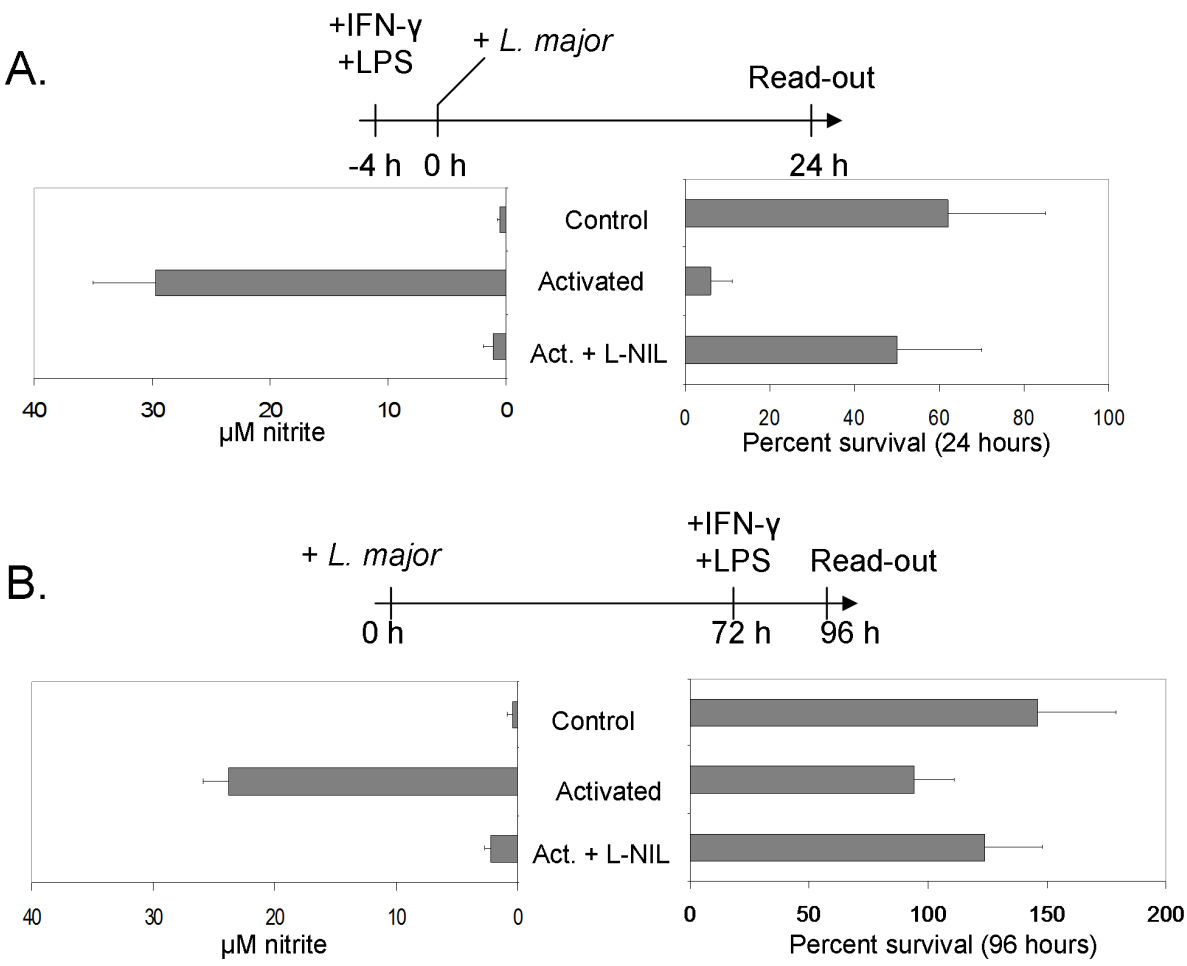

A.
+IFN-γ
+LPS
+ L. major
Read-out
-4 h
0 h
24 h
Control
Activated
Act. + L-NIL
40
30
20
10
0
μM nitrite
0
20
40
60
80
100
Percent survival (24 hours)
B.
+ L. major
+IFN-γ
+LPS
Read-out
0 h
72 h
96 h
Control
Activated
Act. + L-NIL
40
30
20
10
0
μM nitrite
0
50
100
150
200
Percent survival (96 hours)

Figure 8

Vaccinates		
WT persistent	*lpg2-* C57BL/6	*lpg2-* BALB/c
++++	+/-	+++

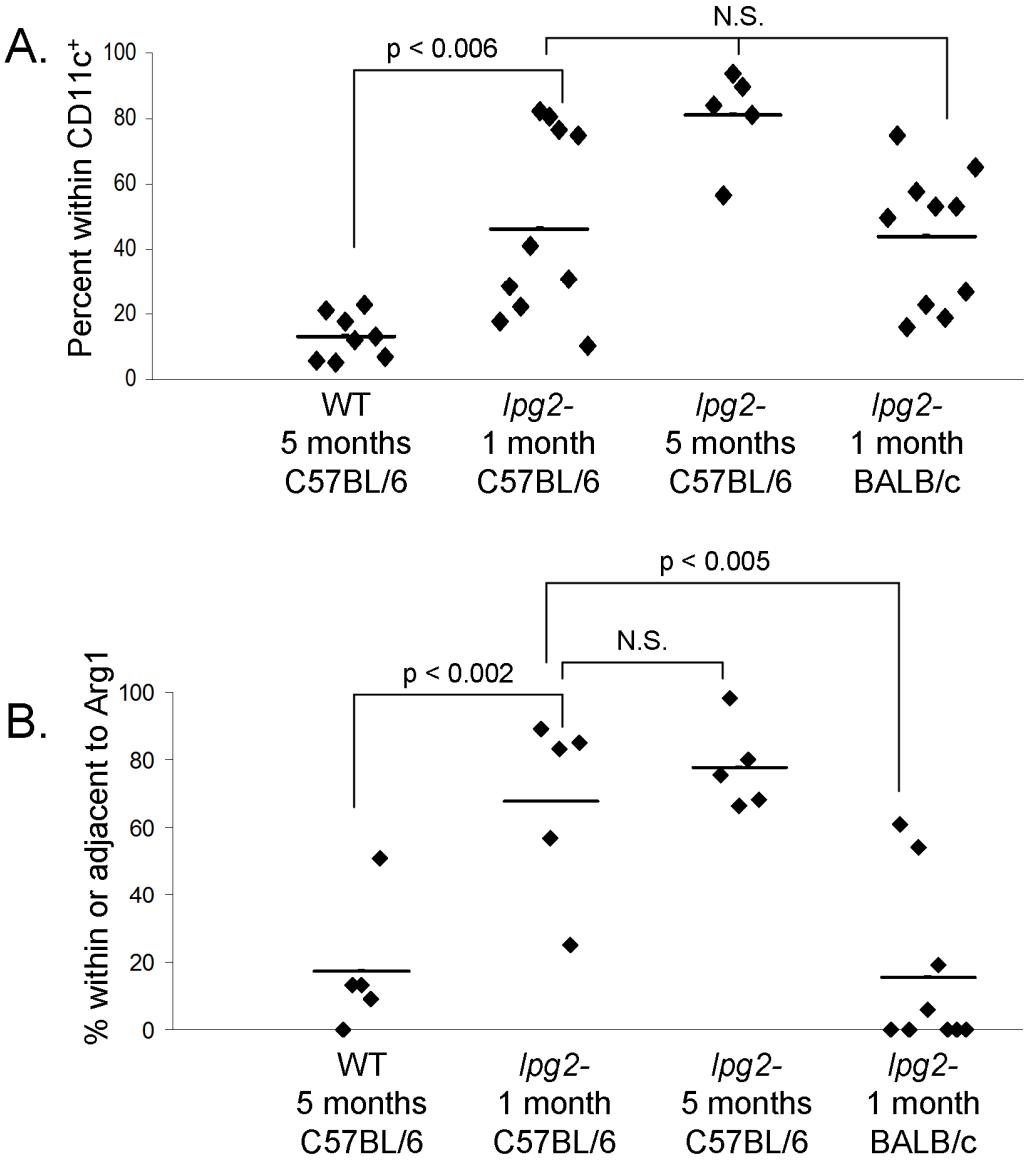
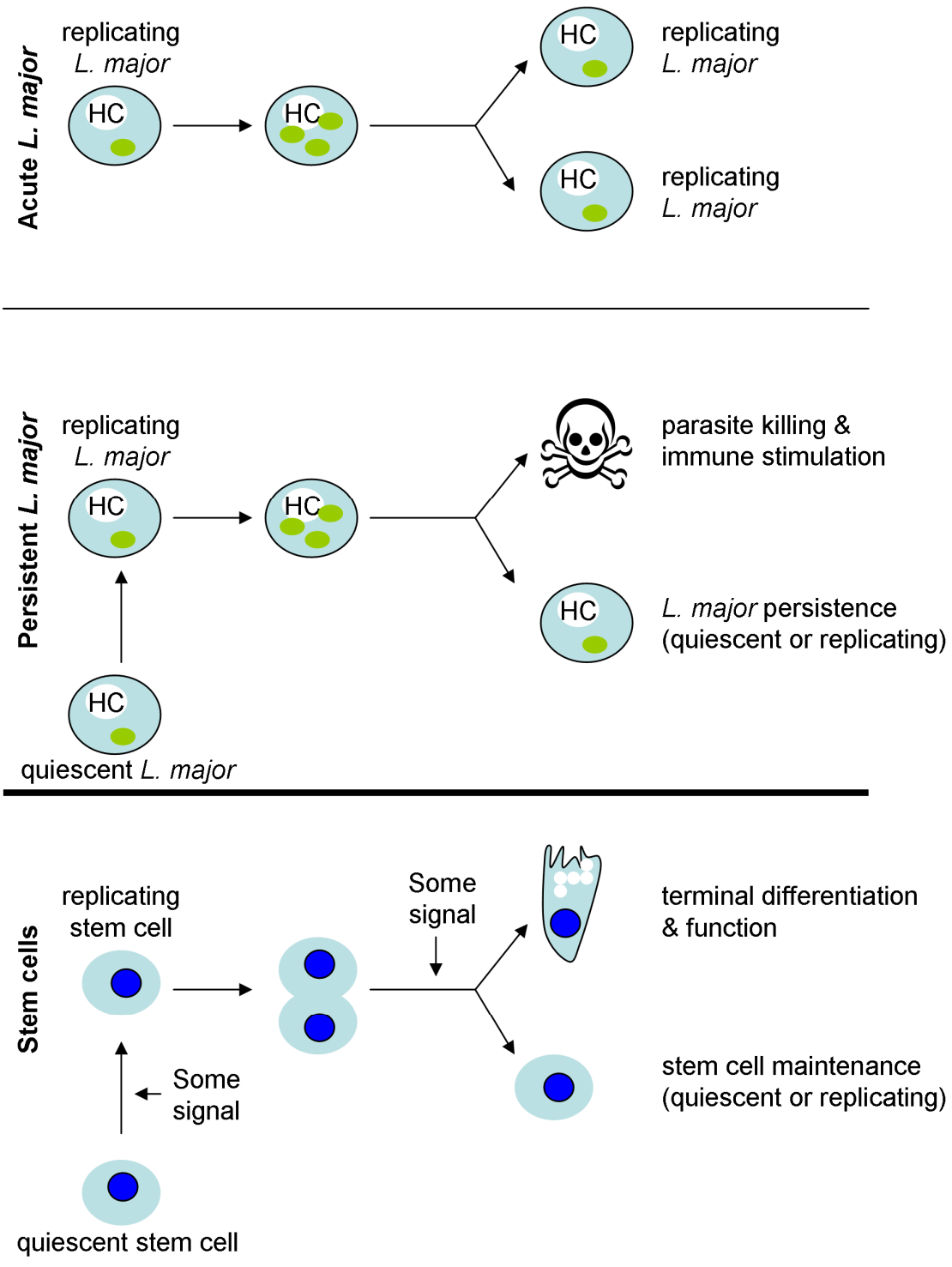
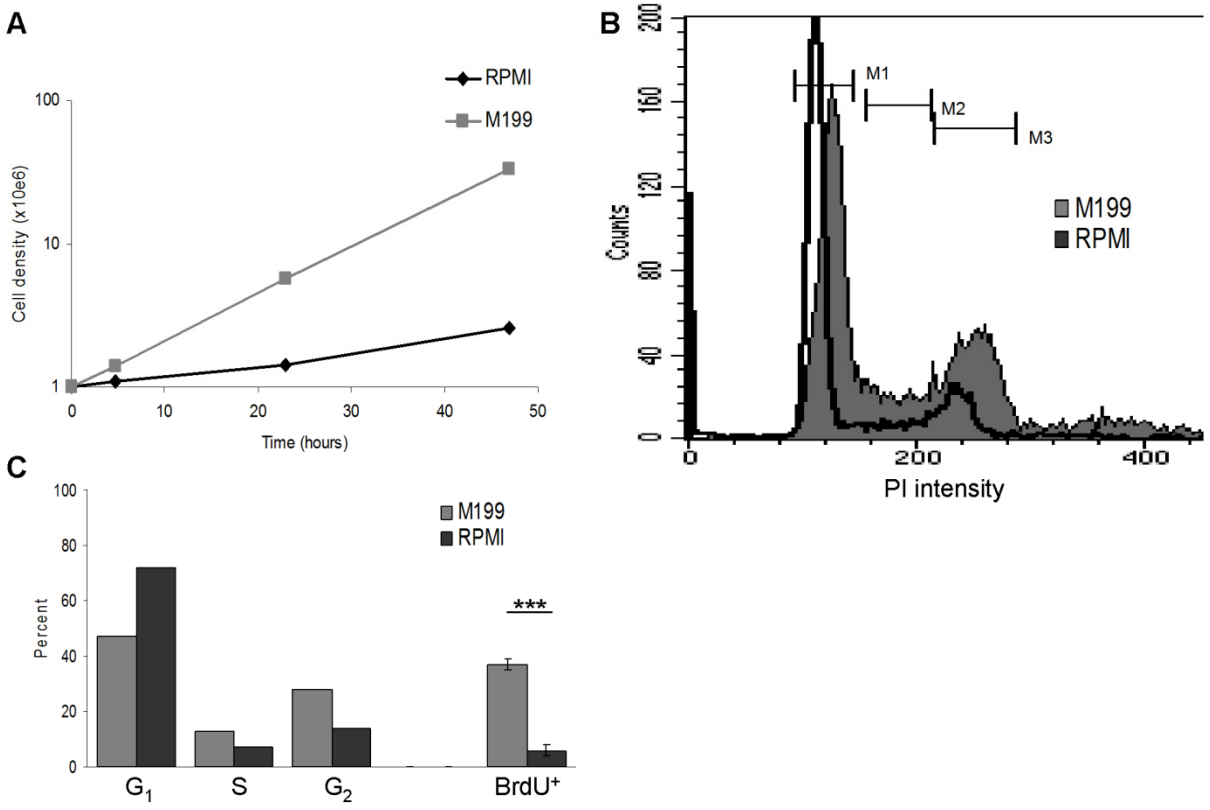


