The Thesis Committee for Christie Hay Certifies that this is the approved version of the following thesis:

Understanding the importance of vaccine formulation in generating protective immune responses against *Leishmania amazonensis* challenge

Committee:

Lynn Soong, M.D., Ph.D., Mentor

Nisha Garg, Ph.D.

Peter C. Melby, M.D.

Tetsuro Ikegami, Ph.D.

Dean, Graduate School

Understanding the importance of vaccine formulation in generating protective immune responses against *Leishmania amazonensis* challenge

by

Christie Lynn Hay, BSc

Thesis

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

The University of Texas Medical Branch

July, 2014

Understanding the importance of vaccine formulation in generating protective immune responses against *L. amazonensis* challenge

Publication No._____

Christie Lynn Hay, degree sought Master of Science

The University of Texas Medical Branch, 2014

Supervisor: Lynn Soong

Leishmaniasis presents with a broad range of clinical manifestations and is caused by infection with *Leishmania* parasites. The life-long immunity found in individuals who naturally healed their infections suggests that development of a vaccine in feasible, although there is not one yet approved for human use. The immune responses in murine models are well characterized, and suggest possible correlates of protection induced by vaccination. However, these immune responses were determined by the prototypical species *L. major*, and are not necessarily found in all *Leishmania* infections. Therefore, we are working towards understanding the correlates of vaccine-mediated protection in *L. amazonensis*, which is required for properly determining protective mechanisms. We tested both conventional and unorthodox vaccine regimens, but were not able to recapitulate the correlates of protection determined by *L. major*. Conversely, we found IFNγ-producing CD8+ T cells correlated with protection, while ineffective vaccines induced a mixed Th1/Th2 immune response. These data can help to establish parameters for determing vaccine efficacy in *L. amazonensis*.

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List of Abbreviations

UTMB	University of Texas Medical Branch
GSBS	Graduate School of Biomedical Science
TDC	Thesis and Dissertation Coordinator
LCL	localized cutaneous leishmaniasis
DCL	diffuse cutaneous leishmaniasis
MCL	mucocutaneous leishmaniasis
APC	Antigen-presenting cell
DC	Dendritic cell
HIV	Human immunodeficiency virus
AIDS	Acquired immune deficiency syndrome
PCR	polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
MΦ	macrophage
PMN	neutrophil
Th-1	T helper 1
Th-2	T helper 2
Treg	regulatory T cells
FoxP3	forkhead box P3
IL-12	interleukin 12
CD3	cluster of differentiation 3

CD4+	cluster of differentiation 4
CD8+	cluster of differentiation 8
IFNγ	interferon gamma
M1	classically activated macrophages
M2	alternatively activated macrophages
iNOS	inducible nitric oxide synthase
RNS	reactive nitrogen species
ROS	reactive oxygen species
IL-4	interleukin 4
IL-10	interleukin 10
TGFβ	transforming growth factor beta
CD8+0	cluster of differentiation 80
CD8+6	cluster of differentiation 86
MHCII	major histocompatibility type two
CD200	cluster of differentiation 200
FDA	Food and Drug Administration
Alum	aluminum salts
HPV	human papilloma virus
AS04	adjuvant system 04
TLR	toll-like receptor
AS03	adjuvant system 03
MPL	monophosphoryl lipid A
LPS	lipopolysaccharide

GM-CSF	granulocyte macrophage colony stimulating factor
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
MyD88	Myeloid differentiation primary response gene (88)
TRIF	TIR-domain-containing adapter-inducing interferon-β
GLA	glucopyranosyl lipid A
ODN	oligodeoxynucleotides
ISA	Incomplete septic adjuvants
PBMC	peripheral blood mononuclear cells
FBS	fetal bovine serum
s.c.	subcutaneous
HRP	horseradish peroxidase
IgG1	immunoglobulin G subclass 1
IgG2a	immunoglobulin G subclass 2a
TMB	3,3',5,5'-Tetramethylbenzidine
PMA	phorbol myristate acetate
PBS	phosphate buffered saline
IFNa/bR	Type one interferon receptor
pDC	plasmacytoid dendritic cells
LbAg	soluble *L. braziliensis* antigens
BSA	bovine serum albumin
NETosis	cell death via the release of neutrophil extracellular traps
CTL	cytotoxic T lymphocyte

CHAPTER ONE: INTRODUCTION

LEISHMANIA PARASITES AND LEISHMANIASIS

Leishmaniasis is a spectrum of clinical diseases caused by infection with *Leishmania* parasites (1). The majority of leishmaniasis cases are the localized cutaneous form, which is characterized by the development of an ulcerated lesion at the site of the sand fly bite (2, 3). The lesion can require several months to heal, and is dependent upon the host immune response. The healed lesion continues to have parasite persistence, whose presence is responsible for the life long immunity against that species of *Leishmania* parasites (1, 4). In the New World, specific species of parasites are able to induce disfiguring secondary forms of the cutaneous disease: diffuse cutaneous and mucocutaneous leishmaniases (5).

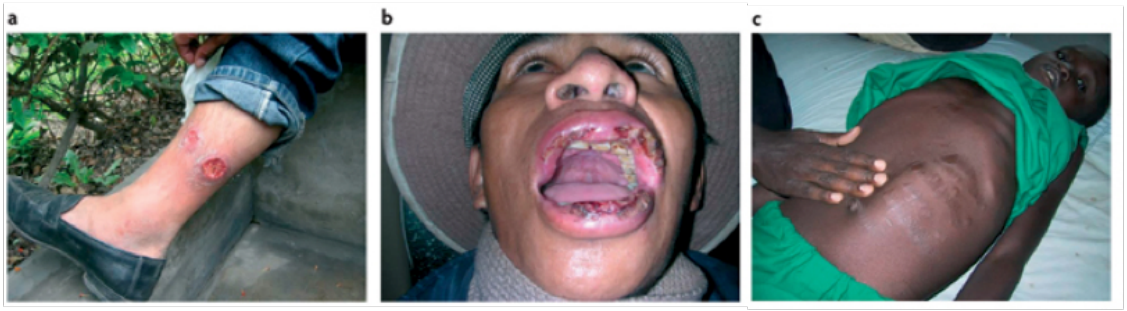

Illustration 1. Clinical manifestations of leishmaniasis (6).

(A) Localized cutaneous leishmaniasis, (B) Mucocutaneous leishmaniasis, (C) Visceral leishmaniasis.

Diffuse cutaneous leishmaniasis (DCL) is characterized by the dissemination of parasites from the original site of infection, leading to the development of non-ulcerated lesions with a high parasite load and minimal CD4+ T cell response; this form can be highly disfiguring (5). DCL is caused by species of the *L. mexicana* complex, including *L. amazonensis, L. mexicana,* and *L. pifanoi* (3). The primary feature of mucocutaneous leishmaniasis (MCL) is the dissemination of parasites to the mucous membranes,

generating a very robust immune response. This parasite-specific response leads to the highly disfiguring damage to the septum, nasal and oral cavity. MCL is caused by species of the *Viannia* complex, notably *L. braziliensis* (3). The most fatal form of leishmaniasis is the visceral disease (VL), which has parasite dissemination to the liver, spleen, and bone marrow. This form is fatal in all cases without treatment, and is caused worldwide by *L. donovani* and *L. infantum* (7).

Leishmaniasis is emerging as an HIV co-infection. Current studies indicate that infection with *Leishmania* spp. can increase the pace at which patients reach AIDS defining events through an undefined mechanism (8). Studies indicate that HIV co-infection increases the events of treatment failure of leishmaniasis and also can lead to relapse of infection. The increase in relapse is related to the HIV-induced alteration of host immune responses to either latent *Leishmania* infections or healed infections (9).

There are four major methods employed for diagnosis of leishmaniasis, each with its limitations and advantages (6). The gold standard for diagnosis is visualization of amastigote-infected macrophages via microscopy. This test will indicate that an infection is ongoing, but it is difficult to get tissue samples from visceral organs for testing (6). Polymerase chain reaction (PCR) can be performed to detect the presence of parasite genetic material, but this method also requires sampling from infected tissues and its versatility is limited by parasite persistence, in which the genetic material is still present after clinical cure (10). An agglutination test to detect parasite antigen in patient urine is being explored, but it appears to have variable sensitivity, depending upon the endemic region. Serological methods will detect the presence of anti-*Leishmania* antibodies circulating, however due to parasite persistence, both diseased and healed individuals will test positive (6). Therefore, current diagnostics are limited in their ability to distinguish between acute infections compared to healed individuals or ones who never developed symptomatic disease.

Leishmaniasis is treated differently depending upon the parasite species, the disease manifestation, and the healthcare infrastructure available to patients. Visceral leishmaniasis is treated in most cases to prevent mortality. LCL with a localized lesion is rarely treated, and this form will self heal over time. MCL and DCL require treatment to prevent disfigurement (11). There are several major treatments widely available. Pentavalent antimonials and their derivatives have been a front line of treatment for over 60 years, as well as the types associated with drug resistance concerns in the Indian subcontinent (12). The next major class of treatment available is amphotericin B and its derivatives. Amphotericin B requires hospital admission and oversight of treatment due to its high degree of toxicity, however due to its mode of action, the development of resistant parasites is highly unlikely (12). Miltefosine is the first orally available drug for leishmaniasis treatment. This drug increases efficacy of the pentavalent antimonials, due to a cooperative effect (11). In all cases, drugs are required for several weeks, which makes them cost prohibitive in most endemic regions (11).

PARASITE LIFE CYCLE IN SAND FLY VECTOR

Leishmania parasites have a digenetic life cycle dependent upon the host: flagellated promastigotes are found in the sand fly gut and non-motile amastigotes reside within parasitophorous vacuoles inside host phagocytic cells (1). When infection is initiated, a female sand fly deposits metacyclic promastigotes during a blood meal. These promastigotes are rapidly internalized by phagocytic cells; including macrophages, dendritic cells, and neutrophils (1). Within macrophages, the promastigotes undergo a morphological change due to environmental shifts, becoming amastigotes. Replication of amastigotes via binary fission occurs primarily in macrophages, within a parasitophorous vacuole, which exhibits characteristics of a phagolysosomal compartment (13). After several rounds of replication, macrophages will rupture, releasing amastigotes into the extracellular space. These amastigotes are internalized by phagocytes and continue to

multiply (1). To complete the transmission cycle, a female sand fly will consume a blood meal from an infected host, containing infected macrophages. The amastigotes convert to promastigotes and are released from macrophages in the sand fly midgut (14).

There are two genera, with approximately 30 species, of sand fly capable of transmitting leishmaniasis: *Phlebotomus* spp. in the Old World and *Lutzomyia* in the New World (15). The sand fly transmission patterns are characterized according to where they feed as well as where they rest, either indoors or outdoors (15). Therefore, *Leishmania* spp. can have distinct transmission cycles, which differ among sand fly species and habitat locations. Generally, in the Old World, anthroponotic transmission occurs, in which humans are believed to be the major reservoir for *Leishmania*. Tranmission between humans is documented for both LCL and VL in the Middle East (16). Zoonotic transmission occurs primarily in the New World, with numerous animals serving as reservoirs. The New World species overall do not generate as high a parasite burden as the Old World species, limiting the ability for infected people to actively transmit to sand flies during a blood meal (17).

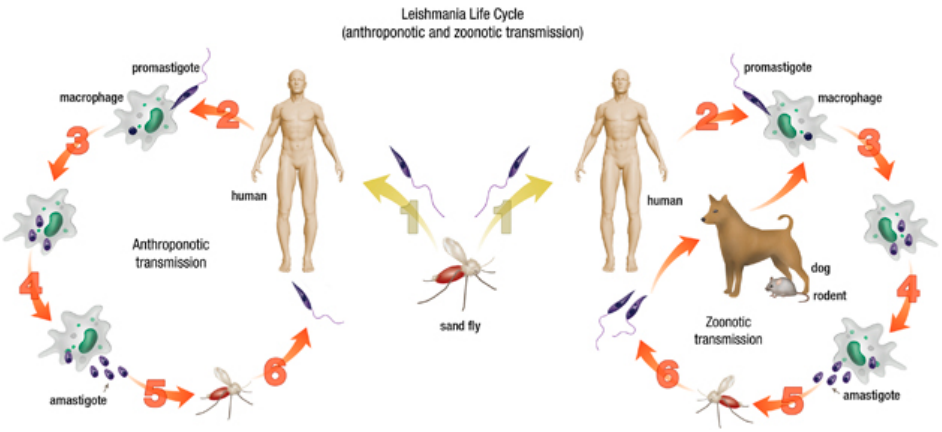

Illustration 2. Life cycle of *Leishmania* with transmission cycles (18).

As leishmaniasis infection occurs through a vector and has multiple host species, there are a many methods to employ to control transmission. These methods are based around three major concepts: control of vector populations, animal reservoir and human

reservoirs (17). Within the domain of controlling vector populations, the two main strategies being employed are application of insecticides and management of environments with high numbers of vectors. Control of animal reservoir populations depends upon the major species and location involved (17). In South America, domestic dogs are major reservoirs for visceral leishmaniasis. Countries have employed strategies to control domestic dog populations (culling programs), block sand fly feeding of dogs (providing insecticide-impregnated dog collars), and reduce infection of dogs (development of effective canine vaccines) (19). In areas that domestic dogs serve as reservoirs, reducing the overall level of infection in dogs will have a significant impact on the human infections (19). To control transmission from human reservoirs, insecticide impregnated fabrics and bed nets at night are being used. Additionally, research into developing a human vaccine is ongoing (17).

IMMUNE RESPONSES GENERATED DURING INFECTION AND PARASITE EVASION STRATEGIES

Infection with *L. major* in murine models has two major trends of disease progression, based upon the immune bias of the mouse used. Mice with a Th1-bias (C57B/6) are highly resistant to infection, and will quickly heal their lesions. However, Th2-biased (BALB/c) mouse backgrounds develop chronic, non-healing lesions (1, 20). This intrinsic dichotomy improved our understanding of immune effector molecules and cell mediated immunity.

In Th1-biased animals, dendritic cells (DC) that present parasite antigen are polarized to produce IL-12. This IL-12 will act on effector CD4+ T cells, and induce a differentiation into IFNγ-producing Th1 T cells. The IFNγ will serve to induce an M1 phenotype in macrophages (20). M1 macrophages are microbicidal, with an increased production of RNS and ROS, which are responsible for parasite killing within the macrophage (21, 22). In Th2 biased animals, effector CD4+ cells are responding to IL-4

secretion, which induces an M2 phenotype in the macrophages (21, 23). These macrophages will produce arginase rather than iNOS, and will secrete IL-10 and TGFb. In the presence of TGFb and IL-10, additional CD4+ T cells can be polarized towards a Treg phenotype. These cells will secrete IL-10 and TGFb, which will promote the M2 phenotype of macrophages as well as parasite growth (23, 24).

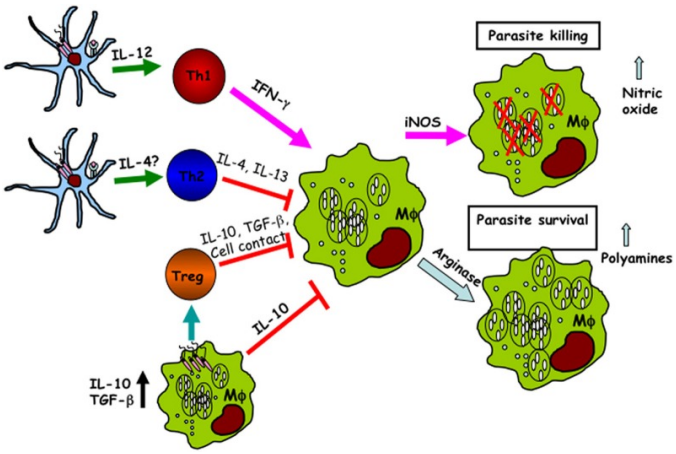

Illustration 3. *Leishmania*-induced immune responses in murine models (25).

In order to induce immune responses that favor infection, *Leishmania* has developed several mechanisms to modulate host immune responses. Even at the initiation of infection, proteins found in sand fly salivary gland extracts help to prevent immune activation (26, 27). The first salivary protein capable of promoting disease identified was a vasodilator named maxadilan, which is related to a mammalian neuropeptide. Maxadilan is capable of modulating inflammatory responses, and typically exacerbates them. In fact, addition of maxadilan alone reversed the disease trends of *L. braziliensis* in mice, allowing for the development of cutaneous lesions (28). An additional protein, Lundep, was identified in *Lutzomyia longipalpis*. This protein is an endonuclease and was shown to promote parasite survival following interactions with neutrophils undergoing NETosis (29).

