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Antibody Dependent Enhancement of Visceral Leishmaniasis

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ANTIBODY DEPENDENT ENHANCEMENT OF VISCERAL LEISHMANIASIS

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by

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ABSTRACT

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Leishmaniasis is a parasitic disease caused by protozoans of the genus *Leishmania*. This vector-born disease, transmitted by biting phlebotomine sandflies, typically manifests in one of three ways. The cutaneous form of the disease is characterized by localized lesions of the skin and is by far the most common manifestation. The visceral form of the disease is caused by parasitic infiltration of internal organs, particularly the spleen, liver, and bone marrow. The mucocutaneous form is caused by parasitic infection of the mucosa in the nose or mouth. While cutaneous leishmaniasis (CL) is often self-healing, visceral leishmaniasis (VL) is fatal if left untreated. Mucocutaneous leishmaniasis (MCL) can lead to severe facial disfigurement if left untreated but is not particularly deadly. The geographic range of the disease is broad, encompassing areas in Asia, Africa, South America, and the warmer regions of Europe and North America. It is estimated nearly 3 million people are infected by any manifestation of the disease each year and roughly a billion people live in endemic areas. Treatments for the disease are available, though demanding treatment regimens and high costs can be prohibitive for many. No vaccine is currently available, but previous exposure generally confers immunity against future exposures to the same

species of *Leishmania.* The antiquated practice of leishmanization takes advantage of this. The process involves the administration of exudate from active CL lesions to an open wound on an individual to be immunized. It is believed this practice is still used in some areas.

Given the fact parasites known to cause CL have considerable geographic overlap with those that cause VL it is possible for people in endemic areas to have exposures to both in their lifetime. This calls into question the disease outcomes for individuals that experience heterologous secondary exposure after recovering from earlier infection. Studies have addressed this question by using *L. major* as a primary infection followed by *L. infantum* challenge in the murine model. Results are contentious, with reports of heterologous protection against *L. infantum* and others reporting disease exacerbation. Observations of increased disease severity hint at the possibility of antibody dependent enhancement of infection (ADE).

Herein we explore the possibility of extrinsic ADE as a result of heterologous exposure to *L. major* and *L. infantum in vitro*. ELISA was done to verify cross reactivity of *L. major* antiserum to antigen derived from both *L. major* and *L. infantum*. Results indicate substantial cross reactivity to the two antigens. This was followed with phagocytosis assays to measure parasite uptake by macrophages *in vitro*. J774 macrophages were combined with either *L. major* or *L. infantum* in the presence of *L. major* antiserum, naïve serum, or no serum. Parasite uptake was measured at several time points using flow cytometry. In the presence of *L. major* antiserum we observed significantly higher rates of *L. major* uptake but no effect on *L. infantum* uptake. Our results would suggest increased disease severity observed by others *in vivo* is not a result

of extrinsic ADE. We hypothesize biased memory T cell responses, possibly as a result of intrinsic ADE caused by Fcγ signaling, could account for disease exacerbation in the animal model.

ACKNOWLEDGMENTS

I am, and will continue to be, so grateful for the opportunities afforded to me by this program, the Biology Department, and most importantly the people who run it. I must thank my cohort for their comradery and support. I will always look back fondly on the memories we made and I am certain the friendships we share will last for years to come. I also owe thanks to biology support staff who assisted me throughout this process. I am very appreciative of the friendly help offered by Kari Linnell in navigating university and department bureaucracy. Jonathan Betz always brightened my day with his unassailable positive attitude and worked hard to help me keep my mice as happy and healthy as possible. Mark Young was absolutely instrumental in helping me complete this project, without him I would have been unable to move forward. He eagerly provided technical and emotional support whenever needed and always served as a sympathetic ear to my research woes.

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Thank you again to all my supporters,

- Alan McNolty

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CHAPTER I

INTRODUCTION

Leishmaniasis is a disease caused by protozoan parasites belonging to the genus *Leishmania*. The genus is comprised of 53 species, around 20 of which are known to infect humans (Akhoundi et al. 2016). The parasite is transmitted by biting phlebotomine sandflies and can be found in the warmer regions of South America, Africa, Europe, and Asia. There are two ways in which *Leishmania* infection will usually manifest; either as cutaneous leishmaniasis (CL) or visceral leishmaniasis (VL). CL is the most common form of infection and is characterized by open, weeping sores. These sores, though grisly in appearance, are often reported to be painless by patients (Borghi et al. 2017). Generally, this type of infection will self-heal in weeks or months (Nadim et al. 1997; Markle & Makhoul 2004). VL causes a more diffuse infection that attacks the internal organs, particularly the spleen and liver. VL is characterized by fever, abdominal swelling, and a dark ashen coloration of the skin. This form of leishmaniasis is usually fatal when left untreated (Alvar et al. 2012).

There are also rarer forms of the disease such as mucocutaneous leishmaniasis (MCL) and post-kala azar dermal leishmaniasis (PKDL). MCL is characterized by parasitization of the mucosal membranes of the nose and mouth. It is most often caused by parasites within the *L. braziliensis* species complex and transmission occurs almost exclusively in South America (reviewed by Torres-Guerrero et al. 2017). PKDL is characterized by a resurgence of disease months or years after recovery from VL. PKDL presents as small papules that can appear all over the body that can sometimes be resolved without treatment (Zijlstra et al. 2003). The manner in which the disease

presents is dependent on the species of *Leishmania* causing the infection as well as host immunophenotype.

The global impact of leishmaniasis is far-reaching. It is estimated that there are up to 3 million new cases each year, leading to as much as 50,000 fatalities annually (Lozano et al. 2012; Mathers, Ezzati & Lopez 2007). The World Health Organization has identified leishmaniasis as a neglected tropical disease in need of further study. Leishmaniasis is also ranked second to malaria as the deadliest parasitic disease in terms of death toll (Mathers, Ezzati & Lopez 2007). The primary risk factor for people living in endemic areas is poverty and the associated living conditions. To be more specific, risk of exposure is higher for people who spend many hours outdoors, people who are not adequately clothed, and people lacking adequate shelter. Increased rates of transmission have also been linked to urbanization, population mobility, environmental change, animal husbandry, and destabilization caused by warfare (Oryan & Akbari 2016).

Effective treatment options for this disease are available, though steep cost and demanding treatment regimens can be prohibitive for those without access to proper medical care. There are also prevention strategies for both individuals and communities. Community prevention generally comes in the form of municipal programs aimed at preventing malaria. Fortuitously, many of the techniques used to control malaria-carrying mosquitoes are also effective against the sandflies that carry *Leishmania* (Reyburn et al. 2000; Claborn 2010; Zofou et al. 2014). Municipal prevention techniques include vector control, environmental management, disease surveillance, and control of animal hosts (Reyburn et al. 2000; Claborn 2010). On the individual level, it is advised that one should use bed nets, dress adequately and use insect repellents if possible

(Claborn 2010). Another, more antiquated method of prevention is leishmanization. This is the practice of collecting exudate from active lesions of those afflicted with CL and administering them to a skin abrasion of another individual to intentionally infect and confer some degree of immunity. This method, though prone to side effects, has been shown to be effective at preventing future cases of CL (Nadim, Javadian & Mohebali 1997; Peters et al. 2009). One issue that has not been addressed is the fact that strains of *Leishmania* known to cause CL and those that cause VL tend to co-occur geographically (Pigott et al 2014). This calls into question the potential outcomes for individuals that have undergone leishmanization or had natural exposure to CL, only to have later exposure to VL.

The present study is certainly not the first to explore the potential for cross protection between *Leishmania* species. Two studies done by a team of Brazilian researchers investigated potential outcomes associated with heterologous exposures to American leishmaniases. They began by producing soluble *Leishmania* antigen (SLA) extracted from *L. amazonensis*, a causative agent of CL. The SLA was used to immunize mice which were later challenged with *L. braziliensis*, a causative agent of MCL. The immunized mice showed increased susceptibility to *L. braziliensis* (Silva et al. 2011). Silva and colleagues noted that the disease exacerbation was associated with high titers of *L. amazonensis* antibody. In a subsequent experiment these researchers replicated their earlier findings and identified a particular antigenic protein present in *L. amazonensis* SLA that is responsible for the deleterious antibody production (Silva et al. 2015).

The topic of potential cross protection through heterologous exposures has also been examined in the context of Old World leishmaniases. There have been a number

studies that examine how previous exposure to *L. donovani* or its constituent antigens can lend protective effects against *L. major* (Mitchell & Handman 1987; Rachamim & Jaffe 1993; Gicheru, Olobo & Anjili 1997). These authors all noted heterologous protection against *L. major*. Indeed, *L. donovani* appears to be a popular subject of interest for its potential to invoke immunity to other species. Other studies have endeavored to find potential cross protection conferred by *L. donovani* against *L. mexicana*, *L. amazonensis*, *L. tropica*, and *L. chagasi* (Dey et al. 2014; Nico et al. 2014; Manson-Bahr 1961; Aguilar-Be et al. 2005). The rationale behind all these studies is solid, though it could be argued they are not necessarily reflective of real-world conditions. Parasites known to cause CL are more geographically widespread than their VL inducing relatives and CL is considerably more prevalent on a global scale (Pigott et al. 2014; Alvar et al. 2012). Additionally, the old practice of leishmanization is likely still used in certain localities and is looked to by some as a viable method of disease prevention (Khamesipour et al. 2005). As recently as the late 1980's Iran implemented mass leishmanization that saw nearly 2 million people undergo inoculation (Nadim et al. 1997). This is all to say that individuals in endemic areas are more likely to have been exposed to/recovered from CL than VL. With this in mind, CL would seem to be a logical starting point when considering potential outcomes of heterologous exposures.

A few inquiries have been structured this way, opting to use exposure to *L. major* as a foundation to study immunological cross reactivity toward VL, particularly *L. infantum*. Though the matter has been investigated by multiple studies, results are discordant. One publication examining the effects of heterologous immunization used *L. major* as an inoculum to confer future protection against *L. chagasi (infantum)* in the

BALB/c model. The authors found no such effect and observed similar parasite burden between naïve animals and those that had been primed with *L. major* (Streit et al. 2001). Another study went down this same path of inquiry, using an initial inoculation of *L. major* followed by challenge with *L. infantum* in the BALB/c model. In this instance, the authors reported an increase in tissue parasite burden as well as detrimental cytokines among the *L. major*-primed group when compared to the naïve control group, indicating disease exacerbation. (Nation, Dondji & Stryker 2012). Another study using passive serum transfer yielded supportive results. Three groups of BALB/c mice were treated with either naïve serum, *L. major* antiserum, or live *L. major*. These groups were then challenged with *L. infantum* and parasitemia was measured. Results indicated substantially higher parasite burdens in recovered individuals and even higher burdens in individuals treated with *L. major* antiserum (Anderson, Dondji & Stryker 2014). The most recent study on the matter followed the same line of inquiry as the others, inoculation with *L. major* followed by challenge with *L. infantum.* However, the C57BL/6 model was used. In this instance the authors noted lower parasite burden in *L. major* recovered animals compared to the naïve control group, suggesting a protective effect (Romano et al. 2015). These contradictory findings speak to complexity of the mechanisms at play and call for further investigation.

The findings of Nation et al. (2012) and those of Anderson et al. (2014) seem to hint at the possibility of antibody dependent enhancement (ADE); a potentiality that is rarely considered in the context of leishmaniasis. ADE is a phenomenon wherein an individual that has had previous exposure to a particular pathogen experiences a more severe infection when later exposed to the same or a closely related pathogen. The

mechanism behind this is dependent on the presence of antibody generated during the first infection. Over time, antibody concentration may fall below levels necessary for neutralization. Alternatively the antibody may be incapable of neutralizing a closely related pathogen. If the pathogen in question targets the cells of immunity the presence of antibody can be counterproductive. When a second infection occurs the non-neutralizing antibody will opsonize the pathogen either directly or by inducing compliment deposition. These opsonized pathogens serve as an attractant for phagocytes which can become infected upon arrival. This process exacerbates the infection by allowing the pathogen more opportunities to take hold while depriving the host of valuable leukocytes. ADE has been implicated in the pathogenesis of other diseases known to attack antigen presenting cells (APCs) such as Dengue virus, yellow fever, Zika virus, and Chikungunya virus (Katzelnick et al. 2017; Gould & Buckley 1989; Bardina et al. 2017; Lum et al. 2018). Given *Leishmania*'s proclivity for APCs, particularly macrophages, it is entirely possible that the presence of non-neutralizing antibodies from a previous exposure could augment rates of infection. It has been demonstrated that the presence of antibody can induce production of detrimental cytokines and exacerbate leishmaniasis (Kima et al. 2000; Anderson & Mosser 2002; Miles et al. 2005; Buxbaum 2008; Deak et al. 2010).

With this in mind, we set out to further investigate the possibility of ADE of *L. infantum* infection after previous exposure to *L. major*. First, *L. major* antiserum cross reactivity to *L. major* and *L. infantum* antigen was demonstrated using ELISA. This was followed up with an *in vitro* experiment to directly measure rates of parasite uptake by host cells. Macrophages of the J774 line were combined with *L. infantum* in the presence

of *L. major* antiserum, control serum, or no serum and prevalence of unbound *Leishmania* was measured at several time points using flow cytometry. Prevalence of infected macrophages were measured at 24 hours. *L. major* antiserum did not induce increased *L. infantum* uptake compared to the controls. Our results would seem to exclude the possibility extrinsic ADE leading to increased parasite entry of host cells. However, intrinsic ADE mediated by Fcγ signaling and associated IL-10 production may still be the cause of increased disease severity in immunized individuals as observed by others.