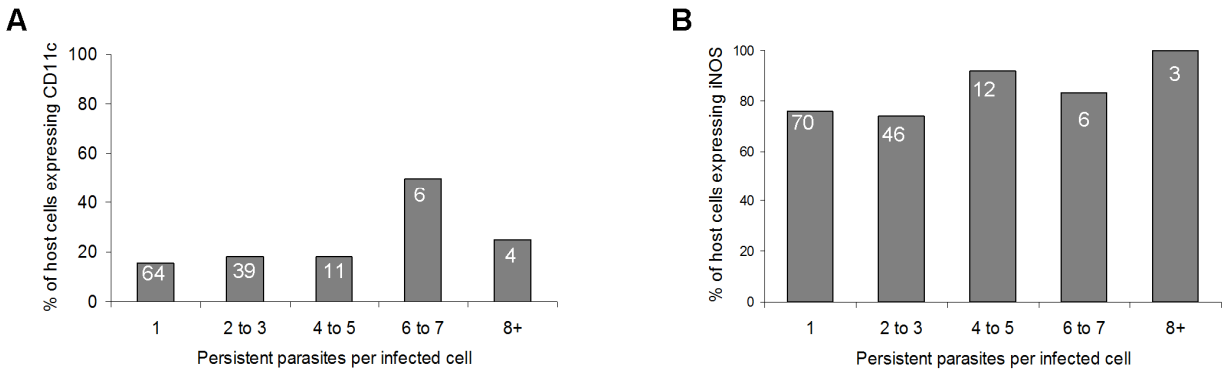
Figure 9


Acute L. major
replicating
L. major
HC
HC
HC
replicating
L. major
HC
replicating
L. major
Persistent L. major
replicating
L. major
HC
HC
parasite killing &
immune stimulation
HC
L. major persistence
(quiescent or replicating)
HC
quiescent L. major
Stem cells
replicating
stem cell
Some
signal
terminal differentiation
& function
Some
signal
stem cell maintenance
(quiescent or replicating)
quiescent stem cell

Supplementary figure S1



Supplementary figure S2



Supplementary figure S3

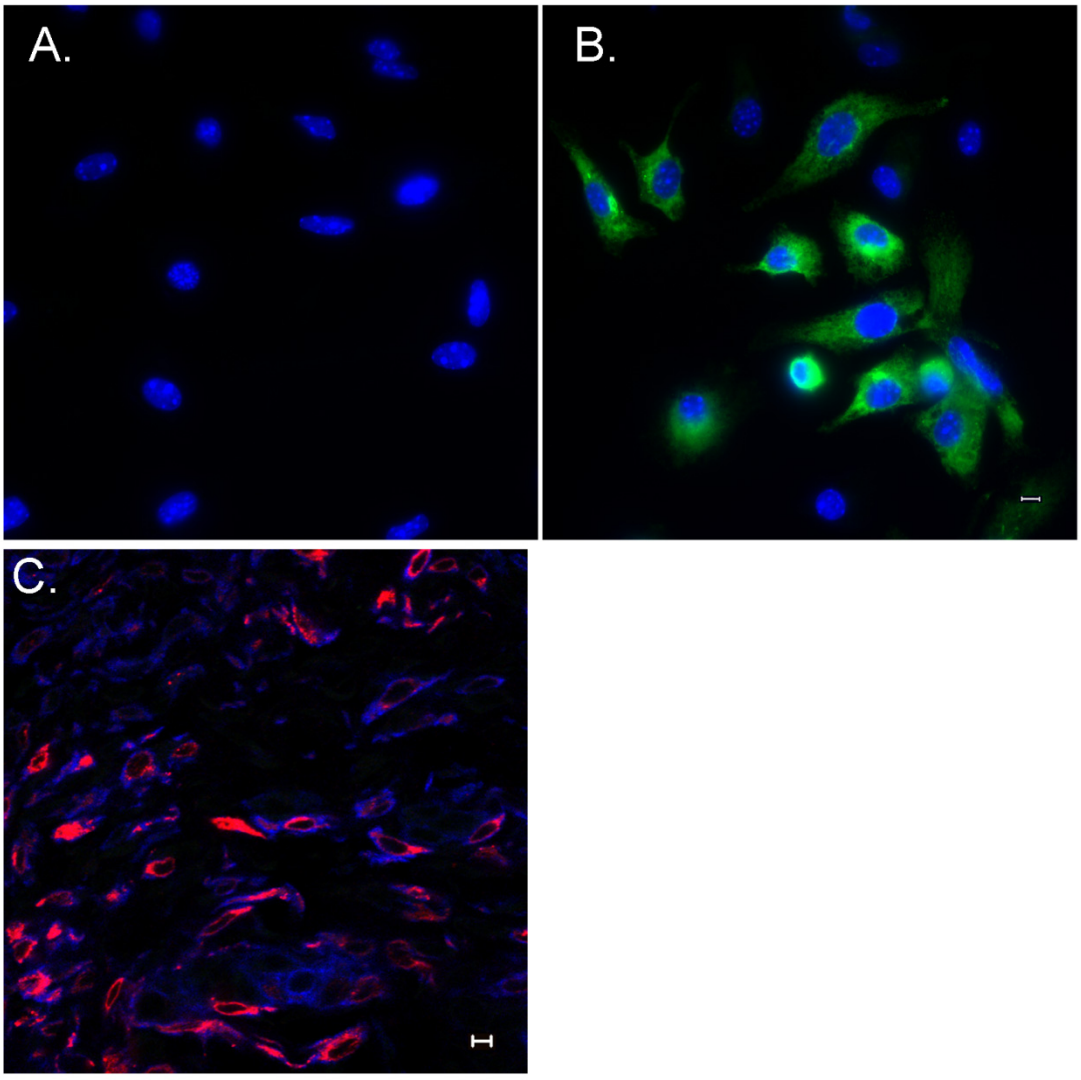


Table 1

Percent of persistent parasites within iNOS$^+$ host cells			
% Total	**% Clusters**	**% Total**	**% BrdU$^+$**
59 ± 15%	61 ± 15%	82 ± 10%	85 ± 13%
N = 3E / 8M / 329 clusters		N = 2E / 3M / 254P	

Table 2

Parasite containing cells: footpad			
	WT (> 5 months)	*lpg2-* (1 month)	P-value
% BrdU$^+$	19 ± 6%	16 ± 3%[A]	N.S.
ER-TR7$^+$	2 ± 3%	0 ± 0%[B]	N.S.
F4/80$^+$	79 ± 12%	85 ± 15%[C]	N.S.
CD11c$^+$	13 ± 7%	46 ± 29%[D]	< 0.006
RELMα$^+$	0 ± 0%	0 ± 0%[E]	N.S.
iNOS$^+$	59 ± 15%	70 ± 22%[F]	N.S.
F4/80$^+$iNOS$^+$	42 ± 14%	52 ± 23%[C]	N.S.
CD11c$^+$iNOS$^+$	10 ± 6%	44 ± 29%[D]	< 0.005
Arg1 (within)	3 ± 3%	37 ± 41%[G]	N.S.
Arg1 (adjacent)	12 ± 11%	35 ± 32%[G]	N.S.
Arg1 (within + adjacent)	15 ± 14%	72 ± 26%[G]	< 0.003
Percent of BrdU$^+$ parasites in iNOS$^+$ host cell	85 ± 13%	80 ± 28%[H]	N.S.
Arg2$^+$	0 ± 0%	0 ± 0%[I]	N.S.
Parasite cluster containing cells: footpad			
F4/80$^+$	73 ± 11%	85 ± 15%[J]	N.S.
iNOS$^+$	61 ± 15%	67 ± 19%[K]	N.S.
CD11c$^+$	17 ± 10%	41 ± 25%[L]	< 0.05

Chapter 4

Concomitant immunity induced by persistent *Leishmania major* does not preclude secondary re-infection: consequences to the maintenance of natural parasite diversity

Michael A. Mandell, and Stephen M. Beverley[1]

[1]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis MO 63110 USA

[1]Corresponding author; Dept. of Molecular Microbiology, Washington University School of Medicine, Campus Box 8230, 660 S. Euclid Ave., St. Louis MO 63110; Tel: 314-747-2630; e-mail beverley@borcim.wustl.edu.

Author contributions

All experiments were performed by M.A.M.

M.A.M and S.M.B. wrote the text.

Abstract

Many microbes have evolved the ability to co-exist for long periods of time within other species in the absence of overt pathology. For potential pathogens, evolutionary biologists have proposed benefits to the microbe from 'asymptomatic persistent infections', most commonly invoking increased likelihood of transmission by longer-lived hosts. Typically asymptomatic persistent infections arise from strong containment by the immune system, accompanied by protective immunity; such 'vaccination' in the presence of a non-sterilizing immune response is termed premunition or concomitant immunity. Here we consider another potential benefit of persistence and concomitant immunity: the 'exclusion' of competing super-infecting strains, in a manner reminiscent of that seen in lyosgenic bacteriophage in prokaryotes. To investigate this in the protozoan parasite *Leishmania major*, which provides a superb model for the study of asymptomatic persistence, we used isogenic lines of comparable virulence bearing independent selectable markers. One was then used to infect genetically resistant mice, yielding infections which healed and progressed to asymptomatic persistent infection; these mice were then super-infected with the second marked line. As anticipated, super-infection yielded minimal pathology, showing that protective immunity had been established. The relative abundance of the primary and super-infecting secondary parasites was then assessed by plating on selective media. The data show clearly that super-infecting parasites were able to colonize the immune host effectively, achieving numbers comparable to and sometimes greater than that of the primary parasite. We conclude that induction of protective immunity does not guarantee the *Leishmania* parasite exclusive occupation of the infected host. This finding has important

consequences to the maintenance and generation of parasite diversity in the natural *Leishmania* infectious cycle alternating between mammalian and sand fly hosts.

Introduction

Persistent host/pathogen relationships are often characterized by a 'stalemate' in which the host neither succumbs to disease nor is able to completely achieve sterile cure. Persistent infections can show varying degrees of pathology, ranging from chronic overt disease to asymptomatic infections (1-5). Especially for asymptomatic persistent infections, a key component is a strong immune response on the part of the host, which is required to keep pathogen numbers in check. In some cases, this immune response also serves to protect against pathology resulting from subsequent re-infection by the same pathogen, a process known as premunition or concomitant immunity (6-8).

Long-term host/pathogen relationships carry with them benefits and risks to both partners, and have been the subject of a considerable study from an evolutionary perspective (9-11). In the case of concomitant immunity, the host benefits by its immune system's ability to control the infection and minimize pathology, as well as protection from disease arising from new infections. However, this comes at the cost of increased risk of disease reactivation, typically arising from immunosuppression or stress (1, 4, 12). From the pathogen's perspective, while concomitant immunity decreases microbial numbers, it may improve the likelihood of transmission due to the increased longevity of the infected host.