One of the first cells promastigotes encounter are neutrophils, which respond differently depending upon the parasite species encountered. With *L. major*, neutrophils were shown to readily phagocytose promastigotes. These parasites survive the killing mechanisms of neutrophils, and ultimately use neutrophils as Trojan horses to avoid immune detection. Once these neutrophils undergo apoptosis, they are ingested by macrophages and shuttled into a phagolysosome. The parasites are able to survive digestion of the neutrophil, and establish infection within macrophages without triggering immune recognition (30). In *L. amazonensis* infection, neutrophils undergo cell death. Neutrophils that have contact with promastigotes undergo NETosis, in which the release of nuclear material can encase the parasites and lead to their death (31). The remaining neutrophils undergo apoptosis, helping to maintain an anti-inflammatory environment.

In addition to neutrophils, promastigotes can infect dendritic cells, a professional antigen presenter. Parasite interactions with DC have different immune outcomes, based upon infecting species, cytokine production and capacity of DC to prime antigen specific T cells (32, 33). Monocyte derived DC efficiently engulf promastigotes, and induce secretion of IL-12p40 and IL-10. In the presence of a second immune stimulus, infected mDC can produce IL-12p70. *L. amazonensis*, however, does not stimulate strong IL-12 production, and activation of IL-12 is transient (23). Additionally, *L. amazonensis* infection of DC have lower levels of activation markers (CD8+0, CD8+6) and are less capable to prime T cells (based on surface expression of MHC II molecules) (34). Therefore, promastigote infection modulates the function of antigen presenting cells in order to reduce immune responses.

In addition to promastigotes, amastigotes encounter DC throughout infection. *L. amazonensis* amastigotes undergo phagocytosis by DC through interactions with DC-SIGN, but this interaction does not induce DC maturation or antigen presentation (32). Amastigote uptake can also be mediated through parasite opsonization and interaction with the Fc-gamma receptor (35). However, this interaction induces IL-10 production

instead of IL-12, which further promotes immune suppression through the induction of IL-10-producing effector T cells and increases parasite growth due to downstream effects on macrophage polarization (35).

There are several mechanisms employed to evade the microbicidal activities of macrophages, the cells in which *Leishmania* reproduce. *Leishmania* enter macrophages via traditional receptor-mediated phagocytosis, using a combination of the complement receptors 1 and 3, the mannose receptor, and fibronectin receptor. The model for this interaction suggests that for *L. amazonensis*, complement receptor 1 is essential for the initial ligation to the macrophage surface, and the fibronectin receptor interaction is necessary for phagocytosis (35). However, this interaction is not sufficient to trigger immune responses in the macrophage. Once inside macrophages, *Leishmania* parasites modulate immune responses. Infection with *Leishmania* leads to a reduction in IL-12 gene transcription and protein levels. In *L. amazonensis* infection, expression of CD200, is induced; CD200 was shown to inhibit iNOS activation and reduce the subsequent microbicidal activity of macrophages (36).

Leishmania amastigotes have evolved to survive and replicate within a specialized vacuole, which has characteristics of a phagolysosome. The parasitophorous vacuole has a pH of approximately 5, which is similar to an acidified phagosome (37). *Leishmania* also has a unique, complex antioxidant system, which has greater reducing ability than the mammalian counter (38). Based on unpublished data, there are differences in the gene copy number of elements of this system between parasite species, which correlates to intracellular survival within activated macrophages.

VACCINE HISTORY AND SUCCESS

In summary, a vaccine against leishmaniasis is considered highly desirable due to the challenges in treating infected individuals. Overall, treatment is difficult due to drug toxicity, expense, and long drug regimens. Additionally, it is known that individuals who

heal their infections develop life-long immunity against that species. Furthermore, through research in animal models, we understand the immune responses required for self-healing rather than disease progression. Due to these factors, there is a great deal of vaccine research in *Leishmania* ongoing.

First generation vaccines

The most successful vaccine strategies against leishmaniasis use antigens from the whole parasite in several different forms (39). Leishmanization, the gold standard for prophylactic vaccine, is the inoculation of an avirulent parasite strain onto a part of the body that is not typically seen when individuals are clothed. Disease pathology normally remains isolated to the site of inoculation, and once the lesion is healed, individuals develop life long immunity (40). Similar to leishmanization, genetically attenuated parasites can be used as a live vaccine. These parasite strains have multiple defined mutations, serving to limit parasite growth within the host but maintain antigen diversity and immune responses (41, 42). However, any attenuated strain can revert to wild type virulence. Additional testing has been done using killed or formalin-fixed parasites (39). Finally, vaccination with lysate generated from promastigotes is also effective, depending upon the parasite species selected and adjuvant choice (40). This strategy is primarily being employed in domestic dogs, to reduce zoonotic transmission in South America (43-45). The advantages of using killed parasite or lysates is that there is no opportunity for reversion to wild type virulence and there are fewer side effects (namely no lesion development due to the vaccine).

Second generation vaccines

Despite the success of first generation vaccine strategies, there was interest in generating vaccines to known antigens, in order to improve safety, reduce side effects, and eliminate the risk of reversion to wild-type. The second generation vaccines are

subunit vaccines, typically targeting one or more immunodominant parasite antigens (46). These antigens were traditionally identified using sera from individuals exposed to infection, but never developed disease symptoms (47). There are several routes of vaccine delivery being explored with multiple adjuvants. Recombinant proteins of the antigens can be generated in cell culture using an inducible expression system (48). Combining these proteins with adjuvants or a particle (such as a liposome) can improve protection while maintaining its safety (49). Several polyprotein vaccines have reached human clinical trials, and are shown to be safe while inducing strong antibody responses (50, 51). Additionally, these antigens can be cloned into mammalian expression plasmids, and deliver the DNA as the vaccine. Cells from the vaccinated individual, primarily skin or muscle, will uptake the plasmid and express the antigen (52). Efficiency of plasmid uptake can be improved using a biolistic delivery device (53). Finally, the target antigens can be cloned into a bacterial or viral vector, which will express the proteins for recognition by DC (46, 54).

Third generation vaccines

The newest vaccine strategy being tested in murine models of leishmaniasis involves a heterologous prime- boost approach. This strategy still uses defined antigens, but combines several delivery platforms throughout the course of immunization. Using this method requires APCs to respond to the antigens in different forms, and generates more robust antigen-specific immune responses compared to second generation vaccines (40, 46). The typical vaccine regimen for third generation vaccines involves priming with plasmid DNA expressing the antigen, then boosting with a viral carrier construct. These vaccines are also adjuvanted to further improve immune responses (55). An additional third generation strategy (although it may soon constitute a fourth generation) is the use of antigen-pulsed dendritic cells in the vaccine regimen. In this strategy, the immune polarization at the DC level is already complete, and these transferred cells will

promote the protective immune response through direct interaction with host CD4+ and CD8+ T cells (56).

HISTORY OF ADJUVANT USAGE

Definition of adjuvant

Adjuvants are defined as any material or chemical that is capable of increasing the immune response to a vaccine antigen, typically measured through the humoral or cellular immunity (57). There are several mechanisms through which adjuvants can promote immune responses. It is believed that proper adjuvanting can function to keep the vaccine antigen at the site of inoculation, and maintain a slow rate of antigen release. Adjuvants can also modify the functions of APCs, in order to promote protective immune responses. These APC will be modified to secrete specific cytokines based on the adjuvant and antigen combination being used (58). Overall, successful adjuvants have demonstrated ability to promote the development of faster, stronger, and longer lasting immune responses.

FDA-approved adjuvants

To date only three adjuvants are approved by the FDA for use in human vaccines. The first adjuvant approved was aluminum salts (alum) in the 1950s. This adjuvant works through a still unknown mechanism to improve vaccine-induced humoral immune responses (59). Overall alum is very safe, and rarely generates severe local reactions. In the HPV vaccine, Cervarix, AS04 was approved. AS04 contains aluminum hydroxide combined with MPL, which is a TLR4 agonist (58, 59). The H1N1 vaccine included the adjuvant AS03, an oil-in-water emulsion (59). Due to the limited number of vaccine adjuvants approved, research is ongoing into new adjuvant strategies, which will be safe and effective at boosting cellular immune responses, in addition to humoral.

Adjuvants in development

RECOMBINANT CYTOKINE ADJUVANTS

The two major cytokines being explored as adjuvants for *Leishmania* are IL-12 and GM-CSF. In a murine model, IL-12 with antigen induced protection against disease via induction of Th1 CD4+ T cells in the lymphoid organs, namely the spleen and lymph nodes. Additionally, mice that received IL-12 had a significant reduction in IL-4, but increased IFNγ production (51, 57). GM-CSF was explored due to the importance of this cytokine in DC function. In preliminary human experiments, GM-CSF with recombinant antigens promoted lesion healing in infected individuals with minimal side effects at the site of inoculation (57).

TLR AGONISTS

Signaling through toll like receptors (TLRs) on the cell surface serves to activate inflammatory genes, which are dependent upon the adaptor molecules present in the signaling cascade. One of the best-studied TLRs in *Leishmania* vaccination is TLR4. TLR4 is activated by the surface component of bacterial cells, lipopolysaccharide (LPS) and activates NFkB through both MyD88 and TRIF, which is essential to its ability to serve as an adjuvant (58). It has been proposed that the initial signaling through MyD88 rapidly activates NFkB and pro-inflammatory cytokine production (namely IL-12 and IFNγ); signaling through TRIF provides long-lasting NFkB activation and IL-6 production (51). Today, several synthetic versions of LPS exist, and have improved safety over the natural molecule. MPL and MPL-SE (MPL in stable emulsion) improved protection in second generation vaccines against *L. major* and *L. donvani* (51). An additional fully synthetic TLR4 agonist was recently developed, glucopyranosyl lipid A (GLA), and has undergone testing against *L. major* in the original formulation as well as in stable emulsion (51).

Signaling through TLR7/8 via use of resiquimod and imiquimod can induce the production of antiviral cytokines in monocytes (IFNa, TNF, and IL-1β) and secretion of

pro-inflammatory cytokines in macrophages (IFNγ and IL-12) (58). Imiquimod was formulated to be a topical cream, which is being explored for use as a therapeutic in conjunction with traditional treatment approaches. This combination was effective in inducing cure of cutaneous lesions, and was well tolerated by patients. Topical imiquimod has been tested as a vaccine adjuvant for both *Leishmania* and *Plasmodium* in murine models, and was found to induce Th1 responses and high antibody titers, which correlated with protection (51, 57).

CpG oligodeoxynucleotides (ODN) induce TLR9 signaling and downstream effects on immune responses depend upon the class of ODN used. Type A ODN induce NK cell activity and promote IFNα release by plasmacytoid DCs (pDC). Type B ODN promote B cell activity and antibody production; induce NK cell activation, and the production of pro-inflammatory cytokines. Type C ODN combine the downstream effects of Type A and Type B (51, 57, 60). In murine studies of *L. major*, the use of CpG ODN Type B as a vaccine adjuvant increased both CD4+ and CD8+ T helper cells, and generated significant protection against LCL (61-63). CpG ODN Type A is effective on its own as an immunomodulator to reduce *L. amazonensis* pathology in rhesus macaques (64, 65). The mechanism for this protection has not been elucidated.

ADDITIONAL ADJUVANT COMPOUNDS

These molecules are adjuvant compounds that do not distinctly activate cellular functions through a TLR, but are actively being pursued in basic research. Saponins are naturally occurring glycosides found in steroid or triterpene that are effective at augmenting multiple cellular processes, but are also used in products available for human consumption (beverages, cosmetics, and pharmaceuticals) (66). As vaccine adjuvants, Quil A and its derivatives are the best studied. These compounds are uniquely able to stimulate Th1 responses as well as enhancing the antigen-specific CTL activity. However, their use in humans has been limited due to injection site reactions and their

molecular instability in aqueous solution (67). In South America, whole parasite lysate vaccines with saponin adjuvants are approved for use in domestic dogs, in an effort to reduce transmission (68-70). These adjuvants are being explored for human use in HIV vaccines and cancer immunotherapy (66).

Montanide Incomplete Sepic Adjuvants (ISA) are a collection of related adjuvants, which are based on specific oils mixed with surfactants. The two oils used most often are a non-metabolizable mineral oil and another metabolizable oil; the two oils can also be combined. Montanide ISA was shown to promote both Th1 type cellular immunity, but also humoral immunity in human safety trials. Overall Montanide ISA is well tolerated; patients only reported pain at the injection site (57). In patients tested, PBMCs had increased proliferation and cytokine secretion (primarily IL-2 and IFNγ). However, in *Leishmania* infection, the effectiveness of this adjuvant is debated. In a non-human primate model, addition of Montanide ISA generated protection, while in mice there was no difference in disease outcome compared to controls (57).

Correlates of vaccine-mediated protection

Due to the abundance of *Leishmania* vaccine testing done in animal models, we have a clear understanding of protective immune responses and can determine the correlates of protection. It has been demonstrated that both CD4+ and CD8+ T cell responses are essential to protection against *Leishmania* (18). In *L. major*, multifunctional CD4+ T cells correlate with vaccine-induced protection. Multifunctional cells are capable of producing more than one proinflammatory cytokine, namely IFNγ, TNFα, and IL-2, and significantly more cells that produce all three simultaneously (71). These multifunctional cells are able to stimulate long-lasting Th1 effector cells. In *L. amazonensis*, CD8+ T cells that produce perforin and IFNγ are essential for vaccine-induced protection (72). Overall, we can conclude that vaccine-induced protection requires a strong Th1-type response, characterized by the secretion of IFNγ.

RATIONALE

Although vaccine studies across all generation have been conducted with *L. amazonensis*, the overall proportion of the studies is quite low (40). It is well established that *L. amazonensis* induces of chronic lesion development in all mouse models tested (73). Furthermore, correlates of vaccine-mediated protection have only been defined in Old World species, specifically *L. major* (71). Therefore, testing of vaccine candidates against *L. amazonensis* challenge must be performed. This would allow for optimization of vaccine regimens in order to generate reduced disease pathology, and further allows for correlates of protection to be characterized. **We hypothesize that vaccine-mediated protection will correlate with in the induction of antigen-specific multifunctional CD4+ T cells.** In aim one, we will test a polyprotein vaccine candidate that generates significant protection against *L. major* and *L. donovani* against our *L. amazonensis* challenge model, and elucidate the immune mechanisms. In aim two, we will use a first generation strategy (soluble protein lysates) and different adjuvant compounds to determine the importance of adjuvants in vaccine-mediated protection. Overall, we will highlight the differences in immune responses and disease pathology due to vaccination and attempt to define correlates of protection against *L. amazonensis*.

CHAPTER TWO: IMMUNIZATION WITH RECOMBINANT POLYPROTEIN ANTIGENS

INTRODUCTION

The majority of vaccine design research is performed in Old World species, and use *L. major* as the prototypical LCL parasite species. However, it is well established that *L. amazonensis* induces quite different host immune responses and responds differently to immune activation (74). Overall, in *L. major*, it is established that Th1-biased immune responses will result in healing while Th2-biased responses encourage lesion progression (1). However, *L. amazonensis* generates mixed immune responses in all mouse models, with resultant progressive lesion development (73, 75).

With this knowledge in mind, it is essential to test vaccine candidates that are protective in *L. major* against *L. amazonensis*. To this end, we are testing a recombinant polyprotein antigen candidate, KSAC, with the TLR4 agonist GLA in our infection model. KSAC was designed against VL, but was partially protective against *L. major* challenge. KSAC is comprised of antigenic regions of four immunodominant proteins: kinetoplastid membrane protein 11, sterol-6-methyltransferase, A2, and cysteine protease. These candidate antigens were identified using medium throughput serological screen of serum from VL patients (47). KSAC with MPL partially protected against both *L. donovani* and *L. major* in mouse models, measured by parasite load in infected tissues. Additionally, KSAC plus MPL increased the frequency of antigen-specific multifunctional CD4+ T cells, which correlated with protection (48). Recently, a fully synthetic TLR4 agonist was developed: glucopyranosyl lipid A (GLA). GLA was shown to signal through both MyD88 and TRIF, which is essential to its ability to activate both

innate and adaptive cells (76). Furthermore, GLA enhances protective immune responses when combined with recombinant protein antigens (76).

The first testing performed using KSAC with GLA compared the protection generated with different route of parasite inoculation, namely needle delivery or through sand fly feeding (77, 78). In all cases, KSAC plus GLA immunization generated significant protection against needle challenge, through the induction of multifunctional CD4+ T cells (77). However, when immunized mice were challenged via sand fly feeding, there are conflicting reports regarding the protection from disease (27, 77, 78). Previous studies have reported differences in protective potential of vaccines when mice are challenged via sand fly feeding, likely due to the impact of sand fly salivary proteins on the immune response (79). Regardless, due the differences in the mechanism behind immunopathology between parasite species, it is possible for vaccines to have limited cross species protection despite high degrees of homology and must be tested individually against all potential parasite species.