CHAPTER II

LITERATURE REVIEW

Discovery and Early Work on *Leishmania*

Major William B. Leishman first observed *Leishmania* in 1900 while serving with the British Army near Kolkata, India (Leishman 1903 A). Leishman was investigating an enigmatic disease thought to be an unusual variety of malaria. He referred to the malady as "Dum-dum fever", so named for a locality near Kolkata that produced numerous cases of the unknown disease. While examining the spleen of a recently deceased Irish soldier, Leishman noted large numbers of ovoid bodies within the splenocytes. He commented on two chromatin containing masses, one considerably larger than the other, within these ovate bodies. Later, while working with a mouse experimentally infected with African trypanosomiasis, Leishman observed these same characteristic chromatin masses. These masses were of course the nuclei and kinetoplasts of the parasites. Based on these observations, Major Leishman correctly postulated that the causative agent of "Dum-dum fever" was a trypanosome.

Figure 1: William B. Leishman 1865-1926.
Credit: Wellcome Collection, London

Working concurrently but independently, Captain Charles Donovan made a similar observation to that of Leishman. He too had been examining the spleens of deceased individuals believed to have died of malaria. He noted the same ovate bodies described by Leishman, but believed them

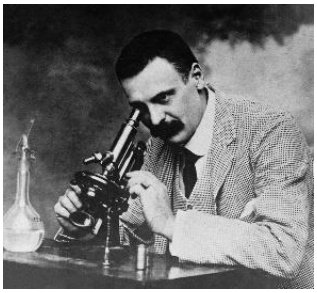

Figure 2: Charles Donovan 1863-1951.
Credit: Wellcome Collection, London

to be an artifact of degraded splenocytes. After reading Leishman's description of the same bodies he was able to see the similarity with his own observations. To confirm, Donovan collected a biopsy from the spleen of a young boy suffering from kala-azar and the same ovate bodies were present (Ross 1903A).

The suggestion that these Leishman-Donovan bodies, as they came to be known, were trypanosomes was initially disputed. Donovan himself doubted they were trypanomastids as did Major Ronald Ross, a correspondent to both Leishman and Donovan. Ross consulted the French physician Alphonse Laveran, a well-regarded expert in microscopic parasites at the time. Laveran suggested that the Leishman-Donovan bodies were a new species, belonging to the order *Piroplasmida* (Ross 1903B). The primary reason for resistance to Leishman's idea that the observed bodies were trypanosomes came from the fact that they lacked flagella; a characteristic believed to be defining of trypanosomes at the time. In a correspondence to Ross, Leishman defended his assertion, stating: ". . . *it is perhaps possible that the life history of some trypanosomata may include a stage of encystment, such a condition known to occur in other members of flagellata* [sic]." (Leishman 1903B, pg. 1377). Leishman's description of "encystment" proved to be prophetic as it seems to allude to the existence of what is now known as the amastigote. Ultimately, Leishman's arguments proved to be compelling. In further correspondences Ross conceded that the Leishman-Donovan bodies could in fact be trypanosomes of a new genus and consequently, proposed the name *Leishmania donovani* for the newly discovered organism (Ross 1903, 1904). *Leishmania*'s trypanomastid

Figure 3: Ronald Ross 1857-1932. Credit: Wellcome Collection, London

classification was further solidified in 1905 when Drs. Chatterjee and Rogers were able to culture *Leishmania* collected from a patient's spleen. After being cultured for three days in a solution of sodium citrate, Chatterjee commented on the presence of "elongated flagellate bodies", making him the first person to view promastigotes (Chatterjee 1905).

It was very soon after this that the nature of cutaneous leishmaniasis was elucidated. The disease then known as "Delhi boil" or "tropical ulcer" had been studied for some time. It is likely that the parasite was first observed in 1884 by Dr. David D. Cunningham. His descriptions of stained and sectioned tissue may indicate the presence of amastigote-like bodies. However, he incorrectly classified the organisms he saw as belonging to "*Monadinae*", a now defunct subclass of slime molds (Cunningham 1885). The American physician John H. Wright made similar observations to those of Cunningham but came to a different conclusion. He declared these small, intracellular bodies to be a new protozoan species which he referred to as "*Helcosoma tropicum*" (Wright 1903).

Despite these earlier misclassifications, German scientist Dr. Max Luhe was able to synthesize the recent findings surrounding the new parasite and formulated a fairly complete understanding of the organism for the time. Luhe commented on Leishman's descriptions of the two chromatin masses within each cell or "cores" as Luhe referred to them. Luhe agreed that the two differently-sized cores are reminiscent of other known trypanosomes. He supported this assertion with Chatterjee and Roger's descriptions of the cultured, flagellated form of the parasite. Luhe was also aware of Wright's new species, "*Helcosoma tropicum*" and stated that it is nearly identical in appearance to organisms described by Leishman. He concluded that the two organisms must be closely

related and proposed renaming Wright's parasite *Leishmania tropica*. He also highlighted key differences between *Leishmania* and known trypanosomes. He noted the lack of an undulating membrane in the organism's motile form as well as the close proximity of the smaller core to the flagellum. In addition to this, Luhe stated that *Leishmania* inhabits marcrophages of the spleen but are never found in "red blood"; an apparent contrast between other trypanosomes commonly observed in peripheral circulation. Based on these dissimilarities, he concluded that *Leishmania* must be a group related to, but distinct from other trypanosomes (Luhe 1906).

Not long after this, *Leishmania* was described in the Americas. The discovery of New World cutaneous leishmaniasis was credited to Italian physician Antonio Carini working with Brazilian researcher, Ulysses de Freitas Paranhos as well as dermatologist Adolpho Lindenberg who worked independently of the former two. The discovery was made during the construction of the Northwest Brazil Railroad when large numbers of workers developed lesions of the skin and mucous membranes (Jogas 2017).

Figure 4: Adolpho Lindenberg 1872-1944. Credit: Wellcome Collection, London

Leishmanial parasites were identified in biopsies and exudates of these so-called "Bauru ulcers" (Lindenburg 1909, Carini & Paranhos 1909). Initially, the causative agent was thought to be *L. tropica* as seen in the Old World, due to morphologically identical parasites and a similar cutaneous manifestation (Carini & Paranhos 1909).

There was, however, dispute over the notion that the infections observed in South America were indeed the same as those in the Old World. In fact, the first objection came from Carini who himself had initially stated the parasites were indistinguishable

from *L. tropica* two years previously. He later commented that mucocutaneous manifestations of the disease were not uncommon in Brazil, a stark contrast to descriptions of Old World CL (Carini 1911).

This notion of dissimilarity between old and New World *Leishmania* was strengthened by a paper published by the Peruvian doctor, Edmundo Escomel. In it he gave the first full description of MCL or what he called espundia. His paper pointed out that mucocutaneous manifestations can appear months or years after a cutaneous episode. He also described a much longer duration for American MCL and CL; sometimes lasting years or never resolving (Escomel 1911). Escomel later sent tissue samples to French scientists Alphonse Laveran and Louis Nattan-Larrier who confirmed that they did contain leishmanial parasites (Laveran & Nattan-Larrier 1912). While they agreed that the parasites did seem to bare subtle differences to *L. tropica*, they were still hesitant to classify the American parasites as a new species. Italian physician, Affonso Splendore, who is also credited with elucidating the nature of MCL, contended that the causative agent of espundia must be a unique species and proposed the name "*L. americana*" (Splendore 1911).

There was considerable back and forth on the issue for some time. Laveran and Nattan-Larrier suggested the causative agent of espundia may be unique to *L. tropica* because of subtle differences in morphology and behavior in culture (Laveran & Nattan-Larrier 1912). Brazilian scientist, Gaspar Vianna observed what he described as a filament within the ovate bodies

Figure 5: Gaspar Vianna 1885-1914. Credit: Biblioteca Virtual Carlos Chagas

of American *Leishmania*. He concluded that since the filament had yet to be described in Old World specimens, his American specimens must be a new species (Vianna 1911). Laveran and Nattan-Larrier responded to this with hesitation, but ultimately agreed it was fair to differentiate the Brazilian parasite based on clinical manifestation rather than morphology, in the same manner *L. tropica* and *L. donovani* had previously been classified. This position was met with some resistance from British physician, Patrick Manson. Manson agreed that while differing geographic distributions and clinical manifestations could point to multiple species, he thought it more likely that clinical manifestation was dependent on the parasite's passage through differing intermediate hosts before being deposited in its human host (Manson 1914). This position may seem somewhat unusual and unsupported by evidence, but even so it was given a measure of credibility thanks to Manson's prominent position in the field of tropical disease. The issue was anticlimactically settled in 1914 when Vianna published a paper referring to the causative agent of espundia as *L. braziliensis*; a classification that has endured.

Phylogeny & Prehistoric Origins

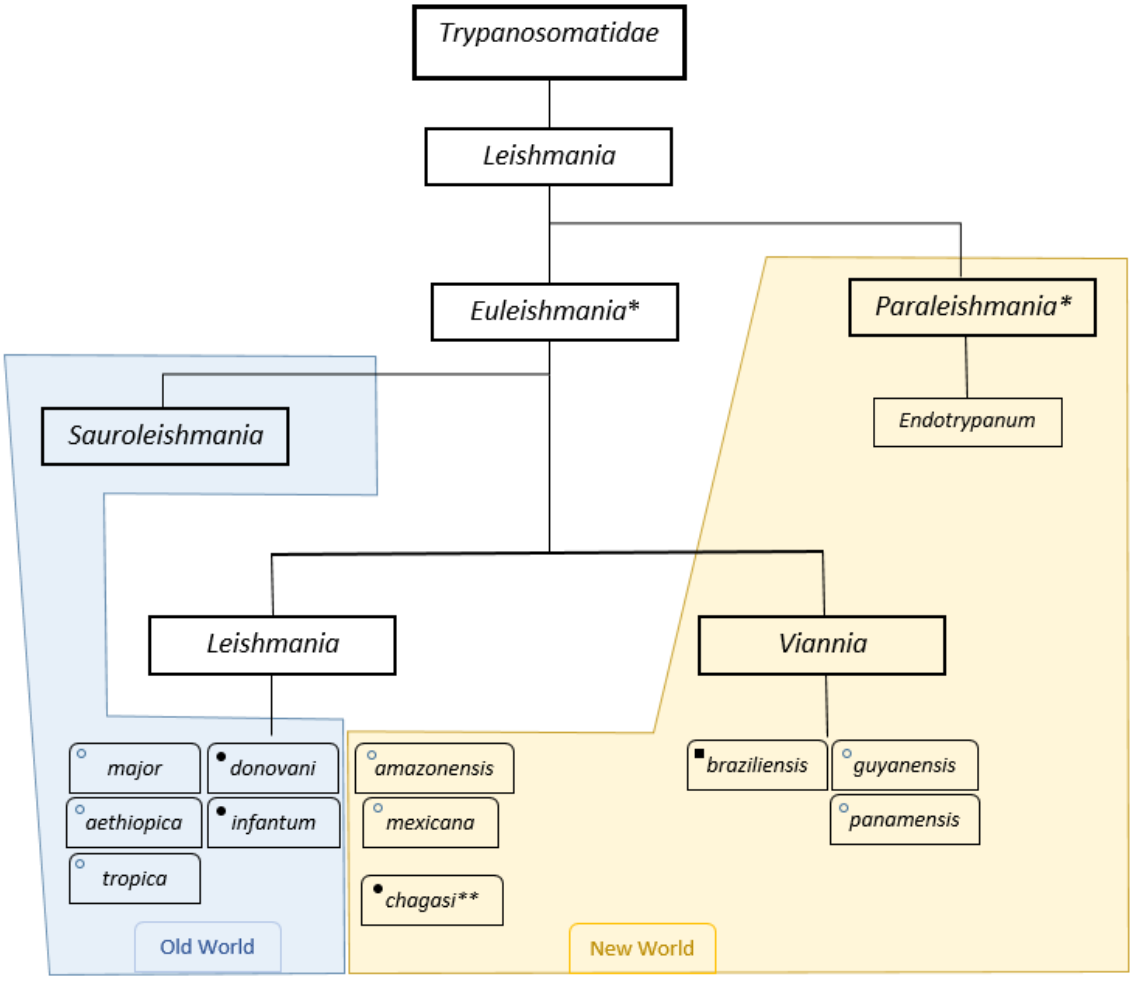

Figure 6: Classification of *Leishmania*. Begins with order Trypanosomatidae followed by genus *Leishmania*, subgenera *Euleishmania* and *Paraleishmania* as proposed by Cupolillo et al. 2000*, subegenera *Leishmania* and *Viannia*, and a non-exhaustive list of species relevant to human health. Blue box indicates Old World species, yellow box indicates New World species. Shapes next to species names indicate disease type: o= CL, •= VL, ■= MCL. (Shaw 1994; reviewed by Akhoundi et al. 2016)
** *L. chagasi* is generally considered synonymous with *L. infantum.*

Leishmania's origins extend deep into prehistory and remain the subject of discourse. The earliest known specimen of an ancestor to *Leishmania* was discovered in an ancient piece of Burmese amber dating to the early Cretaceous, ~110-100 MYA, at the

most recent (Poinar & Poinar 2003). The parasite was found in the digestive tract of the now extinct sandfly species *Palaeomyia burmitis*. The fact that promastigotes, amastigotes, and paramastigotes were observed in the amber sample would suggest that the lineage already had an established dimorphic life history. The ancient parasite was classified as *Paleoleishmania proterus.* DNA analysis has not been carried out due to concerns over destroying the specimen, for this reason it is not immediately clear how *P. proterus* is related to contemporary *Leishmania*.