A second potential benefit to the pathogen is 'exclusivity', in that concomitant immunity may reduce the invasion of the host by other strains or species. In the case of *Schistosoma mansoni*, concomitant immunity may limit intraspecific competition for limited resources (6) (13). A further benefit of exclusivity is transmission, in

guaranteeing transmission of the primary infecting line over that of secondary 'invading' pathogens. In some respects this scenario resembles that proposed for lysogenic bacteriophages, which are generally resistant to super-infection with closely related phage (14).

The protozoan parasite *Leishmania major* provides an excellent model for investigating forces of concomitant immunity and persistence. *L. major* is transmitted to mammalian hosts by the bite of phlebotomine sand flies, and in laboratory mice a range of pathology ensues depending on both the particular parasite and mouse strain (15). Infections of genetically susceptible mice (such as BALB/c) with most *L. major* strains yields a progressive and fatal infection (15). In contrast, infection of genetically resistant mice (e.g. C57BL/6) initially gives rise to a progressive parasitemia and lesion pathology at the site of inoculation similar to that seen in BALB/c mice, but after 4-6 weeks an immune response develops which controls both parasitemia and pathology (15, 16). Notably, the healed mice are effectively vaccinated and resistant to disease pathology from subsequent infections. Following healing, and for the remainder of the host's life, a small number of parasites persist in the skin at the site of inoculation and in the regional lymph node draining that site (17). In keeping with concomitant immunity/premonition paradigm, these persistent parasites appear to be important for the maintenance of an anti-*Leishmania* immune response, as treatment resulting in sterile cure is associated with the loss of immunity (18)(19). The strong protective immunity induced by persistent *Leishmania* is the basis for the ancient practice of leishmanization, in which live, virulent parasites are intentionally inoculated in inconspicuous sites of the body to protect against natural infection and pathology at other sites (20).

Importantly the asymptomatic persistent *Leishmania* infections of C57BL/6 mice fit several criteria relevant to understanding of the benefits and tradeoffs of concomitant immunity. The animals are healthy, and despite the small numbers of persistent parasites (< 1000 / mouse), they can be efficiently transmitted to sand flies (21-23). What has not been solidly addressed previously in the literature is exclusivity; while it is well known that the persistently infected mammalian host is vaccinated from disease pathology, it has not been rigorously shown whether the long lived persistently infected host can be successfully colonized by secondary parasite exposure. Several studies have evaluated the potency of the immune response maintained by persistent parasites by injecting parasites in a primary site, waiting for the lesion to resolve, and then injecting parasites into a secondary site and determining the parasite load there (19, 24-26). The data from these studies would seem to suggest that persistent parasites do not generate "exclusivity" in that viable parasites were recovered from the site of secondary challenge. However, the ability of *L. major* to traffic to sites distant from the site of inoculation (17) raised the possibility that these parasites actually arose from the primary infection, perhaps accentuated by the transient reactivation of parasites at the primary infection site reported by Mendes et al (24).

To assess the question of exclusivity, we generated parasites derived from the same strain of *L. major* of equal virulence but bearing independent drug resistance markers (PHLEO/phleomycin and SAT/nourseothricin). This were then used in the classic infection/challenge persistence model, using the SAT strain as the primary infection, which gave rise to the expected lesion/healing/persistence phenomenon, followed by injection with the PHLEO strain in the opposite foot. The results show clearly that under

these conditions *Leishmania* persistence is not accompanied by 'exclusivity', in that equivalent numbers of both 'primary' and 'secondary' parasites persisted at their respective sites of inoculation. These data suggest that while persistent *L. major* vaccinate its host from disease pathology, it does not confer exclusivity to the acquisition of secondary infecting *Leishmania*. This finding has important consequences to the maintenance and generation of *Leishmania* genetic diversity, including that arising through sexual processes (27, 28).

Materials and Methods

Parasite strains and culture

The generation of both the phleomycin resistant parasites (*SSU:IR1PHLEO-YFP*; referred to here as LmjF PHLEO) and the nourseothricin-resistant parasites (SSU:SAT-TK-LUC; referred to here as LmjF LUC-SAT) used in this study was described previously (29, 30). Parasites were grown at 26˚C in M199 medium (US Biologicals) supplemented with 40 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) pH 7.4, 50 μM adenosine, 1 μg ml^{-1} biotin, 5 μg ml^{-1} hemin, 2 μg ml^{-1} biopterin and 10% (v/v) heat-inactivated fetal calf serum {Kapler, 1990 #126}. Nourseothricin (Jena Bioscience, Jena, Germany) was used at a concentration of 100 μg/ml and phleomycin (Sigma, St. Louis, MO) was used at a concentration of 20 μg/ml. Infective metacyclic-stage parasites were recovered using the density gradient centrifugation method (31).

Mouse infections

Animal studies were approved by the Animal Studies Committee at Washington University (protocol #20090086) in accordance with the Office of Laboratory Animal Welfare's guidelines and the Association for Assessment and Accreditation of Laboratory Animal Care International.

Female C57Bl/6J mice (Jackson Labs) were injected subcutaneously in a hind footpad with 10^5 metacyclic stage parasites. Naïve mice (6-8 weeks old) were injected in the left hind footpad. Secondary injections took place in the right hind footpad at a time point >1

month after primary lesions had resolved. Footpad lesion pathology was measured using a Vernier caliper (Mitutoyo). Luciferase activity was determined as described elsewhere (30). Briefly, mice were given a dose of D-luciferin (150 μg gram^{-1} body weight; Biosynth) in PBS 10 minutes prior to imaging with an IVIS 100 imaging system (Xenogen Corp). Limiting dilution assays were performed as described previously (32), with the addition of phleomycin or nourseothricin as indicated.

Statistics

Data are presented as the arithmetic mean ± the standard deviation. P values were calculated by the Student's t-test.

Results

Development of two genetically marked L. major *with equivalent virulence in resistant mice.*

For this study, we used two *L. major* Friedlin V1 parasites expressing genes conferring resistance to the antibiotics nourseothricin (SAT) or phleomycin (PHLEO). The nourseothricin resistant parasites also express firefly luciferase, and will be referred to hereafter as "LmjF-LUC-SAT", while the phleomycin resistant parasites will be referred to as LmjF-PHLEO. To confirm that the LmjF-LUC-SAT and LmjF-PHLEO parasites were of comparable virulence in mice, 10^5 metacyclic-stage parasites were inoculated into the footpads of naïve C57BL/6 mice (5 mice/group), and the lesion pathology was monitored over time (Fig. 1A). Both lines exhibited disease progression typical of *L. major* / C57BL/6 infections, with lesions developing between 10-17 days post infection and reaching their maximum (~1.4 mm in thickness) around 30 days post infection. Thereafter the lesions declined, and were completely resolved by 130 days post-infection (Fig. 1A). At this time, mice were sacrificed and the parasite titers in the infected feet were enumerated by limiting dilution analysis (Fig. 1B). No significant difference in the number of persistent parasites was seen, with LmjF-LUC-SAT and Lmj-PHLEO showing a similar range (Fig 1B) and mean (25and 32 parasites / foot; $P > 0.45$ by Student's t-test). We judged these lines to be of comparable virulence and suitable for the following tests.

Healed mice were protected against pathology from subsequent challenge

Two experiments were performed in which naïve mice (4-5 mice per experiment) were inoculated with 10^5 purified metacyclic-stage LmjF-LUC-SAT parasites in the left hind footpad primary infection site. These mice formed lesions which spontaneously resolved similar to that shown in Fig. 1A (data not shown). At a time point >1 month later, 10^5 metacyclic LmjF-PHLEO parasites were inoculated into the right hind footpad secondary infection site. Footpad swelling of both the primary (L) and secondary (R) injection sites were then measured over time. We also used *in vivo* imaging of luciferase activity to visualize LmjF-LUC-SAT parasites, as a second probe of whether transient reactivation of primary parasites occurred (24).

As expected in both experiments the mice showed good protection, as evidenced by a reduction in lesion pathology at the secondary 'challenge' site. Although there was some between-experiment variation, in both experiments the lesions generated by the secondary LmjF-PHLEO parasites were significantly smaller and resolved more rapidly than those in naïve mice, (Figure 2A). We saw no evidence of reactivation by the "primary" LmjF-LUC-SAT^R parasite, as judged by either lesion measurement (Figure 2B) or *in vivo* imaging of parasite luciferase (Figure 2C). Interestingly, in expt. 1 we saw a low level of LUC expression in the persistent infection; in other studies we have seen this as well, although in general persistent parasite numbers are insufficient for reliable imaging (Hickerson & Beverley unpublished).

Roughly equivalent numbers of both "primary" and "secondary" parasites persist

We then measured the occurrence of both the primary and secondary- infecting parasites, in both infection sites, by limiting dilution assays at day 87 or 139 post-infection. Total

parasites were assessed by growth in the absence of drug, while LmjF-LUC-SAT was estimated from growth in media containing nourseothricin and LmjF-PHLEO from growth in phleomycin. The results from individual mice from both experiments as well as the global averages are shown in Fig 3.

Parasites were recovered from all primary infection sites, ranging from 14 to 504 parasites/foot, with an average of 282 ± 158 parasites recovered per foot (N = 9). These parasites were exclusively the primary LmjF-LUC-SAT parasite, as they were unable to grow in the presence of phleomycin. In one animal the SAT marker was apparently lost; similar results have been reported in *L. tarentolae* and attributed to the genetic plasticity of the ribosomal RNA locus where gene conversion has been postulated (33), and we have seen this occasionally in other experiments in *L. major*.

Parasites were also recovered from the secondary infection site from 8 of the 9 mice, ranging from 14 to 785 parasites/foot with an average of 119 ± 156 parasites per foot. Importantly, nearly all of the parasites recovered from the secondary infection site were the LmjF-PHLEO parasite inoculated there (99 ± 3%). In only one mouse (#2-5) could colonization of the secondary site by 'primary' infection site LmjF-LUC-SAT parasites be found at all, suggesting that metastasis of parasites from the primary to the secondary sites is relatively infrequent. Importantly, the numbers of 'primary' infection site LmjF-LUC-SAT parasites were not significantly different from that seen for the 'secondary' infection site LMjF-PHLEO parasites ($P > 0.08$, Student's T-test). These data show that despite 'vaccination' as defined by prevention of lesion pathology, the protective immunity is not 'sterilizing' against secondary infections and does not preclude efficient colonization of the infected mouse.

Discussion

A number of factors have been proposed to contribute to the maintenance of pathogens for long periods of time in the host, including an insufficient immune response and the benefits accruing to the pathogen from residing within a longer-lived host thereby increasing the likelihood of transmission (9, 11). In many cases this relationship has progressed to the point where the pathogen infection is asymptomatic, thereby fulfilling the evolutionary dictum that a 'successful pathogen does not kill its host too quickly". Often this asymptomatic persistence is accompanied by protection from disease induced by further infections of the same or related pathogens, a process termed concomitant immunity (6). Such a relationship provides benefits to both the pathogen and the host through increased longevity of the latter (albeit with some risk of reactivation), and increased transmission of the former.

Leishmania provides an attractive system for the study of concomitant immunity (17, 18, 24, 34-36) and here we have used this to consider another potential benefit to the pathogen, one of 'exclusivity'. Exclusivity would favor transmission of the primary infecting pathogen due to reduction in the ability of secondary infecting parasites to establishing in a previously infected host. However, our data show clearly that despite induction of a protective immune response able to mitigate disease pathology, secondary *Leishmania major* infections are nonetheless able to establish themselves effectively in a previously infected host. While this result may have been anticipated from prior studies (19, 25), this is the first time this has been established rigorously for *Leishmania* using genetically marked parasites able to distinguish primary from secondary infections.

In our studies we used an inoculum consisting of 10^5 purified metacyclic parasites. While most sand flies transmit less than 600 parasites to mice, a few transmit up to 10^5 (22). Thus, the dose used here is not an unrealistic one. In addition, other studies using low-dose infections (challenging with 100 metacyclics) yielded results similar to ours in that parasites were harvested from the site of secondary infection (19, 24-26). Similarly, sand flies convey other factors including saliva and secreted parasite molecules such as proteophosphoglycan, both of which typically act to facilitate primary infections (37-40) but which can also engender various protective responses (41-42). Thus future studies may address this phenomenon in the context of natural sand fly transmission and the dissection of the relative roles of sand fly saliva and secreted *Leishmania* molecules such as PPG.

In our study, the immunity generated by persistent parasites was not sterilizing and the average number of "secondary" parasites was not significantly different from that of the "primary" parasites (Fig. 3). Nonetheless, the average number of secondary LmjF-PHLEO parasites was about 2-fold less than the primary LmjF-LUC-SAT parasites, which is consistent with previous studies, such as those of Mendez et al (2004) (24) where 10-fold fewer parasites were isolated from the secondary infection site. As noted earlier, while these authors did not use genetically marked parasites, our studies show that the dissemination of parasites from the primary to the secondary infecting foot is relatively infrequent, being seen in only 1/9 infected mice (Fig. 3, mouse #2-5). Thus, while secondary infecting parasites may be able to gain access to the previously infected host, they may experience a quantitative disadvantage in terms of transmission, especially as the efficiency of sand fly infection following feeding on a persistently infected host is

already low (~10%) (21). Such a quantitative advantage could over evolutionary time provide a strong positive selective force favoring concomitant immunity in addition to the previously mentioned factor of host longevity.