MATERIALS AND METHODS

Mice. Female BALB/c mice were purchased from Taconic Farms (Germantown, NY) and maintained under specific pathogen-free conditions. Mice were 6 to 8 weeks of age when immunizations were initiated. Animal protocols were approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX).

Parasites and *Leishmania* antigen preparation. *L. amazonensis* (MHOM/BR/77/LTB0016) parasites were maintained by regular passage through BALB/c mice. Promastigotes were cultured at 23°C in Schneider's *Drosophila* medium (Invitrogen, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS). For immunization study, stationary phase *L. amazonensis* promastigotes of less than three *in vitro* passages were isolated. To prepare promastigote and amastigote lysates, parasites

were suspended in phosphate-buffered saline and subjected to three freeze-thaw cycles and a 15-minute sonication prior to storage at -20°C.

Immunization with KSAC+GLA-SE and *in vivo* challenge. Groups of five mice were immunized with 10 μg of the KSAC protein with 20 μg of glucopyranosyl lipid A in stable emulsion (**GLA-SE**) in a volume of 100 μL. Low dose studies were immunized with 5 μg of KSAC and 5 μg of GLA in 100 μL. Control groups received either adjuvant alone or phosphate-buffered saline (PBS). Three subcutaneous (s.c.) injections were given at the base of the tail at three-week intervals over a total of six weeks. At three weeks after the last immunization, animals were challenged by s.c. injection of $1x10^6$ stationary phase *L. amazonensis* promastigotes. Lesion development was monitored weekly with a digital caliper (Mitutoyo, Aurora, IL).

ELISA for antigen-specific antibody production. Serum samples were taken from animals at one-week pre-challenge and ten-weeks post-challenge. Antigen-specific ELISAs were performed for the identification of IgG1 and IgG2a specific for KSAC and *L. amazonensis*. Briefly, microtiter plates (Nunc) were coated with 2 μg/mL KSAC or 50 ng/mL *L. amazonensis* promastigote lysate diluted in phosphate buffered saline overnight. Plates were blocked with 10% FBS. Serum samples were diluted 1:100 with PBS and applied to plates. Plates were washed, and horseradish peroxidase (HRP)-conjugated rat-anti mouse IgG1 or IgG2a (BD Pharminogen) was added at a 1:1000 dilution and incubated at room temperature. Plates were detected using TMB substrate. The optical density was determined at 450 nm.

Flow cytometry-based cytokine detection. For detection of surface markers, draining lymph node cells were harvested. After blocking of non-specific binding sites with rat anti-mouse CD16/32, cells were stained with the following specific monoclonal antibodies: PerCpCy5.5-conjugated rat anti-mouse CD3, and FITC-conjugated rat anti-mouse CD4, or FITC-conjugated rat anti-mouse CD8. For detection of intracellular cytokines, draining lymph node cells were isolated ($1x10^6$/mL) were restimulated with

phorbol myristate acetate (PMA)/ionomycin in the presence of GolgiPlug (BD BioSciences) for 4 hours. Subsequently, cells were stained for surface markers, fixed/permeabliized (eBiosciences FoxP3/Transcription Factor Staining Buffer Set), and then incubated for 60 minutes with APC-conjugated rat anti-mouse IFNγ. Data was collected using an Accuri C6 flow cytometer (Accuri Cytometers Inc., Ann Arbor, MI). Flow cytometry data was subsequently analyzed using CFlow version 1.0.227.4 (Accuri Cytometers Inc.).

RNA extraction and RT-PCR analysis. Total RNA was isolated from whole foot tissue from sacrificed mice (RNeasy, Qiagen). Total RNA was reverse transcribed to cDNA using SuperScript III kit according to manufacturer's instructions with the random hexamer primer set (Invitrogen). Relative expression of multiple cytokines was determined using quantative PCR, with annealing temperature of 60°C for all primer sets (80). Data was normalized using the ddCT method (81). Primers utilized are listed in Table 1.

Target	**Forward Primer**	**Reverse Primer**
IFNγ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
TNFα	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
IFNβ	CAGCTCCAAGAAAGGACGAA	GGCAGTGTAACTCTTCTGCAT
INFa/bR	AGCCACGGAGAGTCAATGG	GCTCTGACACGAAACTGTGTTTT
NFkBp50	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG
gapdh	GAGCTGAACGGGAAGCTCAC	ACCACCCTGTTGCTGTAGC
La UbiqH	AACGTGAACAACTGGATGTGCGTC	ATGGTACCAAGCTTGACACATGCC

RESULTS AND DISCUSSION

Immunization with KSAC plus GLA induces antigen-specific antibody production.

Following the published protocol, mice were immunized s.c. with 10 μg of KSAC + 20 μg of GLA or PBS at three-week intervals (48). Two weeks after the final boost, mice were bled to measure antibody production. KSAC- and *L. amazonensis*-specific antibody titers for both IgG1 and IgG2a were increased following immunization with KSAC plus GLA, but were not detectable in PBS immunized mice (Figure 2.1A,B). The levels of IgG1 and IgG2a were similar in KSAC immunized mice, which corroborates the antibody responses detected in prior studies (48, 78). However, this result suggests there is no bias towards a Th1-type response against KSAC antigen. *L. amazonensis*-specific antibodies were also present following immunization with KSAC + GLA (Figure 2.1B). Furthermore, the amount of IgG2a was increased relative to IgG1, suggesting that the vaccine induced a Th1 biased immune response specifically against the parasite antigen. However, the amount of antibody is reduced compared to the KSAC-specific IgG2a titers.

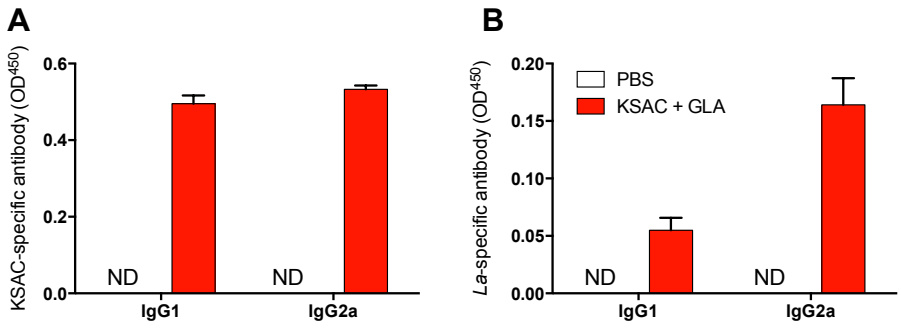

Figure 2.1. Vaccine-induced *L. amazonensis*-specific antibody production.
Mice were immunized at three-week intervals with KSAC polyprotein + GLA or PBS alone for controls. Two weeks following final boost, KSAC specific (A) and *L. amazonensis*-specific (B) IgG1 and IgG2a production was determined using ELISA. (ND = not detectable)

KSAC plus GLA vaccination exacerbates L. amazonensis disease pathology.

Mice were immunized as before (10 μg of GLA with or without 20 μg of KSAC, PBS as control) in 100 μL volume, at three-week intervals. Three weeks following the final boost, mice were challenged s.c. in the footpad with $1x10^6$ *L. amazonensis* stationary phase promastigotes. Through weekly monitoring, we recorded a significant increase in lesion size in mice that received both antigen and adjuvant, while the control groups had similar courses of disease (Figure 2.2A). These results are entirely opposite of those in previous publications (48). However, correlates of *L. amazonensis* vaccine induced protection have not been characterized. It is possible that the vaccine is stimulating too strong of an immune response, leading to increased immunopathology in *L. amazonensis*.

However, parasite burden in the footpad was not significantly different between groups at times measured. At 4.5 weeks post-infection, parasite burdens were similar (Figure 2.2B), but at 10 weeks post-infection, there was a trend of higher parasite load in mice receiving KSAC + GLA (Figure 2.2C). During data analysis, the parasite gene signal is normalized to the host housekeeping gene; using this method, our results are suggesting there is a similar ratio of parasite to host cells in all treatment groups. This would indicate the increased lesion size is not due to solely an increased cellular infiltration, but rather an equal increase in both parasite numbers and corresponding inflammatory cell influx.

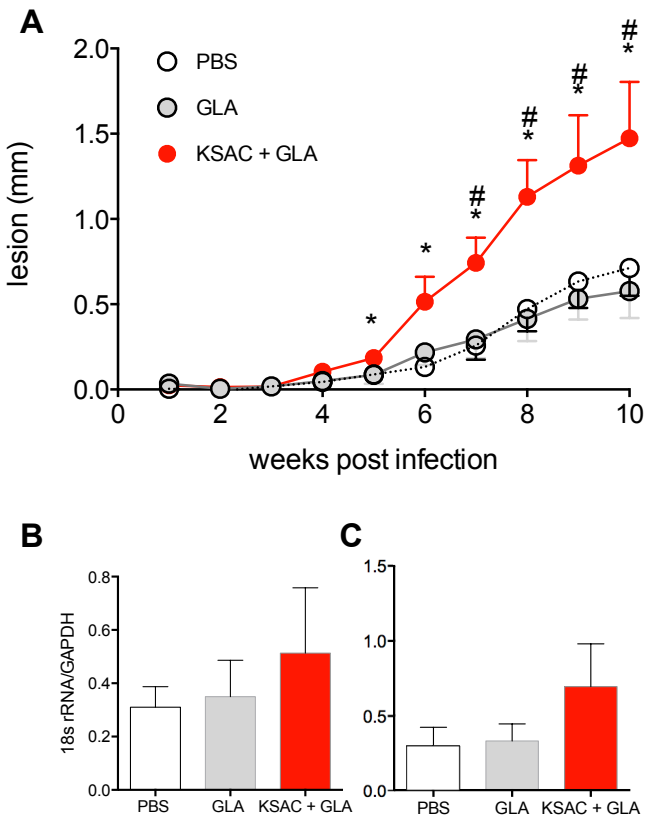

Figure 2.2. Disease progression following immunization.

Mice were challenged at three-weeks following final immunization with $1x10^6$ *L. amazonensis* stationary phase promastigotes. Lesion development was measured weekly (A). Parasite burden at infection site was measured using qRT-PCR at 4.5 (B) and 10 (C) weeks post infection. Statistical analysis was performed using ANOVA. (*, $p \leq 0.05$ between PBS control and KSAC + GLA; #, $p \leq 0.05$ between GLA control and KSAC + GLA)

Infection of KSAC + GLA-immunized mice induces differential antibody responses relative to control animals.

Serum from mice that were infected with *L. amazonensis* following immunization with KSAC plus GLA or PBS alone was used to determine antibody production at 10-weeks post-infection. Mice that received KSAC + GLA maintained high levels of KSAC-specific antibody production, of both IgG1 and IgG2a isotypes (Figure 2.3A). However, levels were similar, indicating the mice continued to have a mixed Th1/Th2 immune response against KSAC antigen. Control mice developed detectable levels of

KSAC-specific antibodies, but the abundance was much lower than immunized (Figure 2.3A). Control mice produced appreciable levels of *L. amazonensis*-specific antibodies, of both isotypes (Figure 2.3B). KSAC + GLA mice induced production of IgG1 relative to antibody levels prior to challenge (Figure 2.1B), but did not increase IgG2a production. Furthermore, relative to control mice, KSAC + GLA immunization induced higher levels of IgG1 and lower levels of IgG2a. These results suggest that *L. amazonensis* infection alone induces a stronger Th1 response compared to the mice immunized prior to challenge.

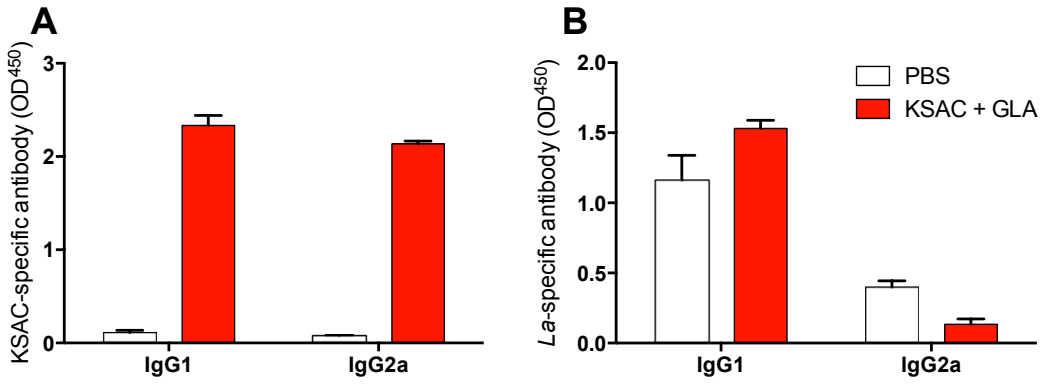

Figure 2.3. Mixed infection-induced antibody production by immunized mice. Mice were immunized at three-week intervals with KSAC polyprotein + GLA or PBS alone for controls, and challenged with *L. amazonensis* three-weeks following final boost. Production of KSAC-specific (A) and *L. amazonensis*-specific (B) antibodies were measured using ELISA at ten weeks post infection.

Cellular immune responses generated during *L. amazonensis* infection due to vaccination with KSAC plus GLA.

Draining lymph node cells from mice that were immunized with KSAC + GLA or PBS alone were used to measure the frequency of cytokine producing cells. Single cell suspensions were generated from popliteal lymph nodes in immunized and PBS control mice. Cells were stimulated four hours *ex vivo* with PMA, ionomycin, and GolgiPlug. Cells were stained for surface markers (CD3, CD4+, and CD8+), fixed and

permeabilized, then stained for intracellular IFNγ. At 4.5 weeks post infection, there were higher frequencies of both CD4+ and CD8+ T cells producing IFNγ in KSAC + GLA mice relative to PBS controls (Figure 2.4A). However, by ten weeks post infection, the increased frequency of CD4+ IFNγ-producing cells was lost (Figure 2.4B). The amount of CD4+ IFNγ+ T cells were nearly identical in control and immunized mice, while the CD8+ IFNγ+ T cell frequency was reduced in the vaccinated mice relative to PBS treated. These results suggest there is a down-regulation of the CD4+ immune responses in immunized mice between weeks 4.5 and 10.

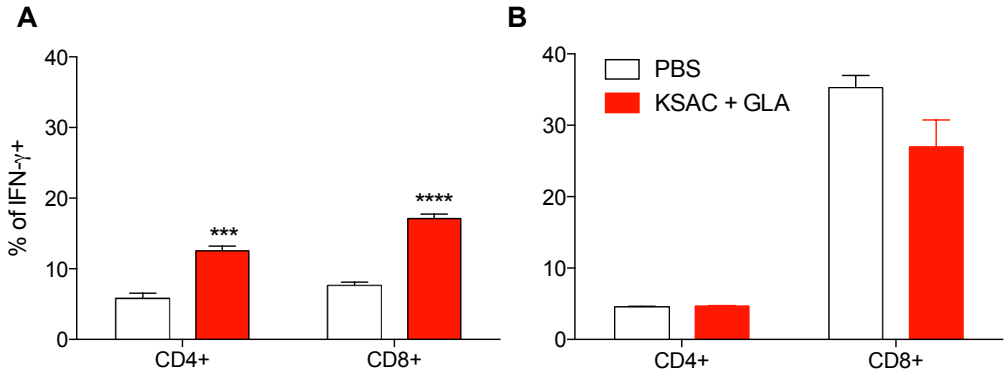

Figure 2.4. IFNγ production in KSAC plus GLA immunized mice.
Production of IFNγ was determined in mice immunized with KSAC plus GLA or PBS at 4.5 (A) and 10 (B) weeks post challenge with *L. amazonensis*. Draining lymph node cells were stimulated *ex vivo* for four hours with PMA, ionomycin, and GolgiPlug, then stained for surface marker expression (CD3, CD4+, and CD8+), fixed and permeablized, then stained for intracellular IFNγ. Statistical analysis was performed using a two-tailed student's t test. (***, $p \leq 0.001$)

Exacerbation of disease is not dependent upon vaccine dosing.

To determine if disease exacerbation is due to the high vaccine dose generating inflammatory responses that are too strong, we reduced the vaccine dose from 10 μg of KSAC + 20 μg of GLA to 5 μg of KSAC + 5 μg of GLA. Mice were immunized at three-week intervals; then challenged with *L. amazonensis* stationary phase promastigotes three weeks following the final boost. We found that disease progression was still

exacerbated in immunized mice through weekly monitoring (Figure 2.5A). This suggests that the vaccine inherently induces aberrant immune responses that do not generate protection against *L. amazonensis*. At 12-weeks post infection, we determined the parasite burden in footpad tissue was higher in the mice receiving KSAC + GLA, although it did not reach statistical significance (Figure 2.5B). Unlike in Figure 2.2, the normalization method applied to determine parasite burden is based on a standard curve of parasite RNA, generating absolute numbers of parasites in the lesion. These results indicate that mice that received the KSAC + GLA vaccine has an increased parasite load in the lesions, although it is not statistically significant due to variation.

