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ABSTRACT

Background and Significance. The leishmaniases are a diverse group of diseases with four major clinical manifestations: cutaneous, mucocutaneous, visceral, and diffuse. Approximately 21 *Leishmania* species and 30 sandfly vectors have been implicated in human leishmaniasis. Leishmaniasis is endemic in 88 countries, 72 are developing countries. Currently, 350 million people live in leishmaniasis-endemic areas around the world. The true incidence of the disease is not known. However, the estimated incidence for visceral leishmaniasis is 500,000 cases per year, and for cutaneous and mucocutaneous leishmaniasis 1.5-2 million cases per year. Visceral leishmaniasis causes 70,000 deaths each year. In the Americas region, leishmaniasis extends from the United States to Argentina excepting Canada, Uruguay and Chile. In 2006, 62,000 leishmaniasis cases were registered affecting mostly Brazil, Colombia, Paraguay, Venezuela, Panama, Ecuador, and Peru. In the United States, cutaneous leishmaniasis has been reported in the southern part of Texas and also among military personnel deployed to Iraq and Afghanistan. The first and second line treatments are extremely toxic. Resistant to some of these drugs is very high in some parts of the world.

The only immunizing intervention shown to be effective for preventing human cutaneous leishmaniasis is Leishmanization (LZ), but is not currently recommended due to a small number of complications, such as non-healing lesions, and cases of immunosuppression following LZ. First generation and second generation vaccines are difficult to standardize from cultured parasites. Third generation vaccines using DNA are one of the most promising new developments in vaccination strategies. The introduction of DNA plasmids into the cells of living hosts has the potential to lead to the generation of both humoral and cellular immune responses

and protective immunity. DNA vaccination provides specificity, stability, safety, and a cost advantage.

A leishmaniasis vaccine should optimally be composed of several molecules in order to attack the survival mechanism of the parasite. This is because *Leishmania* parasites have multiple molecules allowing them to enter the host cells and attack the body. For this study, the molecules selected were biopterin transporter (BT), intracellular adhesive molecules (ICAMs), ORFF, and Amastin. Biopterin Transporter is a potential growth promoter of *Leishmania* parasites. Cell adhesion molecules (ICAMs) allow the survival of the parasite inside the cell. The ORFF gene is reported to induce partial protection against challenge with *L. donovani.* The Amastin family plays a role in adjusting the cytoplasmic pH allowing the survival of *Leishmania* parasites inside the cells. For the present study, the BT, ICAM, ORFF, and Amastin antigens were assembled in the pVAX backbone as a bicistronic plasmid in order to examine the poly-antigen vaccination approach. The ability of these molecules to induce immunity and protection was evaluated using a murine model.

Study Objectives. The two major objectives of this experimental phase 0 trial were: (1) to characterize the immune response induced by the DNA vaccine candidate against *L. mexicana* infection in a murine model, and (2) to determine the efficacy of the DNA vaccine candidate in immunized BALB/c mice challenged with virulent *L. mexicana* promastigotes.

Hypothesis. It was hypothesized that the use of a cocktail DNA vaccine would induce protection against *L. mexicana* infection in BALB/c mice by promoting the activation of protective Th1-related cytokines and decreasing the progression of the disease at infected footpad sites.

Methodology. The experimental design used 29, six-week old BALB/c female mice. The experimental and control groups were housed under identical environmental conditions. The mice were randomized to one of the six experimental or control groups. The three experimental groups were immunized with 100μL of pVAX-BT-ICAM, 100μL of pVAX-ORFF-Amastin, or 100μL of pVAX-BT-ICAM plus pVAX-ORFF-Amastin. The negative control groups were injected with 100μL PBS or 100μL of pVAX. Three weeks after their last immunization, the experimental and control groups were challenged subcutaneously in the left hind footpad with 40 μl of 1 x 10^6 stationary phase virulent *L. mexicana* promastigotes (LV4 strain). The development of footpad lesions at the infection site was measured weekly for eight weeks. Quantitative Real Time Polymerase Chain Reactions (QRT-PCR) assays were run to quantify gene expression of Th1 indicators (Interleukin-2 Tumor Necrosis Factor-α, and Interferon-γ) and Th2 (Interleukin-4) indicator. The data were analyzed using descriptive and bivariate statistical techniques.

Results. A partial protection and Th1 immune response was suggested in the experimental group receiving the pVAX-BT-ICAM plus pVAX-ORFF-Amastin vaccine candidate. This group had the smallest mean footpad lesion ($\bar{x} = 0.465 \pm 0.10$). There was found statistically significant reduction in footpad thickness when this group was compared to the other experimental groups [pVAX-BT-ICAM ($P = 0.000$) or pVAX-ORFF-Amastin ($P = 0.032$)] and to the negative controls [PBS ($P = 0.032$) or pVAX ($P = 0.015$)]. The group vaccinated with pVAX-BT-ICAM plus pVAX-ORFF-Amastin had a Th1 cytokine profile (high levels of IL-2, TNF-α, and IFN-γ) which is associated with immune protection against *L. mexicana*. However, there was also an increased IL-4 expression. For this group, no statistically significance differences were found between this group cytokine profiles ($P = 0.376$).

Conclusion. Only the pVAX-BT-ICAM-I plus pVAX-ORFF-Amastin vaccine formulation provided partial protection in BALB/c mice. This vaccine formulation was able to decrease parasite burden better than single bicistronic plasmids at the end of the eight-week follow-up period. The lymphocytes of the group vaccinated with pVAX-BT-ICAM-I + pVAX-ORFF-Amastin had a predominant Th1 immune response by showing higher levels of IFN-γ, TNF-α, and IL-2. However, this protective cytokine profile was reduced by the presence of IL-4 which correlates to a partial protection observed after the eight-week follow-up. Hence, in order to successfully attack *L. mexicana*, the vaccine must be composed of several antigens. Further studies need to be conducted to support these findings as well as to evaluate the efficacy of this vaccine in other *Leishmania* species causing cutaneous leishmaniasis clinical form.