Consequences of 'nonexclusive' parasitism to parasite diversity and vaccination strategies.

The finding that the immunity induced by primary *L. major* infections protects against pathology without being sterilizing also has implications for the generation and maintenance of *Leishmania* diversity. In regions where *Leishmania* is endemic, mammalian hosts are likely subjected to multiple independent infections (43, 44). Over time, this may result in the host being persistently infected with several genetically distinct parasite lines. Once established, mixed infections could then potentially be passed to sand flies, which have recently been shown to be the site of parasite genetic exchange experimentally (28). Since the frequency of sand flies bearing *Leishmania* in natural populations is relatively low (often just a few per cent) (45-47), maintenance of mixed populations in persistent infections may act to increase the frequency at which sand flies acquire mixed infections, which thereafter undergo genetic exchange and generate diversity. While genetic exchange occurs relatively infrequently on a per *Leishmania* cell basis ($<10^{-4}$; (28)), *Leishmania* populations in sand flies are sufficient to yield hybrid parasites at high frequencies (25% or greater per fly; (28) Sacks & Beverley, unpublished data). Thus, the lack of 'exclusivity' even in the presence of protection against disease pathology may result in increased opportunities for genetic exchange and the emergence of new disease phenotypes in nature.

Our data also have some consequences to vaccination strategies. Currently the 'healed' mouse is considered a 'gold standard' for the maintenance of effective immunity against disease pathology, and the generation of live-attenuated parasite lines that persist without pathology while immunizing against virulent challenge has been a priority in vaccine research (48, 49). Our data suggest that such an approach would likely allow virulent parasites from subsequent natural infections to establish their own persistent infections which would then pose a risk of reactivation. This may provide further impetus for the development of vaccines conferring sterilizing, long-lasting protection against both pathology and parasitemia.

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Figure legends

Figure 1. The LmjF-LUC-SAT and LmjF-PHLEO show comparable virulence in infections of resistant mice. C57BL/6 mice (5 per group) were infected with 10^5 metacyclic stage LmjF-LUC-SAT or LmjF-PHLEO *L. major*. N = 1 experiment. A) Measurements of lesion pathology (increase in footpad thickness). Error bars show the standard deviation. B) Persistent parasites. 130 days post infection, mice were sacrificed and the number of parasites persisting in the footpads was enumerated by limiting dilution assay. Horizontal bars show the geometric mean.

Figure 2. Mice persistently infected with LmjF-LUC-SAT show protection from disease pathology by secondary challenge with LmjF-PHLEO parasites. Mice (4-5/group) were inoculated with 10^5 metacyclic LmjF-LUC-SAT parasites in the left hind footpad (primary site), following which they developed lesions and then went on to heal (as shown in Fig. 1A). At least one month after resolution of the primary lesions, each mouse was inoculated in the right hind footpad (secondary site) with 10^5 metacyclic LmjF-PHLEO parasites. In these experiments time 0 is when the secondary inoculation was performed unless otherwise indicated. For all plots, error bars show the standard deviation (n = 4 or 5 in expt. 1 or 2 respectively). A) Lesion pathology at the secondary injection site. The dashed line represents the average of the data presented in Fig. 1A for infections of naïve mice with LmjF LUC-SAT and LmjF-PHLEO for comparison. B) Monitoring of reactivation of pathology at the site of primary infection (left foot). By measuring total footpad thickness. C) Monitoring of reactivation of the primary LmjF-

LUC-SAT parasites at the primary (♦,■) or secondary (◊,□) infection sites site by bioluminescent imaging of luciferase expression *in vivo*; experiment 1 (♦,◊); experiment 2 (■,□). The upper grey dashed line represents the level of luciferase activity normally seen in C57BL/6 mice at peak parasitemia (~7 x 10^7 photons per second), and the lower grey dashed line shows the background typical in these experiments (~5 x 10^3 photons per second).

Figure 3. Retention of both primary and secondary infecting parasites following secondary challenge despite protection from disease pathology. The graph plots the number of persistent parasites present in sites of primary and secondary *Leishmania* infections >10 weeks post secondary challenge as assessed by limiting dilution analysis in unselective (white bar), nourseothricin-containing (gray bars; resistance mediated by *SAT* marker) or phleomycin-containing (black bars; resistance mediated by *PHLEO* marker) as described in the methods. The number of parasites in the primary infection site (LmjF-LUC-SAT inocula) is displayed in the top graph, and the number of parasites in secondary infection site (LmjF-PHLEO inocula) foot is displayed in the bottom graph. The numbers between the two graphs represent the mouse identification number (experiment number-mouse number). "Avg." represents the mean for all mice.

Figure 1

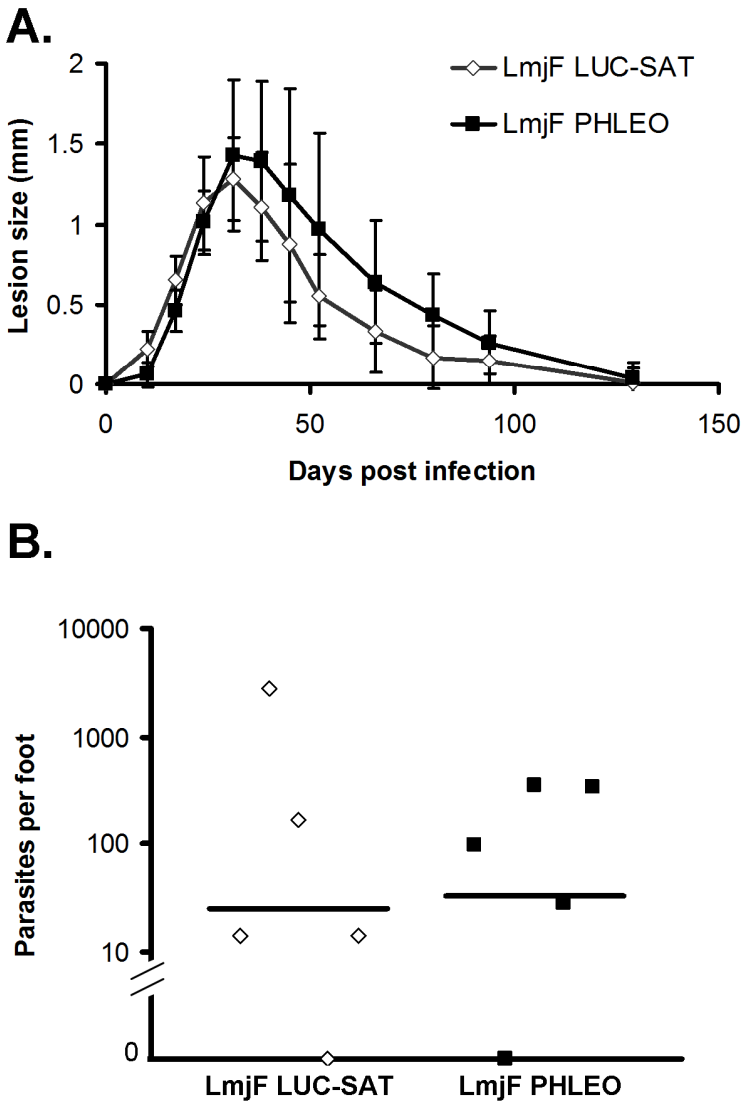

A.
LmjF LUC-SAT
LmjF PHLEO
Lesion size (mm)
2
1.5
1
0.5
0
0
50
100
150
Days post infection
B.
Parasites per foot
10000
1000
100
10
0
LmjF LUC-SAT
LmjF PHLEO

Figure 2

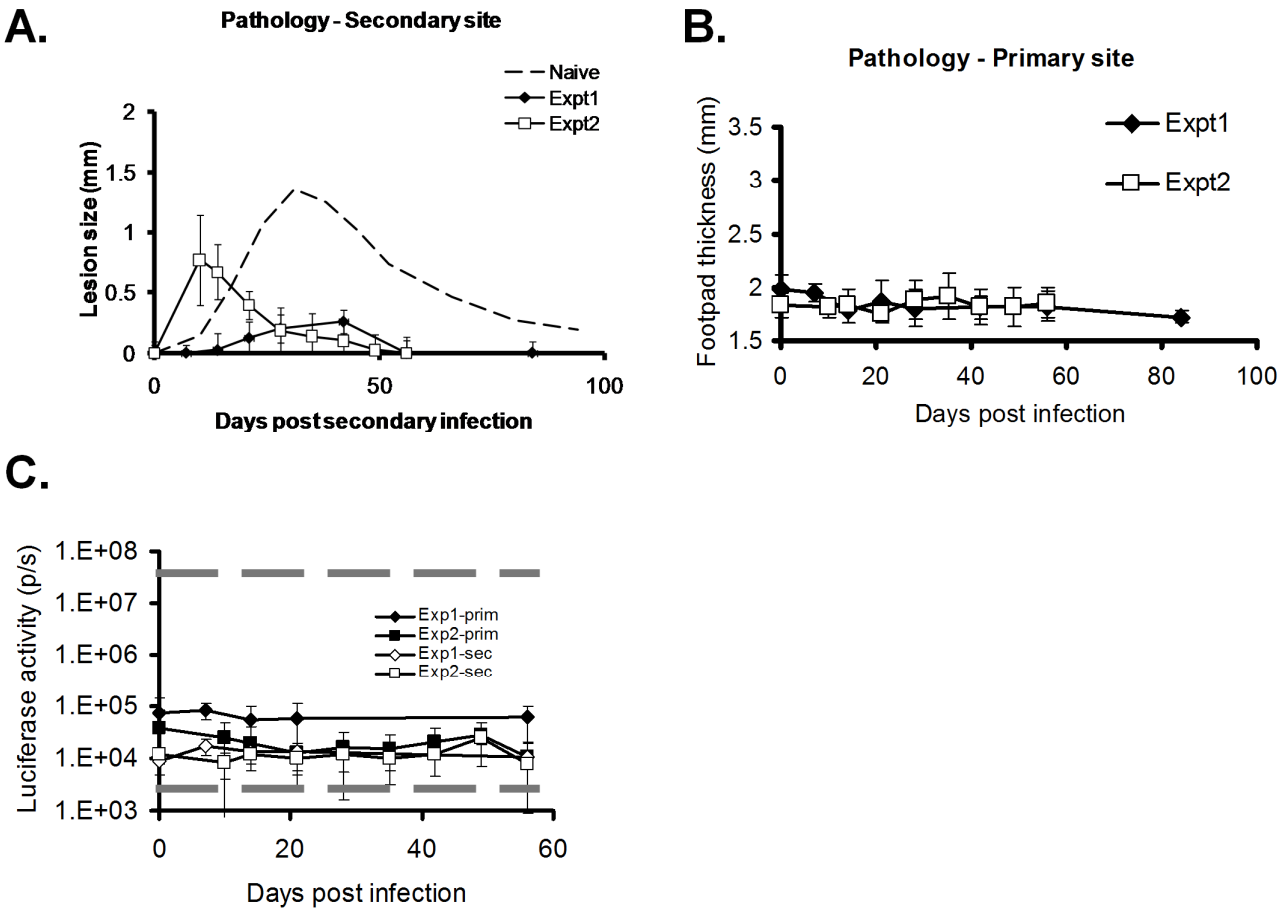
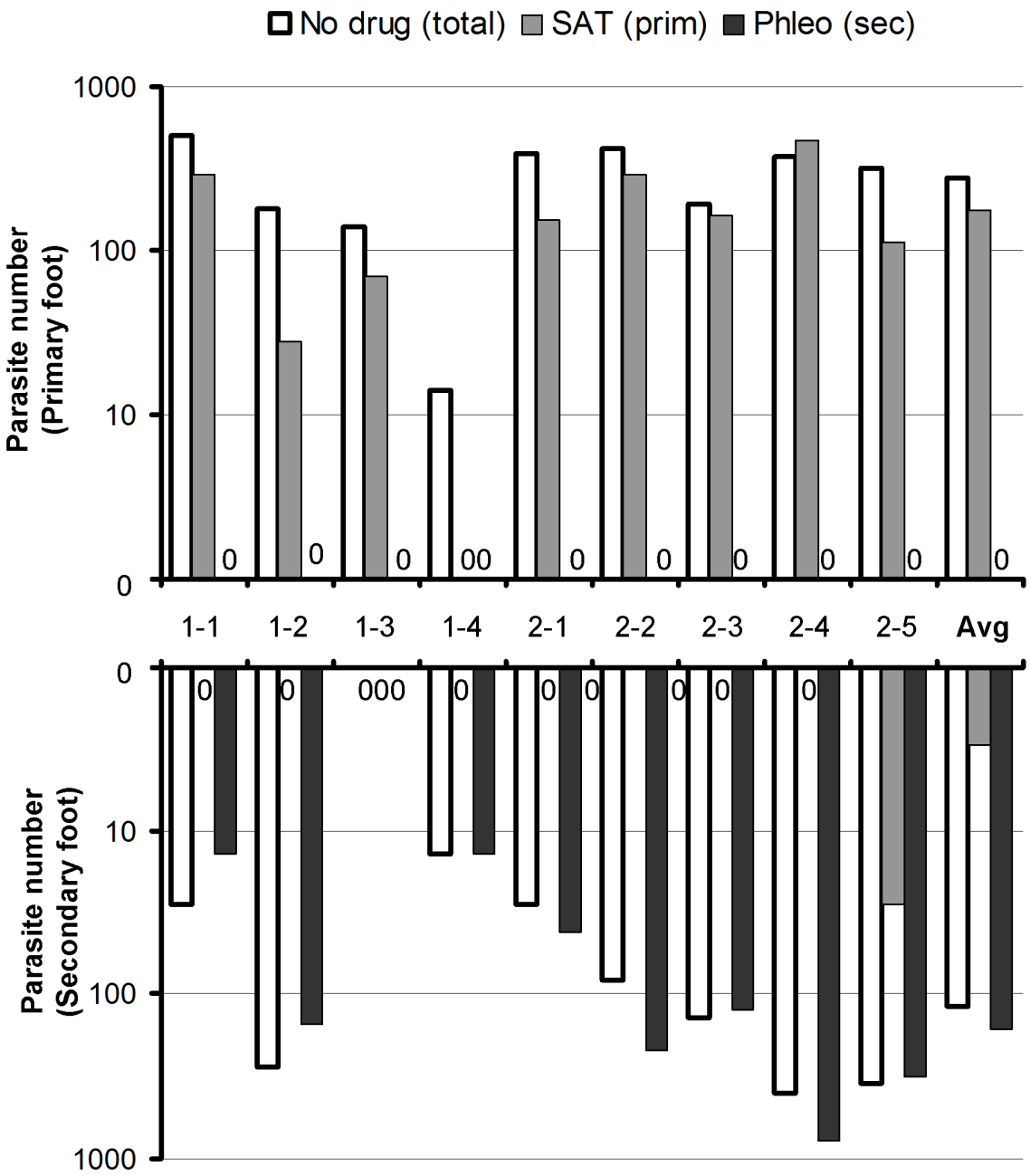