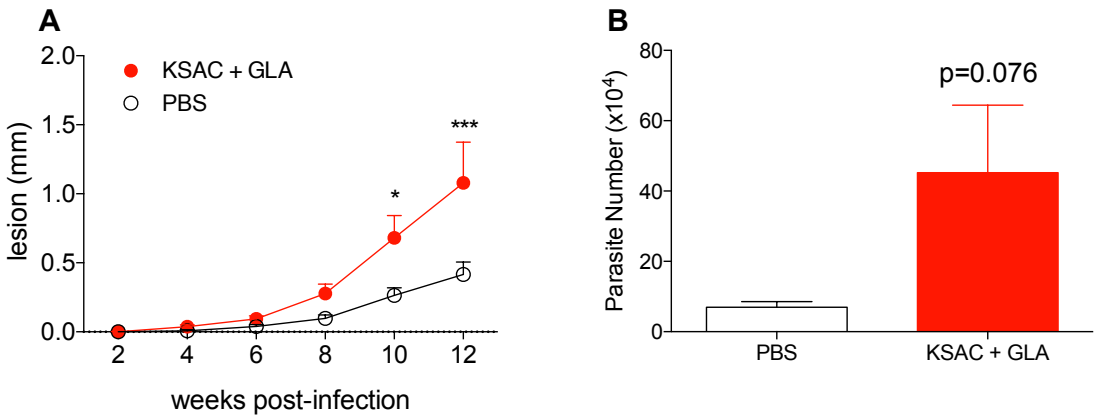

Figure 2.5. KSAC plus GLA exacerbates *L. amazonensis* disease, even at reduced dose. Mice were immunized at three week intervals with lower amounts of vaccine (5 µg KSAC antigen, 5 µg GLA). Mice were challenged at three-weeks following final immunization with $1x10^6$ *L. amazonensis* stationary phase promastigotes. Lesion development was measured weekly (A). Parasite burden at infection site was measured using qRT-PCR at 12 weeks post infection (B). Statistical analysis was performed using a two-tailed student's t test. (*, $p \leq 0.05$; ***, $p \leq 0.001$)

Frequency of multifunctional CD4+ T cells generated during *L. amazonensis* infection due to vaccination with low-dose KSAC plus GLA.

Recent studies performed in *L. major* demonstrate that the frequency of multifunctional CD4+ cells correlates with the level of protection generated by vaccination (71). This trend was demonstrated in KSAC + GLA experiments (48, 77,

78); therefore, we wanted to determine if our *L. amazonensis* model generates these multifunctional CD4+ cells, and their correlation with disease outcomes. Mice immunized with the low dose vaccine regimen were infected with *L. amazonensis* for 12 weeks and the immune responses determined. We isolated popliteal lymph node cells, and stimulated *ex vivo* for four hours with PMA, ionomycin, and GolgiPlug. Cells were stained for FACS analysis; first cells were stained with Live Dye to gate out dead cells, then surface markers (CD3, CD4+) were stained, cells were fixed and permeabilized, and intracellular staining (IFNγ, TNFα, IL-2) was performed. We found a small population of cells that produced all three cytokines (IFNγ, IL-2, and TNFα), however this population appeared slightly reduced in immunized mice (Figure 5.5). Cells that were double positive for IFNγ and TNFα were significantly increased in immunized mice, but remaining populations were unchanged between treatments. These results suggest that any response induced by vaccination is reduced throughout the course of disease. As we have not tested for multifunctional CD4+ T cells only in the context of immunization, we cannot assume this population is up-regulated in our model. Therefore, the vaccine may be exacerbating disease due to a lack of robust multifunctional CD4+ cells. However, we detected the production of Th2- and Th17- type antigen-specific cytokine production by cultured splenocytes. In our model, KSAC + GLA up-regulated the production of Th2-biased cytokines (IL-5, IL-6, and IL-13). We also detected IL-17 production, which would suggest in our model we have a very mixed T cell population (data not shown). This mixed immune response may also be contributing to disease exacerbation. Therefore, it is possible that a vaccine that induces a maintained multifunctional CD4+ cell response would correlate with vaccine-mediated protection.

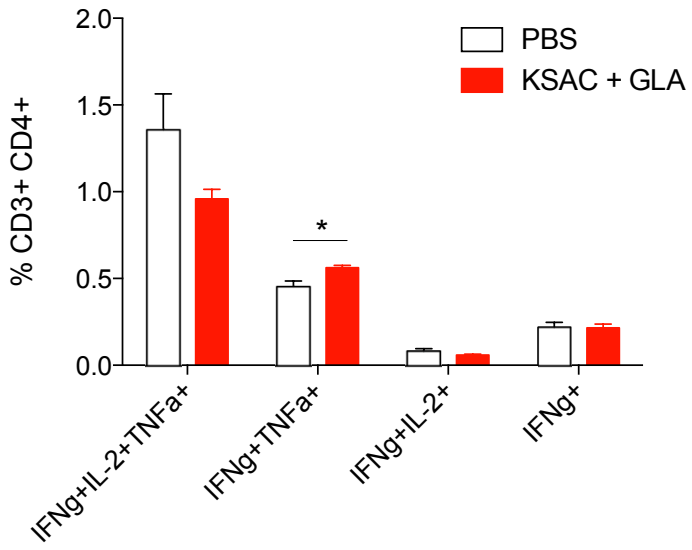

Figure 5.5. CD4+ T cell cytokine production induced by KSAC + GLA during infection. At 12 weeks post-infection, draining lymph node cells were stimulated *ex vivo* and the cytokine production in CD4+ T cells was measured using FACS. Statistical analysis was performed using a two-tailed student's t test. (*, $p \leq 0.05$)

Measurement of immune responses in situ.

To determine the types of immune responses ongoing at the site of infection, we performed qRT-PCR of specific cytokine targets. Interestingly, there was no significant difference in the expression levels of major inflammatory cytokines, including IFNγ, TNFα, IL-6 and IL-10 (Figure 5.6). This would suggest that differential cytokine production is not mediating the phenotype seen in immunized mice. However, we did find an up-regulation of the Type 1 IFN receptor (IFNa/bR) as well as NFkBp50. We previously reported that mice deficient in IFNa/bR had reduced parasite burdens in the lesion, which correlated with increased neutrophil influx relative to wild-type mice (82). We hypothesize that enhanced signaling through the IFNa/bR could exacerbate disease pathology through a reduction in innate immune responses. Additionally, NFkBp50:p50 homodimers are known to be strong repressors of gene signaling (83), and in *L. amazonensis* these homodimers reduce iNOS expression (84).

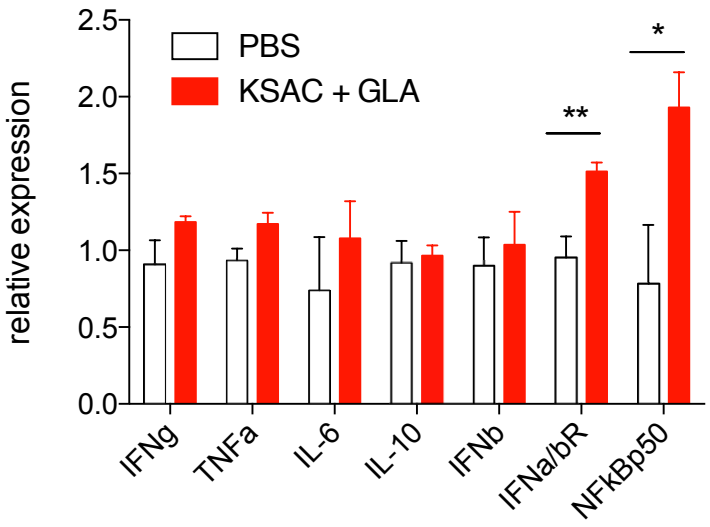

Figure 5.6. Cytokine expression within infected footpad tissues.
Expression of inflammatory markers was determined using qRT-PCR of RNA isolated from infected footpads at 12 weeks post challenge. Statistical analysis was performed using a two-tailed student's t test. (*, $p \leq 0.05$; ***, $p \leq 0.001$)

Overall, we can conclude it is absolutely essential to test candidate vaccines in multiple models of *Leishmania* infection, especially when the immune responses generated are quite diverse (74). In our model, immunization with KSAC + GLA exacerbated disease pathology, regardless of the antigen and adjuvant dosing tested. Despite having IFNγ production early in infection, the cytokine production was not maintained. There was a reduction in the multifunctional CD4+ T cells, which correlated with vaccine efficacy in *L. major* and *L. donovani* infection models (48, 71). Only two molecules appear to correlate with disease phenotype: IFNa/bR and NFkBp50. We do not know the exact mechanism being employed by each, but it could involve innate cell infiltration and reduced macrophage activation (82).

CHAPTER THREE: IMMUNIZATION WITH TWO DIFFERENT TLR AGONISTS

INTRODUCTION

L. braziliensis is a major causative agent of not only LCL, but also MCL in Brazil (5). MCL is characterized by an extremely strong immune response directed towards parasite antigens (5). Due to parasite dissemination, the host immune response can cause extensive tissue damage to nasal and oral cavities (3). This phenotype is correlated to the antigenic potential of *L. braziliensis* proteins, which induce significantly more T cell proliferation than *L. amazonensis* antigens. In fact, *L. amazonensis* antigens induce T cell anergy and apoptosis (85). Prior *L. braziliensis* infection, which is self-limiting and healing in mice, can generate partial protection against *L. amazonensis* challenge, which correlates to increased expansion of antigen-specific CD4+ T cells relative to *L. amazonensis* infection alone (86, 87). Therefore, we have selected soluble *L. braziliesis* antigens as a vaccine antigen.

As an adjuvant, we chose to compare the TLR4-agonist GLA-SE with the TLR9-agonist CpG ODN type A. CpG ODN type A acts directly on pDC to induce Type 1 interferon production, namely IFNa and IFNb, as well as upregulation of costimulatory molecules, including MHC I and II, CD4+0 and CD8+6 (60). Although CpG ODN type A alone was demonstrated to be an effective immunotherapeutic in non human primates, the mechanism that mediates this has not been determined (64, 65). We can hypothesize that the upregulation of type 1 IFN is involved, as the IFNa/bR signaling regulates the expression of iNOS, whose protein product is essential to the intracellular killing capacity of macrophages (88).

GLA in stable emulsion enhances the migration of Langerhans cells, but not dermal DC when injected intradermally. Furthermore, GLA-SE significantly increased the production of pro-inflammatory cytokines, including IL-1b, IL-6, and IL-8. CD4+ T cells activated by DC in mice injected with GLA-SE have increased levels of proliferation, although the IFNγ production was unchanged (89). Adjuvanting a polyprotein vaccine antigen with GLA-SE induced protection against both LCL and VL in a needle challenge model of disease (77, 78). However, in our model of *L. amazonensis*, this vaccine regimen exacerbated disease pathology. It is unclear from our results whether the antigen, adjuvant, or the specific combination is responsible for this phenotype.

In this study, we sought to understand the role of vaccine formulation, specifically adjuvant selection, in vaccine efficacy. CpG and GLA are both being promoted for use in human vaccines against a number of different infections, with GLA currently being used in vaccine trials. Additionally, the use of an unorthodox antigen allows us to determine if the vaccine failure in Chapter 2 was due to antigen selection or the adjuvant being used.

MATERIALS AND METHODS

Mice. Female BALB/c mice were purchased from Taconic Farms (Germantown, NY) and maintained under specific pathogen-free conditions. Mice were 8 - 10 weeks of age when immunizations were initiated. Animal protocols were approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX).

Parasites and Leishmania antigen preparation. *L. amazonensis* (MHOM/RAT/LV78) virulence was maintained by regular passage through BALB/c mice. *L. braziliensis* (MHOM/BR/79/LTB111) was maintained by regular passage through golden Syrian hamsters. Promastigotes were cultured at 26°C in Schneider's *Drosophila* medium (SERVA), pH 7.0 supplemented with 20% FBS and 50 μg/mL

gentamicin (Sigma). To prepare soluble *L. braziliensis* antigens (LbAg), parasites at day 7 of culture were suspended in phosphate-buffered saline, subjected to three freeze-thaw cycles and a 15-minute sonication in an ice bath prior to filter sterilization. Lysates were stored at -80°C. *L. amazonensis* antigens from axenic amastigotes were prepared using the same method. To initiate infection, bulk stationary phase *L. amazonensis* promastigotes of less than three *in vitro* passages were isolated.

Ajuvants. GLA-SE adjuvant was generously provided by Dr. S. Reed (Infectious Disease Research Institute, Seattle, WA). The adjuvant was provided as a ready to use oil-in-water emulsion and was used at a concentration of 5 μg/mouse. Synthetic oligodeoxynucleotides were purchased from Invivogen (San Diego, CA) and were used at a concentration of 25 μg/mouse.

Immunization and challenge of mice. Groups of five mice were immunized with 50 μg of LbAg with or without GLA or CpG ODN type A in a volume of 100 μL. Control groups received phosphate-buffered saline. Three s.c. injections were given at the base of the tail at three-week interval. Three weeks after the last immunization, animals were challenged by s.c. injection of either $1x10^6$ purified metacyclic promastigotes or $3x10^6$ stationary phase *L. amazonensis* promastigotes in right footpad. Lesion development was monitored weekly via footpad measurement with a digital caliper (Mitutoyo, Aurora, IL), and calculated by subtracting the uninfected contralateral footpad measurement from the infected footpad. The total number of parasites was determined using qRT-PCR as described below.

ELISA for specific antibody production. Sera samples was isolated at three-days post final immunization, four-weeks post-infection, and 14-weeks post-infection. Antigen-specific ELISAs were performed for the identification of IgG1 and IgG2a specific for *L. braziliensis* and *L. amazonensis* antigens. 96-well microtiter plates (Nunc) were coated with 50 μL of 50 ng/mL promastigote lysate diluted in carbonate/bicarbonate coating buffer (pH 9.6) and incubated at 4°C overnight. Plates were washed and blocked

overnight at 4°C with 100 μL/well of 10% bovine serum albumin (BSA). Eight two-fold serial dilutions were performed on serum samples beginning at 1:100 in PBS and applied to plates, then incubated overnight at 4°C. Plates were washed, and streptavidin-conjugated rat-anti mouse IgG1 or IgG2a (Abcam) was added at a concentration of 1μg per mL, incubated at room temperature for 1 hour, then detected with streptavidin peroxidase (Abcam) at 1:100 dilution. Plates were detected using TMB substrate, and reaction terminated using 0.6N H2SO4. The optical density was determined at 450 nm.

Cell stimulation. At designated time points, spleens and draining lymph nodes were removed aseptically and cell suspensions were prepared by homogenizing organs through cell strainers (40-μm pore size). Spleen cells were treated with red cell lysis buffer, washed twice, resuspended in cRPMI (containing 10% heat inactivated FBS, 50uM B-mercaptoethanol, sodium pyruvate, pen/strep, geneticin), and plated in 48-well flat bottom plates at 1 x 10^6 cells/well. Cells were stimulated for 72 hours with LbAg (50ug/mL) or anti-CD3 plus recombinant IL-2. Negative controls received medium alone. Supernatants were collected for measurement of cytokine release. Lymph node cells were washed twice and plated in 48-well flat bottom plates at 1 x 10^6 cells/well. Cells were stimulated for 6 hours with PMA/ionomycin in the presence of GolgiPlug (BD Biosciences).

Flow cytometry. All mouse-specific monoclonal antibodies were purchased from BD Biosciences: Pacific Blue- conjugated anti-CD4+, FITC- conjugated anti-CD8+, PercpCy5-conjugated anti-CD25, PE-conjugated FoxP3 and APC-conjugated IFNγ. Briefly, cells were washed, stained for specific surface markers, fixed/permeabilized with a Cytofix/Ctyoperm kit (eBiosciences), and then stained for intracellular molecules. For detection of intracellular cytokines, draining lymph node cells were isolated (1x10^6/mL) were restimulated with phorbol myristate acetate (PMA)/ionomycin in the presence of GolgiPlug (BD BioSciences) for 4 hours. Spleen cells were were cultured with GolgiPlug for the final six hours of 72 hour *in vitro* culturing. Data was collected using a BD

LSRfortessa (BD Biosciences) and analyzed using Flow Jo v9.7.5 (TreeStar, San Carlos, CA).

RNA extraction and RT-PCR analysis. Total RNA was isolated from foot pad lesion of sacrificed mice (RNeasy, Qiagen). Generated cDNA was used in qPCR reactions with primers targeting the single copy number ubiquitin hydrolase gene (forward 5'-AACGTGAACAACTGGATGTGCGTC-3' and reverse 5'-ATGGTACCAAGCTTGACACATGCC-3') [32]. The parasite load per foot was determined based on a standard curve of parasite RNA.