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CHAPTER I: BACKGROUND AND SIGNIFICANCE

1.1 *Leishmania* Species

Leishmaniasis is a chronic infection caused by obligated intracellular protozoan parasites of the genus *Leishmania.* Leishmaniasis causes several clinical forms resulting in the formation of ulcerative skin lesions, partial or total destruction of the mucous membranes, or damage of internal organs (Schwartz et al., 2006). The taxonomic classification is the following: Phylum: Sarcomastigophora; Order: Kinetoplastida; Family: Trypanosomatidae; Genus: *Leishmania* and; Subgenus: *Leishmania* and *Viannia* (Banuls et al., 2007). The two subgenera are separated on the basis of the parasite location in the vector's intestine (Jeronimo et al., 2006). Species belonging to the subgenus *Viannia* develop in the hindgut before migrating to the midgut and foregut of the vector. But, species belonging to the subgenus *Leishmania* only occupy the midgut and foregut of the vector. Isoenzyme analysis, molecular methods, and monoclonal antibodies are used to define species complexes within the subgenera. Species of the subgenus *Viannia* correspond to those found in the New World including *L. V. braziliensis, L. V. panamensis, L. V. guyanensis,* and *L. V. peruviana*, while those in the subgenus *Leishmania* correspond to the Old World including *L. L. donovani, L. L. tropica, L. L. major, L. L. aethiopica, L. L. infantum,* and New World *L. L. mexicana, L. L. venezuelensis, L. L. amazonensis,* and *L. L. chagasi* (Banuls et al., 2007).

1.2 *Leishmania mexicana*

L. mexicana is widely distributed throughout Central and South America, up through Mexico, South Texas and more recently North Texas and Oklahoma (Drisdelle, 2007). In Mexico, cutaneous leishmaniasis is caused by *L. mexicana* and is endemic in 22 states. The most

affected areas are the southern and southeastern states of the country, mainly Veracruz, Tabasco, Chiapas and Yucatán (Sanchez-Garcia et al., 2010). In 2008, within the Dallas-Fort Worth area, 9 patients presented with cutaneous leishmaniasis; 2 of these cases were caused by *L. mexicana* species (Wright et al., 2008).

1.3 *Leishmania* Morphology

Leishmania parasites exist in two main morphological forms: amastigote and promastigote. Amastigotes are round or oval bodies ranging from 2-4 μm in diameter with an internalized flagellum (Jeronimo et al., 2006). Amastigotes are located in the parasitophorous vacuoles of the host's macrophages. The promastigotes exist as elongate, 10-15 μm, flagellated forms, having a flagellum measuring 15-28 μm in length (Jeronimo et al., 2006).

1.4 Life Cycle

All species follow the same parasitic life cycle, alternating between the above-mentioned forms (promastigote and amastigote) (Bellatin et al., 2002). Human infection begins with the injection of promastigotes to the mammalian host during a blood meal (Figure 1). Once the promastigotes are inside the host, these will be phagocytized by macrophages. Inside macrophages, promastigotes transform into amastigotes. Amastigotes divide by simple division and move to infect other mononuclear phagocytic cells (CDC, 2009). The sandfly infection stage begins when the female sandfly becomes infected when feeding on the blood of an infected individual or an animal reservoir host. At this stage, parasites reach the intestinal track within sandfly as amastigotes (Jeronimo et al., 2006). Amastigotes are liberated into the fly midgut, and within few hours are transformed into promastigotes. Then, promastigotes migrate forward to the pharynx, buccal cavity, and mouthparts of the sandlfy. At 8 to 20 days, promastigotes are

released into the bite wound during the sandfly's blood meal (Wittner & Tanowitz, 2000). If female sandflies ingest infected blood, the life cycle will repeat (Drisdelle, 2007).

1.5 Clinical Manifestations

There are four major clinical manifestations of leishmaniasis: cutaneous, mucocutaneous, diffuse cutaneous, and visceral.

Cutaneous leishmaniasis (CL) varies by clinical severity and appearance, and time to cure (Reithinger et al., 2007). The lesions appear within several weeks or months after the bite of an infected sandfly, and it is frequently self-healing. Species causing CL are: *L. major, L. tropica, L. aetipica*, *L. panamensis, L. mexicana, L. braziliensis, L. guyanensis, L. amazonensis*, and *L. peruviana*.

Mucocutaneous leishmaniasis (MCL) usually develops as a complication of cutaneous leishmaniasis in which parasites spread from the skin lesion via lymphatic vessels and blood to reach the upper respiratory tract mucosa. Untreated lesions can result in deformities caused by the destruction of the oral-nasal and pharyngeal cavities (Ahluwalia et al., 2004). Mucocutaneous leishmaniasis is usually caused by *L. braziliensis* and *L. panamensis*.

Diffuse cutaneous leishmaniasis (DCL) occurs in individuals with defective cell-mediated immune response. Lesions do not ulcerate, but instead nodules or plaques are formed because amastigotes disseminate to skin macrophages forming a large number of lesions (Wittner & Tanowitz, 2000). This form of leishmaniasis has no known cure. Diffuse cutaneous leishmaniasis is caused by *L. mexicana* and *L. amazonensis*.

Visceral leishmaniasis (VL) is the most severe clinical form. Parasites infect macrophages of the liver, spleen and bone marrow. Patients become gradually ill over a period of a few months. The disease is usually fatal if left untreated with first, second, or third line drugs

(Boelaert et al., 2000). Untreated VL is fatal in 75 to 85 percent of infantile and 90 percent of adult cases. Visceral leishmaniasis is caused by *L. donovani, L. infantun* in the Old World, and by *L. chagasi* in the New World.

1.6 Vector

The vector for leishmaniasis is a female sandfly of the genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World. The sandflies are 2-3 mm long and usually found in tropical and temperate zones (Sharma & Singh, 2008). The sandfly larvae require organic matter, heat, and humidity for the growth, development, and reproduction. They live in moist soil, stonewalls, garbage dumps, and cracks in house walls. Typically, sandflies have a crepuscular activity after feeding at early night or early morning while the host is asleep (Campbell, 2000). However, the hours in which sandflies are most active depends upon the species. In Mexico, the only proven vector of *L. mexicana* is *Lu. olmeca olmeca* (Sanchez-Garcia et al., 2010). In the United States, two vectors have been identified for *L. Mexicana*: *Lu. Anthophora and Lu. diabolica* (Gonzalez et al., 2010).