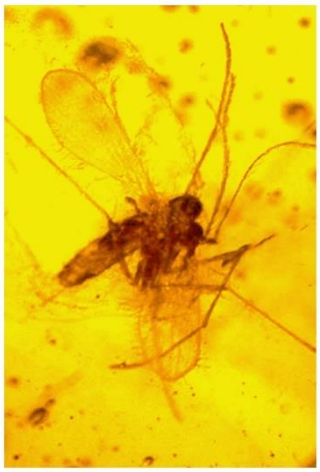

Figure 7: Early Cretaceous Myanmar amber sandfly, *Palaeomyia burmitis.* Credit: George Poinar Jr. (2014)

The earliest fossil evidence of phlebotomine sandflies is congruent with the aforementioned amber-encased *Leishmania* specimens. The oldest specimens that can be confidently classified as phlebotomine were found in Baltic and Lebanese ambers dating to the early Cretaceous, 110-150 MYA (Hennnig 1972; Azar et al. 1999). The appearance of phlebotomine ancestors in the fossil record also corresponds nicely to the proliferation of early mammals roughly 170 MYA (Rowe 2017). Additionally, these timeframes are supported by molecular evidence. Operator metric analysis of 9S and 12S mitochondrial rRNA can provide rough estimates as to the timeframe over which sequence changes occur. Once such analysis including *L. tarentolae, T. cruzi, T. brucei*, and *Leptomonas sp*. Indicated that *Leishmania* likely diverged from *Trypanosoma* 110 ± 30 MYA (Lake et al. 1988).

Figure 8: Procyclic promastigotes of *Paleolishmania proterus* developing in the midgut of sandfly shown in figure 7. Credit: George Poinar Jr. (2014)

Work done using nuclear rRNA yielded supportive results. Analysis of nuclear rRNA using *L. major, L. donovani, Endotrypanum sp., B. caudatus, T. cruzi, T. brucei, Phytomonas sp., E. gracilis,* and *Leptomonas sp.* was done to elucidate relationships between the genera and fossil evidence was used to calibrate divergence timeframes (Fernandes, Nelson & Beverley 1993). These results indicate *Leishmania* and *Endotrypanum* diverged at least 90 MYA. Collectively, fossil and molecular evidence strongly point to the emergence of *Leishmania* sometime in the mid to late Cretaceous. However, the geographical origin of the parasite and how it spread across the globe is still a matter of debate.

The earliest hypothesis as to the geographic origin of the parasite suggests that it evolved in the Palearctic region (Lysenko 1971; Kerr 2000; Kerr, Merkelz & Mackinnon 2000). Current understanding of the fossil record indicates that murine rodents and ancestors of phelobotomine sandflies also evolved in the Palearctic, supporting the notion that *Leishmania* may have emerged there also (Norwalk 1999; reviewed by Lewis 1982). It has been hypothesized that burrows of these early rodents were sufficiently warm and humid to be conducive of sandfly development (Kerr 2000). This would have allowed ample opportunity for a parasite to potentially jump from one species to the other, given their close proximity to one another. The Palearctic hypothesis goes on to suggest that the parasites and their hosts eventually crossed the Bering land bridge into the Nearctic 56-34 MYA (Hopkins 1959). After the land bridge submerged, the American sandflies and their corresponding parasites became isolated in the Nearctic some 34-23 MYA (Kerr 2000). The ancestors of American sandflies and Leishmania we then able to make their way into the Neotropics when the Isthmus of Panama formed ~3 MYA, joining the North

and South American continents. Critics of the Palearctic origin hypothesis have suggested that the greater diversity among American *Leishmania* would point to the genus having originated in the New World (Croan, Morrison & Ellis 1997; Lainson & Shaw 1987). This is not necessarily the case though, as it has been shown that the rate of speciation in a given setting is not constant and is influenced by other factors such as geographic isolation, behavioral isolation, and changes in climate or geology (Sepkoski 1998). The Palearctic hypothesis does become somewhat confounding when trying to explain why New World species belonging to the subgenus *Leishmania* are more closely related to their Old World counterparts rather than the exclusively American subgenus *Viannia*.

The Palearctic hypothesis as championed by Lysenko and Kerr was later rebutted by the Neotropical hypothesis as put forth by Noyes. According to this hypothesis, *Leishmania* diverged from *Endotrypanum* 53-65 MYA (Noyes 1998; Noyes et. al 2000). *Endotrypanum* is only known to parasitize sloths of the New World and is transmitted by sandflies of the genus *Lutzomyia* (Christensen & Herrer 1976). Given their ancient relationship, overlapping biogeography, and shared vector it could be inferred that *Leishmania* and *Endotrypanum* have a shared origin in the Neotropic. The hypothesis goes on to state that proto-Leishmanial species were likely monoxenous parasites of early sandflies. As these sandflies developed hematophagy, their parasites may have become accustomed to mammalian blood. For this reason, it is suggested that sloths were the first mammalian host for *Leishmania* as they have lower body temperatures, serve as hosts for both *Leishmania* and *Endotrypanum*, and were prevalent during the mid-Cenozoic (Noyes 1998). The parasite is said to have then adapted to porcupines where the lineage

again split. This gave rise to *L. hertigi*, known to parasitize only New World porcupines, and the progenitor of modern *Leishmania* (Noyes 1998). From here the infected porcupines could have introduced the parasite to the Nearctic, after which it was carried into the Palearctic by an unknown mammal. The Neotropical hypothesis has been criticized by some who point to indications that porcupines were not present in the Nearctic until after the formation of the Isthmus of Panama, 3 MYA (Nowak 1999, Marshall et al. 1982). This is half a million years after the Bering land bridge would have become inundated and glaciated, meaning *Leishmania* and its associated hosts would have been unable to carry it into the Palearctic. There is also debate as whether or not *Lutzomyia* was present in the Neotropic at the specified time. The earliest known *Lutzomyia* specimen, which also was found to carrying *Paleoleishmania*, came from a piece of Dominican amber dated to 20-30 MYA (Poinar 2008). As previously mentioned, the earliest Old World specimens are in excess of 100 million years old (Poinar 2004). Thus, the fossil record would also seem to refute the Neotropical hypothesis.

The supercontinent hypothesis is the most recent hypothesis as to the geographic origin of *Leishmania* and would seem to carry the strongest support at this time. According to this hypothesis, the progenitors of subgenera *Leishmania* and *Viannia* were originally separated during the break-up of Gondwana 140-120 MYA (Momen & Cupolillo 2000). When the rift that became the Atlantic Ocean formed, the lineage that would become *Viannia* and *Paraleishmania/Endotrypanum* was isolated on the South American continent while the predecessors to the subgenera *Leishmania* and *Sauroleishmania* were left on the African continent. This hypothesis also provides a

plausible explanation for the presence of species representative of subgenus *Leishmania* in the New World. Momen and Cupolillo speculate that, given the similarities between *L. major* and the *L. mexicana* complex, there must have been a second introduction of *Leishmania* into the New World (Lainson & Shaw 1987; Momen & Cupolillo 2000). Proponents of the supercontinent theory propose that an *L. major*-like parasite was carried across the Bering land bridge during the Eocene, some 56-34 MYA (Hopkins 1959). This second introduction can account for the anomalous presence of the *L. mexicana* complex.

In a discussion regarding geographic origins of *Leishmania* it is worth mentioning the peculiarity of *L. chagasi*. A fair number of studies have shown that *L. chagasi* and *L. infantum* are practically indistinguishable as evidenced by comparison of morphology, in vitro behavior, isoenzyme analysis, monoclonal antibody binding assays, and genetic analysis (Braga et al. 1986; Kreutzer, Souraty & Semko 1987; Grimaldi, David & McMahon-Pratt 1987; Beverley, Ismach & McMahon-Pratt 1987; Kuhls et al. 2017). The most well-supported and seemingly logical explanation for the uncanny similarity would be that Old World *L. infantum* was introduced to the New World either by European colonists, African slaves, or imported animals (Momen, Grimaldi & Deane 1987; Mauricio, Stothard & Miles 2000).

Immunology & Course of Infection

Given *Leishmania*'s deep evolutionary history as an endoparasite, it is unsurprising that it has developed an impressive array of armaments and defenses used to subvert and evade host immune responses. Though, the converse is also true. Since

Leishmania's mammalian hosts coevolved alongside it, they too have a number of methods to contend with the parasite. Consequently, the battle between the two organisms is often a close match and the outcome of infection, particularly in humans, can be unpredictable. The infection process is governed by a complex exchange between parasite and host and is unique to the *Leishmania* strain and the host's immunological phenotype. The present review will explore potential interactions between host and parasite throughout the progression of infection.

Manipulation of sandfly vector

Of course the process of infection begins when an infected sandfly stops to take a blood meal from a vertebrate host. In preparation for reaching infective maturity, the parasites move to the anterior midgut of their sandfly host. At this point they are incapable of passing beyond the stomodeal valve, which serves to prevent the sandfly from regurgitating its gut contents. The parasites then secrete lytic enzymes capable of degrading chitin, of which the stomodeal valve is composed (Schlein, Jacobson & Messer 1992). The parasites will also secrete promastigote secretory gel (PSG), a thick proteophosphoglycan-containing substance, into the foregut and mouth parts of the sandfly (Rogers & Bates 2007). The PSG plug inhibits feeding and induces regurgitation. These modifications to the host physiology increase the tenacity with which infected sandflies bite and causes them to regurgitate *Leishmania*-laden PSG into the dermis of the vertebrate host, increasing the probability of establishing infection.

More often than not, the course of infection ends here. In humans, the vast majority of *Leishmania* exposures fail to establish symptomatic infection. This can be evidenced by observations that, of the roughly one billion people living in endemic areas,

the estimated global incidence of VL is 202,000-389,000 and the estimated incidence of CL is 690,000-1.2 million (Alvar et al. 2012). For both diseases combined, this works out to a prevalence of .09-.15% for populations in endemic areas. The low rate of successful infections can likely be attributed to effective host immune response and the relatively small inocula incurred via natural transmission- often less than 1000 promastigotes per bite (Kimblin et al. 2008).

Complement

Upon entry into a mammalian host, *Leishmania* must first avoid destruction by complement. It has been generally accepted that amastigotes and log phase promastigotes are considerably more vulnerable to complement than stationary phase, metacyclic promastigotes (Puentes et al. 1988; Noronha et al. 1998; Dominguez et al. 2002). However, more recent studies indicate that complement takes a heavy toll, even on metacyclic promastigotes. In one study it was shown that, upon exposure to human serum, a given promastigote will be fully complement-bound within one minute and 85-95% will be killed within 2.5 minutes (Dominguez et al. 2002). This general trend was true for *L. major*, *L. amozonensis, L. donovani, and L. infantum*. These findings are congruent with a similar study on *L. infantum* which also showed ~90% of promastigotes were killed within minutes upon exposure to human serum (Moreno et al. 2007). However, promastigotes of the viscerlizing species would seem to be more resistant to destruction by complement than the cutaneous species, a trait that likely enhances their ability to establish systemic infection (Hoover et al. 1984; Dominguez et al. 2002).

It has been generally accepted that the alternative pathway is the most responsive to *Leishmania* and the most likely to be invoked upon exposure (Mosser & Edelson 1984;

Puentes et al. 1988; Wozencraft & Blackwell 1987). This has been demonstrated by observing parasite lysis using C2 and C4 deficient sera as a means of inhibiting the classical and mannose-binding lectin (MBL) pathways to isolate the effects of the alternative pathway (Mosser & Edelson 1984; Dominguez et al. 2002). There is also compelling evidence that the classical pathway plays a significant role in elimination of *Leishmania* from blood serum. It has been shown that *in vitro* exposure to immune serum destroys ~50% more amastigotes in a given time frame than naïve serum (Hoover et al. 1985). It was later shown that IgM, even in non-immune serum, is primarily responsible for enhanced complement activation and destruction of parasites (Navin, Krug & Pearson 1988). Another study concluded that, in normal human serum, the alternative and classical pathways operate in tandem but ~85% of parasite-bound C3 was deposited as a result of activation of the classical pathway by anti-*Leishmania* antibody in non-immune serum (Dominguez et al. 2002). Additionally, it has been shown that MBL will bind *Leishmania* and induce complement activation *in vitro*, though it is not clear if the MBL pathway plays a major role in parasite clearance *in vivo* (Green et al. 1994; Ambrosio & Messias-Reason 2005).

If an invading promastigote is to have any hope of establishing infection it must first overcome the onslaught of host complement. *Leishmania* carries a number of defenses for protection against complement, some of which are better understood than others. These defense mechanisms are located on the cell surface and include lipophosphoglycan (LPG), the metalloprotease glycoprotein 63 (GP63), and leishmanial protein kinase-1 (LPK-1). It should be noted that LPG is differentially expressed throughout the parasite's life cycle and serves many purposes. Experimentation with

LPG deficient *L. major* indicates significant detriment to the parasite's ability to resist lysis by complement and survive in the phagolysosome (Spath et al. 2003). Metacyclic promastigotes have LPG polymers that are twice as long, on average, as those expressed in amastigotes, possibly enhancing their complement survival (McConville et al. 1992). Parasites which lack functional genes for GP63 have been shown to be more sensitive to lysis by complement when compared to their wild-type counterparts (Joshi et al. 1998). This same study demonstrated that GP63 deficient *L. major* is not crippled in its ability to survive in the sandlfy vector, infect host macrophages, or induce symptomatic infection in BALB/c mice; this would suggest the protein is specialized for complement subversion. Despite being used to deflect the destructive effects of complement, GP63 avidly binds C3 and catalyzes production of iC3b (Russell 1987; Brittingham et al. 1995). Using this ingenious maneuver the parasite is able to avoid aggravating a complement cascade while still benefitting from the opsonizing properties of iC3b to gain entry to phagocytes using CR3. In addition, it would appear that promastigotes have a fail-safe mechanism to prevent the full insertion of a membrane attack complex (MAC) by inducing the spontaneous dissociation of C5b9 (Puentes et al. 1990). It is however still unclear as to how the parasite is able to buck the MAC. Lastly, LPK-1 is capable of phosphorylating C3, C5, and C9 effectively rendering them inactive and preventing invocation of the classical and alternative pathways (Hermoso et al. 1991; Forsberg et al. 1990).