RESULTS AND DISCUSSION

Immunization with LbAg generates distinct antibody titration profiles dependent upon adjuvant.

Prior to infectious challenge, *L. braziliensis* specific IgG1 and IgG2a titers were determined to elucidate the systemic immune responses being generated. Using sandwich ELISA, serum antibody titers were measured from serum of immunized mice at three days after the final boost. These results indicate that immunization with LbAg with or without CpG and GLA generate a robust antibody response. For IgG1 titers (Figure 3.1A), immunization with LbAg combined with CpG or GLA generate comparable antibody levels. IgG1 production in mice immunized with antigen alone was marginally reduced compared to adjuvanted animals. Control mice, which only received PBS, had levels of IgG1 identical to naïve controls (data not shown). Immunization with LbAg combined with CpG generated the highest levels of IgG2a (Figure 3.1B); LbAg or LbAg with GLA generated similar IgG2a production. However, upon dilution, the antigen alone group had slightly higher levels of IgG2a compared to LbAg plus GLA. As seen in IgG1, IgG2a levels were similar to naïve controls (data not shown).

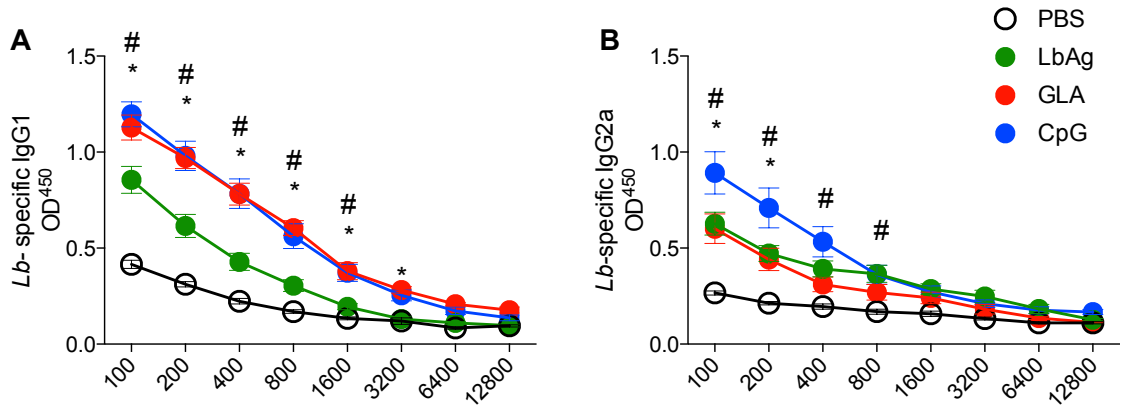

Figure 3.1. *L. braziliensis* specific IgG1 and IgG2a

Mice were immunized s.c. with soluble *L. brazilensis* antigens with or without GLA or CpG ODN. Control groups received PBS. Mice were boosted twice at 3-week intervals. Three days after the last boost, parasite reactive IgG1 (A) and IgG2a (B) isotypes were measured on sera. Two-way ANOVA was performed for statistical analyses (*, significant difference between immunization with GLA and PBS control; #, significant difference between immunization with GpG ODN and PBS control)

Immunization with LbAg plus CpG ODN induces strong Th1-type effector cell responses.

In order to elucidate the cellular responses, splenocytes from immunized mice were stimulated *in vitro* with anti-CD3 and recombinant IL-2 for 72 hours. In the final six hours of stimulation, GolgiPlug was added to induce protein sequestration. CD4+ and CD8+ T cells producing IFNγ and frequency of Foxp3+ T cells were measured using FACS analysis. Mice immunized with LbAg + CpG induced the highest frequency of IFNγ producing cells, with nearly two-fold increases over PBS control in both CD8+ (Figure 3.2A) and CD4+ (Figure 3.2B) cells. LbAg alone and LbAg with GLA had similar frequencies of IFNγ producing cells to the PBS control. Splenocytes cultured from LbAg combined with CpG vaccinated mice also induced a marginal increase in the frequency of Foxp3 Treg cells, while other treatment conditions were largely similar. To further characterize the cellular immunity generated, Il-4 and IL-10 release was measured through ELISA of the supernatant from splenocytes cultured with LbAg for 72 hours.

Only mice immunized with LbAg plus GLA had detectable IL-4 release (Figure 3.2D), indicating an ongoing *Leishmania* specific Th2 immune response with the TLR4-based adjuvant. Levels of IL-10 release were largely similar across immunization groups (Figure 3.2E), suggesting FoxP3+ Treg in LbAg plus CpG are producing different effector molecules.

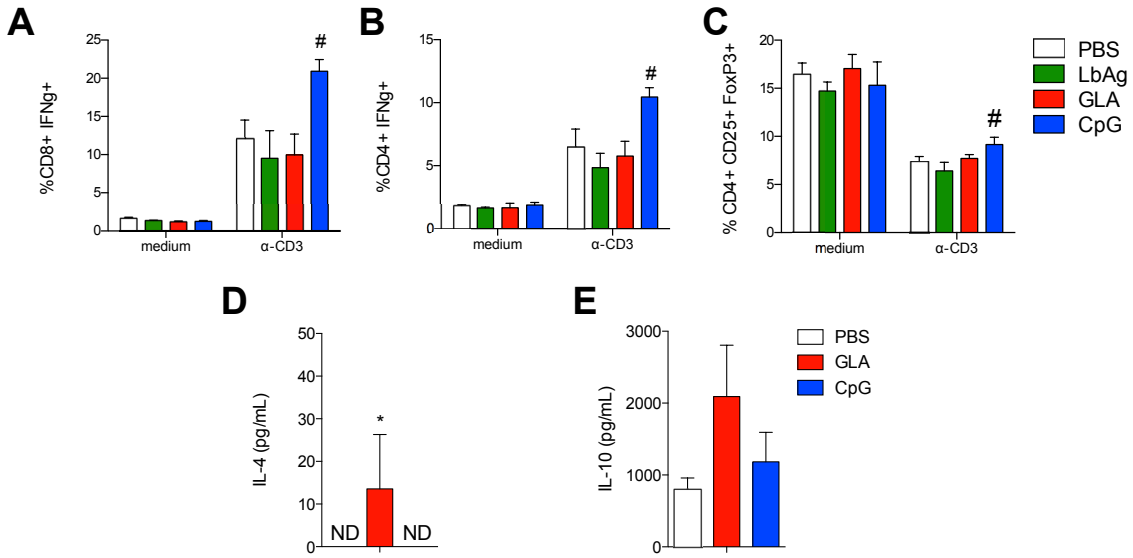

Figure 3.2. Immune responses generated by vaccination regimens.

Three days after the last boost, cells from individual spleens were prepared and stimulated *in vitro*. (A,B,& C) To detect intracellular cytokines, splenocytes were stimulated with α-CD3 plus IL-2 for 72 hours; cells were pulsed with GolgiPlug for the final six hours of incubation. The release of IL-4 (D) and IL-10 (E) was measured in supernatants removed after 72 hours of culture in presence of *L. braziliensis* soluble antigens. (*, significant difference between immunization with GLA and PBS control) ND= not detected.

Partial protection to *L. amazonensis* is generated by immunization with LbAg plus CpG

To test the efficacy of these vaccine regimens, we infected immunized mice with $1x10^5$ purified metacyclic *L. amazonensis* promastigotes three weeks following final boost. Through monitoring the lesion progression, we found that LbAg plus GLA had a

similar disease course to PBS control (Figure 3.3A). However, immunization with LbAg plus CpG generated partial protection from disease, displaying significantly smaller lesions at 14-weeks post challenge. Parasite load in the infected feet was measured through a qRT-PCR-based assay. Again we found similar parasite burdens in PBS and reduced in mice the received LbAg plus GLA (Figure 3.3B), and parasite load was significantly reduced in LbAg plus CpG compared to PBS controls.

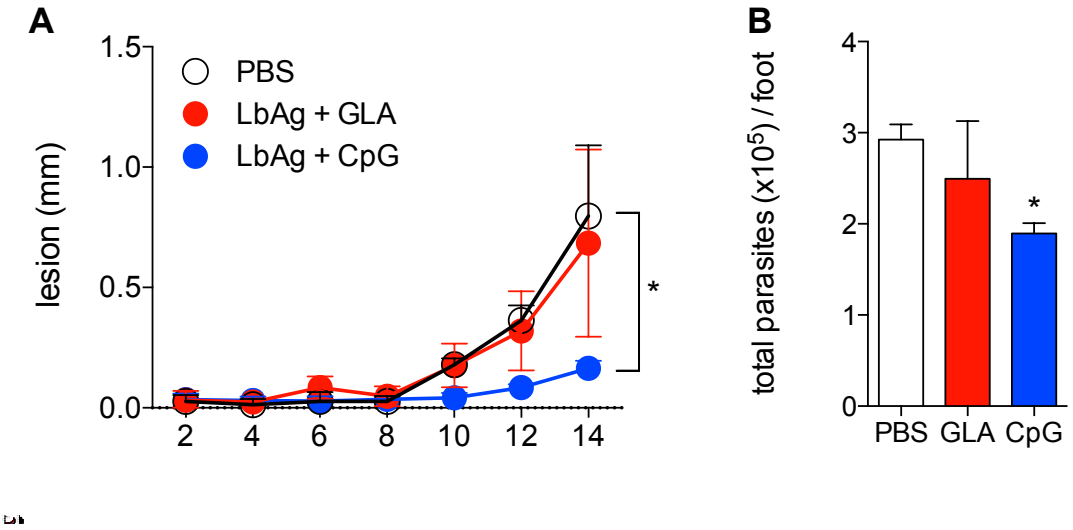

Figure 3.3. Immunization with LbAg plus CpG partially protects against *L. amazonensis*. Three weeks after the last boost, mice were challenged s,c, with $1x10^5$ purified metacyclic phase *L. amazonensis* promastigotes. (A) Lesion progression was monitored weekly using a digital caliper. (B) At 14 weeks post-infection, parasite burden was determined using qRT-PCR. Data are presented as mean ± SEM.

Antibody responses measured in immunized mice after 4-weeks of infection

To determine which cellular population was mediating protection in mice immunized with LbAg plus CpG, we analyzed immune responses at two time points: 4.5 and 10 weeks post infection. Mice were immunized, then three weeks following final

boost, challenged with $3x10^6$ bulk stationary phase *L. amazonensis* promastigotes. This infection dose contrasted from the one used previously due to differences in parasite isolation. As metacyclic promastigotes are only approximately ten percent of total stationary phase promastigotes, our infectious dose was changed to reflect the number in this population.

L. amazonensis specific IgG1 and IgG2a were measured using sandwich ELISA in serum samples isolated from immunized mice at 4 weeks following infection. The highest amount of IgG1 production was found to be in mice immunized with LbAg alone. LbAg plus GLA and LbAg plus CpG induced largely similar IgG1 responses (Figure 3.4A). All immunization conditions produced significantly more IgG1 than the infection control group. IgG2a production was highly similar between all groups measured (Figure 3.4B). However, mice immunized with LbAg plus CpG had a marginally higher amount of IgG2a throughout the dilution series.

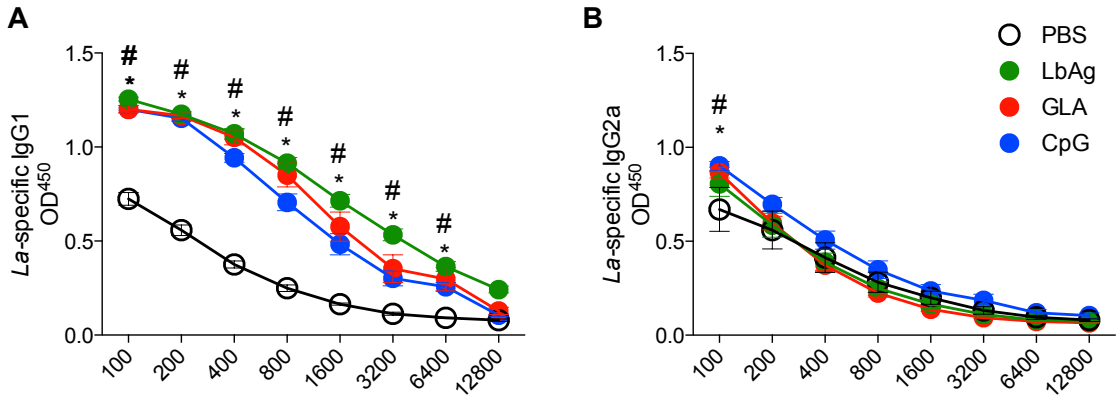

Figure 3.4. Antibody production in immunized mice at 4-weeks following *L. amazonensis* challenge.

Three weeks after the last boost, mice were challenged s,c, with $3x10^6$ bulk stationary phase promastigotes. At 4-weeks post infection, IgG1 (A) and IgG2a (B) titers were measured on sera subjected to eight 2-fold dilutions starting at 1:100. Data are presented as the mean OD of 5 mice ± SEM. Two-way ANOVA was performed for statistical analyses (*, significant difference between immunization with GLA and PBS control; #, significant difference between immunization with GpG ODN and PBS control)

Cell mediated immune responses generated at 4-weeks of infection in immunized mice

To determine the cellular responses, lymph node cells from immunized mice that were infected for four weeks were stimulated *ex vivo* with PMA/ionomycin combined with GolgiPlug. After six hours, frequencies of cell populations were determined using flow cytometry. The popliteal and inguinal lymph nodes were tested separately due to their proximity to the site of infection or the site of immunization, respectively. In the popliteal lymph node, there was no significant difference in CD8+ IFNγ-producing cells (Figure 3.5A). However, in the inguinal lymph node, we detected a significant increase in frequency of IFNγ+ CD8+ cells in mice initially immunized with LbAg + CpG. CD4+ IFNγ+ cell (Figure 3.5B) and Foxp3+ Treg cell (Figure 3.5C) frequency did not have significant differences between immunization conditions by four weeks of infection. These data suggest that the CD8+ IFNγ producing cells are more important to vaccine efficacy than CD4+ T cells against *L. amazonensis*. However, we cannot determine if the lack of CD8+ IFNγ producing cells in the popliteal lymph node is due to reduced migration of DC from the inguinal lymph node in response to vaccination or if the CD8+ responses at the popliteal lymph node are down-regulated by four weeks post infection due to parasite factors.

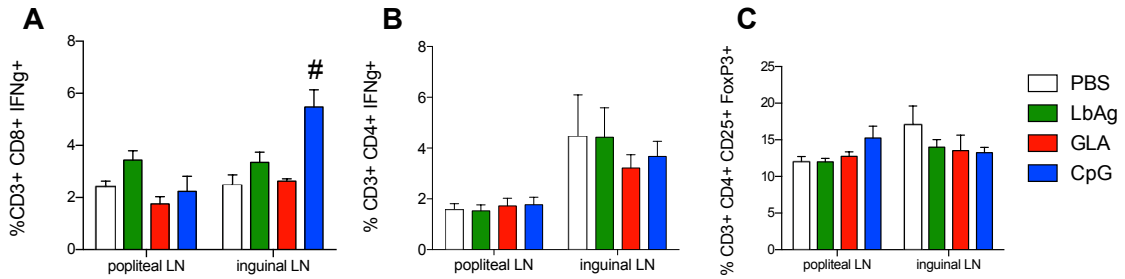

Figure 3.5. Cytokine production in lymph nodes at 4-weeks post *L. amazonensis* challenge.

At 4-weeks post infection, infection draining (popliteal) and immunization draining (inguinal) lymph node cells were isolated. (A, B, & C) For intracellular staining, lymph node cells were stimulated for 6 hours with PMA, ionomycin, and GolgiPlug. Data shown are the mean of five biological replicates. Statistical analysis was performed using ANOVA. (#, $p \leq 0.05$)

High dose parasite challenge abrogates vaccine-mediated protection.

Based upon the change in parasite isolation procedure, we infected immunized mice with a greater number of total parasites. However, we discovered our isolation procedure for purified metacyclic promastigotes reduced the ability of *Leishmania* to infect bone marrow derived macrophages (data not shown). As a result, we performed this second experiment with a much higher infectious challenge than previously. Rather than $1x10^5$ purified metacyclic promastigotes, we challenged with $3x10^6$ bulk stationary phase promastigotes. While monitoring infection, we measured the loss of protection that was previously measured with LbAg plus CpG (figure 3.6A). This treatment had lesions of identical size to the PBS control. In fact, with higher parasite doses, LbAg alone and LbAg plus GLA exacerbated disease relative to control (figure 3.6A). Determination of the parasite load in the infected footpad was done using qRT-PCR, as described previously (80). We found that the parasite load was very similar across treatment groups, once sample differences were taken into account (Figure 3.6B). As the overall parasite load in the footpad was unchanged between groups, disease outcomes may be due to differences in immunopathology.