1.7 Reservoir

The reservoirs for human leishmaniasis in endemic areas of the Old World include humans as well as numerous rodent and canine species (Sharma & Singh, 2008). However, in the New World, leishmaniasis is a zoonotic disease. Reservoirs include forest rodents, sloths, armadillos, and dogs (Gramiccia & Gradoni, 2005). For *L. Mexicana*, *Ototylomys phyllotis* (the tree rat) is the primary host in Belize and the Yucatan peninsula in Mexico (Gramiccia & Gradoni, 2005; Saliba & Oumeish, 1999). In Texas, *Neotoma micropus* (the wood rat), has been found to be naturally infected with *L. mexicana* (McHugh et al., 2003). According to McHugh

and associates (2003), the first known the human cases of leishmaniasis were attributed to residence in or travel to the habitat in which *N. micropus,* the reservoir host in Texas, lives.

1.8 Epidemiology

The leishmaniases are a diverse group of diseases. Approximately 21 *Leishmania* species and 30 sandfly vectors have been implicated in human leishmaniasis (Desjeux, 2004). Leishmaniasis is endemic in 88 countries, 72 are developing countries. Currently, 350 million people live in leishmaniasis-endemic areas around the world (WHO, 2012a). The true incidence of the disease is not known. However, the estimated incidence for visceral leishmaniasis is 500,000 cases per year, and for cutaneous and mucocutaneous leishmaniasis is 1.5-2 million cases per year (WHO, 2012a). Visceral leishmaniasis causes 70,000 deaths each year (Reithinger et al., 2007).

In the Americas region, leishmaniasis extends from the United States to Argentina excepting Canada, Uruguay and Chile (CDC, 2010). In 2006, 62,000 leishmaniasis cases were registered in the Americas affecting mostly Brazil, Colombia, Paraguay, Venezuela, Panama, Ecuador, and Peru (PAHO, 2007). More than 5,000 visceral leishmaniasis cases were reported, most of these from Brazil (WHO, 2012a).

In the United States, cutaneous leishmaniasis has been reported in the southern part of Texas and also among military personnel deployed to Iraq and Afghanistan (Leder & Weller, 2010). Approximately 700 cases due to *L. major* have been reported for U.S. military personnel serving in Iraq (CDC, 2009). In 2008, within the Dallas-Fort Worth complex, nine individuals were diagnosed with cutaneous leishmaniasis (Wright et al., 2008). There are several reasons the burden of the disease might be underestimated. For instance, the site of transmission within endemic areas are often discontinuous, numerous cases are undiagnosed, misdiagnosed or

unreported, especially in places without access to medical facilities, and public health authorities receive poor quality and quantity of information from the field (Desjeux, 2004).

1.9 Modes of Transmission and Risk Factors

Cutaneous leishmaniasis is most commonly transmitted through the bite of an infected sandfly vector. It can also occur through accidental laboratory exposure but this is rare (Wittner & Tanowitz, 2000). In the New World, the main risk factors related to cutaneous leishmaniasis are urbanization, deforestation and new settlements, agricultural development with the building of dams and new irrigation schemes, and for military exercises (Desjeux, 2001; CDC, 2009).

1.10 Immune Response

Leishmania parasites normally reside within macrophages and dendritic cells because these serve as safe habitats (Bogdan et al., 1996). Parasites survive and replicate inside these cells by modulating the normal antimicrobial machinery of the macrophages and dendritic cells (Selvapandiyan et al., 2012). They are able to enter macrophages cells via receptor-mediators including complement receptor 1 (CR1) and complement receptor 3 (CR3). These allow cell entry without activation of oxidative burst (Alexander et al., 1999). The survival of the parasites is also mediated by *Leishmania* surface molecules which appear to inhibit superoxide and nitric oxide species (Bellatin et al., 2002). Thus, *Leishmania* parasites are able to interfere with various steps of the inflammatory process, which might delay or even block the development of a protective immune response (Bogdan & Rollinghoff, 1998).

Leishmania parasites rely on the phagocytic capacity of host cells in order to gain entry into the cell (Rittig & Bogdan, 2000). For all *Leishmania* species, immunologists have found that macrophages, dendritic cells, CD4+ type 1 T helper cells type one (Thl), cytokines such as Interferon-gamma (IFN-γ), Tumor Necrosis Factor-alpha (TNF-α), Interleukin-2 (IL-2), and

transcription factors such as Interferon-Regulatory-Factor-l (IRF-I), are key for the control of infections and for a long-lasting protective immunity (Bogdan & Rollinghoff, 1998).

Previous studies have identified two immunoregulatory subsets of murine CD4+ cells influencing the outcome of infection (Pirmez et al., 1993). The first immunoregulatory subset is T helper cells type one (Th1). T helper cells type 1 related cytokines secretions appear to be crucial for host control of parasite burden and clinical cure (Castellano et al., 2009). For this kind of immune response, Th1 cells enhance cell-mediated immune responses by activating macrophages to kill or inhibit the growth of *Leishmania*. The Th1 response results in mild or self-curing disease. T helper cells type one (Th1) secrete IFN-γ, TNF-α, and IL-2 (Roberts, 2005). The second one is T helper cells type 2 (Th2) immune response. T helper cells type 2 enhance humoral immunity and down regulate cell-mediated immune response (Th1), which results in disease progression and dissemination of *Leishmania* infection. These Th2 cell populations secrete Interleukin-4 (IL-4), Interleukin-5 (IL-5), and Interleukin-10 (IL-10), Interleukin-13 (IL-13) (Roberts, 2005).

The production of IFN-γ leads to intracellular death of amastigotes through a common effector mechanism present in activated macrophages (Roberts, 2005). Interferon-gamma (IFN-γ) (from Th1 cells) mediate macrophage activation, nitric oxide production and parasite killing (Alexander & Bryson, 2005). Furthermore, TNF-α is a potent pro-inflammatory mediator related to tissue damage events and inflammatory cell recruitment to the local site of infection (Castellano et al., 2009). However, IL-2 does not mediate macrophage activation directly. It is responsible for inducing T helper cells proliferation as well as exerting broad immunoregulatory effects on T cells and natural killer cells, and stimulating IFN-γ secretion. Interleukin-2 (IL-2)

also induces protective effects against intracellular infections and has been used as treatment in infected patients (Murray et al., 1993).