Neutrophils

The first host cell *Leishmania* parasites encounter is most often a neutrophil (van Zandbergen et al. 2004; Carlsen et al. 2013; Salei et al. 2017). Most comprehensive studies into the interaction between the parasite and neutrophils have only been carried out in recent years and understanding of the matter is still being refined. To be sure, the nature of this interaction is complex and would sometimes seem contradictory. For example, of four studies examining the effect of artificial neutrophil depletion prior to infection with *L. major* in the BALB/c model, two showed a protective effect (Tacchini-Cottier et al. 2000; Ribeiro-Gomes et al. 2004) and two showed a detrimental effect (Lima et al. 1998; Chen et al. 2005). Clearly these conflicting reports have value and allude to a much more nuanced mechanism at play.

Neutrophils are drawn to the site of infection immediately and reach their highest concentration between 12 and 24 hours post infection (Sousa et al. 2014). Both promastigotes and amastigotes are readily phagocytosed by neutrophils and are able to avoid destruction in lysosomes (Carlsen et al. 2013; Salei et al. 2017; Mollinedo et al. 2010). The parasite avoids destructive measures by barricading itself in non-lytic compartments that do not readily fuse with antimicrobial granules (Gueirard et al. 2008; Mollinedo et al. 2010). It should be pointed out that *Leishmania* does not always survive within the neutrophil. Gueirard et al. (2008) describe both "spacious" compartments, which are fatal, and "tight" compartments which allow survival. Interestingly, the safe "tight" compartments show markers commonly found on endoplasmic reticula, hinting at a potential mechanism of phagolysosome avoidance. The ability to dodge the

phagolysosome has been shown to be LPG dependent, though the exact mechanism has yet to be elucidated (Gueirard et al. 2008).

Infected neutrophils appear to delay apoptosis as long as days after phagocytosing *Leishmania.* During this time they secrete MIP-1β, a macrophage attractant (Aga et al. 2002; van Zandbergen et al. 2004). There is evidence to suggest that *Leishmania* can use neutrophils as a "Trojan horse" to gain entry to their ultimate target, the macrophage. An elegant study by van Zandbergen et al. (2004) demonstrated that *L. major* taken up by human neutrophils was able to survive but not replicate or revert to the amastigote form. It was demonstrated in the same study that these infected neutrophils were phagocytosed by macrophages in which replicative infection was established *in vitro*. What truly occurs *in vivo* is not yet entirely clear. It would seem that the interplay between parasite, neutrophil, and macrophage is heavily context dependent with regard to parasite species, host immunophenotype, and the nature of exposure.

Macrophage Entry

The nature of *Leishmania*-macrophage interactions has a robust body of literature behind it. When it comes to entering the macrophage, "silent entry" is imperative for parasite survival. This is a task easier said than done. The macrophage carries an array of cell surface receptors designed to detect, phagocytose, and degrade pathogens. For this reason, *Leishmania* has developed means to activate said receptors in just the right manner to allow for entry into the host cell while not provoking formation of a fully activated phagolysosome.

Complement receptors

As previously mentioned, the process often begins with the parasite allowing the deposition of iC3b on its surface. The iC3b serves as a non-provocative opsonin that acts on complement receptor (CR) 3(Mosser & Edelson 1985; Brittingham et al. 1995). In the presence of serum, *Leishmania* has been shown to bind CR1 and CR3 as both metacyclic and log phage promastigotes (Rosenthal et al. 1996; Ueno et al. 2009). The early and late phase promastigotes are capable of binding both receptors and gaining entry, however log phase parasites are drawn deep into the macrophage and degraded while metacyclics enter but remain near the cell membrane and resist degradation (Ueno et al. 2009). It is unclear why CR1 is involved as blocking this receptor would not appear to affect parasite entry. However, blocking CR3 causes a marked decrease in parasite internalization (Rosenthal et al. 1996; Wilson & Pearson 1988).

Toll-like receptors

Toll-like receptors (TLRs) play a pivotal role not only in macrophage infection, but also development of an adaptive response (Faria, Reis & Lima 2012). Inquiry into the role of TLRs with regard to leishmaniasis began by observing the course of infection in C57BL/6 mice lacking MyD88, the downstream signaling protein of all but one TLR. The mice showed greatly impaired resistance to the disease compared to wild type controls, indicating TLRs do have involvement in disease progression (Muraille et al. 2003). Further experimentation indicated that promastigote derived LPG acts as a ligand for TLR2 (Veer et al. 2003). The same study also showed that activation of TLR2 by LPG did induce signal transduction via MyD88 leading to phosphorylation of MAPK. Strangely, activation of TLR2 seemed to initiate opposing processes. Resultant MAPK

activation induced nuclear translocation of NF-κB and gave rise to pro-inflammatory TNF-α but also increased expression of SOCS-1 and SOCS-3 (Veer et al. 2003). It is possible that the opposing pro-inflammatory and anti-inflammatory responses to MAKP phosphorylation will tilt one way or the other, giving rise to the characteristic Th1 and Th2 adaptive responses, and ultimately determining disease outcome.

The works described above focused largely on MyD88 signaling which were most useful in constructing an understanding of TLR involvement in *Leishmania* infection. However, this did not provide a complete picture, as TLR3 uses TRIF as a signaling molecule rather than MyD88 and TLR4 has both MyD88 dependent and independent pathways (Johnson, Li & Pearlman 2008; Laird et al. 2009). To address this, another study examined the effect of knocking out TLRs 2 and 3 as well as the downstream signalers MyD88 and IRAK-1 in IFN-γ-primed macrophages. It was demonstrated that all four proteins are involved in TNF-α secretion and nitric oxide (NO) production, and by extension, parasite clearance (Flandin, Chano & Descoteaux 2006). The authors went on to report that silencing either TLR2 or 3 reduced parasite uptake by macrophages. It was also reported that host cells lacking TLR3 were considerably more susceptible parasite induced death than those that carried the receptor (Flandin, Chano & Descoteaux 2006). Paradoxically, these receptors appear to promote recovery, but are also permissive for initial infection. This would suggest that TLR3 plays a role in parasite detection and clearance, though it is not clear what component of *Leishmania* is detected by the receptor.

Additionally, there is evidence indicating TLR4 is involved in parasite clearing processes. TLR4 knockout C57BL/6 mice show increased parasitemia and recovery time

(Kropf et al. 2004). The glycosphigophospholipid antigen of *L. donovani* appears to act as a ligand for TLR4 as well as *L. mexicana* derived proteoglycolipid complex P8 (KIarmakar et al. 2012; Whitaker et al. 2008). Both these molecules seem to elicit a protective response. Another study demonstrated that neutrophil elastase, an inflammatory protease, can act as a ligand for TLR4 (Ribeiro-Gomes et al. 2007). This suggests a possible *in vivo* mechanism for TLR4 activation via neutrophils. Infection by the sandfly would attract phagocytes and initiate cytokine production. Amid the morass of inflammation, neutrophils can shed their inflammatory elastase, activating TLR4 on macrophages and inducing microbicidal activity (Ribeiro-Gomes et al. 2007).

TLR9 also seems to have involvement in *Leishmania* infection, though current research suggests that it contributes to signaling with dendritic cells (DCs) and development of the adaptive response (Fakher et al. 2009; Weinkopff et al. 2013). The receptor's role during the acute phase of infection, if any, has yet to be elucidated.

Other receptors

Experimentation has demonstrated that the mannose-fuccose receptor (MFR) plays a role in parasite entry. Blocking this receptor reduces parasite uptake by ~40% compared to the control (Wilson & Pearson 1988). Furthermore, data would indicate that CR3 and MFR may have a synergistic effect on parasite uptake (Wilson & Pearson 1988). *Leishmania* has also been shown to engage in apoptotic mimicry. Apoptotic cells illicit a sedated response from macrophages and thus mimicking this would be a logical stratagem for the parasite. Indeed, the parasites utilize exposed phosphatidylserine (PS) residues to masquerade as apoptotic cells to induce their own uptake while minimizing the possibility of a violent response from the macrophage (Balanco et al. 2001;

Wanderley et al. 2006). Exposure to PS promotes production of IL-10 and TGF-β, down regulates TNF-α, IL-1, IL-12, and NO production, all while generally making the macrophage more permissible to quiet infiltration (Voll et al. 1997; Balanco et al. 2001). The manner in which PS is used is dependent upon life stage of the parasite.

Promastigotes employ a fascinating tactic by which two subpopulations exist within a given inoculum: those that carry PS and those that do not. PS^+ promastigotes appear to undergo apoptosis as an altruistic gesture meant to induce a non-inflammatory response from the macrophage, allowing their PS^- siblings to enter and establish residence in the host cell (Wanderley et al. 2009). There is some debate as to whether promastigotes actually contain PS or a similar phospholipid such as phosphatidic acid, phosphatidylethanolamine, phosphatidylglycerol or phosphatidylinositol (Weingärtner et al. 2012). Never the less, the final outcome is the same.

Amastigotes, on the other hand, coat themselves in PS without the need for any actual apoptosis (Balanco et al. 2001; Wanderley et al. 2006). Interestingly, the amount of cell surface PS expressed by parasites seems to be responsive to host immunity. Amastigotes derived from BALB/c mice carry considerably more PS than those derived from C57BL/6 (Wanderley et al. 2006). Furthermore, cytokine alteration shows direct correlation with amount of PS exposure while NO synthesis is inversely correlated with PS exposure (Wanderley et al. 2006).

Survival in the Host Cell

Phagosome interference

If *Leishmania* parasites manage to gain entry into a host marcrophage, they must still contend with the unforgiving conditions they find there. Survival for the parasite depends on avoiding or subverting the acidic pH, reactive oxygen species (ROS), and especially NO found in the phagolysosome (Liew et al. 1990; Carneiro et al. 2016). Promastigotes specialize in bridging insect-mammalian transmission and host cell entry. They are poorly equipped to survive long term within a host macrophage are thus obliged to assume their amastigote form to avoid destruction. To achieve this the parasite must buy time while contained in the relative safety of phagosome before fusion with the lysosome occurs. The multipurpose LPG coat of promastigotes is known to dampen formation of the phagolysosome, possibly by altering the curvature and rigidity of the phagosomal membrane (Desjardins & Descoteaux 1997; Miao et al. 1995). Promastigote derived LPG is used to further ensure survival by interfering with the membrane trafficking protein, synaptotagmin V (Syt V). Syt V recruits proton-ATPase pumps to the phagosome to drive down internal pH. Thus, by interfering with Syt V, the parasite is effectively able to prevent acidification of the phagosome (Vinet et al. 2009). These impediments to phagosome development seek to ensure successful reversion to the amastigote form.

Defense against reactive oxygen and nitrogen species

One of the primary dangers to *Leishmania* shortly after cell entry is ROS (Murray 1982). ROS are produced by NADPH oxidase which is assembled from both cytosolic and membrane proteins (Huang & Kleinberg 1999). Two key proteins, $p47^{phox}$ and

p67^{phox}, necessarily must translocate to the phagosomal membrane in order to create the NADPH complex (Leo et al. 1996). It has been shown that *L. donovani* is capable of stopping translocation of p47^{phox} and p67^{phox} by preventing their phosphorylation by protein kinase C (Dey et al. 2005; Lodge, Diallo & Descoteaux 2006). The latter study indicated that the effect appeared to be dependent on LPG of promastigotes. However, Dey et al. (2005) witnessed a similar reduction in ROS production when amastigotes were used, despite LPG expression being altered and significantly down regulated in amastigotes (Turco & Sacks 1991). This is either evidence of LPG's potency or possibly implies a LPG-independent mechanism of NADPH interference.

Even after the parasite has achieved the amastigote form and established residence in the cell, it must remain diligent to ensure continued survival. Part of this involves the continued effort to blunt production of ROS and NO. Parasites produce ROS neutralizing enzymes generally referred to as peroxidoxins. Two peroxidoxins isolated from *L. chagasi*, LcPxn1 & 2 have been shown to protect against different types of ROS (Barr & Gedamu 2003). LcPxn1 in particular also has the ability to neutralize peroxynitrite, a reactive nitrogen species produced from the combination of superoxide with NO (Barr & Gedamu 2003; reviewed by Pacher, Beckman & Liaudet 2007). The parasite also defends against ROS by actively neutralizing it using super oxide dismutase (Ghosh, Goswami & Adhya 2003). In macrophages NO is enzymatically produced from arginine but the parasite also requires arginine for ornithine synthesis. For these reasons, *Leishmania* actively robs the host cell of available arginine, minimizing the host's ability to muster a NO response while satisfying its own need for the amino acid (Acuña et al. 2017).

Resource sequestration

Leishmania is known to steal more than just arginine from the host cell. The parasite requires exogenous sphingolipids in order to transition from promastigote to amastigote (Zhang et al. 2003). The host sphingolipids are gleaned for this purpose, remodeled, and incorporated into parasite membrane components (Zhang et al. 2005). Sphingolipid theft appears to be purely an act of sabotage for *L. mexicana* which is fully capable of synthesizing its own. This is in contrast to *L. major*, for which the pilfered membrane components are vital (Ali, Harding & Denny 2012).