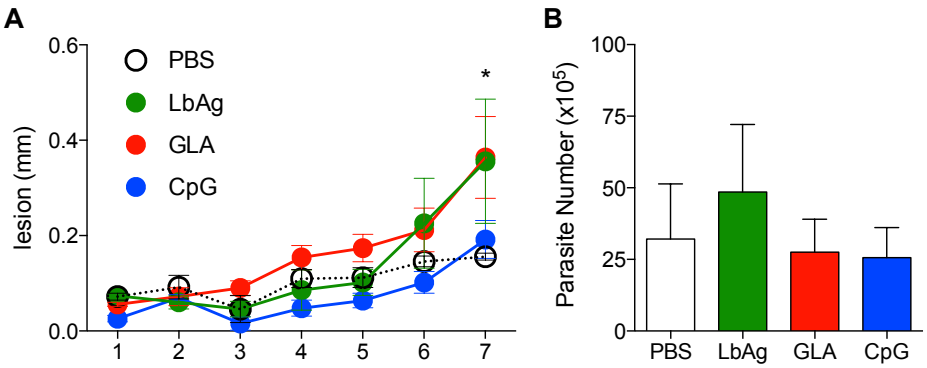

Figure 3.6. Vaccine-mediated protection is dependent upon parasite challenge dose. Mice were immunized with LbAg, LbAg plus GLA, LbAg plus CpG, or PBS as a control. Three weeks following final boost, mice were challenged with a high dose of bulk stationary phase *L. amazonensis* promastigotes ($3X10^6$/footpad). Lesion development was monitored weekly (A) and parasite load determined seven weeks post infection (B).

High dose challenge induces differential antibody production due to infection

Antibody titers were measured at seven weeks post challenge with a dose of $3x10^6$ infectious *L. amazonensis*. *L. amazonensis*-specific IgG1 titration was similar between PBS controls and the vaccines with adjuvants (Figure 3.7A). Of note, mice that were immunized with LbAg alone had higher levels of parasite-specific IgG1. This would suggest that LbAg immunized mice favored a Th2-type immune response during active infection. *L. amazonensis*-specific IgG2a was similar between PBS controls, LbAg alone, and mice immunized with LbAg plus CpG (Figure 3.7B). The differences in IgG1 levels would correlate to PBS and LbAg plus CpG having a less biased Th2 response. However, LbAg alone mice have a strong Th2 bias in their immune responses. Mice that received LbAg plus GLA, however, have a reduction in parasite specific IgG2a. This suggests that the mice generated immune responses favoring Th2 polarization. Furthermore, these data indicate that the exacerbated phenotype can be due to both a polarized Th2 response (GLA) or mixed immune responses that favor Th2 (LbAg).

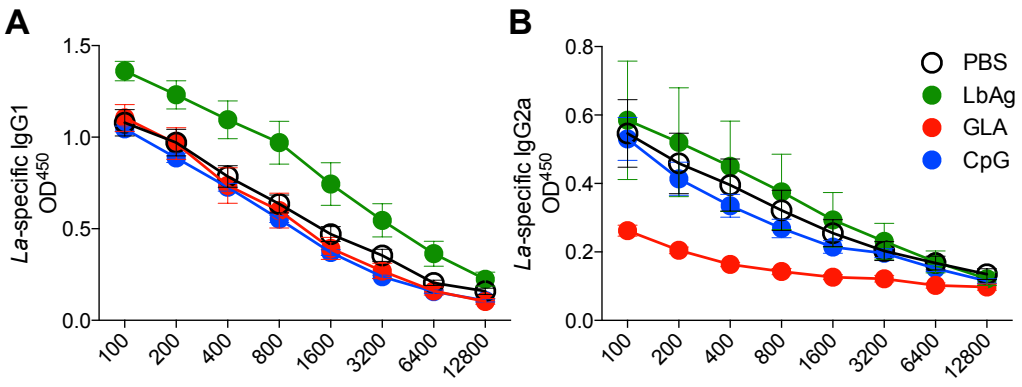

Figure 3.7. Antibody production profiles in immunized mice at seven-weeks post infection.

Serum antibody production of IgG1 (A) and IgG2a (B) was measured in immunized mice receiving LbAg, LbAg plus GLA, LbAg plus CpG, and PBS controls. Serum was isolated at seven weeks post infection and titration determined using sandwich ELISA.

Cellular immune responses detected at seven weeks post-infection

At seven weeks post infection, draining lymph nodes were isolated and the cellular populations determined using *ex vivo* stimulation and FACS analysis. The popliteal lymph node was used due to its proximity to the site of infection, while inguinal is the draining lymph node of the immunization. At four weeks post infection, we detected an increase in the CD8+ IFNγ-producing cells. However, by seven weeks post infection, IFNγ production by CD4+ and CD8+ cells is largely similar (Figure 3.8 A, B). Additionally, we detected a similar frequency in Foxp3+ Treg cells (Figure 3.8C). These results suggest that overall immune responses are regulated by the infection, and any phenotype induced by immunization is undetectable. Furthermore, these results do not indicate that a specific cell population is associated with disease exacerbation.

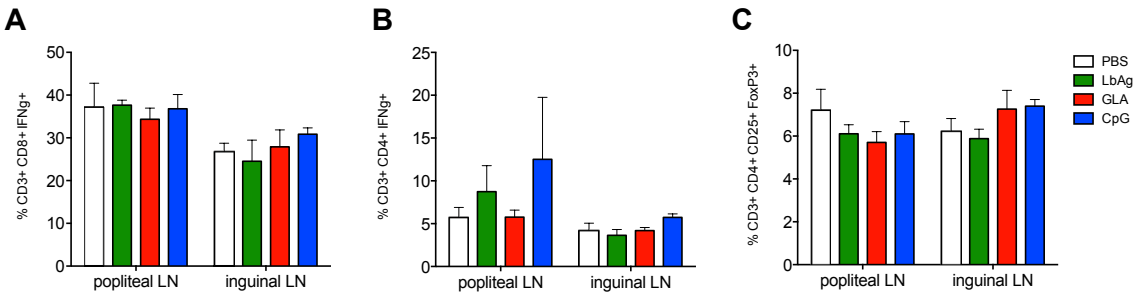

Figure 3.8. Cellular immune responses are down-regulated by seven weeks post infection. At seven weeks post infection, draining lymph node cells were isolated and the cellular populations determined using *ex vivo* stimulation and FACS analysis. Intracellular production of IFNγ was determined in CD8+ (A) and CD4+ (B) cells, as well as frequency of FoxP3+ Treg cells (C).

Our results indicate that there are clear differences in vaccine efficacy based on the adjuvant selected in addition to challenge dose used. We determined that TLR9-based adjuvants (CpG ODN) are able to generate greater protection than a TLR4-based adjuvant (GLA). LbAg plus CpG ODN induced a Th1-biased immune response, with high frequencies of IFNγ producing T cells, and significantly reduced lesion sizes in low dose parasite challenge. At the high challenge dose, LbAg plus CpG did not exacerbate disease, but had a mixed Th1/Th2 immune response. Our results suggest that testing physiologically relevant parasite doses is essential to determining vaccine efficacy, and that higher doses of parasite may lead to the exclusion of relevant vaccine candidates.

DISCUSSION

Our purpose for this study was to determine the importance of vaccine formulation on protection against *L. amazonensis* in our mouse infection model. We studied two different vaccine formulations: varying doses of recombinant polyprotein antigen with TLR4 agonist and soluble *Leishmania* proteins with TLR4 or TLR9 agonists. Overall, we sought to understand the immune responses required for protection in *L. amazonensis*, and predicted they would be similar to *L. major*. However, we found that a vaccine regimen, which generates good protection against *L. major* did not reduce disease pathology in *L. amazonensis*. We found this vaccine regimen increased pathology, suggesting there are different correlates of protection between species. Furthermore, we found distinct patterns in immune activation and vaccine-mediated protection between the types of TLR agonist used, suggesting adjuvant selection plays an important role in generating protection.

Vaccine-mediated protection or pathogenesis

Immunization with KSAC plus GLA generates significant protection against infection against *L. major* (48, 78). This protection is measured through reduction in both the lesion size and parasite load in the infected tissues (48). Additionally, this protection correlated with the induction of multifunctional CD4+ T cells, as previously reported to correlate with protection in *L. major* (48, 71). Furthermore, these multifunctional CD4+ T cells were still upregulated and correlating to protection against needle-challenged mice at four weeks post infection (77). However, challenging mice via sand fly bite abrogated this protection, but still generated a robust IFNγ response (77). Conversely, these mice had an increase in IL-17 secretion and neutrophil influx,

suggesting that the immune response generated was mixed and insufficient to protect (77). In our *L. amazonensis* model, there was an exacerbation of disease in KSAC plus GLA immunized mice. We measured a significant increase in lesion size relative to control, and there was a trend of increased parasite burden in the infected tissue. Using a lower-dose vaccine regimen, we did not detect multifunctional CD4+ T cells at the time of sacrifice (12 weeks post infection). There are several possible explanations to explain this result.

Firstly, in our mouse model, the vaccine regimen may not be inducing multifunctional CD4+ T cells as robustly as the *L. major* models. It is possible the differences in mouse facility and provider may alter basal immune responses sufficiently to prevent their induction. In Goto *et al*., mice were purchased from Charles River Laboratories, while Peters *et al* used mice from The Jackson Laboratory (48, 77). Our laboratory uses mice purchased from Taconic. It is known that the intestinal microbiota can vary substantially between providers, as well as the animal facility in which the mice are housed (90). Specifically, Ivanov *et al*. demonstrated that Taconic mice contain more segmented filamentous bacteria, which led to a Th17-skewing of their immune responses (90). Therefore, it is possible the Taconic mice we used were unable to induce a robust CD4+ T cell response. One way to test this would be to repeat the studies in Jackson mice, or from another producer.

Secondly, the vaccine could have generated the robust multifunctional CD4+ T cells response, but due specifically to *L. amazonensis* infection, the immune responses were insufficient to protect at12 weeks post-challenge. Overall, the purpose of a multifunctional CD4+ T cell response is to produce significantly more IFNγ than single producing effector cells (71). However, *in vitro, L. amazonensis* was shown to not only survive this IFNγ-rich environment, but that IFNγ can actually enhance intracellular amastigote replication in conjunction with other immune factors (91). Therefore, even in situations where the IFNγ secretion is high, it is possible that *L. amazonesis* will not be

killed by macrophages. Additionally, immune modulation by *Leishmania* amastigotes is well characterized (92). Specifically, alterations to NF-kB signaling in *L. amazonensis* infected macrophages results in less IFNγ-responsive transcription factors entering the nucleus (93). This reduced signaling prevents RNS production, while promoting arginase transcription, permitting parasite growth (93).

Additionally, we cannot rule out the possibility that the proteins in the KSAC vaccine generate varying degrees of protective immune responses during infection with different *Leishmania* species. All proteins were reported to be immunogens in Old World species (36, 47, 94-96), but had limited study in New World. Immunization with A2 alone induced a mixed Th1/Th2 immune response, characterized by high levels of both IFNγ and IL-4, which was protective against *L. donovani* (97). However, *L. amazonensis* infection induces a similar mixed immune response, and uses it to promote its own growth (92). Additionally, there was a vaccine-mediated induction of parasite specific IgG1 and IgG2a, further indicating a mixed immune response (97). We previously demonstrated that antibody production can play a pathogenic role in *L. amazonensis* via promotion of T cell activation and cellular recruitment, increasing the amount of immunopathology (98). Therefore, the high titers of mixed antibody response generated in our model could promote lesion development, and partially explain the disease exacerbation.

Furthermore, the differences of expression *in situ* of specific markers also suggests at a mechanism. We detected increased levels of IFNa/bR and NF-kBp50 subunit. We previously demonstrated that a deficiency in type I interferon signaling through IFNa/bR results in reduced lesion pathology and a reduction in parasite load. We detected a significant increase in neutrophil influx following parasite injection, and the neutrophils promoted parasite killing (82). Extrapolating from these results, we can predict that our KSAC plus GLA immunized mice had more type 1 interferon signaling through its increase in transcription of the receptor, which could result in less neutrophil

recruitment and a reduced capacity to kill *Leishmania* parasites. NF-kBp50 is one NF-kB subunit that does not have a transactivation domain, and only possesses a repressor domain. By forming heterodimers with other NF-kB subunits, NF-kBp50 is able to assist in transcription activation. However, NF-kBp50 can also form homodimers, which only serve a repressive function (83, 99, 100). *L. amazonensis*, promotes a conversion from the classically activating NF-kBp65/p50 heterodimer into the repressive NF-kBp50/p50 homodimer, another example of cell signaling regulation induced by infection. Additionally, it was determined that this homodimer is acting on the iNOS promoter to prevent gene transcription (84). Therefore, through reducing the amount of RNS, infected macrophages will be unable to kill intracellular parasites.

The current study did not demonstrate a mechanism for the exacerbated disease, but there is data to support multiple different pathways. Overall, we could conclude that the KSAC vaccine should not be explored for New World species of *Leishmania*, due to the potential of disease exacerbation. Further studies can be done using different polyprotein antigens with GLA or studying KSAC with a different vaccine adjuvant. It is possible that different adjuvanting will permit the KSAC polyprotein to protect against infection with New World species.

Comparison of adjuvants: GLA versus CpG

In a side-by-side comparison, we found that CpG ODN type A with the antigen selected is better able to confer protection in immunized mice against *L. amazonensis* infection than the TLR4-based adjuvant GLA. In a study performed immediately following vaccination, we found that protection correlated with IFNγ-producing effector T cells, both CD4+ and CD8+. At four weeks post-infection, we found only the IFNγ production by CD8+ T cells maintained at the lymph node proximal to immunization. These results suggest that the vaccine containing CpG ODN is inducing a prolonged effector CD8+ T cell response.

These results are consistent with reported mechanisms of vaccine-mediated protection in *L. amazonensis*. An increased frequency of CD4+ and CD8+ T cells producing IFNγ were found at the site of infection in protected mice. Additionally, perforin was essential for mediating vaccine-induced protection (72). In our study, we did not detect a prolonged CD4+ T cell response, and the duration of the CD8+ response was unclear. At four weeks post challenge, we detected a significant increase in CD8+ IFNγ-producing cells only in the inguinal lymph node, which received the bulk of the antigen from the immunization. In the popliteal lymph node, proximal to infection, we did not detect an increase in CD8+ cells. However, by ten weeks post-challenge, CD8+ T cell IFNγ production was identical in both lymph nodes in the group that received CpG ODN as the adjuvant. This suggests two possible mechanisms. First, CD8+ T cell responses were down modulated by *L. amazonensis* infection through multiple different mechanisms, especially at the DC level (92). In this situation, CD4+ and CD8+ T cells would not receive activation signals from DC, and therefore would not expand in response to antigen. Second, that the DC are unable to migrate from the inguinal lymph node to the popliteal, and carry out their functions at the site of infection. If this is occurring, we expect less CD4+ and CD8+ T cell activation. However, as mice with increased CD8+ T cell IFNγ production are generating protection from infection, it is less likely that the cells are unable to migrate. Another limitation of our study is that we did not investigate additional effector functions beyond IFNγ. In the future, perforin and granzyme secretion should also be measured, in order to fully formulate the correlates of protection.

This strong Th1 response can be enhanced by the inclusion of CpG ODN in the vaccine formulation through differing signaling mechanisms relative to GLA. TLR4-based agonists have been thoroughly explored as vaccine adjuvants due to the unique signaling mechanism employed. TLR4 engagement is able to signal through two different adaptor molecules, inducing very robust immune responses. TRIF signaling,

from TLR4 receptors in intracellular organelles, induces the production of type 1 IFN, especially IFNb. Surface bound TLR4 signals through MyD88, which activates N-FkB and subsequent pro-inflammatory cytokine production. However, it is known that the signaling pathway activated during immunization is also dependent upon antigen (101). Although we do not know which signaling pathway is being induced in our immunization, we can assume it is helping to upregulate IL-4 production in mice that received GLA.

TLR9-based agonists are being developed as immunotherapeutics and vaccine adjuvants due to their ability to stimulate strong Th1 immune responses. TLR9 signals exclusively through MyD88, inducing pro-inflammatory cytokine production (101). The immune response generated is dependent upon the type of CpG ODN being used. The CpG ODN used in our study, Type A, strongly induce NK cell activation as well as IFNa production by plasmacytoid DC (101). In addition to IFNa, pDC exposed to CpG will experience a synergism between CD4+0L and the CpG ODN to upregulate IL-12, which is essential to generating Th1-type immune responses (102). TLR9 is also present on NK cells, and CpG will induce NK cell activation, resulting in the production of IFNγ and TNFα (103). Between upregulating IL-12, IFNγ, and TNFα, mice immunized with CpG are primed for robust Th1-type responses, which are required for the protective phenotype observed.