A.
Pathology - Secondary site
Naive
Expt1
Expt2
Lesion size (mm)
2
1.5
1
0.5
0
0
50
100
Days post secondary infection
B.
Pathology - Primary site
Expt1
Expt2
Footpad thickness (mm)
3.5
3
2.5
2
1.5
0
20
40
60
80
100
Days post infection
C.
Luciferase activity (p/s)
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
Exp1-prim
Exp2-prim
Exp1-sec
Exp2-sec
0
20
40
60
Days post infection

Figure 3


No drug (total)
SAT (prim)
Phleo (sec)
Parasite number (Primary foot)
1000
100
10
0
0
0
0
00
0
0
0
0
0
0
1-1
1-2
1-3
1-4
2-1
2-2
2-3
2-4
2-5
Avg
Parasite number (Secondary foot)
0
10
100
1000
0
0
000
0
0
0
0
0
0

Chapter Five

Conclusions and future studies

This chapter was entirely written by Mike Mandell.

Introduction

During the course of my thesis work, I sought to address three main questions. The first question, as presented in chapter 2, was whether metacyclic-stage parasites differentiate into the amastigote stage within dendritic cells and whether the promastigote-specific virulence factor LPG is lost within these cells or retained for potential subsequent transfer to macrophages. Chapters 3 and 4 focus on the life-long asymptomatic persistent infections that occur following healing of lesion pathology. The focus of chapter 3 is primarily to address whether or not persistent parasites replicate as well as to determine their localization. In addition, I sought to determine if the attenuated *lpg2*- line resembled wild type persistent parasites so as to be a model of parasite persistence that would facilitate future studies by decreasing the lag time between mouse infections and experiments. Finally, in chapter 3 I asked whether the immunity maintained by persistent *L. major* is sufficiently strong to be sterilizing against super-infecting parasites, thus gaining the persistent parasites exclusive ownership of and transmission from their host. The main conclusions from these studies are presented here in the following three sections. The remaining sections highlight a number of interesting questions raised by the studies presented in this dissertation that should be the focus of future work. The first of these involve testing aspects of the stem immunogen model of concomitant immunity that was presented in chapter 3 and that will be discussed again below. In addition, two other future aims are presented, both dealing with the capacity of *lpg2*- *L. major* to vaccinate BALB/c, but not C57BL/6 mice, against virulent challenge (1, 2).

The timing of amastigogenesis-related changes in L. major *is variable, depending on the host cell type infected, allowing for the retention of the LPG, a virulence factor critical to parasite establishment in mice* (chapter 2)

My studies showed that the timing of some developmental changes that are associated with parasites differentiating from the infectious metacyclic stage to the intracellularly replicating amastigote stage differs depending on the type of host cell that is infected. In these studies, I infected peritoneal macrophages, bone marrow macrophages, and bone marrow dendritic cells with metacyclic stage parasites and compared the sequence and timing of parasite differentiation as assessed by six markers that are differentially expressed between the two stages of the parasite life cycle. In general, the sequence of amastigote marker induction was similar in all three cell types and amastigote-like phenotypes for each marker individually were present on at least some parasites in all cell types by 24 hours after infection. These data suggest that the parasites do not remain metacyclics within the different cell types, including within dendritic cells which are one of the first types of cells to be infected *in vivo* (3). In fact, amastigote marker induction was similar in all three host cell types in terms of the down-regulation of paraflagellar rod (PFR), the induction of two unidentified amastigote-specific antigens, and in the induction of a YFP transgene which is down-regulated in amastigotes.

However, fewer parasites within the bone marrow derived cells re-entered the cell cycle as assessed by BrdU incorporation and lost LPG expression as compared with the reference peritoneal macrophages. By 48 hours post-infection, most parasites within peritoneal macrophages were LPG-negative. In contrast, 37% or more of the parasites within the bone marrow cells retained high-level LPG expression at 72 hours after

infection. These data imply that the timing of differentiation as assessed by these markers is somewhat plastic and dependent on the type of host cell infected. Alternatively, the parasites within bone marrow derived cells may undergo an incomplete differentiation in which relatively early developmental changes take place and then the parasites arrest their development prior to cell cycle re-entry and LPG loss. Future work could focus on what differences between the three host cell types result in the differences seen here.

These data are important for two reasons. First, they suggest that, under *in vivo* conditions in which parasites appear to be transferred from the first cell types infected (including dendritic cells) to macrophages by ~48 hours after infection, macrophages are likely to encounter promastigote-specific virulence factors such as LPG which are important in the establishment phase of infection. Second, the fact that parasite differentiation can be slowed down or even arrested *in vitro* suggests that parasites may also be able to do this *in vivo*, thus prolonging the duration in which LPG and potentially other promastigote-specific virulence factors could interact with host cells to promote parasite establishment. In addition to my data, one of the main contributions of my work that is presented in chapter 2 is the evaluation of cytological markers of differentiation, as such reagents are potentially useful to the field. Uses for these markers could include identifying environmental signals that are either positive or negative regulators of differentiation or as read-outs for genetic studies of amastigogenesis.

Studies of the replication and localization of persistent WT and lpg2- L. major *in mice* (chapter 3)

As the number of persistent parasites within a mouse remains roughly constant over time, I asked whether persistent parasites were replicating or in a quiescent state. My studies show that persistent parasites do indeed replicate. Continual parasite replication despite constant parasite numbers strongly implies that persistent parasites must also undergo destruction. Although persistent parasites do replicate, it appears that they do so less than do acute-phase parasites. Further studies suggested that this difference in replication is attributable to the presence of a slow or non-replicating sub-population of persistent parasites which is not detected in the acute phase. As I was unable to find parasites within persistently infected tissue that were obviously undergoing destruction, I assume that all of the parasites which were visualized, including those that appear to be poorly replicating, are alive. Further studies, which will be described later in this chapter, are necessary to test this assumption in order to rule in our out the existence of a quiescent sub-population. Until that time, I propose that persistent parasites can be classified into three sub-groups: replicating persistent, quiescent persistent, and terminal persistent (parasites that are destined for destruction).

An obvious question arising from the classification of persistent parasites into these three groups (replicating, quiescent, and terminal) is whether the different classes of parasites are found within different host cell types. The localization of persistent parasites has been a somewhat controversial issue in the field, at least in part resulting from the “safe cell” hypothesis that was proposed by Bogdan et al (2000) (4). This model assumes that macrophages would be unfit host cells for persistent *Leishmania* owing to their ability to express high levels of iNOS. Instead, Bogdan showed that some persistent parasites in lymph nodes were within fibroblasts, which typically were iNOS-negative. These

fibroblasts were proposed to be a crucial reservoir in which parasites could reside during the persistent phase. Although no other study has been published that supports or refutes the safe cell model, this study has been cited 87 times and the safe cell model has to some extent become dogma. Contrary to Bogdan's data, I found very few persistent parasites within fibroblasts and instead found that the vast majority of persistent *L. major* to be within macrophages and dendritic cells in both footpad and lymph node tissue. This finding does not support the essentiality of fibroblasts as safe cells for persistent *Leishmania*. In addition, although many of the infected host cells expressed iNOS, the parasites within $iNOS^+$ cells appeared to be viable and replicating, calling into question the central assumption of the safe cell model that iNOS-expressing host cells cannot support parasite survival. Therefore, my data do not support a safe cell model in which persistent parasites "hide-out" from iNOS-expressing cells within fibroblasts.

Instead, my data suggest that persistent infections are likely quite dynamic systems driven by constant parasite replication in which progeny parasites can have one of two fates. Some parasites go on to infect a new macrophage or dendritic cell in which they survive as either replicating or quiescent forms, thus maintaining the persistent infection. Other progeny go on to face destruction in other macrophages or dendritic cells, which keeps parasite numbers from increasing. I propose that antigens from those killed parasites could be presented and may have a role in stimulating the immune system and consequently maintaining protective immunity. If this is the case, the replicating persistent parasites are crucial both for their own self-regeneration as well as constantly providing a continual immune boost in the form of progeny parasites that are destined for killing. In chapter 3, I compare this model with stem cells, in which a stem population

replicates with some daughter cells remaining pluripotent and maintaining the stem cell population while other daughter cells become terminally differentiated. As will be discussed later in this chapter, future work should focus on testing whether or not parasite killing is important for antigen presentation by infected cells.

Another aim of the work presented in chapter 3 was to compare the replication and localization of attenuate *lpg2*- parasites, which previously have been proposed as a model that would allow for more rapid establishment of persistent infections (5), with what is seen with wild type persistent parasites. *lpg2*- parasites resembled persistent WT parasites in terms of the percent of parasites labeling with BrdU, indicative of similar replication. Furthermore, my analysis suggests that both quiescent and replicating sub-populations of *lpg2*- exist in mice. Similar to wild type persistent parasites, *lpg2*- parasites are found within macrophages and dendritic cells, many of which express high levels of iNOS protein. However, I observed two differences in the nature of the host cells infected by *lpg2*-: first that a higher percentage of *lpg2*- parasites were found in DCs, and second, in that more *lpg2*- -infected host cells or a host cell directly adjacent to them expressed arginase 1 (Arg1) that was seen with persistent WT parasites. Arg1 association correlated inversely with the ability of *lpg2*- to vaccinate mice against virulent challenge, as the association between *lpg2*- and Arg1 was seen in C57BL/6 mice which are not vaccinated by *lpg2*-, but was not seen in BALB/c mice, which are strongly protected by *lpg2*- (1, 2). Taken together my data supports the use of *lpg2*- as a model of WT persistence, especially in the BALB/c mouse.

Do persistent parasites use their host's protective immunity to exclude super-infecting competitors? (Chapter 4)

A number of potential benefits have been proposed for pathogens which immunize their hosts. In chapter 4, I proposed another such benefit that persistent *L. major* might achieve through protecting their hosts against subsequent infection: that of exclusivity. If the immunity that is maintained by persistent parasites is sufficient to prevent the establishment of super-infecting parasites, then the first parasite to infect a host would gain a monopoly on that host, which from then on could only transmit the genome of the first parasite to infect. The ability of a parasite to gain exclusivity would be highly selective and would be predicted to move quickly through a population. Using genetically marked parasites of equivalent virulence, I showed that persistent parasites do not gain exclusivity, but rather that super-infecting parasites can become established and persist in similar numbers within their host. These data suggest that, in nature, a host could potentially be infected with parasites derived from multiple independent infections, with the parasites potentially being genetically and phenotypically distinct from each other. Such mixed infections could then be passed on to sand flies, which have been shown to support sexual recombination of *L. major* (6).

Is there a quiescent sub-population of persistent parasites and, if so, do the quiescent persistent parasites differ from replicating persistent parasites or acute-phase amastigotes?

As discussed above, my data suggest that there are two populations of persistent parasites, one that is actively replicating and one that appears to be replicating slowly if at all. One interpretation of this is that the slow replicating population represents a group of viable parasites that replicate slowly either because they are in sub-optimal conditions for growth or because they have exited the cell cycle and are in some sort of arrest or stasis.