Iron is the object of a similar skirmish between parasite and host. Iron cations are used by *Leishmania* as a cofactor for protective superoxide dismutase and are necessary for parasite growth (Paramchuk et al. 1997). Ironically, divalent iron ions are also required by macrophages in order to produce the very ROS which the parasites must defend against (reviewed by Bedard & Krause 2007). *Leishmania* is certainly not the only pathogen that benefits from iron and consequently macrophages have evolved iron efflux pumps to deprive their endosomes of divalent iron (Gruenheid et al. 1999). In macrophages these transporters are natural resistance associated macrophage protein (Nramp) 1 & 2. Nramp1 exists in lysosomal membranes while Nramp2 is found in most other endosomes (Gruenheid et al. 1997; Jabado et al. 2002). *Leishmania* employs its own iron sequestration mechanisms including heme transporter LHR1, ferrous transporter LIT1, and ferric to ferrous iron reductase LFR1 (Miguel et al. 2013; Huynh, Sacks & Andrews 2006; Flannery et al. 2011).

Intercellular Signaling Interference

With its immediate security ensured, the parasite must now direct its efforts to evading and sabotaging the adaptive immune response; an endeavor for which *Leishmania* is extraordinarily well-equipped. The parasite employs a number of techniques to avoid drawing the attention of T cell lymphocytes and hinder their response.

MHC Interference

To prevent invocation of an effective adaptive response a logical first step would be to silence any attempts by the host cell to communicate distress signals. To that end, *Leishmania* has several strategies to interfere with MHC II signaling. It begins by actively internalizing the MHC II molecules lining the phagosome in which it resides (Lang et al. 1994; De Souza Leao et al. 1995). The parasite is apparently able to draw the MHC II deep within its cytoplasm where it is degraded by cysteine proteases. Of course the parasite can only access MHC lining the parasitophorous vacuole and so further countermeasures are needed.

Another strategy is to interfere with antigen loading onto MHC II. It is known that macrophages lose their ability to display *Leishmania* specific antigen via MHC II as the parasites transition from promastigotes to amastigotes (Kima et al. 1996; Prina et al. 1996). The impaired antigen presentation is specific to leishmanial antigen but expression of MHC II and use of costimulatory signals are unaffected (Kima et al. 1996). Further research would suggest the process of antigen sequestration is mediated by GP63 (Matheoud et al. 2013). This research showed GP63 was able to cleave SNARE proteins vital to proper phagosome function. The crippled phagosomes were unable to properly

process and transport antigen for loading onto MHC I. It is not clear if this same process is used to subvert MHC II.

The host cell is prepared to counter these attacks on MHC signaling. When the concentration of presentable antigen is very low, antigen presenting cells (APCs) are known to construct low-solubility lipid rafts rich with MHC II. These rafts are meant to stimulate T cells more effectively than would be possible with a more diffuse distribution of MHC (Huby, Dearman & Kimber 1999; Anderson, Hiltbold & Roche 2000). However, *Leishmania* anticipates this countermeasure and acts accordingly. Infected macrophages have been shown to have increased membrane fluidity, resulting in less cohesive MHC rafts (Chakraborty et al. 2005). Affected cells were shown to be considerably less capable of stimulating T cells. Another study suggests a possible mechanism for decreased membrane fluidity. The authors demonstrated LPG is capable of intercalating into host cell membranes and disrupting lipid rafts (Winberg et al. 2009). However, this research focused on lipid rafts associated with phagosomal maturation, thus it is not known if the same process affects MHC rafts.

Alteration of cytokine signaling

The fate of *Leishmania* within the host ultimately depends on the elicited T cell response. T cell responses are driven by cytokine signaling and for this reason the parasite takes active measures to influence a more favorable outcome. It has been known for some time that the parasite is able to subvert IFN-γ signaling to discourage the Th1 response. The method employed is disruption of JAK/STAT signaling to render infected cells less responsive to stimulation by IFN-γ (Nandan and Reiner 1995; Blanchette et al. 1999). Others have shown infected macrophages display impaired secretion of IL-12, a

key Th1 cytokine (Reiner et al. 1994; Weinbeber et al. 1998). The mechanism behind this is not known with certainty. Weinbeber et al. (1998) noted that IL-12 mRNA transcription did not appear to be affected by infection. Expression of NO was also unaffected (Reiner et al. 1994). A more recent study found a link between IL-12 inhibition and CR3 activation but noted that functionality of MAPK and NF-κB signaling as well as transcription factors ETS and IRF were all unaffected (Ricardo-Cartier et al. 2013). Though the parasite's mode of IL-12 interference is not fully understood, it could be linked to preferential induction of IL-10 in infected cells. Two studies done on the cytokine production of infected cells reported preferential production of IL-10 (Chandra & Naik 2008; Meddeb-Garnaoui, Zrelli & Dellagi 2009). In both cases IL-10 production had an inverse correlation with IL-12 secretion. This was attributed to suppression of TLR2 mediated signaling by inhibition of MAPK P^{38} phosphorylation accompanied by increased phosphorylation of ERK1/2 (Chandra & Naik 2008).

Chemokine modulation

There is good evidence to suggest *Leishmania* is capable of altering the localized chemokine environment of lesions. A thorough study conducted by Katzman & Fowell (2008) examined the expression of several chemokines in the dermis of *L. major* infected mice compared to mice receiving ovalbumin (OVA) with adjuvant. In the early stages of infection the authors noted down regulation of most chemokines except CCL7, a potent M2 macrophage attractant (Xuan et al. 2014). A subsequent experiment was done in which mice received *L. major* in one ear and OVA+adjuvant in the other. Later, mice were challenged with OVA+adjuvant. Elevated levels of IL-4 were exhibited in the infected ears as was selective recruitment of Th2 cells (Katzman & Fowell 2008). Other

studies have examined chemokine receptor expression during infection. One inquiry on the matter demonstrated sustained increases in CCR1, CCR7, and CXC4 (Pinheiro et al. 2006). The purpose of this may be to direct the host cell toward chemokine signatures indicative of conditions prosperous for the parasite. Alteration of chemokine receptor density also appears to be species dependent. *L. major* induces greater upregulation of CCR2, CCR3, and CCR5 than *L. donovani*, perhaps owing the localized nature of *L. major* infection (Matte & Olivier 2002). *Leishmania* is also capable of secreting its own chemotactic agents. It was demonstrated that promastigote culture medium contains a chemotactic factor that attracts neutrophils (van Zandbergen et al. 2002). The reason for attracting these neutrophils may be to use them as an intermediate host cell, serving as a staging point prior to infecting macrophages.

It is also worth mentioning the curious case of macrophage migration inhibitory factor (MIF). The nature of this protein is poorly understood despite being expressed by, and acting on, a broad range of cells. It appears to spur the induction of a wide range of proinflammatory cytokines and receptors (reviewed by Rosado & Rodriguez-Sosa 2011). Researchers working to construct the *L. major* genome noted the presence of genes encoding a *Leishmania* derived MIF (LMIF) which somewhat resembles that found in mammals (Ivens et al. 2005). It is strange that *Leishmania* would express this proinflammatory protein, especially given MIF's ability to induce NO production in macrophages (Lan et al. 1997). Early research as to the relevance of host derived MIF reported antiparasitic effects driven by ROS and TNF-α (Jüttner et al. 1998). Another study using MIF deficient mice produced supportive results, showing increased susceptibility to disease (Satoskar et al. 2001). This begs the question as to why *L. major*

would express a protein that seems to contribute to its own demise. This was addressed in a study using LMIF knockout parasites in mice. The authors noted substantially reduced parasitemia in animals harboring knockout parasites, which were twice as vulnerable to macrophage killing as their wild-type counterparts (Holowka et al. 2016). These authors postulated that LMIF depletes protective T cells while others have suggested LMIF serves the parasite by preventing apoptosis of infected macrophages (Kamir et al. 2008). Conflicting reports between outcomes associated with host derived MIF and LMIF highlight the need for further research into differing effector functions of the two proteins.

In the context of leishmaniasis, the presence of M2s is associated with disease exacerbation. It has been demonstrated that BALB/c mice lacking IL-4R exhibited slowed disease progression and improved microbicidal activity when infected by *L. major* (Holscher et al. 2006). In humans, post kala-azar dermal leishmaniasis (PKDL) is associated with a preponderance of M2s in peripheral circulation (Mukhopadhyay et al. 2015). Expression of markers associated with the M2 profile were found to be 13-14 times higher in PKDL patients but, reassuringly, M2 markers returned to base line after treatment with antimony or Miltefosine (Mukhopadhyay et al. 2015). Another study noted that mice can be chemically treated to induce proliferation of M2s which corresponded with increased severity of CL in both the BALB/c and C57BL6 models (Vellozo et al. 2017). Experimentation with genetically attenuated strains of *L. major* have shown considerable polarization to M1 phenotype compared to wild-type parasites (Bhattacharya et al.) These findings imply parasite-mediated polarization of macrophage

phenotype, suggesting yet another mechanism by which *Leishmania* is able to influence host immunity.

Current Perspectives on Adaptive Immunity

The Th1/2 paradigm

No discussion regarding host-*Leishmania* interaction would be complete without mention of the Th1/2 paradigm. By the 1970's it was known that different inbred mouse strains experienced markedly different outcomes with *L. major* infection (Nasseri & Modabber 1979). Though the underlying mechanism behind host specific vulnerability remained elusive. Nearly a decade later a landmark paper by Scott et al. (1988) elucidated the issue. The authors observed that susceptible BALB/c mice could be partially immunized against *L. major* using injections of SLA (soluble *Leishmania* antigen). They hypothesized that the key to protective immunity against *L. major* was dependent on T cell activation. In the hopes of identifying which components in particular were responsible for protective effects, they fractionated the SLA to examine the constituents. These fractions were assayed to measure their stimulatory effects on T cells. Two fractions activated T cells but only one was protective. From here they were able to identify the cytokines associated with protective and non-protective states of T cell activation. While the Th1/2 designations had been established at the time, substantially less was understood about their roles in disease. They observed that IL-2 and IFN-γ were associated with Th1 activation and disease healing, while IL-4 and IL-5 were associated with Th2 activation and disease progression. Other researchers would go

on to produce supportive results, further cementing the protective role of IFN-γ and the detrimental role of Th2 cytokines (Heinzel et al. 1989; Castellano et al. 2009; Kopf et al. 1996). The observations made by Scott and colleagues not only helped establish a foundational understanding of immunity to *Leishmania* but also shaped the general understanding of adaptive immunity.

At first glance this is a very straightforward model for understanding outcomes of leishmaniasis. Unfortunately, the reality of the matter is not so tidy. The Th1/2 dichotomy as summarized above largely applies to only *L. major* infection in the BALB/c strain. The general understanding of disease outcomes has evolved and become incredibly nuanced. Other reviewers have noted that disease outcomes are heavily context dependent and are influenced by species of parasite, host phenotype, mode of infection, and organs/body site sampled (reviewed by Mears et al. 2015; Loria-Cervera & Andrade-Navaez 2014; Loeuillet, Bañuls & Hide 2016; Alexander & Brombacher 2012). Though the daunting minutia of study-specific results may seem confounding, there is value in identifying overarching trends.

One such trend is the universally beneficial effects of IFN-γ (Belosevic et al. 1989; Harms et al. 1989; Robersts 2005; Castellano et al. 2009; Nylén & Gautam 2010; Alexander & Brombacher 2012; Kumar et al. 2014; Solano-Gallego et al. 2016). The anti-leishmanial property of IFN-γ can be attributed to its ability to induce NO production in macrophages (Ding, Nathan & Stuehr 1988; Tötemeyer et al. 2006). IL-12 is also considered a protective cytokine, owing to its ability to induce expression of IFN-γ (Munder et al. 1998; Darwich et al. 2009). IFN-γ also stimulates production of IL-12, allowing the two cytokines to form a positive feedback loop with each other (Ma et al.

1996).

TNF-α is also widely regarded as protective against CL (Liew et al. 1990; Kemp et al. 1999; Wilhelm et al. 2001; Tripathi, Singh & Naik 2007; Fromm et al. 2016). As with IFN-γ, TNF-α is associated with elevated NO production in macrophages, leading to greater parasite clearance (Lin et al. 1994; Fonseca et al. 2003). However the role of TNF-α is somewhat more ambiguous in the context of VL. Though it has been associated with clearance of VL (Tumang et al. 1994; Murray et al. 2000), deprivation of TNF-α has also been shown to have no effect on parasite burden *ex vivo* (Singh et al. 2016). Other researchers have correlated increased splenic tissue damage in wild-type mice when compared to TNF-α knockout mice (Engwerda et al. 2002). TNF- α has also been associated with more severe manifestations of American leishmaniases (Castes et al. 1993; Da-Cruz et al. 1996). However these observations were made in a clinical setting with no regard for the causative agent. Conclusions were drawn based on observed decreases in TNF-α after treatment, which could just as well be a return to baseline cytokine expression after recovery.