1.11 *Leishmania* Relevant Molecules

Leishmania parasites have multiple molecules allowing them to enter the host cells and attack the body (Banuls et al., 2007). These molecules include glycosylphosphatidylinositol (GPI), glycosylphospholipid (GIPL), lipophosphoglycans (LPG), phosphoglycans (PG), leishmanolysin (gp63), and cysteine proteases (CPs) (McGwire et al., 2002). All of these molecules appear to play important roles in *Leishmania* infection of macrophages since they are involved in the binding, migration and release of the parasite in the sandfly midgut and in the modulation of resistance to lysis by the host complement (McGwire et al., 2002). Lipophosphoglycans (LPG) are also responsible for the binding and uptake by macrophages, modulating macrophage signal transduction, resistance to oxidative attack, and for allowing the parasite to establish successful infections (Beverley & Turco, 1998). Leishmanolysin (gp63) is involved in the intracellular survival of the parasite within host macrophages (McGwire et al., 2002). For this study, the molecules selected were biopterin transporter (BT), intracellular adhesive molecules (ICAMs), ORFF, and Amastin.

Biopterin transporter is the molecule encoding the transport of biopterin (BT). Biopterin transporter (BT) may be a potential growth promoter for *Leishmania* cells. The exact role of BT is unknown. Biopterin transporter (BT) is essential for parasite survival, because this gene encodes the transport of biopterin which is an essential nutrient for the parasite (Lemley et al., 1999). Cell adhesion molecules (CAMs) play a key role in stabilizing and strengthening cell-to-matrix and cell-to-cell interactions (Chiang et al., 2002). Additionally, ICAMs interact with receptors in macrophages allowing the survival of the parasite inside the cell (Ng et al., 2009).

Previous studies have identified purified recombinant ORFF (rORFF) protein in the serum from patients with VL. After immunization of mice with rORFF protein, investigators were able to demonstrate partial protection against challenge with *L. donovani* (Sukumaran et al., 2003). Researchers also were able to identify ORFF in the multigene LD1 locus in all *Leishmania* isolates: the LD1 locus is a 27.5-kb region of chromosome 35 (Ghosh et al., 1999).

Amastins belong to a large family of surface proteins and have been discovered in the *Leishmania* genus (Rafati et al., 2006). There are approximately 45 members of the Amastin gene family dispersed through the *Leishmania* genome. Amastin usually code for small proteins composed of about 200 amino acids (Rochette et al., 2005). Due to its relative hydrophobic sequence and its localization in the membrane, the Amastin family plays a role in proton or ion traffic across the membrane adjusting the cytoplasmic pH for the survival of *Leishmania* parasites inside the cells (Rafati et al., 2006). Nonetheless, the role of the Amastin family with respect to *Leishmania* virulence remains unclear. A study conducted by Rafati et al. (2006) showed an Amastin antibody response specific for the active stage of visceral leishmaniasis patients. However to date, no attempts have been made to utilize this molecule as a cutaneous leishmaniasis vaccine candidate.

1.12 Available Treatments

The first line drug treatments for cutaneous and visceral leishmaniasis were first developed over 60 years ago (Croft et al., 2006). Currently, no single drug is able to cure all clinical forms of leishmaniasis (Croft et al., 2006; Minodier & Parola, 2007; Reithinger et al., 2007). Disease prognosis varies by species and the immune status of the human host (Croft & Coombs, 2003). Unfortunately, species identification using culture and isoenzymatic examination methods is difficult and time consuming (Minodier & Parola, 2007). In addition

new rapid diagnostic tools, such as genomic amplification by the polymerase chain reaction, are still not widely available (Minodier & Parola, 2007). Clinicians usually initiate treatment by assuming patients were infected with the parasite living in that specific geographical setting (Reithinger et al., 2007). Few drugs are available to treat leishmaniasis and these have various shortcomings.

The first line treatment is pentavalent antimony. This has long been the cornerstone of anti-leishmaniasis chemotherapy and still plays a leading role in treatment (Minodier & Parola, 2007; Ouellette et al., 2004). However, resistance to this drug is very high in some parts of the world, particularly in North East India (Ouellette et al., 2004)

Second-line drugs for leishmaniasis include pentamidine isethionate and amphotericin B but these are used less often due to toxicity and cost issues (Minodier & Parola, 2007). Pentamidine isethionate is used in South America for cutaneous leishmaniasis, especially for CL caused by *L. guyanensis* (Minodier & Parola, 2007). Amphotericin B (AmB) has been used in place of antimonial drug treatment. The polyene AmB is a second-line antifungal drug used to treat systemic fungal infection (Ouellette et al., 2004). AmB is extremely toxic and is associated with severe side effects. However, newer lipid formulations of AmB appear less toxic while maintaining anti-leishmaniasis properties (Minodier & Parola, 2007). These new lipid AmB formulations are unaffordable for a majority of affected populations in endemic developing countries (Ouellette et al., 2004).

Paromomycin and localized controlled heat (LCH) have been used as topical treatments for CL (Croft et al., 2006; Minodier & Parola, 2007). An ointment prepared with 15% paramomicyn sulfate and with 12% methylbenzonium chloride was found to be effective in treating CL cause by *L. mexicana* in Belizian patients, and *L. mexicana* and *L. braziliensis* in

Guatemalan patients (Minodier & Parola, 2007). In other study, ointments prepared with 15% paramomicyn sulfate plus 12% methylbenzonium chloride (PR-MBCL) and 15% paramomicyn sulfate plus 10% urea (PR-U) were compared to meglumine antimoniate (MA) (Armijos et al., 2004a). After 12 weeks, the proportion of clinically cured participants in the MA (91.7%) compared to PR-MBCL (79.3%) and PR-U (70%) groups was found to be not statistically significant different. Localized controlled heat (LCH) has been used to treat *L. mexicana* infections. The FDA has approved the use of the ThermoMed® (ThermoSurgery Technologies, Inc, Phoenix, Arizona) device which delivers localized radio frequency-generated heat directly to the lesion by placing prongs onto it (Minodier & Parola, 2007). The procedure is painful and requires the application of a local anesthetic. Heat may be controlled locally and it is applied for 30 seconds, reaching temperatures up to 50°C. It was reported as effective in treating *L. mexicana* affecting Guatemala and Mexico with 90% healing in two months but this study was not a controlled clinical trial (Velasco-Castrejon et al., 1997).