Another interpretation is that these parasites do not undergo replication as assessed by BrdU labeling because they are the parasites that are dead or dying, and were not identified as such previously because the assays used in chapter 3 (TUNEL and observation of morphological integrity) are simply the wrong assays of parasite destruction. Thus, an important question is whether or not the parasites that are herein referred to as quiescent persistent are actually alive. Perhaps the best approach to demonstrate viability would be through some kind of metabolic labeling that would label all living parasites but would not label host cells. One possible approach takes advantage of a tunable protein expression system in which a reporter protein such as DsRed fused to a destabilization domain (DD) can be stabilized in the presence of a small molecule that can be administered to mice (7, 8). Mice could be infected with transgenic parasites expressing DD-DsRed. Once the infections have entered the persistent phase, the mice could be dosed with the small molecule Shield1, which would stabilize the DD and allow for DsRed protein to accumulate within parasites that are actively undergoing transcription and translation (in other words, alive). As discussed in chapter 3, the quiescent parasites tended to be in host cells containing one to three parasites, and so the absence of DsRed protein in parasites within such host cells relative to what is seen in host cells containing four or more parasites would indicate that the parasites that I refer to as quiescent are in fact dead. Alternatively, if DsRed-positivity of the parasites is independent of the number of parasites per infected cells, this would argue the opposite.

If the quiescent persistent parasites are shown to be viable, the next question to be addressed is whether or how they differ from replicating persistent parasites. Potentially, quiescent persistent parasites differ from replicating persistent parasites only in terms of

replication, possibly because the replicating persistent parasites are in an environment more suitable to rapid replication. Alternatively, the quiescent persistent parasites may in fact constitute a different stage of the parasite life cycle in a manner consistent with quiescent *Toxoplasma*, herpes viruses, or *Mycobacteria*, all of which have gene expression profiles that are specifically devoted to persistence (9-11). To address this question, the gene expression profiles of quiescent persistent parasites (enriched in small clusters) versus replicating persistent parasites (enriched in large clusters) could be determined by microarray after RNA isolation by laser capture procedures. Additionally, similar studies could be performed to compare the gene expression profile of persistent parasites with that of acute-phase amastigotes. The purpose of these studies would be to first, determine if *Leishmania* also has a stage of its life cycle that is specifically devoted to persistence, and second, to identify markers that would distinguish persistent parasites from acute-phase parasites or quiescent persistent parasite from replicating persistent parasites.

Is parasite killing important for effective antigen presentation?

As has been mentioned elsewhere in this dissertation, persistent parasites have a role in maintaining protective immunity (12, 13). Presumably, a key component of this role is constant presentation of parasite antigens to the immune system. In the stem immunogen model, I propose that some parasites become "terminally differentiated" (are killed) and that the antigens that are presented are derived from these parasites rather than the replicating "stem" *Leishmania*. It is reasonable to assume that host cells containing dead *Leishmania* may more effectively present antigen than do host cells containing live parasites, as live *Leishmania* have been shown to inhibit antigen presentation by their

host cells (14-16). However, this assumption has not been proven and it is possible that just as much antigen is presented by host cells containing healthy, replicating parasites as is presented by host cells that contain antigens from parasites that they had previously killed. Therefore, future studies should determine if intracellular parasite killing is a prerequisite for effective antigen presentation and T cell stimulation as this question has not been directly addressed in the literature. For these studies, macrophages or dendritic cells could be infected with transgenic parasites expressing the model antigen OVA and with thymidine kinase derived from herpes simplex virus. Some of the infected cells could then be cultured in the presence of gangcyclovir, which should kill the parasites but have minimal effects on the host cells. If host cells containing killed parasites more efficiently present antigen, then OVA-specific OT-II T cells should proliferate more after co-culture with the gangcyclovir-treated infected cells than after co-culture with the untreated infected cells.

What is the role of host arginase 1 in vaccine failure of lpg2- *-infected C57BL/6 mice?*

When *lpg2*- -infected BALB/c mice are 'challenged' with virulent parasites, they are able to prevent the virulent parasites from replicating to high titers and are protected from disease pathology (1). However, *lpg2*- parasites are not sufficient to confer such resistance to C57BL/6 mice unless the parasites are co-injected with CpG DNA as an adjuvant (2). Data presented in chapter 3 demonstrates that association of parasites with host arginase 1-expressing cells was a negative correlate of immunity to pathology. In other words, a higher percentage of parasites in (C57BL/6 harboring *lpg2*-) which were shown to be poorly protected in other studies, were either within or adjacent to an Arg1-expressing host cell than were parasites within strongly protected C57BL/6 mice

harboring WT persistent parasites or BALB/c mice harboring *lpg2*- parasites (Table 5-1). While this correlation does not prove that Arg1 expression results in vaccine failure, there is ample evidence in the literature that suggests such a causal relationship is plausible (17-24).

Like all arginases, Arg1 catalyzes the hydrolysis of L-arginine resulting in the production of urea and L-ornithine, which is the precursor for polyamine synthesis (25). Cells expressing high levels of Arg1 have been shown to quickly deplete L-arginine from culture media and are thought to also have the capacity to deplete arginine in their micro-environment *in vivo* (26). L-arginine depletion has been shown to diminish effector T-cell function by decreasing the number of T-cell receptors present on the cell surface and by preventing T-cell proliferation in response to antigen stimulation (23, 26, 27). These results have been confirmed in *L. major*-specific T-cells, which were also shown to express lower amounts of interferon-γ upon antigen stimulation under conditions of arginine-deprivation (24). In addition, depletion of essential amino acids including arginine is reported to induce naïve T-cells to adopt a regulatory phenotype (20). As adoptive transfer of *Leishmania*-specific Treg cells leads to reactivation of persistent *L. major* infections (28), an increase in these cells would also likely be disease-promoting in the event of virulent *L. major* challenge. Arginine depletion may also have a role in preventing the generation of nitric oxide (NO), an effector molecule required for the control of *L. major* infection *in vitro* and *in vivo* (29-31). NO is generated through the enzymatic activity of iNOS, which uses L-arginine as a substrate (27). Numerous studies demonstrate that reduction in arginine availability results in corresponding reductions in NO output by iNOS-expressing cells (27, 32-35). In addition to these effects of arginine

depletion in iNOS activity, arginine depletion has also been demonstrated to have effects on NO production by decreasing iNOS expression (22).

Another consequence of elevated levels of Arg1 expression in association with lpg2- parasites could be an increase in the amounts of polyamines which appear to be limiting for *L. major* early in mouse infection, thus facilitating parasite growth (27, 34, 36). In addition to being potentially beneficial to the parasite in terms of nutrition, polyamines have also been shown to attenuate iNOS expression and function (17-19).

Some of the mechanisms mentioned above by which Arg1 expression could result in vaccine failure by *lpg2*- (e.g. effects on NO production or providing polyamines to "challenge" parasites) act directly upon the parasite's environment and thus depend on the up-regulation of Arg1 in or the recruitment of Arg1-expressing cells to the challenge site, a point which has not yet been established. Other mechanisms, such as those involving T-cells, act indirectly on the parasite's environment and would not require close association between parasite-infected cells at the challenge site and $Arg1^+$ cells.

Future work should aim to increase the size of the data-set demonstrating a correlation between Arg1-association and vaccine failure. While my data suggests such a correlation exists, more data points are needed to firmly conclude this point. To this end, experiments could be performed to determine the level of Arg1 association with *lpg2*- in C57BL/6 mice that have been treated with CpG DNA as an adjuvant and that are strongly protected against pathology from new infections. In addition to *lpg2*-, several other *L. major* lines exist which persist indefinitely without causing pathology (e.g. *fbp*- or *iscl*-), but studies regarding their ability to vaccinate different strains of mice has not been

published (37, 38). Studies could be performed with these parasite lines to determine their vaccination status in different mouse strains and the level of Arg1 association. If after these studies the correlation between Arg1 association and vaccine failure is still apparent, future work should focus on determining if Arg1 association is causative of vaccine failure as outlined below.

To determine if the observed Arg1-association causes vaccine failure, the ability of *lpg2*-parasites to vaccinate Arg1-deficient mice and their WT litter-mates of the appropriate mouse strain (C57BL/7) could be assessed. Fortunately, such mice have been generated: Arg1 has been deleted from most macrophages and neutrophils in *Arg1* $^{flox/flox}$; *LysMcre* mice (39). If Arg1 is responsible for vaccine failure in C57BL/6 mice, then arginase-deficient *Arg1* $^{flox/flox}$; *LysMcre* mice should be protected from virulent challenge by prior *lpg2*- infection while their wild type siblings would remain susceptible.

If Arg1 expression is found to be responsible for vaccine failure, future work should focus on understanding the mechanism by which this occurs. As mentioned above, the "direct" effects of Arg1 depend on its localization at the challenge site. Thus, if Arg1 results in vaccine failure by attenuating NO production or increasing the amount of polyamines available to the parasites, then the expression of Arg1 within the challenge site of *lpg2*- -infected C57BL/6 mice should be greater than that of "immune" *lpg2*- -infected BALB/c mice. If this is the case, future studies could focus on potential differences between "immune" and non-imnune" *lpg2*- -infected mice in terms of iNOS expression and NO production at the challenge site.

If *lpg2*- vaccinates Arg1 conditional knock-out mice but not their WT litter-mates, then this system will allow for a good way to determine if Arg1 affects T-cell function by the use of adoptive transfer experiments of T-cells from *lpg2*- -infected *Arg1* $^{flox/flox}$; *LysMcre* mice and WT *Arg1* $^{flox/flox}$ mice into naïve mice followed by virulent *L. major* challenge. As previous studies have demonstrated that such transfers of T-cells from immune mice confer protection to naïve mice (40), the T-cells from the "immune" *Arg1* $^{flox/flox}$; *LysMcre* mice would be expected to be protective. If Arg1-induced vaccine failure results from altered T-cell phenotypes and not from the "direct" effects described above, then adoptive transfer of T-cells from *lpg2*- -infected *Arg1* $^{flox/flox}$ would not be expected to confer protective immunity. If this is indeed the result, then modifications of this adoptive transfer system could be made to further define how Arg1 affects T-cells resulting in vaccine failure.

Use of lpg2- *vaccination model to identify anti*-Leishmania *effectors in addition to NO*

As mentioned above, nitric oxide is clearly important in controlling *L. major* infections *in vitro* and *in vivo* (29-31). As such, cells expressing iNOS have been considered likely sites of parasite destruction (4, 41-43). However, in Chapter 3 I present data showing that both *lpg2*- and WT persistent parasites were capable of surviving and replicating within host cells expressing high levels of iNOS. This surprising result raises the possibility that while NO is necessary for *Leishmania* killing, it may not be sufficient and other effector molecules in addition to NO are also required.

As described above and in table 5-1, infection of C57Bl/6 mice with *lpg2*- parasites does not lead to protective immunity against subsequent virulent challenge while *lpg2*-

infection of BALB/c mice does confer protection. Thus, it is reasonable to assume that the host cells containing *L. major* at the challenge site in "immune" mice (*lpg2*- // BALB/c) differ in some way relevant to *Leishmania* killing from *L. major*-containing cells in "non-immune" mice (*lpg2*- // C57BL6). One possible difference is in terms of iNOS expression. However, my data show that ~70% of *lpg2*- parasites are within $iNOS^+$ host cells in either BALB/c ("immune") or C57BL/6 ("non-immune") mice. If the phenotype of infected cells at the challenge site resembles that of cells at the site of primary *lpg2*- infection, then the levels of iNOS expression by cells at the challenge site may not differ with vaccination status. Differences in the ability of *Leishmania*-containing cells to kill parasites in "immune" versus "non-immune" mice despite similar levels of iNOS expression and NO generation would imply that other factors are also involved. As such, a global comparison of the infected host cells at the challenge site between "immune" and "non-immune" mice may present a good system to identify these factors.

Some of the differences between the *Leishmania* containing cells in "immune" versus "non-immune" mice could be in terms of the activity of constitutively-expressed proteins which are normally in an inactive form but are activated under conditions of infection by post-translational modification. Alternatively, the relative expression of genes involved in *Leishmania* killing may differ between *Leishmania*-containing cells in "immune" mice versus those in "non-immune" mice. I propose to use a microarray-based approach to focus on the latter class of factors. For these studies, I will use C57BL/6 that have been simultaneously injected with *lpg2*- parasites and with CpG DNA as an adjuvant and which are strongly protected against virulent challenge (2) as the "immune" mice, thus

eliminating mouse strain as a potentially confounding variable in comparisons with "non-immune" *lpg2*- -infected mice of the same strain.

Several control experiments must be performed prior to trying to obtain RNA from infected cells. First, it will be important to establish the appropriate time frame at which parasite killing is occurring in "immune" mice but not in "non-immune" mice. It will also be important to eliminate the possibility that the difference in the ability of "immune" versus "non-immune" mice to kill *L. major* results merely from the relative inability of "non-immune" mice to generate NO at the challenge site. If the "immune" and "non-immune" *lpg2*- -infected mice generate comparable levels of NO following virulent challenge, then experiments can proceed.