The characteristic Th2 cytokines such as IL-4, IL-5, and IL-13, are typically considered conducive to parasite persistence but forthcoming research indicates the reality of the matter is more complex. Though IL-4 has been associated with negative outcomes during *L. major* infection (Chatelain, Varkila & Coffman 1992) it does appear to have some role in Th1 development. Evidence would suggest that IL-4 is associated with higher levels of IFN-γ and can induce IL-12 secretion by DCs during initial stages of infection (Biedermann et al. 2001; Gurung & Poudel 2018). Interestingly IL-4Rα, a receptor shared by IL-4 and IL-13, appears to have some role in resistance to *L. major*

(Mohrs et al. 1999). The role of IL-4 in visceral disease is also multifaceted. Studies using IL-4 deficient mice infected by *L. donovani* have produced mixed results. IL-4 has been shown to confer either modest protection or little to no effect on parasite burden (Stäger et al. 2003; Satoskar, Bluethmann & Alexander 1995; Alexander et al. 2000). The latter study also hints at a potential role for IL-4 in recovery from VL. Under treatment with antimonial drugs, mice lacking IL-4 have an impaired ability to resolve infection compared to wild-type animals (Alexander et al. 2000). IL-13 has considerable functional overlap with IL-4 due to the two cytokines having shared structural motifs and receptor homologies (Zurawski et al. 1993; Mueller et al. 2002). For this reason, IL-13 very closely mimics the effects of IL-4 during *Leishmania* infection. As with IL-4, IL-13 is implicated in progression of CL, both Old World and New World (Coêlho et al. 2010; Matthews et al. 2000; Bourreau et al. 2001; Alexander et al. 2002). IL-13 also appears to impart some protection against *L. donovani* (Murray et al. 2006; McFarlane et al. 2011). Publications on the roles of IL-5 are somewhat sparse. Studies have provided indirect evidence for a potentially protective role for IL-5 in *L. amazonensis* and *L. infantum* infection (Watanabe et al. 2004; Mary et al. 1999). However, further study is required before serious conclusions can be drawn with regard to IL-5.

The growing body of knowledge regarding Th1/2 responses to leishmaniasis would indicate that the old presupposition of Th1 being protective and Th2 being detrimental is not entirely accurate. As mentioned above, Th2 cytokines can have protective effects and may be involved in regulating Th1 development. It is the opinion of this reviewer that it is not necessarily the presence of a Th2 response that is

disadvantageous, but rather the absence of a Th1 response that leads to increased disease severity.

Roles for other CD4 T cell subtypes

Since the Th1/2 CD4 cell subtypes were first described by Mosmann et al. (1986), other subsets of T helper cells have been identified. Of particular note are Th17 and T_{reg} cells. Th17 cells, first described by Harrington et al. (2005), differentiate upon activation by IL-6 and TGF-β or IL-21 (Mangan et al. 2006; Bettelli et al. 2006; Nurieva et al. 2007). Characteristic Th17 effector cytokines include IL-17, IL-21, and IL-22 (Park et al. 2005; Korn et al. 2007; Chung et al. 2006). Th17 cells are involved in autoimmunity and it has been theorized their evolutionary purpose is to mount fierce inflammatory reactions against extracellular pathogens that cannot be adequately dealt with by Th1 or Th2 cells (reviewed by Korn et al. 2009). Due to this destructive capability, the Th17 response is often associated with tissue damage and this would seem to be the case in CL. The presence of Th17 cells and their constituent cytokines has been implicated in increased severity of infection caused by *L. major*, *L. mexicana*, and *L. guyanensis* (Kostka et al. 2009; Anderson et al. 2009; Pedraza-Zamora et al. 2017; Hartley et al. 2016). Th17 cells appear to have a generally protective effect against VL. A number of publications have reported protective effects against *L. donovani* and *L. infantum* (Pitta et al. 2009; Ghosh et al. 2012; Nascimento et al. 2015; Quirino et al. 2016). This could be explained, at least in part, by increased neutrophil recruitment to affected organs. IL-17 has been linked to increased expression of CXCL1, a neutrophil chemoattractant (Liang et al. 2007). A more recent study was able to directly link disease resistance to neutrophil infiltration mediated by IL-17 (Quirino et al. 2016).

Historically, IL-6 has been considered a Th2 cytokine and has been studied as such. Though IL-6 has since been reclassified as a Th17-promoting cytokine the body of work behind it is valuable. Observational clinical studies on VL have associated IL-6 with symptomatic leishmaniasis and more severe forms of disease (de Lima, Peiro & Vasconcelos 2007; dos Santos et al. 2016; Ansari, Ramesh & Salotra 2006). However, these studies are correlational and cannot show causation between IL-6 and disease progression. Similar clinical studies on American leishmaniasis show a link between active disease and IL-6 production, but again it is unclear if IL-6 is effectual in disease progression (de Lima, Peiro & Vasconcelos 2007; Gomes et al. 2014; Espir et al. 2014). This is in contrast to experimental studies which, with some exception (Murray 2008), suggest a mildly protective role against Old World CL and VL (Titus et al. 2001; Stäger et al. 2006; Kling et al. 2011).

T_{reg} cells, as the name would imply, are functionally characterized by their role in down-regulating inflammatory responses via secretion of IL-10 (Rubtsov et al. 2008; Chaudhry et al. 2011). One might expect that reduced inflammation may be beneficial, at least during CL infection, but this does not seem to be the case. T_{reg} cells and IL-10 are associated with parasite persistence in infection by *L. major*, *L. donovani*, and *L. guyanensis* (Belkaid et al. 2002; Kane & Mosser 2001; Murphy et al. 2001; Singh et al. 2012; Rai et al. 2012; Bourreau et al. 2009). However, experimental depletion of T_{reg} cells would indicate that they do play a role in preventing pathology caused by inflammatory damage resulting from *L. panamensis* infection (Ji et al. 2005). A similar outcome was observed after using adoptive transfer of T_{reg} cells specifically immunized to *L. amazonensis* (Ji et al. 2005). T_{reg} cells and IL-10 are also thought to be responsible

for non-sterile cure observed in recovered subjects (Belkaid et al. 2002; Mendez et al. 2004; Falcão et al. 2014). Prevention of full parasite clearance may initially seem undesirable, however these same studies demonstrated that the minute parasite persistence afforded by T_{reg} cells and IL-10 helps confer lasting immunity to future exposures.

CD8 T cells

The role of CD8 cells in leishmaniasis could be described as "a double edged sword". Conventional wisdom would suggest that CD8s are beneficial, given their cytotoxic ability, but this does not seem to be the case. Heavily cytolytic CD8s, as measured by granzyme expression, have been correlated with more severe lesions caused by CL in both humans and mice (Moll et al. 1991; Faria et al. 2009). This correlation was further examined using perforin deficient mice. The absence of perforin did not affect lesion development caused by *L. major* (Conceição-Silva et al. 2006). This effect was further examined using Rag1 knockout mice, which lack both T and B cells. After infection with *L. braziliensis*, Rag1 -/- mice received transfers of wild-type or perforin knockout CD8s. Mice that received perforin-free CD8s exhibited reduced lesion development, suggesting a link between CD8 mediated cytolytic activity and tissue damage caused by CL (Novais et al. 2013). It should however be noted that reduced lesion development in Rag1 knockout mice does not correlate with decreased parasite burden, only reduced pathology (Belkaid et al. 2002; Novais et al. 2013). Disease progression associated with CD8 cells has also been linked to inflammasome activation mediated by IL-1β (Novais et al. 2017)

CD8 cells are not exclusively harmful by any measure. A number of studies have demonstrated beneficial effects associated with the presence of CD8s in both CL and VL (Stern et al. 1988; Müller et al. 1993; Alexander, Kaye & Engwerda 2001; Belkaid et al. 2002; Uzonna, Joyce & Scott 2004; Murray et al. 2015). The protective effect offered by CD8 cells is tied to their ability to induce IFN-γ secretion (Müller et al. 1993; Belkaid et al. 2002; Uzonna, Joyce & Scott 2004; Murray et al. 2015). Uzonna, Joyce & Scott demonstrated this particularly well in their publication (2004). They first demonstrated the adoption of a Th2 response in separate groups of knockout mice that had been deprived of either CD8 cells or β-2 microglobulin, a component of MHCI. Infected CD8 knockout mice were then given either CD8 cells from wild-type mice or IFN-γ knockout mice. Those that received wild-type, IFN-γ competent CD8s exhibited recovery; while the group that received IFN-γ deficient CD8s showed lesion development and parasitemia comparable to the CD8 knockout group. Cytotoxic T cells are also believed to contribute to lasting immunity after immunization or recovery from leishmaniasis. CD8s mediate immunity to secondary exposure by mounting a memory response characterized by heavy secretion of IFN-γ (Müller et al. 1993; Rhee et al. 2002).

B cells

Current literature regarding the role of B cells in leishmaniasis suggests that these lymphocytes are inconsequential at best. One study used mice lacking competent B cells to investigate what outcome this may have on *L. donovani* infection. The knockout mice exhibited reduced parasite burdens compared to the controls, but at the cost of somewhat greater liver pathology (Smelt et al. 2000). This same study linked this liver damage to neutrophil activity independent of T cells. They also observed no difference in

antiparasitic capability of CD4 cells from B cell deficient mice and wild-type. These results would suggest that the increased disease severity associated with presence of B cells is unrelated to CD4 activity. Another research group has also reported negative outcomes of *L. donovani* infection in presence of intact B cells. They observed B cell activation mediated by endosomal TLRs is responsible for a weakened Th1 response and hypergammaglobulinemia (Silva-Barrios et al. 2016). The authors go on to link B cell TLR action with subsequent secretion of IL-10 and type-I IFN. They suggest that stimulation by these cytokines upregulates TLR expression, creating a positive feedback loop. Studies on American CL with regard to B cells have produced inconclusive results. One study using *L. panamensis* reported smaller lesions in B cell deficient animals but also feeble IFN-γ production compared to the control (Wanasen, Xin & Soong 2008). Another group described an interplay between human derived CD4 cells and B cells in the presence of *L. panamensis* antigen that leads to substantial upregulation of some eleven cytokines (Rodriguez-Pinto, Saravia & McMahon-Pratt 2014). What influence this may have on disease is not known.

Macrophage subtype

Macrophages are often associated with innate immunity but they do play a part in shaping the adaptive response and are heavily influenced effectors of adaptive immunity. Studies examining the effect of macrophage subtype on *Leishmania* infection are scarce but the concept is worth discussing. It was first observed in 1962 that intracellular infection by *Listeria* could induce lasting changes in the phenotype and microbicidial activity in macrophages independent of serum immunity (Mackaness 1962). It was noted that peritoneal macrophages from recovered mice were markedly more resistant to death

and had enhanced pathogen-killing capability. More interesting still, the immune macrophages retained their abilities even after sterile cure, though resistance began to diminish after three weeks. The resistance did not disappear entirely, however. Mackaness postulated that this depends "…*upon an ability to generate a new population of resistant cells from a residuum of specifically sensitized macrophages or macrophage precursors still surviving in the tissues*…" (Mackaness 1962, pg. 405).

Later, these differing macrophage profiles were tied to the Th1/2 paradigm of acquired immunity and in 2000 the designations of M1/2 were coined (Mills et al. 2000). It has been pointed out that the M1/2 classification has some inconsistencies and limitations (Murray et al. 2014; Martinez & Gordon 2014). Without an improved systematic approach to macrophage activation the M1/2 paradigm will have to do for now. M1 macrophages are associated with type 1 inflammation, tumor resistance, and killing of intracellular pathogens while M2s can be immune regulatory, involved with wound healing, type 2 inflammation, and extracellular parasite killing (Mantovani et al. 2004). Generally speaking, M1 macrophages correspond to the Th1 cytokines TNF-α, IFN-γ, and/or bacterial lipopolysaccharide. M2 macrophages on the other hand are activated by the Th2 cytokines IL-4, IL-13, IL-1R ligands, and IL-10 (Mantovani et al. 2004). Ideally, a host's physiology will be able to balance the competing processes throughout the course of infection in order to clear offending pathogens while minimizing self-harm.

Summary

The genus *Leishmania* was initially discovered and identified at the turn of the 20th century by British medical officers working in India. Much earlier references to

leishmaniasis exist, but it was the work of William B. Leishman, Charles Donovan, and Ronald Ross that was able to link kala-azar to this particular parasite. They were also the first to determine that the parasites were trypanomastids. Later, Max Luhe was able to classify the causative agents of both CL and VL as being *Leishmania*. The parasites where then discovered in the Americas and described by Antonio Carini, Ulysses de Freitas Paranhos, and Adolpho Lindenberg. American *Leishmania* was determined to be distinct from Old World *Leishmania* by Edmundo Escomel and Gaspar Vianna.

The genus *Leishmania* is subdivided into multiple subgenera. The two that are relevant to human health are subgenera *Leishmania* and *Viannia*. The subgenous *Viannia* exists exclusively in the New World while subgenous *Leishmania* is represented in both the Old World and New World. This raises questions as to the geographic origin of the parasite and how it came to be distributed across the globe. A number of theories have been put forth but the hypothesis that carries the most support at this time suggests that the ancestors of modern *Leishmania* evolved on the supercontinent of Gondwana. Subgernera *Leishmania* and *Viannia* were isolated from one another as the Atlantic Ocean widened. It is believed that subgenous *Leishmania* was introduced to the New World via the Baring land bridge during the Eocene. This introduction is hypothesized to have given rise to members of the *L. mexicana* species complex. It also believed that *L. infantum* was introduced to the New World very recently, during colonization by Europeans.

Leishmaniasis is a highly nuanced disease, the outcome of which depends on multiple factors including parasite species, host immunophenotype, and host immune status. The interplay between host immunity and parasite virulence factors is complex

and our understanding of it is continually being refined. During initial exposure, *Leishmania* uses a variety of methods to establish infection and survive innate immunity. The process begins within the sandfly vector, wherein the parasite alters host physiology to encourage more tenacious feeding and increase chances of successful transmission. The parasite then works to survive an innate response by preferentially allowing opsonization by iC3b, prevention of MAC formation, and neutrophil manipulation. *Leishmania* must then gain silent entry to macrophages through selective activation of particular compliment receptors, TLRs, MFR, and apoptotic mimicry. It avoids destruction in the phagosome by preventing fusion with the lysosome, impeding production of ROS and NO, and scavenging resources for survival.