1.13 Development of Vaccines

Vaccination is considered the most promising intervention for preventing cutaneous leishmaniasis in humans (Blackburn, 2006). A main rationale for the development of a vaccine for CL is that individuals who have previously had leishmaniasis are resistant to subsequent re-infection (Reithinger et al., 2007). So far, there are no approved vaccines for leishmaniasis. However, many investigators have been working on the development of a CL vaccine (Coler & Reed, 2005; Khamesipour et al., 2006; Requena et al., 2004). The only immunizing intervention shown to be effective for preventing human CL is Leishmanization (LZ), but is currently not recommended by WHO (Reithinger et al., 2007). Leishmanization consists of injecting live-attenuated parasites in humans (Khamesipour et al., 2005). Leishmanization was originally

developed for used in Iran and Israel but is not currently practiced by either country (Handman, 2001; Khamesipour et al., 2005). In Iran, a national LZ campaign was halted due to a small, but unacceptable number of complications such as non-healing lesions (Khamesipour et al., 2005). In Israel, it was ceased due to some cases of immunosuppression resulting in reduced responsiveness to pediatric diphtheria, pertussis, and tetanus vaccines following LZ (Khamesipour et al., 2005).

Leishmaniasis vaccines are categorized as first generation, second generation, and third generations. First generation leishmaniasis vaccines are made with killed whole parasites (Modabber, 1995; Noazin et al., 2008). In general, these types of vaccines are relatively easy to produce and low in cost. The primary disadvantage, of first generation vaccines, is the complexity of standardizing vaccines derived from cultured parasites. Studies have been conducted in Colombia, Ecuador, Venezuela, Brazil, Iran and Sudan with varying results using first generation vaccines (Armijos et al., 1998; Mayrink et al., 2002; Velez et al., 2005).

A randomized, double-blind clinical trial was conducted in Colombia in order to examine the immunogenicity and safety of three doses of a monovalent *L. amazonensis* (L-8 Brazilian strain) vaccine with Bacille Calmette-Guèrin (BCG) used as an adjuvant against CL (Velez et al., 2005). Participants were followed for twelve months. The overall incidence of CL was similar in both the vaccinated and placebo groups (7.8% vs. 6.8%). Although the vaccine was safe and immunogenic, it did not provide significant protection against CL infection.

Safety, immunogenicity and efficacy of two intradermal doses of a triple species vaccine was assessed using two doses of BCG in Ecuador (Armijos et al., 1998). The promastigotes used in the study were originally collected from the lesions of patients living in the study area and were mixed with BCG to construct the vaccine (*L. braziliensis*, *L. guyanensis*, and *L.*

amazonensis). Twelve months of follow-up revealed an efficacy of 73%. Subjects were followed for 4 more years (Armijos et al., 2003). The vaccine provided protection between the 13th and 18th month, with an efficacy of 56.5%. However, from the 19th to the 60th month no statistical significance was found different between the vaccine and control arm subjects. Later, a randomized, blinded study was also conducted in Ecuador to examine the safety, immunogenicity, and efficacy of two doses of an autoclaved-killed, whole cell *Leishmania amazonensis* vaccine (IFLA/BR/67/PH8) with Bacille Calmette-Guèrin (BCG) used as an adjuvant against CL infections (Armijos et al., 2004b). The overall incidence of CL was similar in both the vaccinated and placebo groups (2.0% vs. 1.3%). However, the vaccine did not offer significant protection against CL infection.

Second generation vaccines consist of defined protein antigens or recombinant protein components. Only one second generation leishmaniasis vaccine has been evaluated in phase 1 and 2 clinical trials in humans (Coler & Reed, 2005). Three vaccine-candidate antigens were selected to test a vaccine against CL and MCL (Skeiky et al., 2002). The molecules, LmSTI1, TSA and LeIF, were selected because these are found in most *Leishmania* species causing human disease (Skeiky et al., 1995). They also have been shown to elicit a Th1-type immune response in mice and human cells (Probst et al., 1997; Skeiky et al., 1995; Webb et al., 1996). The vaccine appeared to be safe and a majority of recipients showed a Thl immune response 4 weeks after the last immunization.

Third generation vaccines using DNA are one of the newest developments in vaccination strategies (Pavlenko et al., 2004). The introduction of DNA plasmids into the cells of living hosts has the potential to generate both humoral and cellular immune responses and protective immunity (Dunning, 2009; Palatnik-de-Sousa, 2008; Ulmer et al., 1996). Some of the reported

advantages of DNA vaccination include: specificity, non-immunogenicity of the plasmid vector, mammalian post-translational modification, stability, safety, cost advantage, and generic manufacturing (Dunning, 2009; Palatnik-de-Sousa, 2008; Ulmer et al., 1996).

Single antigen and multiple antigen DNA vaccines have been developed. Single antigen DNA vaccines produce a stronger Th1 immune response, longer-lasting immunity, and/or a better protection against the progression of the disease (Aguilar-Be et al., 2005; Iborra et al., 2003; Rafati et al., 2006; Sjolander et al., 1998a; Sjolander et al., 1998b). Moreover, multiple antigen DNA vaccines have proven to be more effective; however, this efficiency dependents on the antigen combination (Dumonteil et al., 2003; Iborra et al., 2004; Mendez et al., 2002; Mendez et al., 2001; Rosado-Vallado et al., 2005). Dumonteil and associates (2003) investigated the efficacy of DNA vaccines by encoding *L. mexicana* antigens gp63, gp46, CPb, and LACK with the purpose of determining the usefulness of DNA vaccines and define the most useful antigens against *L. mexicana*. The study results suggested that DNA vaccines may be a useful strategy since these offer partial protection against *L. mexicana* infection (Dumonteil et al., 2003). Previous Phase 0 studies using pVAX-BT-ICAM and pVAX-ORFF-Amastin have shown partial protection in susceptible BALB/c mice (Armijos et al., 2007).