Interestingly, our protection was actually dependent upon the number of parasites used in the challenge. Our original study used purified metacyclic promastigotes, which we determined have a reduced ability to infect macrophages. Due to this, we do not know how many infectious parasites were inoculated into mice, but we can surmise it is likely several logs less than intended. This study had similar disease development between mice immunized with antigen plus GLA and PBS controls, but the CpG adjuvanted mice were significantly protected. In a second study, we chose to use a higher number of bulk stationary phase promastigotes, which we know are able to infect

macrophages. This time the protection mediated by vaccination with antigen plus CpG was abrogated, and lesions were similar to PBS control. However, mice receiving antigen alone or with GLA had larger lesions throughout the course of disease. Studies are ongoing to determine if the protection found in the pilot study can be repeated using a lower challenge dose of stationary phase promastigotes.

Overall, these results suggest that several factors can contribute to vaccine-mediated protection. Firstly, adjuvant choice is essential to mediating protection, and that TLR9-based adjuvants are more effective in our model. However, challenge dose can affect vaccine efficacy; therefore, it is important to use biologically relevant doses rather than extremely high doses.

Concluding Remarks

This work sought to determine the importance of vaccine formulation, both the antigens selected as well as adjuvant used, on mediating protection against *L. amazonensis*. In our first study, we found that a promising vaccine can actually exacerbate lesion development, regardless of the dose used. Although we have not determined the mechanism, it is due to both enhanced parasite replication as well as immunopathology. In our second study, we demonstrated that adjuvant selection can be critical in mediating protection. We have evidence supporting an essential role for CD8+ T cells in protection.

VITA

BIOGRAPHICAL:

Christie Hay was born in Allentown, PA on April 5, 1987 to Brenda Edwards (Crush) and Claude Hay. Following graduation from Whitehall High School, she attended Cedar Crest College, studying Genetic Engineering. After one year of working as a research assistant at Duke University, she was accepted into the Microbiology and Immunology PhD program at University of Texas Medical Branch. Christie earned several awards, including a McLaughlin Predoctoral Fellowship, Edith and Robert Zinn Presidental Scholarship, Sealy Center for Vaccine Development travel award and the Alan C. Brasier award for mentor of the year in the Ball High School Bench Tutorials program.

RESEARCH EXPERIENCE:

3/2011 – 7/2014, UTMB, Department of Microbiology and Immunology
Thesis work was testing and developing new vaccine strategies for New World species *L. amazonensis*. Collaboration with IDRI allows the testing of KSAC polyprotein antigen with TLR4- based adjuvants *in vivo*. Comparison of TLR4- and TLR9-based adjuvant with soluble *L. braziliensis* antigens *in vivo*.
1/2007 - 5/2009, Cedar Crest College, Allentown, PA 18104
Independent research into the roles of four putative alpha-1,3-glucanases in *C. neoformans*, predicted to play a role in virulence.
6/2007 - 8/2007, Cedar Crest College, Allentown, PA 18104
Ten-week internship with Dr. Richard Kliman that focused on design of DNA sequencing primers for *C. neoformans* to determine codon bias.

PROFESSIONAL WORK HISTORY:

7/2009 - 7/2010, Duke University Medical Center, Durham, NC 27710
I assisted ongoing research in the lab focusing on the role of transcription factors on the virulence of *Cryptococcus neoformans*.

In February 2010 paper, I conducted intracellular growth experiments using macrophage cell line.

In June 2010 paper, I developed the cell lines used in *Saccharomyces cerevisiae* experiments.

5/2008 - 7/2009, Deibel Laboratories, Bethlehem, PA 18018
The lab performs quality control testing for food and environmental samples.

AWARDS & HONORS:

9/2013, Awarded Edith and Robert Zinn Presidental Scholarship.
7/2013, Awarded McLaughlin Predoctoral Fellowship; 9/1/2013 – 8/31/2015.
5/2013, Robert C. Brasier Award for outstanding performance as a Bench Tutorials: Scientific Research and Design Mentor; mentored Rachel Mathers 9/2012 - 6/2013.
7/2012, UTMB Sealy Center for Vaccine Development Travel Award to attend the Keystone Symposium entitled "Immunological Mechanisms of Vaccination" in Ottawa, Canada in 12/2012
8/2008, Pennsylvania Academy of Sciences Undergraduate Research Grant ($500)

PUBLICATIONS:

Henard C., E.D. Carlsen, **C. Hay**, P. Kima, L. Soong. *Leishmania amazonensis* amastigotes highly express a tryparedoxin peroxidase (TXNPx) isoform that increases parasite resistance to macrophage antimicrobial defenses and fosters parasite virulence. *Manuscript* at R3 in PLoS Pathogens.

Carlsen, E.D., **C. Hay**, C. Henard, P Vsevolod, N. Garg, L. Soong. *Leishmania amazonensis* amastigotes trigger neutrophil activation, but resist neutrophil microbicidal mechanisms. Infection and Immunity. 2013 August; doi: 10.1128/IAI.00770-13

O'Meara, T. R., **C. Hay**, M. S. Price, S. Giles, and J. A. Alspaugh. *Cryptococcus neoformans* Histone Acetyltransferase Gcn5 Regulates Fungal Adaptation to the Host. Eukaryotic Cell. 2010 August; 9(8): 1193–1202.

O'Meara, T. R., D. Norton, M. S. Price, **C. Hay**, M. F. Clements, C. B. Nichols, and J. A. Alspaugh. Interaction of *Cryptococcus neoformans* Rim101 and Protein Kinase A Regulates Capsule. PLoS Pathogens. 2010 February; 6(2): e1000776

ABSTRACTS:

Hay, C., ED Carlsen, HL De Matos Guedes, Y Liang, CA Henard, L Soong. "Efficacy of TLR-based adjuvants in vaccine against Leishmania amazonensis in mice" 2014 IHII/McLaughlin Colloquium, April 2014, Galveston, TX, poster

Hay, C., ED Carlsen, C Henard, H Nakhasi, and L Soong. "Leishmania Vaccine Development and Protection" 2013 IHII/McLaughlin Colloquium, April 2013, Galveston, TX, poster.

Hay, C., ED Carlsen, C Henard, H Nakhasi, and L Soong. "Leishmania Vaccine Development and Protection" Immunological Mechanisms of Vaccination, December 2012, Ottawa, Ontario, Canada, poster.

Hay, C., Y Chen, ED Carlsen, and L Soong. "Identification of novel *Leishmania braziliensis* antigens using an immunoinformatics approach" 2012 IHII/McLaughlin Colloquium, April 2012, Galveston, TX, poster.

Hay, C., Y Chen, ED Carlsen, and L Soong. "Identification of *L. braziliensis* novel antigens and vaccine candidates using a bioinformatics approach" The Sealy Center for Vaccine Development: The Changing Landscape of Vaccine Development: Vaccines for Chronic Diseases, February 2012, Galveston, TX, poster

.

Reese, A. J., **C. Hay**, L. Horvath, and S. Rennoll, "Investigation of four alpha(1,3)glucanases from *Cryptococcus neoformans"* 109th American Society for Microbiology meeting, Philadelphia, May 2009, poster.

Rennoll, S., **C. Hay**, L. Horvath, and A. J. Reese, "Investigation of four alpha(1,3) glucanases from *Cryptococcus neoformans,"* 85th Pennsylvania Academy of Sciences, March 2009, Camp Hill, PA, poster.

Resling, J., **C. Hay**, and A. J. Reese, "Using RNA interference and over-expression methods to study the role of alpha(1,3)glucanase in the opportunistic fungal pathogen *Cryptococcus neoformans,"* 84th Pennsylvania Academy of Sciences, April 2008, Harrisburg PA, poster.

Permanent address: 425 Sixth Street, Whitehall, PA, 18052.

This dissertation was typed by Christie Hay.

WORKS CITED

1. Kaye, P., and P. Scott. 2011. Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol* 9: 604-615.
2. Alvar, J., I. D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin, M. den Boer, and W. L. C. Team. 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7: e35671.
3. Reithinger, R., J. C. Dujardin, H. Louzir, C. Pirmez, B. Alexander, and S. Brooker. 2007. Cutaneous leishmaniasis. *Lancet Infect Dis* 7: 581-596.
4. Murray, H. W., J. D. Berman, C. R. Davies, and N. G. Saravia. 2005. Advances in leishmaniasis. *Lancet* 366: 1561-1577.
5. Silveira, F. T., R. Lainson, C. M. De Castro Gomes, M. D. Laurenti, and C. E. Corbett. 2009. Immunopathogenic competences of Leishmania (V.) braziliensis and L. (L.) amazonensis in American cutaneous leishmaniasis. *Parasite Immunol* 31: 423-431.
6. Chappuis, F., S. Sundar, A. Hailu, H. Ghalib, S. Rijal, R. W. Peeling, J. Alvar, and M. Boelaert. 2007. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* 5: 873-882.
7. P, C., D. P, G. MG, J. J, K. A, M. A, M. P, N. MB, S. A, and S. CJ. 2006. Tropical Diseases Lacking Adequate Control Measures: Dengue, Leishmaniasis, and African Trypanosomiasis. In *Disease Control Priorities in Developing Countries*, Second Edition ed. J. DT, B. JG, M. AR, and e. al., eds, Washington D.C.
8. Wolday, D., N. Berhe, H. Akuffo, P. Desjeux, and S. Britton. 2001. Emerging Leishmania/HIV co-infection in Africa. *Med Microbiol Immunol* 190: 65-67.
9. Alvar, J., P. Aparicio, A. Aseffa, M. Den Boer, C. Cañavate, J. P. Dedet, L. Gradoni, R. Ter Horst, R. López-Vélez, and J. Moreno. 2008. The relationship between leishmaniasis and AIDS: the second 10 years. *Clin Microbiol Rev* 21: 334-359, table of contents.
10. Boggild, A. K., A. P. Ramos, D. Espinosa, B. M. Valencia, N. Veland, C. Miranda-Verastegui, J. Arevalo, D. E. Low, and A. Llanos-Cuentas. 2010. Clinical and demographic stratification of test performance: a pooled analysis of five laboratory diagnostic methods for American cutaneous leishmaniasis. *Am J Trop Med Hyg* 83: 345-350.
11. Croft, S. L., and P. Olliaro. 2011. Leishmaniasis chemotherapy--challenges and opportunities. *Clin Microbiol Infect* 17: 1478-1483.
12. Matlashewski, G., B. Arana, A. Kroeger, S. Battacharya, S. Sundar, P. Das, P. K. Sinha, S. Rijal, D. Mondal, D. Zilberstein, and J. Alvar. 2011. Visceral leishmaniasis: elimination with existing interventions. *Lancet Infect Dis* 11: 322-325.
13. Russell, D. G., S. Xu, and P. Chakraborty. 1992. Intracellular trafficking and the parasitophorous vacuole of Leishmania mexicana-infected macrophages. *J Cell Sci* 103 (Pt 4): 1193-1210.

14. Schlein, Y., R. L. Jacobson, and G. Messer. 1992. Leishmania infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. *Proc Natl Acad Sci U S A* 89: 9944-9948.
15. Sharma, U., and S. Singh. 2008. Insect vectors of Leishmania: distribution, physiology and their control. *J Vector Borne Dis* 45: 255-272.
16. Reithinger, R., M. Mohsen, and T. Leslie. 2010. Risk factors for anthroponotic cutaneous Leishmaniasis at the household level in Kabul, Afghanistan. *PLoS Negl Trop Dis* 4: e639.
17. Stockdale, L., and R. Newton. 2013. A review of preventative methods against human leishmaniasis infection. *PLoS Negl Trop Dis* 7: e2278.
18. Kumar, R., and C. Engwerda. 2014. Vaccines to prevent leishmaniasis. *Clinical and Translational Immunology* 3.
19. Otranto, D., and F. Dantas-Torres. 2013. The prevention of canine leishmaniasis and its impact on public health. *Trends Parasitol* 29: 339-345.
20. Bogdan, C., A. Gessner, W. Solbach, and M. Röllinghoff. 1996. Invasion, control and persistence of Leishmania parasites. *Curr Opin Immunol* 8: 517-525.
21. Murray, P. J., and T. A. Wynn. 2011. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 11: 723-737.
22. Murray, P. J., and T. A. Wynn. 2011. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 11: 723-737.
23. Liu, D., and J. E. Uzonna. 2012. The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. *Front Cell Infect Microbiol* 2: 83.
24. Biswas, S. K., and A. Mantovani. 2010. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* 11: 889-896.
25. Liu, D., and J. E. Uzonna. 2012. The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. *Frontiers in Cellular and Infection Immunology* 2: 8.
26. Oliveira, F., P. G. Lawyer, S. Kamhawi, and J. G. Valenzuela. 2008. Immunity to distinct sand fly salivary proteins primes the anti-Leishmania immune response towards protection or exacerbation of disease. *PLoS Negl Trop Dis* 2: e226.
27. Gomes, R., and F. Oliveira. 2012. The immune response to sand fly salivary proteins and its influence on leishmania immunity. *Front Immunol* 3: 110.
28. Morris, R. V., C. B. Shoemaker, J. R. David, G. C. Lanzaro, and R. G. Titus. 2001. Sandfly maxadilan exacerbates infection with Leishmania major and vaccinating against it protects against L. major infection. *J Immunol* 167: 5226-5230.
29. Chagas, A. C., F. Oliveira, A. Debrabant, J. G. Valenzuela, J. M. Ribeiro, and E. Calvo. 2014. Lundep, a sand fly salivary endonuclease increases Leishmania parasite survival in neutrophils and inhibits XIIa contact activation in human plasma. *PLoS Pathog* 10: e1003923.
30. Peters, N. C., and D. L. Sacks. 2009. The impact of vector-mediated neutrophil recruitment on cutaneous leishmaniasis. *Cell Microbiol* 11: 1290-1296.
31. Guimarães-Costa, A. B., M. T. Nascimento, G. S. Froment, R. P. Soares, F. N. Morgado, F. Conceição-Silva, and E. M. Saraiva. 2009. Leishmania amazonensis

promastigotes induce and are killed by neutrophil extracellular traps. *Proc Natl Acad Sci U S A* 106: 6748-6753.
32. Soong, L. 2008. Modulation of dendritic cell function by Leishmania parasites. *J Immunol* 180: 4355-4360.
33. Baldwin, T., S. Henri, J. Curtis, M. O'Keeffe, D. Vremec, K. Shortman, and E. Handman. 2004. Dendritic cell populations in Leishmania major-infected skin and draining lymph nodes. *Infect Immun* 72: 1991-2001.
34. Brandonisio, O., R. Spinelli, and M. Pepe. 2004. Dendritic cells in Leishmania infection. *Microbes Infect* 6: 1402-1409.
35. Ueno, N., and M. E. Wilson. 2012. Receptor-mediated phagocytosis of Leishmania: implications for intracellular survival. *Trends Parasitol* 28: 335-344.
36. Cortez, M., C. Huynh, M. C. Fernandes, K. A. Kennedy, A. Aderem, and N. W. Andrews. 2011. Leishmania promotes its own virulence by inducing expression of the host immune inhibitory ligand CD200. *Cell Host Microbe* 9: 463-471.
37. Ndjamen, B., B. H. Kang, K. Hatsuzawa, and P. E. Kima. 2010. Leishmania parasitophorous vacuoles interact continuously with the host cell's endoplasmic reticulum; parasitophorous vacuoles are hybrid compartments. *Cell Microbiol* 12: 1480-1494.
38. Manta, B., M. Comini, A. Medeiros, M. Hugo, M. Trujillo, and R. Radi. 2013. Trypanothione: a unique bis-glutathionyl derivative in trypanosomatids. *Biochim Biophys Acta* 1830: 3199-3216.
39. Palatnik-de-Sousa, C. B. 2008. Vaccines for leishmaniasis in the fore coming 25 years. *Vaccine* 26: 1709-1724.
40. Nagill, R., and S. Kaur. 2011. Vaccine candidates for leishmaniasis: a review. *Int Immunopharmacol* 11: 1464-1488.
41. Selvapandiyan, A., R. Dey, S. Gannavaram, I. Lakhal-Naouar, R. Duncan, P. Salotra, and H. L. Nakhasi. 2012. Immunity to visceral leishmaniasis using genetically defined live-attenuated parasites. *J Trop Med* 2012: 631460.
42. Dey, R., P. K. Dagur, A. Selvapandiyan, J. P. McCoy, P. Salotra, R. Duncan, and H. L. Nakhasi. 2013. Live Attenuated Leishmania donovani p27 Gene Knockout Parasites Are Nonpathogenic and Elicit Long-Term Protective Immunity in BALB/c Mice. *J Immunol*.
43. Borja-Cabrera, G. P., F. N. Santos, F. B. Santos, F. A. Trivellato, J. K. Kawasaki, A. C. Costa, T. Castro, F. S. Nogueira, M. A. Moreira, M. C. Luvizotto, M. Palatnik, and C. B. Palatnik-de-Sousa. 2010. Immunotherapy with the saponin enriched-Leishmune vaccine versus immunochemotherapy in dogs with natural canine visceral leishmaniasis. *Vaccine* 28: 597-603.
44. Araújo, M. S., R. A. de Andrade, L. R. Vianna, W. Mayrink, A. B. Reis, R. Sathler-Avelar, A. Teixeira-Carvalho, M. C. Andrade, M. N. Mello, and O. A. Martins-Filho. 2008. Despite Leishvaccine and Leishmune trigger distinct immune profiles, their ability to activate phagocytes and CD8+ T-cells support their high-quality immunogenic potential against canine visceral leishmaniasis. *Vaccine* 26: 2211-2224.
45. Grenfell, R. F., E. A. Marques-da-Silva, M. C. Souza-Testasicca, E. A. Coelho, A. P. Fernandes, L. C. Afonso, and S. A. Rezende. 2010. Antigenic extracts of Leishmania braziliensis and Leishmania amazonensis associated with saponin