Infection is maintained by subversion of the adaptive immune response. CD4 T cell activation is critical for parasite clearance. A Th1 response, dominated by IFN-γ production is beneficial and conducive of cure. The Th2 response, historically thought to be detrimental, can actually confer protective effects under certain circumstances. It is a lack of Th1 activity that leads to injurious effects, rather than the presence of Th2 activity. Contemporary understanding of leishmaniasis includes roles for other CD4 T cells, particularly Th17 and T_{reg}. Th17 cells are associated with tissue damage during CL, likely owing to their powerful inflammatory abilities. On the other hand, Th17 cells seem to protect against dissemination of VL. T_{reg} cells are associated with parasite persistence but also play a role in limiting inflammatory tissue damage. T_{reg} cells are also partially responsible for preventing sterile cure, which allows for lasting immunity to future exposures. CD8 T cells cause tissue damage resulting from their cytolytic activities but also aid in recovery by spurring IFN-γ production. B cells seem to play a

minimal role in leishmaniasis, and can be considered to have mildly negative to neutral effects on disease progression.

Despite having been discovered and identified well over a century ago, *Leishmania* still perplexes modern researchers. From its ambiguous evolutionary origins and world-wide dispersal, to its complex interactions with host immunity, there is still much to learn about this unique and prolific parasite.

CHAPTER III

METHODS

Animals

BALB/c mice were originally procured from Simonsen Laboratories (Gilroy, CA) and propagated in the CWU vivarium. They were housed under controlled light and temperature conditions with food and water provided *ad libitum*. All procedures were carried out in compliance with IACUC approved protocols #A01170.

Cells

J774 murine macrophage line (ATCC TIB-67™) was cultured in high glucose DMEM with sodium pyruvate (GE Healthcare Bio-Sciences, USA) fortified with 6mM L-glutamine and 10% heat-inactivated FCS (Gemini, Sacremento, CA). Cells were incubated at 37^{o}C in a humidified atmosphere with 5% CO_2.

Leishmania parasites were cultured in Schneider's complete insect medium (Sigma-Aldrich, USA) with 15% heat-inactivated FCS and incubated at 24^{o}C. Prior to culture, parasites were kept in liquid nitrogen and virulence was maintained by passage through BALB/c mice. Both *L. major*, strain MHOM/IL/79/LRC-L251 and *L. infantum*, MHOM/ES/92/LLM-320 were originally provided by Dr. Diane McMahon-Pratt (Yale School of Medicine, New Haven, CT).

ELISA

Soluble *Leishmania* antigen (SLA) was prepared by washing parasites and resuspending in PBS. The suspension was then frozen overnight at -18^{o}C to lyse cells. The solution was thawed and homogenized using sonication (Misonix, Farmingdale, NY)

at setting 12 for 3-15 second intervals. SLA was adjusted to a concentration of 1mg/ml and 100µl per well was added to a 96-well plate. The antigen was allowed to set overnight at 4°C. The plate was then washed five times with PBS+.05% Tween20 (PBS-T). Blocking buffer was made using PBS-T+1% milk and 200ul was added to each well and allowed to set for two hours on the benchtop. The plate was again washed and 100ul serum solution was added to each well and allowed to set overnight at 4°C. Serum from three control animals and three infected animals was diluted 1:50 and 1:100 in blocking buffer and each dilution was measured in duplicate. The plate was then washed and 100µl secondary antibody solution was added to each well. Goat anti-mouse IgG conjugated with horse radish peroxidase (HRP) was used as secondary antibody and diluted 1:3000 in blocking buffer, according to manufacturer's instructions (Bio-rad, Hercules, CA). After a two hour incubation at room temperature, plates were washed again and 100µl HRP substrate solution (Thermo-Fisher, Rockfield, IL) was added to each well. Absorbance at 405nm was then measured every five minutes for 30 minutes using a BioTek plate reader (Winooski, VT). A two-tailed Student's T test assuming unequal variances was used to compare serum affinity for *L. major* and *L. infantum* SLA.

Fluorescent microscopy

To demonstrate progressive infection of cells, fluorescent staining was used for photomicroscopy. Cells were harvested, enumerated, reacted, fixed, and stained as described for flow cytometry. In addition to PI being used to stain nuclei and kinetoplasts, membranes were counter stained using fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA), 1µg/ml (Vector Labs, Burlingame, CA). Stains were allowed to set for 30 minutes in darkness and at room temperature. Photos

were taken using a Leica DMRB microscope with Leica application suite software. (Wetzlar, Germany).

Phagocytosis assays

Prior to *in vitro* experimentation, serum used to treat cells was produced and harvested as follows: *L. major* was cultured until determined by visual inspection that metacyclic promastigotes predominated the medium. Parasites were removed from the medium via centrifugation at 1000 rpm for ten minutes. Metacyclic promastigotes were then isolated using a Percoll (Sigma-Aldrich, St. Louis, MO) density gradient as previously described (Ahmed et al. 2003). Promastigotes were then suspended in sterile PBS to a concentration of 10^6 cells/ml. A 10µl dose, delivering 10^4 promastigotes was injected into the left rear paw of BALB/c mice. The infection was allowed to progress for twelve months prior to serum collection. Control serum was collected from age/sex matched animals.

J774s and *Leishmania* were harvested and enumerated using a hemocytometer. Treatments of either no serum, naïve serum, or *L. major* antiserum were administered to *Leishmania* using 1µl serum to 1ml cell culture. Serum was added directly to culture medium and allowed to incubate for 30 minutes. Concentration of J774s was adjusted to 1.25×10^5 and reacted with *Leishmania* at a 1:10 ratio in cDMEM. Reacting cells were incubated at 37^{o}C in a humidified atmosphere with 5% CO_2. Samples were collected in triplicate at 0, 12, 18, and 24 hours. Once collected, samples were incrementally fixed to minimize clumping, first with 35% EtOH and then in 70% EtOH.

Prior to analysis, samples were resuspended in PBS and adjusted to a concentration of 5×10^5 cells per milliliter. Cells were stained using propidium iodide

(PI), 1µg/ml. Flow cytometry was carried out using an S3 cell sorter (Bio-Rad, Hercules, CA). A multiparametric protocol was devised to quantify unbound parasites via forward scatter, side scatter, and PI fluorescence. Data on 3 X 10^4 events were collected from each sample. Data were then analyzed using FlowJo software (FlowJo, Ashland, OR). Statistical significance was determined using Graphpad Prism software (La Jolla, CA) to carry out an ANOVA test coupled with Tukey's comparison.

CHAPTER IV

RESULTS

Serum cross reactivity

To verify the cross reactivity of *L. major* antibody against *L. infantum* antigen an ELISA was performed (Figure 9). Wells were coated with both *L. infantum* SLA as well as *L. major SLA* to serve as positive control. Results indicate substantial cross reactivity between the two parasites.

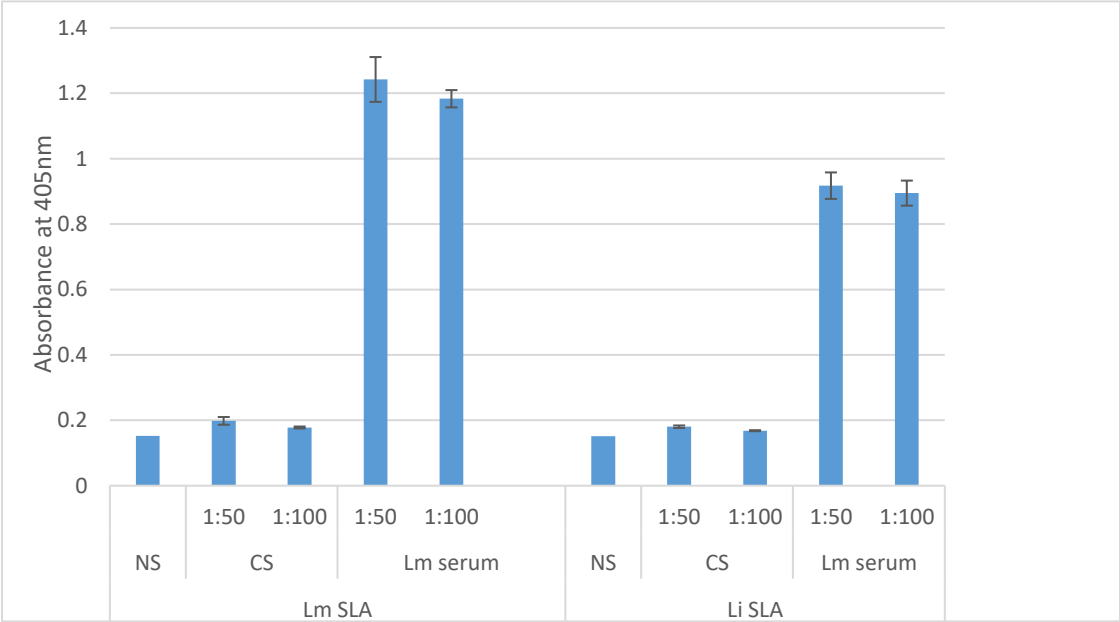

Figure 9: ELISA results measuring affinity of *L. major* antiserum for *L. major* and *L. infantum* soluble *Leishmania* antigen. Each bar represents serum derived from one individual. Sera were measured in duplicate and bars connote the average of measurements. Serum dilutions of 1:50 and 1:100 were used. NS= no serum control, CS= control serum, Lm= *L. major*, Li= *L. infantum*

Phagocytosis assays

In vitro experiments were performed to determine whether *L. major* antiserum will increase rates of parasite uptake by host macrophages. J774 monocytes were combined with *L. major* in the presence of *L. major* antiserum, naïve serum, or no serum.

Samples were collected at 0, 12, 18, and 24 hours after combining host cells and parasites. Progression of infection was verified using fluorescent microscopy (Figure 10). Data were gathered in histogram format and free parasites were quantified (Figure 11). As expected, parasite uptake in the presence of immunized serum was significantly higher than with control serum or no serum (Figure 12, 14A).

Having established proof of principle, the same experiment was carried out using *L. infantum*. Under these same conditions, no increased parasite uptake was observed in the presence of *L. major* antiserum (Figure 13, 14B).

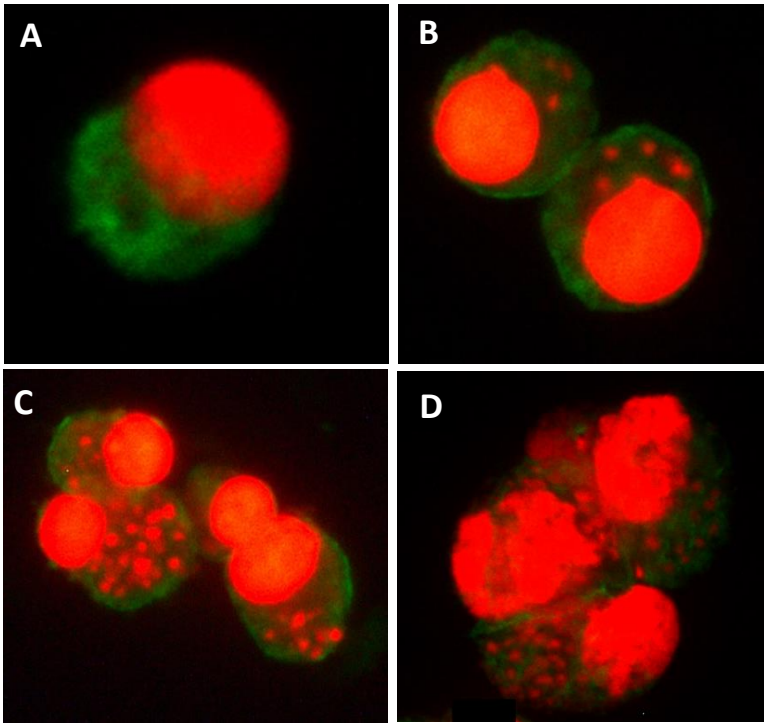

Figure 10: Progression of *L. major* infection of J774s. Photos depict- **A:** uninfected cell, **B:** recently infected cell, **C:** heavily infected cells, **D:** Heavily infected cells nearing death. Nuclei and kinetoplasts were stained red with propidium iodide. Membranes stained green with FITC- conjugated wheat germ agglutinin.