Sukumaran et al. (2003) conducted a study for the purpose of examining the possibility of using ORFF molecule for the development of a DNA vaccine. Their study findings suggested that DNA vaccination with the ORFF molecule induced in a significant protection of BALB/c mice against infection with *L. donovani* (Sukumaran et al., 2003). Protection reached its maximum level at four weeks after infection, although immune memory persisted only eight weeks. Hence, the ORFF molecule from the LD1 locus of *Leishmania* could represent an

effective vaccine strategy against *L. donovani* infection. Many investigators have agreed that a successful *Leishmania* vaccine will need to be based on multiple antigens (Dumonteil, 2007).

1.14 Study Rationale

Cutaneous leishmaniasis is a global public health threat. An estimated of 350 million persons live in leishmaniasis-endemic areas and 1.5-2 million new cases occur each year (WHO, 2012a). Cutaneous leishmaniasis, along with other clinical forms, is one of 17 neglected tropical diseases identified by the World Health Organization (WHO, 2012a). Leishmaniasis is a disease of poverty associated with socioeconomic disparities and environmental degradation such as deforestation, urbanization, dam-building, and monocrop agriculture (WHO, 2012b). Although the cutaneous form of leishmaniasis rarely fatal, it has been linked with significant adverse such as psychological consequences (e.g. low self-esteem, stress, anxiety, depression) (Weigel et al., 1994; Weigel, 1996), social stigma and discrimination (Weigel et al., 1994; Weigel, 1996), reduced work productivity (Weigel et al., 1994), reduced marriage prospects in females (Weigel et al., 1994), secondary bacterial infection risk (Weigel et al., 1994), and child growth stunting and iron-deficiency anemia (Weigel et al. 1995).

Current treatments for leishmaniasis are highly toxic and expensive. Drug-resistance has been increasing in many parts of the world (Chakravarty & Sundar, 2010). Vaccination is considered as the most effective type of preventive intervention (Blackburn, 2006). The use of attenuated vaccines is not recommended because of the risk of reverting to virulent parasite forms (Handman, 2001). Attenuated vaccines also have the potential for pathogenicity in immunocompromised patients such as aging persons or those with TB or HIV/AIDS (Kedzierski, 2010). Furthermore, the transport of these types of vaccines to remote areas where leishmaniasis is endemic can be difficult because of their cold storage requirements and need for mixing on site

with adjuvant (Handman, 2001). In contrast, DNA vaccines offer potential higher specificity, non-immunogenicity of the vector (plasmid), mammalian post-translational modification, better stability, safety, cost advantage, and relatively easy manufacturing technology (Dunning, 2009; Palatnik-de-Sousa, 2008; Ulmer et al., 1996). To date, most of the work done conducted on DNA vaccines has used murine models, and these have shown some level of protection against tuberculosis, smallpox, and other intracellular pathogens in mice models (Hunt, 2010).

Cutaneous leishmaniasis caused by *L. mexicana* is a good candidate for a DNA vaccine since the immune system is able to generate memory cells, thus preventing re-infection (Alexander & Bryson, 2005; Kemp et al., 1998). In order to be successful, the vaccine should optimally be composed of several molecules attacking the different survival mechanisms used by the parasite (Dumonteil, 2007). The present study used BT, ICAM, ORFF, and Amastin antigens assembled in the pVAX backbone as a bicistronic plasmid in order to examine the promising poly-antigen vaccination approach. The ability of these molecules to induce immunity was evaluated in this study using a murine model.

CHAPTER II: STUDY AIMS AND HYPOTHESES

2.1 Study Objectives

The major objective of the experimental study was to investigate the efficacy of a DNA vaccine in preventing murine leishmaniasis caused by *Leishmania mexicana*.

2.2 Specific Aims

The first aim was to characterize the immune response induced by the DNA vaccine candidate against *L. mexicana* infection in a murine BALB/c model. This was accomplished by carrying out immunological assays to measure Th1 and Th2 cytokine levels in the immune response against *L. mexicana* infection in BALB/c mice.

The second aim was to determine the efficacy of the DNA vaccine candidate in immunized BALB/c mice challenged with virulent *L. mexicana* promastigotes. This was accomplished by monitoring progression of the lesion in the infection site.

2.3 Hypothesis

It was hypothesized that the use of a cocktail DNA vaccine would induce protection against *L. mexicana* infection in BALB/c mice by promoting the activation of protective Th1-related cytokines and decreasing the progression of the disease at infected footpad sites.

CHAPTER III: METHODS AND MATERIALS

3.1 Study Approval

The study protocol was approved by the UTEP Institutional Biosafety Committee Recombinant DNA Protocol (IBC) on March 14, 2005 (Protocol #2005-02) and the UTEP Institutional Animal Care and Use Office on September 7, 2006 (Protocol # A-200606-3)

3.2 Plasmid Design

The vaccine should optimally be composed of several molecules in order to successfully attack the survival mechanisms of the parasite and using only one gene would potentially allow the parasite to adapt more easily. The following four gene segments were selected because their role in important aspects of *Leishmania* survival. Biopterin Transporter is a potential growth promoter of *Leishmania* parasites (Lemley et al., 1999). Cell adhesion molecules (ICAMs) interact with receptors in macrophages allowing the survival of the parasite inside the cell (Ng et al., 2009). The ORFF gene is reported to induce partial protection against challenge with *L. donovani* (Sukumaran et al., 2003). The Amastin family plays a role in proton or ion traffic across the membrane adjusting the cytoplasmic pH for the survival of *Leishmania* parasites inside the cells (Rafati et al., 2006).