partially protects BALB/c mice against Leishmania chagasi infection by suppressing IL-10 and IL-4 production. *Mem Inst Oswaldo Cruz* 105: 818-822.
46. Singh, B., and S. Sundar. 2012. Leishmaniasis: vaccine candidates and perspectives. *Vaccine* 30: 3834-3842.
47. Goto, Y., L. Y. Bogatzki, S. Bertholet, R. N. Coler, and S. G. Reed. 2007. Protective immunization against visceral leishmaniasis using Leishmania sterol 24-c-methyltransferase formulated in adjuvant. *Vaccine* 25: 7450-7458.
48. Goto, Y., A. Bhatia, V. S. Raman, H. Liang, R. Mohamath, A. F. Picone, S. E. Vidal, T. S. Vedvick, R. F. Howard, and S. G. Reed. 2011. KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. *Clin Vaccine Immunol* 18: 1118-1124.
49. Tafaghodi, M., A. Khamesipour, and M. R. Jaafari. 2011. Immunization against leishmaniasis by PLGA nanospheres encapsulated with autoclaved Leishmania major (ALM) and CpG-ODN. *Parasitol Res* 108: 1265-1273.
50. Coler, R. N., and S. G. Reed. 2005. Second-generation vaccines against leishmaniasis. *Trends Parasitol* 21: 244-249.
51. Raman, V. S., M. S. Duthie, C. B. Fox, G. Matlashewski, and S. G. Reed. 2012. Adjuvants for Leishmania vaccines: from models to clinical application. *Front Immunol* 3: 144.
52. Campbell, K., H. Diao, J. Ji, and L. Soong. 2003. DNA immunization with the gene encoding P4 nuclease of Leishmania amazonensis protects mice against cutaneous Leishmaniasis. *Infect Immun* 71: 6270-6278.
53. Hallengärd, D., B. K. Haller, A. K. Maltais, E. Gelius, K. Nihlmark, B. Wahren, and A. Bråve. 2011. Comparison of plasmid vaccine immunization schedules using intradermal in vivo electroporation. *Clin Vaccine Immunol* 18: 1577-1581.
54. Liu, M. A. 2010. Immunologic basis of vaccine vectors. *Immunity* 33: 504-515.
55. Jayakumar, A., T. M. Castilho, E. Park, K. Goldsmith-Pestana, J. M. Blackwell, and D. McMahon-Pratt. 2011. TLR1/2 activation during heterologous prime-boost vaccination (DNA-MVA) enhances CD8+ T Cell responses providing protection against Leishmania (Viannia). *PLoS Negl Trop Dis* 5: e1204.
56. Remer, K. A., C. Apetrei, T. Schwarz, C. Linden, and H. Moll. 2007. Vaccination with plasmacytoid dendritic cells induces protection against infection with Leishmania major in mice. *Eur J Immunol* 37: 2463-2473.
57. Mutiso, J. M., J. C. Macharia, and M. M. Gicheru. 2010. A review of adjuvants for Leishmania vaccine candidates. *J Biomed Res* 24: 16-25.
58. Coffman, R. L., A. Sher, and R. A. Seder. 2010. Vaccine adjuvants: putting innate immunity to work. *Immunity* 33: 492-503.
59. 2014. Vaccines, Blood & Biologics. In *Common Ingredients in U.S. Licensed Vaccines*. **U.S. Food and Drug Administration**, Silver Springs, MD.
60. Klinman, D. M. 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol* 4: 249-258.
61. Iborra, S., N. Parody, D. R. Abánades, P. Bonay, D. Prates, F. O. Novais, M. Barral-Netto, C. Alonso, and M. Soto. 2008. Vaccination with the Leishmania major ribosomal proteins plus CpG oligodeoxynucleotides induces protection against experimental cutaneous leishmaniasis in mice. *Microbes Infect* 10: 1133-1141.

62. Rhee, E. G., S. Mendez, J. A. Shah, C. Y. Wu, J. R. Kirman, T. N. Turon, D. F. Davey, H. Davis, D. M. Klinman, R. N. Coler, D. L. Sacks, and R. A. Seder. 2002. Vaccination with heat-killed leishmania antigen or recombinant leishmanial protein and CpG oligodeoxynucleotides induces long-term memory CD4+ and CD8+ T cell responses and protection against leishmania major infection. *J Exp Med* 195: 1565-1573.
63. Stacey, K. J., and J. M. Blackwell. 1999. Immunostimulatory DNA as an adjuvant in vaccination against Leishmania major. *Infect Immun* 67: 3719-3726.
64. Verthelyi, D., M. Gursel, R. T. Kenney, J. D. Lifson, S. Liu, J. Mican, and D. M. Klinman. 2003. CpG oligodeoxynucleotides protect normal and SIV-infected macaques from Leishmania infection. *J Immunol* 170: 4717-4723.
65. Verthelyi, D., and D. M. Klinman. 2003. Immunoregulatory activity of CpG oligonucleotides in humans and nonhuman primates. *Clin Immunol* 109: 64-71.
66. Sun, H. X., Y. Xie, and Y. P. Ye. 2009. Advances in saponin-based adjuvants. *Vaccine* 27: 1787-1796.
67. Garçon, N., and M. Van Mechelen. 2011. Recent clinical experience with vaccines using MPL- and QS-21-containing adjuvant systems. *Expert Rev Vaccines* 10: 471-486.
68. Aguiar-Soares, R. D., B. M. Roatt, H. G. Ker, N. Moreira, F. A. Mathias, J. M. Cardoso, N. F. Gontijo, O. Bruna-Romero, A. Teixeira-Carvalho, O. A. Martins-Filho, R. Corrêa-Oliveira, R. C. Giunchetti, and A. B. Reis. 2014. LBSapSal-vaccinated dogs exhibit increased circulating T-lymphocyte subsets ($CD4^+$ and $CD8^+$) as well as a reduction of parasitism after challenge with Leishmania infantum plus salivary gland of Lutzomyia longipalpis. *Parasit Vectors* 7: 61.
69. Roatt, B. M., R. D. Aguiar-Soares, J. Vitoriano-Souza, W. Coura-Vital, S. L. Braga, R. Corrêa-Oliveira, O. A. Martins-Filho, A. Teixeira-Carvalho, M. de Lana, N. Figueiredo Gontijo, M. J. Marques, R. C. Giunchetti, and A. B. Reis. 2012. Performance of LBSap vaccine after intradermal challenge with L. infantum and saliva of Lu. longipalpis: immunogenicity and parasitological evaluation. *PLoS One* 7: e49780.
70. Vitoriano-Souza, J., N. Moreira, D. Menezes-Souza, B. M. Roatt, R. D. de Oliveira Aguiar-Soares, F. A. Siqueira-Mathias, J. M. de Oliveira Cardoso, R. C. Giunchetti, R. G. de Sá, R. Corrêa-Oliveira, C. M. Carneiro, and A. B. Reis. 2013. Dogs immunized with LBSap vaccine displayed high levels of IL-12 and IL-10 cytokines and CCL4, CCL5 and CXCL8 chemokines in the dermis. *Mol Immunol* 56: 540-548.
71. Darrah, P. A., D. T. Patel, P. M. De Luca, R. W. Lindsay, D. F. Davey, B. J. Flynn, S. T. Hoff, P. Andersen, S. G. Reed, S. L. Morris, M. Roederer, and R. A. Seder. 2007. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* 13: 843-850.
72. Colmenares, M., P. E. Kima, E. Samoff, L. Soong, and D. McMahon-Pratt. 2003. Perforin and gamma interferon are critical CD8+ T-cell-mediated responses in vaccine-induced immunity against Leishmania amazonensis infection. *Infect Immun* 71: 3172-3182.
73. Childs, G. E., L. K. Lightner, L. McKinney, M. G. Groves, E. E. Price, and L. D. Hendricks. 1984. Inbred mice as model hosts for cutaneous leishmaniasis. I.

Resistance and susceptibility to infection with Leishmania braziliensis, L. mexicana, and L. aethiopica. *Ann Trop Med Parasitol* 78: 25-34.
74. McMahon-Pratt, D., and J. Alexander. 2004. Does the Leishmania major paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? *Immunol Rev* 201: 206-224.
75. Neal, R. A., and C. Hale. 1983. A comparative study of susceptibility of inbred and outbred mouse strains compared with hamsters to infection with New World cutaneous leishmaniases. *Parasitology* 87 (Pt 1): 7-13.
76. Coler, R. N., S. Bertholet, M. Moutaftsi, J. A. Guderian, H. P. Windish, S. L. Baldwin, E. M. Laughlin, M. S. Duthie, C. B. Fox, D. Carter, M. Friede, T. S. Vedvick, and S. G. Reed. 2011. Development and characterization of synthetic glucopyranosyl lipid adjuvant system as a vaccine adjuvant. *PLoS One* 6: e16333.
77. Peters, N. C., S. Bertholet, P. G. Lawyer, M. Charmoy, A. Romano, F. L. Ribeiro-Gomes, L. W. Stamper, and D. L. Sacks. 2012. Evaluation of recombinant Leishmania polyprotein plus glucopyranosyl lipid A stable emulsion vaccines against sand fly-transmitted Leishmania major in C57BL/6 mice. *J Immunol* 189: 4832-4841.
78. Gomes, R., C. Teixeira, F. Oliveira, P. G. Lawyer, D. E. Elnaiem, C. Meneses, Y. Goto, A. Bhatia, R. F. Howard, S. G. Reed, J. G. Valenzuela, and S. Kamhawi. 2012. KSAC, a defined Leishmania antigen, plus adjuvant protects against the virulence of L. major transmitted by its natural vector Phlebotomus duboscqi. *PLoS Negl Trop Dis* 6: e1610.
79. Peters, N. C., N. Kimblin, N. Secundino, S. Kamhawi, P. Lawyer, and D. L. Sacks. 2009. Vector transmission of leishmania abrogates vaccine-induced protective immunity. *PLoS Pathog* 5: e1000484.
80. Carlsen, E. D., C. Hay, C. A. Henard, V. Popov, N. J. Garg, and L. Soong. 2013. Leishmania amazonensis amastigotes trigger neutrophil activation but resist neutrophil microbicidal mechanisms. *Infect Immun* 81: 3966-3974.
81. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45.
82. Xin, L., D. A. Vargas-Inchaustegui, S. S. Raimer, B. C. Kelly, J. Hu, L. Zhu, J. Sun, and L. Soong. 2010. Type I IFN receptor regulates neutrophil functions and innate immunity to Leishmania parasites. *J Immunol* 184: 7047-7056.
83. Elsharkawy, A. M., F. Oakley, F. Lin, G. Packham, D. A. Mann, and J. Mann. 2010. The NF-kappaB p50:p50:HDAC-1 repressor complex orchestrates transcriptional inhibition of multiple pro-inflammatory genes. *J Hepatol* 53: 519-527.
84. Calegari-Silva, T. C., R. M. Pereira, L. D. De-Melo, E. M. Saraiva, D. C. Soares, M. Bellio, and U. G. Lopes. 2009. NF-kappaB-mediated repression of iNOS expression in Leishmania amazonensis macrophage infection. *Immunol Lett* 127: 19-26.
85. Pinheiro, R. O., E. F. Pinto, A. B. Benedito, U. G. Lopes, and B. Rossi-Bergmann. 2004. The T-cell anergy induced by Leishmania amazonensis antigens is related with defective antigen presentation and apoptosis. *An Acad Bras Cienc* 76: 519-527.

86. Vargas-Inchaustegui, D. A., L. Xin, and L. Soong. 2008. Leishmania braziliensis infection induces dendritic cell activation, ISG15 transcription, and the generation of protective immune responses. *J Immunol* 180: 7537-7545.
87. Xin, L., J. L. Wanderley, Y. Wang, D. A. Vargas-Inchaustegui, and L. Soong. 2011. The magnitude of CD4(+) T-cell activation rather than TCR diversity determines the outcome of Leishmania infection in mice. *Parasite Immunol* 33: 170-180.
88. Diefenbach, A., H. Schindler, N. Donhauser, E. Lorenz, T. Laskay, J. MacMicking, M. Röllinghoff, I. Gresser, and C. Bogdan. 1998. Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity* 8: 77-87.
89. Schneider, L. P., A. J. Schoonderwoerd, M. Moutaftsi, R. F. Howard, S. G. Reed, E. C. de Jong, and M. B. Teunissen. 2012. Intradermally administered TLR4 agonist GLA-SE enhances the capacity of human skin DCs to activate T cells and promotes emigration of Langerhans cells. *Vaccine* 30: 4216-4224.
90. Ivanov, I. I., K. Atarashi, N. Manel, E. L. Brodie, T. Shima, U. Karaoz, D. Wei, K. C. Goldfarb, C. A. Santee, S. V. Lynch, T. Tanoue, A. Imaoka, K. Itoh, K. Takeda, Y. Umesaki, K. Honda, and D. R. Littman. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139: 485-498.
91. Qi, H., J. Ji, N. Wanasen, and L. Soong. 2004. Enhanced replication of Leishmania amazonensis amastigotes in gamma interferon-stimulated murine macrophages: implications for the pathogenesis of cutaneous leishmaniasis. *Infect Immun* 72: 988-995.
92. Pereira, B. A., and C. R. Alves. 2008. Immunological characteristics of experimental murine infection with Leishmania (Leishmania) amazonensis. *Vet Parasitol* 158: 239-255.
93. Abu-Dayyeh, I., K. Hassani, E. R. Westra, J. C. Mottram, and M. Olivier. 2010. Comparative study of the ability of Leishmania mexicana promastigotes and amastigotes to alter macrophage signaling and functions. *Infect Immun* 78: 2438-2445.
94. Basu, R., S. Bhaumik, J. M. Basu, K. Naskar, T. De, and S. Roy. 2005. Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-sensitive and -resistant strains of Leishmania donovani that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. *J Immunol* 174: 7160-7171.
95. Bhaumik, S., R. Basu, S. Sen, K. Naskar, and S. Roy. 2009. KMP-11 DNA immunization significantly protects against L. donovani infection but requires exogenous IL-12 as an adjuvant for comparable protection against L. major. *Vaccine* 27: 1306-1316.
96. Fernandes, A. P., M. M. Costa, E. A. Coelho, M. S. Michalick, E. de Freitas, M. N. Melo, W. Luiz Tafuri, D. e. M. Resende, V. Hermont, C. e. F. Abrantes, and R. T. Gazzinelli. 2008. Protective immunity against challenge with Leishmania (Leishmania) chagasi in beagle dogs vaccinated with recombinant A2 protein. *Vaccine* 26: 5888-5895.

97. Ghosh, A., W. W. Zhang, and G. Matlashewski. 2001. Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against Leishmania donovani infections. *Vaccine* 20: 59-66.
98. Wanasen, N., L. Xin, and L. Soong. 2008. Pathogenic role of B cells and antibodies in murine Leishmania amazonensis infection. *Int J Parasitol* 38: 417-429.
99. Caamaño, J., and C. A. Hunter. 2002. NF-kappaB family of transcription factors: central regulators of innate and adaptive immune functions. *Clin Microbiol Rev* 15: 414-429.
100. Bowie, A., and L. A. O'Neill. 2000. Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* 59: 13-23.
101. Duthie, M. S., H. P. Windish, C. B. Fox, and S. G. Reed. 2011. Use of defined TLR ligands as adjuvants within human vaccines. *Immunol Rev* 239: 178-196.
102. Krug, A., A. Towarowski, S. Britsch, S. Rothenfusser, V. Hornung, R. Bals, T. Giese, H. Engelmann, S. Endres, A. M. Krieg, and G. Hartmann. 2001. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur J Immunol* 31: 3026-3037.
103. Sivori, S., M. Falco, M. Della Chiesa, S. Carlomagno, M. Vitale, L. Moretta, and A. Moretta. 2004. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc Natl Acad Sci U S A* 101: 10116-10121.