RNA from infected host cells of both "immune" and "non-immune" mice would then be harvested by laser-capture procedures at the earliest time-point at which parasite killing is seen in "immune" mice, and differences in gene expression would be detected by microarray. While such an approach would miss those proteins whose anti-*Leishmania* activity is affected by post-translational modification, it may identify novel anti-microbial pathways that may also be important for the control other intracellular pathogens in addition to *Leishmania*.

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Table 5-1

	C57BL/6 WT persistent No adjuvent	C57BL/6 *lpg2-* No adjuvent	BALB/c *lpg2-* No adjuvent	C57BL/6 *lpg2-* CpG DNA
	"immune"	"non-immune"	"immune"	"immune"
Arg1 association (primary foot)	-/+	+++	-/+	N.D.
Arg1 association (secondary foot)	?	?	?	?
iNOS association (primary foot)	+	+	+	N.D.
iNOS association (secondary foot)	?	?	?	?

Appendix I

Localization studies of the amastigote-specific antigens recognized by mAB T17 and mAB T18

Michael A. Mandell, Wandy L. Beatty, and Stephen M. Beverley[1]

[1]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis MO 63110 USA

Author contributions

M.A.M performed experiments and wrote the text.

W.L.B performed experiments.

S.M.B. made comments on the text.

Introduction

One approach for identifying novel *Leishmania* factors involved in mammalian virulence is by the identification of molecules that are specifically expressed by the amastigote stage of the parasite's life cycle, as such molecules are unlikely to have roles in cellular "house keeping" or in sand fly virulence. Additionally, molecules with amastigote-specific expression are good candidates for reverse genetic studies, as their synthesis is unlikely to be essential to promastigote-stage parasites, which is the stage of the parasite life cycle most easily cultured in a laboratory setting and in which transfection experiments are typically performed (1). More than two decades ago, Charles Jaffe developed monoclonal antibodies that specifically recognize *Leishmania major* amastigotes, but neither the molecules recognized by these antisera, nor their functions, have been determined (2). In Chapter 2 of this thesis, I developed the use of two of these antisera, referred to as T17 and T18, as "amastigote markers". In that work, we showed by immunofluorescence microscopy that the two amastigote-specific antisera had different sub-cellular localization patterns, and hence recognized different antigens. We also found that the T17 and T18 reactive molecules are induced by 8 hours after infection of host cells, ultimately labeling >90% of the parasites by 11 hours post-infection. In this work, we further describe the sub-cellular localization of the molecules recognized by these two antisera by immunoflourescence and immunoelectron microscopy and identify conditions that result in high level expression of the T17 and T18 antigens in by >40% of parasites axenic culture for proteomic analysis.

Materials and Methods

Parasite culture

L. major Friedlin V1 strain (MHOM/IL/80/Friedlin) parasites expressing YFP (yellow fluorescent protein; *SSU:IR1PHLEO-YFP*) were generated as described elsewhere (3). These parasites were grown at 26˚C in M199 medium (US Biologicals) supplemented with 40 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) pH 7.4, 50 μM adenosine, 1 μg ml^{-1} biotin, 5 μg ml^{-1} hemin, 2 μg ml^{-1} biopterin and 10% (v/v) heat-inactivated fetal calf serum (1). For experiments in which amastigote marker induction was assayed in the absence of host cells, 10 ml of parasites that had been in stationary-phase for two days ($2X10^8$ cells) were pelleted and resuspended in 30 ml pre-warmed RPMI 1640 (Invitrogen) and kept in a 37˚C for 24 hours with 5% CO_2.

Macrophage infections

Peritoneal macrophages (PEMs) were elicited by a peritoneal injection of potato starch into female C57Bl/6J mice (6-10 weeks old; Jackson Labs) harvested as described, plated on glass coverslips, and maintained in DMEM (Invitrogen) containing 10% FCS and 2 mM L-glutamine in a 37˚C incubator with 5% CO_2 (4). The day after the PEM isolations, infective metacyclic-stage parasites were recovered using the density gradient centrifugation method (5) and opsonized with serum from C5-deficient mice prior to being added at a parasite to PEM ratio of 5:1. Extracellular parasites were removed 2 hours after infection by extensive washing. Infected PEMs were provided with fresh media daily, and samples were prepared for microscopy 3 days after infection.

Antibodies, immunofluorescence staining and microscopy

T17 and T18 antisera were provided by C. Jaffe as lyophilized mouse ascites fluid. For these studies we used T17 that was prepared 1/7/1996 and T18 that was prepared 2/8/1991.

Samples of infected PEMs on coverslips were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 minutes. *In vitro* "differentiated" parasites were were fixed in 4% (w/v) PFA in phosphate-buffered saline (PBS) for 2 minutes, and then the parasite/PFA suspension was diluted 10-fold in PBS. Samples were washed in PBS, and then blocked and permeabolized in PBS containing 5% (v/v) normal goat sera (Vector labs) and 0.1% (v/v) Triton-X-100 for 30 min (blocking buffer). Parasite nuclei were then stained with a pool of rabbit antibodies raised against *L. major* histones H_2A, $H_2A_{variant}$, H_2B, H_3, and H_4 (pooled at a ratio of 3:2:3:3:1 by titer) and used at a dilution of 1:750 in blocking buffer (Wong and Beverley, in preparation). T17 or T18 antibodies were diluted 1:400 in blocking buffer. After a one hour incubation in primary antibodies, unbound antibody was washed off in PBS and primary antibodies were detected with Alexafluor488 goat anti-mouse and Alexafluor555 goat anti-rabbit (Invitrogen, both used at a concentration of 2 μg ml^{-1}). DNA was detected with Hoechst 33342 (Invitrogen, used at a concentration of 5 μg ml^{-1}) for wide-field microscopy or TOPRO-3 (Invitrogen, used at a concentration of 2 μM) for confocal microscopy. After a 40 minute incubation, samples were washed with PBS and mounted in ProLong Gold (Invitrogen).

In vitro "differentiated" parasites were were fixed in 4% (w/v) PFA in phosphate-buffered saline (PBS) for 2 minutes, and then the parasite/PFA suspension was diluted

10-fold in PBS. Fixed parasites were then spun down and resuspended in PBS at a concentration of 8 X 10^6 parasites per milliliter. Parasites (4 X 10^6) were then adhered to glass cover slips by centrifugation. Blocking, permeabilization, and antibody staining was then performed as described above.

Microscopy was performed on an Olympus AX-70 wide-field fluorescence microscope or a Zeiss 510 META confocal laser scanning microscope. Cutoffs for saturation and background levels were adjusted with Photoshop software (Adobe).

Immuno-electron microscopy

These studies were performed by Wandy Beatty in the Microbiology imaging facility. For immunolocalization by transmission electron microscopy, infected cells were fixed in 4% paraformaldehyde/0.05% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100mM PIPES/0.5mM MgCl2, pH 7.2 for 1 hr at 4˚C. Samples were infiltrated overnight in the cryoprotectant 2.3M sucrose/20% polyvinyl pyrrolidone in PIPES/MgCl2 at 4˚C. To permeabilize cells for antibody labeling samples were plunge-frozen in liquid nitrogen and subsequently thawed in PBS at room temperature. This technique was confirmed to permeabilize the host cell membrane and intracellular organelle membranes. Samples were probed with the primary antibodies at 1:250 dilutions followed by FluoroNanogold anti-mouse Fab (1:250; Nanoprobes, Yaphank, NY) and silver enhancement (Nanoprobes HQ silver enhancement kit). Samples were washed in phosphate buffer and post-fixed in 1% osmium tetroxide (Polysciences Inc., Warrington, PA) for 1 hr. Samples were then rinsed extensively in dH20 prior to *en bloc* staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 hr.

Following several rinses in dH20, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA). All pre-labeling experiments were conducted in parallel with omission of the primary antibody. These controls were consistently negative at the concentration of Nanoprobes-conjugated secondary antibodies used in these studies.

Results and discussion

T17 epitope localizes to the amastigote flagella and surface and is also found in vesicles within the host cell cytoplasm

To determine the localization of the antigen recognized by mAB T17, we stained PEMs that had been infected for 72 hours with mAB T17, as well as antisera raised against *L. major* histone proteins to localize parasite nuclei, and with Hoechst 33342 to detect DNA (Figure 1A). The most intense T17 reactivity presented as a line at the parasites anterior end proceeding outward from the kinetoplast to the anterior pole of the cell. This staining pattern is consistent with mAB T17 recognizing the amastigote flagella, the length of which is almost entirely within the flagellar pocket (6). Less intense staining was also seen on the surface of amastigotes. This staining pattern was apparent on 91% of T17+ parasites scored (N = 222 parasites). As shown in Figure 1B and C, this localization pattern was also found by immuno-electron microscopy.

Interestingly, mAB T17 reactivity was not limited to the parasite itself, but was also showed a punctate staining pattern within the cytoplasm of infected, but not uninfected, macrophages (Figure 2A). Immuno-electron microscopy images revealed that, in many cases, the T17 antigen was concentrated just outside the phagolysosomal membrane at the distal tip of the amastigote flagellum (Figure 2B) and was also found within membranous compartments further away from the parasite-containing phagolysosome (Figure 2C).

In summary, these results suggest that the mAB T17 antigen localizes predominantly to the amastigote flagellum, and is somehow translocated to the parasite's surface and out of

the phagolysosome. These data are provocative when considered in the context of two recent studies from other laboratories. First, Reiner's laboratory has suggested that a number of *Leishmania* proteins are delivered to the host cell within 'exosomes', which could modulate immune responses by host cells (7, 8). One concern from these data is that the exosome composition when evaluated by proteomics is qualitatively similar to that of total *Leishmania* proteins, albeit with modest quantitative differences. Second, Gluenz et al (2010) have proposed the existence of a 'flagellar' synapse in the parasites orient their flagella such that the distal tip is in intimate contact with the phagolysosomal membrane (6). Connecting these results is the observation of exosomes budding off of the distal tip of *Chlamydomonas* flagella (9), suggesting a secretory role for this organelle. Potentially antigen T17 represents an example of a stage-specific parasite 'cargo' delivered by the flagellar route. This will be pursued in the future by the Beverley laboratory.

T18 epitope localizes to amastigote surface and a novel structure at the parasite's posterior pole

Figure 3 shows the localization of the antigen recognized by mAB T18 on amastigotes. The parasite's surface clearly is recognized by the antibody. In addition, most parasites (78%, N = 187) have a region of intense staining on their posterior end, which is defined as the end of the cell furthest from the kinetoplast DNA network (Figure 3A). 29% of these parasites have an additional region of staining located between the parasite nucleus and anterior pole (Figure 3B). In general, immunoEM studies yield results consistent with what is seen by fluorescence microscopy, with antibody labeling present on the parasite surface as well as labeling a ring of electron-dense material at the parasite's posterior

pole (Figure 3C & D). However, the T18 reactivity detected between the anterior pole and the parasite nucleus that was seen by fluorescence microscopy was not detected by immunoEM studies.

There are very few reports of organelles at the posterior end of *L. major* amastigotes. One candidate for the structure recognized by mAB T18 is the "megasome", a lysosome-like organelle that has been described in New-World Leishmania species such as *L. amazonensis.* This organelle, which is found near the posterior end of these parasites, has been reported to degrade host MHC class II (10, 11). However, in electron micrographs, amastigote megasomes do not resemble the electron-dense rings seen in our images, and instead appear to be large, mostly open, vacuoles containing some electron dense material (10). Thus the structure/region recognized by T18 may define a cellular structure that has not been described previously.

Efforts to identify T17 and T18 antigens

The localization patterns of the antigens recognized by T17 and T18 are sufficiently interesting to warrant studies directed towards the identification of the molecules recognized by these antisera. One challenge is that Jaffe reported that these antisera do not identify parasite molecules in western blotting (2). He did show that they were able to immunoprecipitate several amastigote molecules, and from labeling studies inferred these were proteins (2). Another challenge is the generation of sufficient amastigote lysate for these studies, as isolation of lesion amastigotes from mice is costly, labor intensive, and often results in substantial contamination with molecules of host origin. Unfortunately, *L. major* does not give rise to culturable axenic amastigotes, despite efforts by our or other

laboratories. Previous work and preliminary studies I performed suggest that these antibodies do not cross-react with amastigotes from other *Leishmania* species (2), precluding the use of 'axenic' amastigotes from species such as *L. tropica, L. braziliensis*, *L. mexicana* or *L. donovani* as a source of antigen. As such, an approach in which the expression of the T17 and T18 antigens could be expressed by parasites in the absence of host cells would be extremely beneficial.

To simulate conditions under which *L. major* might differentiate into amastigotes, we diluted stationary-phase parasite cultures into commercially available RPMI. 24 hours later, 40-60% of the parasites presented an amastigote-like morphology (round with no visible flagella) and were reactive with T17 and T18 (Figure 4) and were "positive" for some of the other amastigote differentiation markers described in Chapter 2 (data not shown). Interestingly, the use of other media conditions which had previously be shown to successfully to induce other *Leishmania* species such as *L. donovani* to differentiate into amastigote-like forms and support their subsequent replication (11) was much less successful at inducing T17 and T18 reactivity.