The plasmids used in the present study were previously designed by Armijos and associates using pVAX1 backbone (2007) (Figure 2 and Figure 3). The PCNV serves as the promoter for BT and ORFF, meanwhile the internal ribosome entry site (IRES) reassures expression of ICAM and Amastin. The restrictive enzymes *Neh-I*, *Bam H1*, *Pst-1* and *Xba-1* present in pVAX1 were digested to insert the four *Leishmania*'s gene segments. The stop codon is BGHpA, whereas pUCORI is the stop codon. Kanamycin resistance gene is use for the

selection in *E. coli* as well as to minimize allergic responses in hosts. The BT, ICAM, ORFF, and Amastin genes were PCR-amplified from the *L. mexicana* genome and inserted into the cloning site of pVAX1 (Invitrogen, cat No: V26020). A eukaryotic bicistronic expression vector, pVAX1 was specifically designed for the development of vaccines as per FDA recommendations for plasmids used in DNA vaccines for preventable infectious diseases in humans (FDA, 1996).

3.3 Experimental Animals

The experimental (phase 0) design study used 29, six-week old BALB/c female mice (Jackson Laboratory). The BALB/c mouse strain was selected for this study because of its documented susceptibility to infection with *L. mexicana* (Padigel et al., 2003). The animals were randomized to the experimental (n = 14) and the control (n = 15) groups. All animals were housed under the same environmental conditions in the pathogen-free Vivarium located in the UTEP Biology Building.

3.4 Study Protocol

There were three experimental and three control groups in the study (Table 1). All groups were injected in the hind leg quadriceps using a one milliliter syringe with a 30 gauge, ½-inch needle (BD Ultra-Fine needle^{TM}). The experimental groups were immunized with 100μL of the corresponding plasmid. The negative controls were injected with 100μL of PBS and pVAX, respectively. The phosphate buffered saline (PBS) is not going to induce an immunological response or provide protection to mice. The pVAX vector alone does not provide protection against leishmaniasis (Masih et al., 2011). The groups received a total of three doses. Each dose was administered every three weeks.

Three weeks after the last immunization, the experimental and control groups were challenged subcutaneously in the left hind footpad with 40 μl of 1 x 10^6 stationary phase *L.*

mexicana promastigotes. The virulent LV4 strain was provided by Dr. Eric Dumonteil, Universidad Autonoma de Yucatan. The development of footpad lesions at the infection site was measured weekly for eight weeks. The measurements were expressed as the difference between the thickness of the infected versus contra-lateral non-infected footpad on the same experimental animal.

The experimental and control groups were sacrificed eight weeks after their initial challenge with *L. mexicana* promastigotes in order to extract lymphocytes required to examine the hypothesized the immunological response induced by the vaccines. Carbon dioxide inhalation was used to euthanize the mice. As per the accepted protocol, the animals remained in the CO_2 chamber for approximately a minute, and death was verified by lack of cardiac pulse and dilated pupils (AVMA, 2007)

3.5 Quantitative Real Time Polymerase Chain Reaction (QRT-PCR)

RNA Homogenization/Extraction/Purification (RiboPure™ Kit). Lymph node cells were used to measure the immune response generated by the vaccine. Lymph node cells were homogenized in 1ml TRI Reagent. The homogenate was incubated for five minutes at room temperature, which was subsequently centrifuged 12,000 x g for 10 minutes at 4ºC. Supernatant was transferred to a new tube. Two hundred microliters of chloroform was added per tube and vortexed at maximum speed for 15 seconds. Tubes were incubated at room temperature for five minutes and centrifuged at 12,000 x g for 10 minutes at 4ºC. Two layers were formed after tubes were centrifuged. Then, four hundred microliters of the top layer was transferred to a new 1.5ml microcentrifuge tube. Two hundred microliters of 100% ethanol was added and vortexed for five seconds. The sample was passed through a filter cartridge and centrifuged at 12,000 x g for 30 seconds at room temperature. The flow-through was discarded and filter was returned to

cartridge. Five hundred microliters of wash solution was added to the filter cartridge-collection tube assembly. Once again, the cartridge-collection tube assembly was centrifuged for 30 seconds at room temperature and flow-through once more discarded and returned to original collection tube. The cartridge-collection was washed once more followed by centrifugation for 30 seconds at room temperature. The filter cartridge was placed in a new collection tube and 50µl of Elution Buffer was added to filter column. Incubation at room temperature for two minutes followed. The filter column was centrifuged for 30 seconds to elute the RNA from the filter.

cDNA Reverse Transcription Reaction (iScript™ cDNA Synthesis Kit). Ten microliters of a 2X RT master mix was pipetted into each individual tube. Ten microliters of RNA sample was added into each tube and pipetted up and down. The tubes were centrifuged briefly to spin down contents and to eliminate air bubbles. The tubes were then placed in a thermal heater (Table 2). Upon completion, samples were stored at the -80ºC freezer.

RT-PCR plate (Applied BioSystems™). The cytokines analyzed were TNF-α, IL-2, IL-4, and IFN-γ. TaqMan probes were used for each of the cytokines and β-actin served as the reference gene. Each well in a PCR plate included an amount of cDNA equivalent to 500 ng of RNA, 1 µL TaqMan probe, 10 µL TaqMan Universal Master Mix, and an amount of nuclease free water bringing the total volume in the well up to 20 µL. Once the plate was filled with the samples to be analyzed, it was placed in an iCycler iQ™ Optical Module thermal cycler manufactured by BIO RAD with the appropriate thermal cycler program (Table 3).

3.6 Sample Size

The UTEP's Institutional Animal Care and Use Committee recommend using a small number of animals for ethical reasons (OLAW, 2002). The study was originally designed to have

eight mice per group. However, the number of mice was reduced in certain groups due to unforeseen technical difficulties. Specifically, the *L. mexicana* strain did not arrive on time. The mice groups had previously received the three doses of the vaccine making a start time a high priority. Thus, a decision was made by the supervising faculty, research mentor to use selected mice for another investigation so as not to lose the mice. Thus, by the time the *L. mexicana* strain arrived, those mice were already allocated to another study which reduced the sample size in some groups.

3.7 Statistical Analysis

Data obtained by the QRT-PCR followed the Mean $2^{-\Delta\Delta}$CT (Livak) Method in order to obtain normalized expression ratios (Livak & Schmittgen, 2001). Data obtained on the weekly progression of the foodpad lesions and the QRT-PCR were analyzed using descriptive statistics in SPSS (Version 19). Data following a normal distribution were analyzed using the Repeated Measures ANOVA. Data not normally distributed were analyzed using the Kruskal-Wallis Test. Data are presented as the mean ± S.E.M.