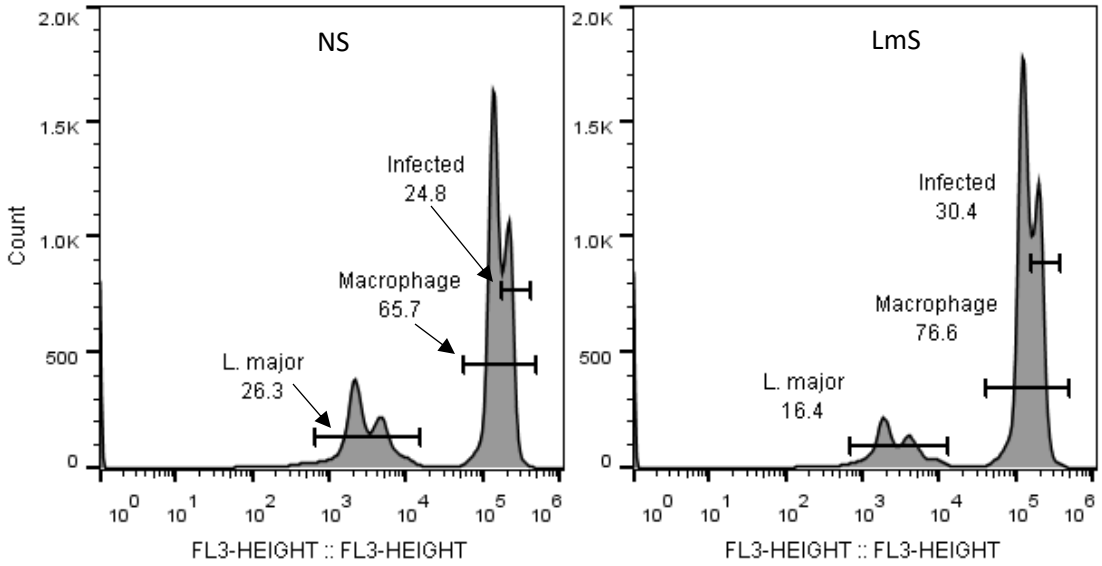

Figure 11: Sample of raw data used to construct graphs shown in figures 12 & 13. Histogram data showing peaks that correspond to free parasites and host macrophages. Gates denote the percentage of events within the selected range and correspond to free parasites and infected macrophages. Both panels depict measurements taken at 24 hours after cells were combined. NS= no serum, LmS= *L. major* antiserum.

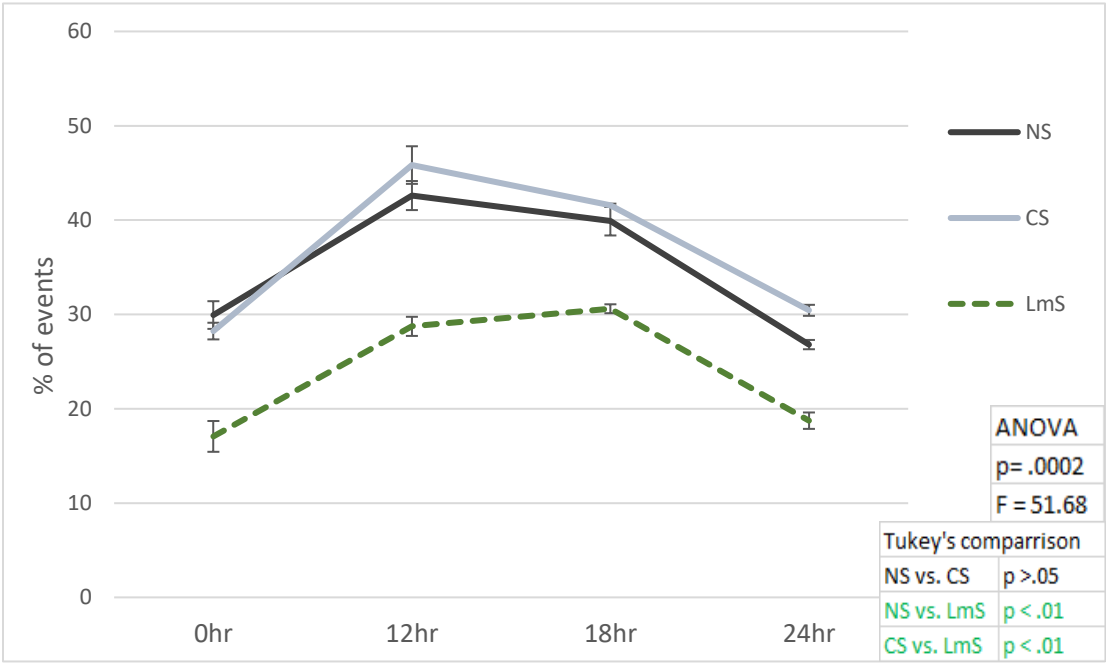

Figure 12. Free *L. major* in the presence of *L. major* antiserum, control serum, and no serum. Lines represent uninternalized *L. major* with standard error. NS= no serum, CS= control serum, LmS= *L. major* antiserum.

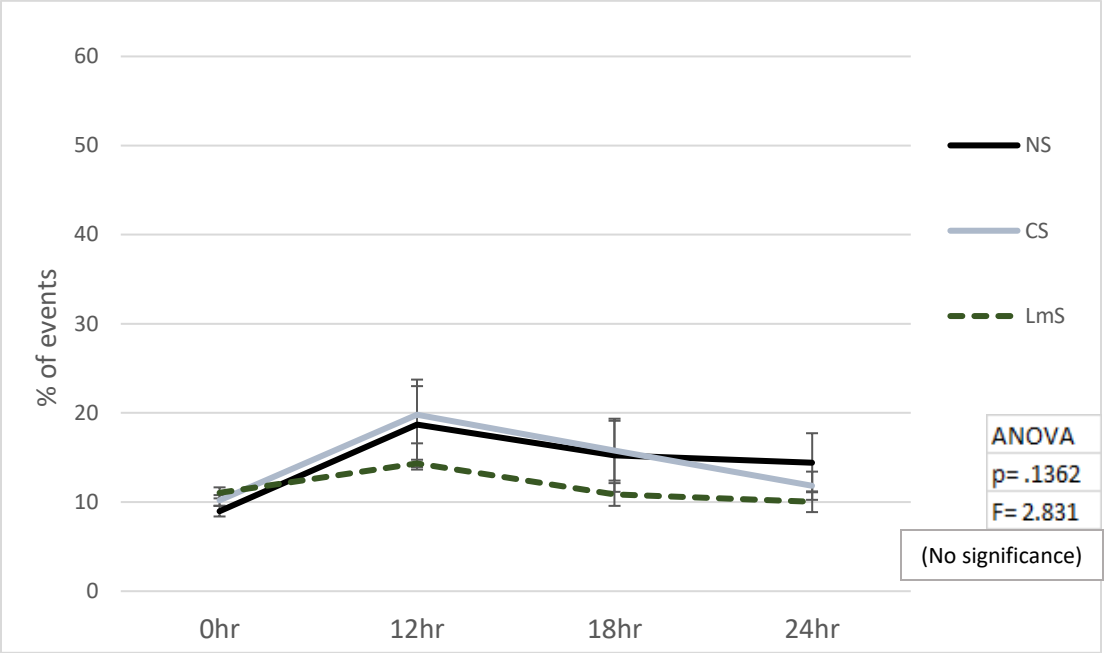

Figure 13. Free *L. infantum* in the presence of *L. major* antiserum, control serum, and no serum. Lines represent uninternalized *L. infantum* with standard error. NS= no serum, CS= control serum, LmS= *L. major* antiserum.

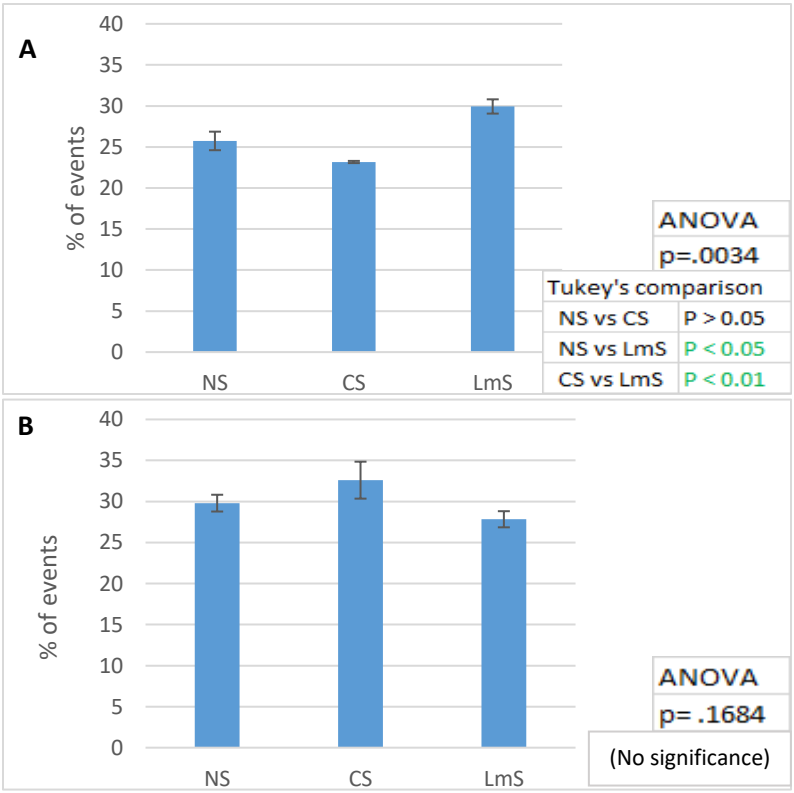

Figure 14. Infected macrophages at 24 hours. Bars represent averaged percentage of events corresponding to infected macrophages at 24 hours with standard error. **A:** cells infected by *L. major*, **B:** cells infected by *L. infantum*. Panels A & B correspond with figures 12 & 13, respectively. NS= no serum, CS= control serum, LmS= *L. major* antiserum.

CHAPTER V

DISCUSSION

Using ELISA, we demonstrated substantial cross reactivity of *L. major* antiserum against *L. infantum* SLA (Figure 9). This was to be expected as previous works have demonstrated not only significant cross reactivity between *Leishmania* species, but even between other genera of *Trypanosoma* (Malchiodi et al. 1994; Vexenat, Santana & Teixeira 1996; Vale et al. 2009). This result establishes a basic premise of this study; the presence of cross reactive antibody is requisite if ADE is occurring in this model. We then moved onto the phagocytosis assays. These were designed to determine if *L. major* antiserum induces increased uptake of *L. infantum in vitro*. Allowing parasites, host cells, and serum interact *in vitro* enables us to attribute any increased parasite uptake to extrinsic ADE. Extrinsic ADE is generally defined increasing rates of infection purely through the effect of opsonization, allowing a pathogen to bind or come in close proximity to leukocytes, leading to higher rates of pathogen internalization. Intrinsic ADE is characterized by host cells becoming more receptive and less hostile toward potential attackers. It is caused by changes in cell signaling, especially increased IL-10 produced as a result of Fcγ-FcγR signaling (Anderson, Gerber & Mosser 2002).

To address the possibility of extrinsic ADE we proceeded to carry out phagocytosis assays to measure uptake of parasites by J774 macrophages in the presence of either naïve serum, *L. major* antiserum, or no serum. The amount of unbound/uninternalized parasites was measured using flow cytometry. To validate the technique, uptake of *L. major* in the presence of *L. major* antiserum was first observed (Figure 12). We noted significantly lower numbers of free parasites treated with

antiserum compared to those treated with naïve serum or no serum, as expected. We also observed significantly higher rates of *L. major* infection among host cells in the presence of *L. major* antiserum (Figure 14A). This process was repeated to measure changing amounts of free *L. infantum* under the same conditions. Interestingly, *L. major* antiserum had no effect on *L. infantum* uptake or numbers of infected host cells (Figure 13&14B). This result would suggest that the heterologous disease exacerbation observed *in vivo* (Nation, Dondji & Stryker 2012; Anderson, Dondji & Styker 2014) cannot be attributed to extrinsic ADE.

This potentially leaves two explanations as to why increased parasite burden was observed by others *in vivo*. The first would of course be intrinsic ADE. In short, the circulating antibody opsonized the parasites, attracting macrophages which bound the antibody, inducing IL-10 production through Fcγ signaling (Anderson, Gerber & Mosser 2002). The IL-10 in turn down regulated the microbicidal response of macrophages allowing for easier entry and more hospitable conditions for *L. infantum* (Kane & Mosser 2001). The reason this effect was not observed *in vitro* could be attributed to a lack of other leukocytes to reinforce effector cytokine functions.

The supposition that intrinsic ADE is at play fails to address the findings of Romano et al. (2015) who observed protective effects against *L. infantum* after immunization with live *L. major*. These authors opted to use the C57BL6 model, known for its predisposition for the Th1 adaptive response and relative resistance to Old World CL (Scott 1988 & 1989; Heinzel et al. 1991). Knowing this, it may come as no surprise that C57BL6s are able to quell leishmaniasis, given their propensity toward the typically protective cytokines IFN-γ and TNFα (Heinzel et al. 1989; Wilhelm et al. 2001).

However, in consideration of our current results and the observations of others, we hypothesize that biased memory T cell responses can account for these seemingly conflicting observations. It has been shown that macrophages are capable of activating CD4 cells and influencing whether they will be directed toward the Th1 or Th2 phenotype (Anderson & Mosser 2002). Macrophages from resistant and vulnerable mouse strains tend to have correspondingly biased phenotypes (Buchmüller-Rouiller & Mauël 1986). These biased macrophages are classified as M1 and M2 and have cytokine profiles analogous to those of Th1 and Th2 CD4 cells respectively (Mills et al. 2000). Anderson & Mosser (2002) eloquently demonstrated that CD4 cells will retain the bias of the macrophages which originally activated them. Furthermore, CD4s will react to repeated exposures to presented antigen in the same manner they did during first exposure, even in the absence of biasing conditions, and even when activated by different types of APC (Anderson & Mosser 2002). In light of this information, we postulate that the heavily biased adaptive responses of the BALB/c and C57BL6 models will give rise to correspondingly biased T cell populations after first exposure to *L. major*. These T cells will then retain their biases, whether protective or detrimental, and react in the same manner upon subsequent exposure to similar antigens. In short, the systems are biased to begin with and these biases are only reinforced with subsequent exposures. Additional experimentation examining T cell polarization as a result of activation by macrophages within these biased models is necessary to further elucidate this issue.

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