In collaboration with Igor Alameida's lab at the University of Texas El Paso, these conditions are being employed to generate large batches of cells expressing the T17 and T18 antigens. Lysate from these parasites will be subjected to immunoprecipitations with the two monoclonal antisera with the ultimate goal of antigen identification by MS. If these experiments are successful, reverse genetic experiments will be conducted in our lab to generate parasites that are unable to synthesize the molecules recognized by T17 and T18, which will be used as tools to help ascertain the function of these molecules in the parasite life cycle.

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Figure legends

Figure 1. Localization of T17 reactvitiy. A-C) PEMs that had been infected for 3 days with *L. major* were fixed as described in methods. A) Infected cells were stained with rabbit antisera that recognize parasite histones and mouse mAB T17. Primary antibodies were detected with Alexafluor488-conjugated anti-mouse antisera (green) and Alexaflour555-conjugated anti-rabbit antisera (red). DNA was stained with Hoechst 33342 (blue). Images were captured using wide-field fluorescence microscopy, scale bar represents 2 μm. B-C) Immuno-electron micrograph of infected PEMs stained with mAB T17, in which T17-reactivity is indicated by silver granule deposition. Scale bar represents 0.5 μm. Abbreviations: N = parasite nucleus, K = kinetoplast, A = flagellar axoneme, P = posterior end.

Figure 2. T17 reactivity is seen in the cytoplasm of infected host cells. A) Confocal micrograph of uninfected PEMs (left) or PEMs that had been infected for 3 days with *L. major.* Samples were stained to detect parasite histones (red), the T17 antigen (green), and DNA (blue). Bright green punctate staining is absent in uninfected PEMs, but present in some infected PEMs. B) T17 reactivity is primarily on parasite flagella, but also appears to exit phagolysosome and enter PEM cytoplasm at the flagellar distal tip. C) In this image, T17 reactivity is primarily on the parasite flagella and surface (upper left hand corner) but also within a membrane-bound compartment within PEM cytoplasm (arrow). Scale bar represents 0.5 μm. Abbreviations for B and C: N = parasite nucleus, K = kinetoplast, A = flagellar axoneme.

Figure 3. Localization of T17 reactvitiy. A-C) PEMs that had been infected for 3 days with *L. major* were fixed as described in methods. A) Infected cells were stained with rabbit antisera that recognize parasite histones and mouse mAB T18. Primary antibodies were detected with Alexafluor488-conjugated anti-mouse antisera (green) and Alexaflour555-conjugated anti-rabbit antisera (red). DNA was stained with Hoechst 33342 (blue). Images were captured using wide-field fluorescence microscopy, scale bar represents 2 μm. B) Confocal slice of a parasite as stained in (A) showing surface and posterior labeling with mAB T18 (DNA labeling is not shown). Intracellular staining between the nucleus and the anterior end of the parasite is also visible. Scale bar represents 2 μm. C-D) Immuno-electron micrograph of infected PEMs stained with mAB T18 in which T18 reactivity is detected by silver granule deposition. C and D) Zoomed-in image of parasite posterior. Arrows indicate the amastigote-specific structure recognized by mAB T18 and arrowheads indicate surface labeling. Scale bar represents 0.5 μm. Abbreviations: N = parasite nucleus, K = kinetoplast, FP = flagellar pocket.

Figure 4. Induction of T17 and T18 reactivity in axenic culture. Representative images of stationary-phase parasites that had been subjected to 37˚C in RPMI media for 24 hours and stained with rabbit antisera raised against *L. major* histone proteins (red) and either mAB T17 (left) or mAB T18 (right) shown in green. Scale bar represents 5 μM.

Figure 1

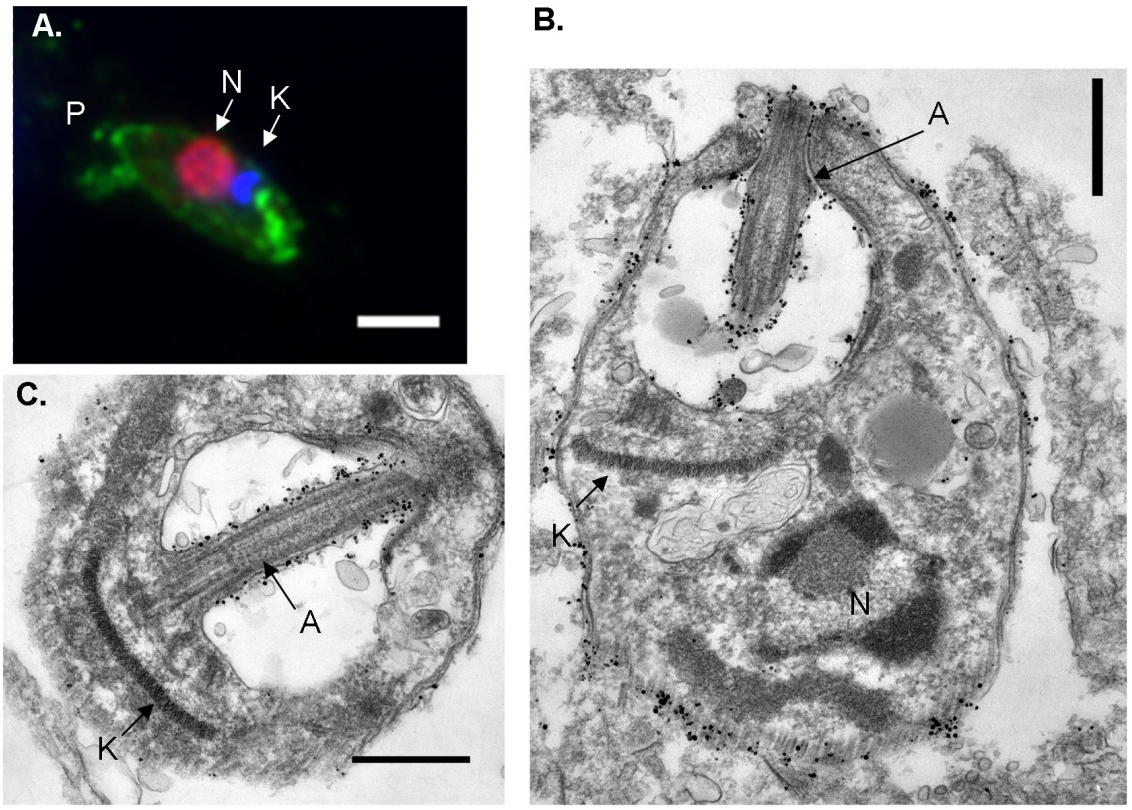

Figure 2

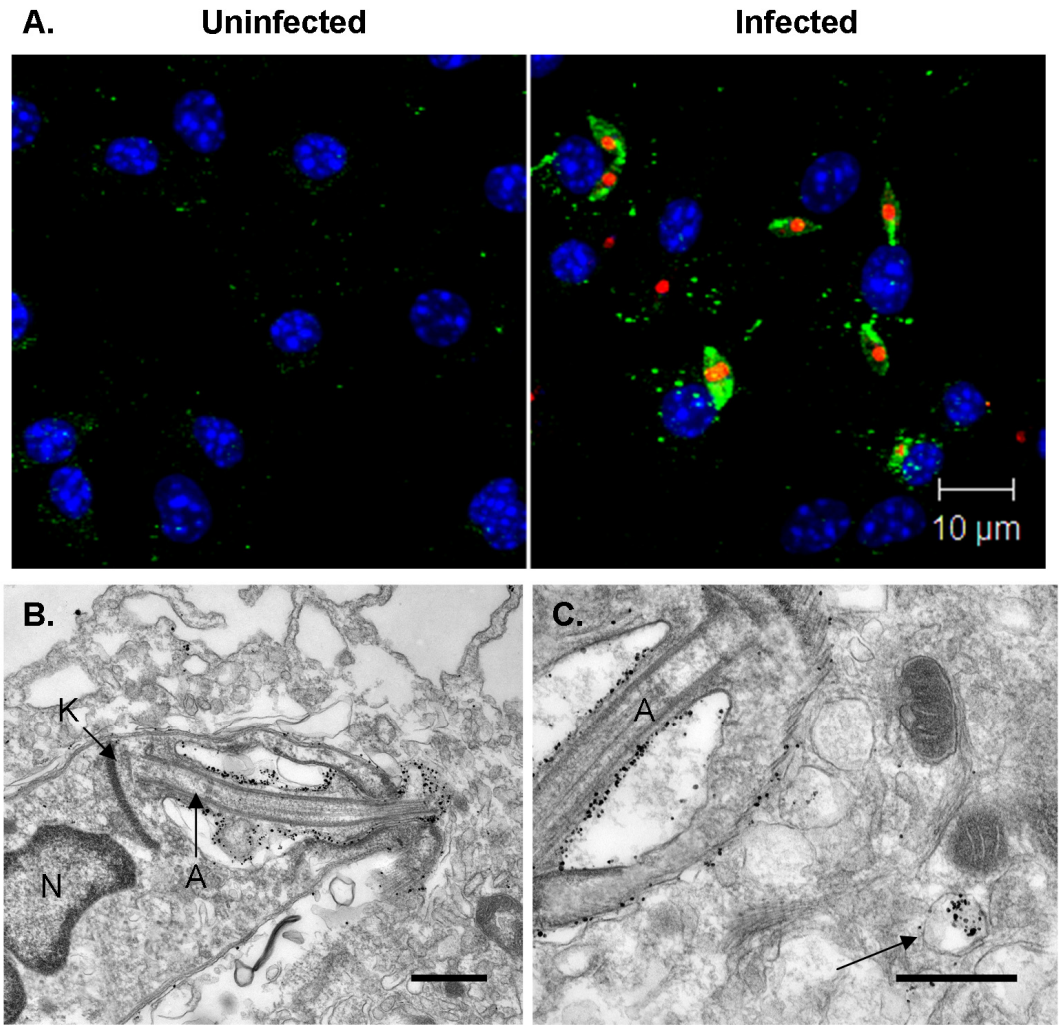

Figure 3

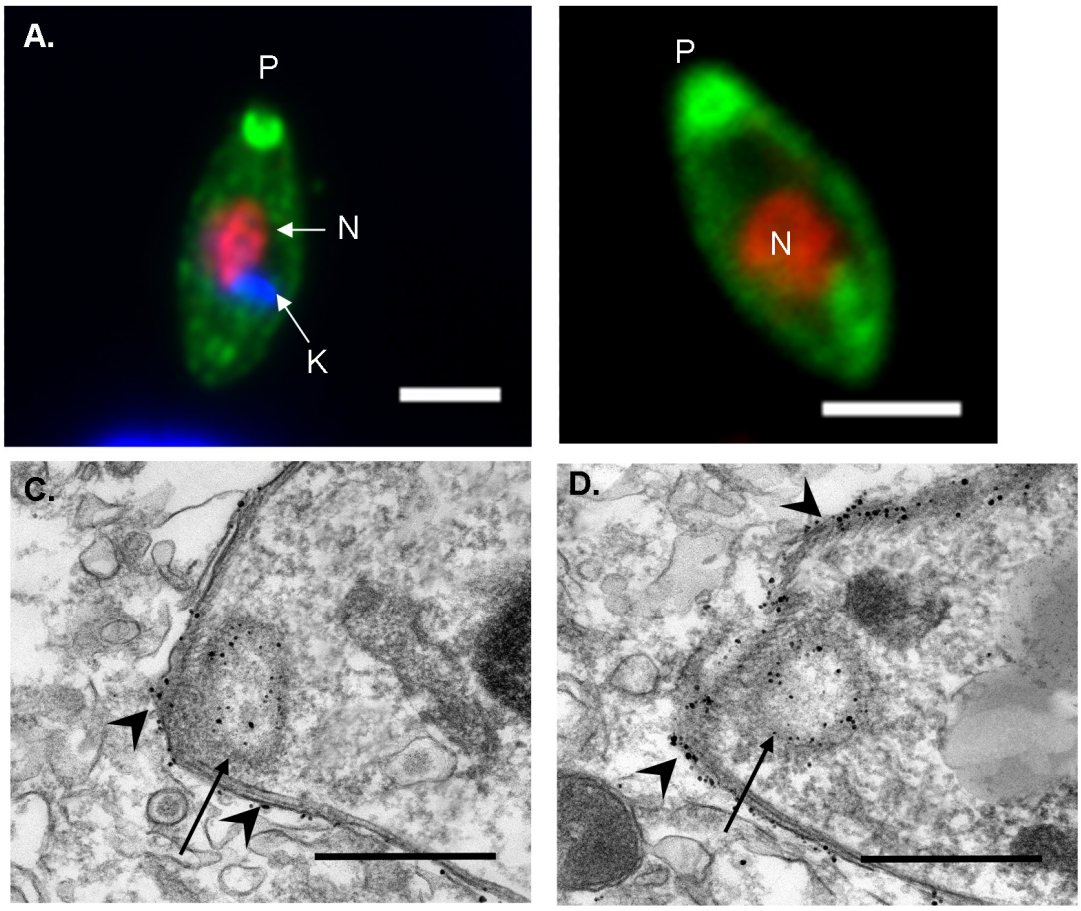

Figure 4