CHAPTER IV: RESULTS

4.1 Protective Effect of the DNA Vaccine

In order to determine whether vaccination with pVAX-BT-ICAM, pVAX-ORFF-Amastin, or pVAX-BT-ICAM plus pVAX-ORFF-Amastin was able to confer protection, vaccinated mice and controls were challenged with 40 µl of 1 x 10^6 stationary phase *L. mexicana* promastigotes on their left footpads as discussed in the methodology section. The lesion progress was measured weekly with a digital caliper. Lesion size was expressed as the mean difference between the infected footpad and the contra-lateral uninfected footpad. Repeated measures ANOVA using a generalized linear model was used for statistical comparison between groups.

At eight weeks, Group 3 (pVAX-BT-ICAM plus pVAX-ORFF-Amastin) had the smallest mean difference footpad swelling between the infected and contra lateral footpad (Table 4). The results for Group 3 (pVAX-BT-ICAM plus pVAX-ORFF-Amastin) showed a statistically significant reduction in footpad thickness when compared to the groups injected with single bicistronic plasmids [(Group 1) pVAX-BT-ICAM ($\bar{x}$ = 0.940mm ± 0.151; P = 0.000) or (Group 2) pVAX-ORFF-Amastin ($\bar{x}$ = 0.521mm ± 0.151; P = *0.032*)] and to the negative controls [PBS ($\bar{x}$ = 0.465 ± 0.135; P = *0.032*) and pVAX ($\bar{x}$ = 0.489mm ± 0.130; P = *0.015*)] (Figure 4). Hence, the results suggested that pVAX-BT-ICAM plus pVAX-ORFF-Amastin appeared to provide partial protection in mice.

4.2 Immune Response to the DNA Vaccine

A robust Th1 immune response is required to confer adequate protection against *Leishmania* infection (Alexander & Bryson, 2005; Dumonteil et al., 2003). In order to characterize the type of immune response generated by pVAX-BT-ICAM plus pVAX-ORFF-

Amastin, the relative expression of IL-2, TNF-α, IFN-γ and IL-4 from regional lymph nodes was analyzed using quantitative RT-PCR (Applied Biosystems^{TM}). Kruskal-Wallis Test was used for statistical comparison between groups.

The results of the study showed a mixed Th1/Th2 immune response for Group 1 (pVAX-BT-ICAM) (Figure 5). The cytokines profiles of Group 1 were compared but there was not statistical difference (*P = 0.367*). Moreover, Group 1 was compared to negative controls, PBS and pVAX, but no statistically significant differences were found (*P = 0.432* and *P = 0.592*).

For Group 2 (pVAX-ORFF-Amastin) findings suggested the activation of a Th2 immune response indicating the progression of the disease (Figure 6). Although, the differences between cytokine profiles of Group 2 did not achieve statistical significance (*P = 0.856*). Group 2 was also compared to negative controls, PBS and pVAX, but no statistically significant differences were found (*P = 0.247* and *P = 0.372*).

Group 3 (pVAX-BT-ICAM plus pVAX-ORFF-Amastin) exhibited a Th1 cytokine profile (high IFN-γ, TNF-α, and IL-2 levels), but there was also an increased IL-4 expression (Figure 7). For this group, no statistically significance differences were found between the cytokine profiles (*P = 0.376*). Likewise, there was not found statistically significant differences when Group 3 was compared to negative controls, PBS (*P = 0.221*) and pVAX (*P = 0.372*).

For the negative controls, Group 4 (PBS) and Group 5 (pVAX), a mixed Th1/Th2 immune response is insinuated as well (Figure 8 and 9). However, there was no statistical difference between the cytokine profiles of Group 4 (*P = 0.715*) or Group 5 (*P = 0.602*).

CHAPTER V: DISCUSSION

To date, there are no approved vaccines for leishmaniasis. However, substantial efforts have been done for the development of a vaccine (Coler & Reed, 2005; Khamesipour et al., 2006; Requena et al., 2004). DNA or recombinant leishmaniasis vaccine candidates must be composed of several antigens in order to affect the parasite survival mechanism (Dumonteil, 2007). The mixture of antigens should stimulate different layers of the immune responses and exert a synergistic effect against target microorganism (Ahmed et al., 2009; Dumonteil et al., 2003; Dunning, 2009). Hence, this present study used BT, ICAM, ORFF, and Amastin antigens assembled in the pVAX backbone as a bicistronic plasmid in order examine this poly-antigen vaccination approach.

A major finding for this study was that vaccine candidate given to Group 3 (pVAX-BT-ICAM-I plus pVAX-ORFF-Amastin) provided a partial protection and immunogenicity in BALB/c mice. This group had the smallest mean difference in footpad thickness and this protection can be associated with the activation of Th1-related cytokines (high levels of IL-2, TNF-α, and IFN-γ). Similar patterns were observed in the study carried out by Dumonteil, et al (2003). The progression leishmaniasis was followed for eight weeks noticing and BALB/c mice immunized with the cocktail vaccine (GP63 plus GP46 plus CPb) developed smaller lesions than those mice immunized with single-antigen vaccines or PBS. Another study using antigens LACK, TSA, and LmSTI1 found that cocktail vaccines generated significantly higher levels of protection in BALB/c mice (Ahmed et al., 2009). In general, investigators have found that the immunogenicity of any anti-leishmaniasis vaccine is measured by its capability to induce an efficient Th1 immune response, which is strongly associated with the protection of mice

challenged with virulent *Leishmania s.p.* (Ahmed et al., 2009; Dumonteil et al., 2003). However, the protective cytokine profile for Group 3 (pVAX-BT-ICAM-I plus pVAX-ORFF-Amastin) was diminished by the activation of a Th2 immune response (high levels of IL-4). The presence IL-4 correlates with the partial protection observed after the eight-week follow-up. It also demonstrates that at the beginning of the trial this group had a high Th1 immune response which was later on downregulated by the increasing levels of IL-4 marking the progression of the disease.

Group 1 (pVAX-BT-ICAM) had the highest mean difference in footpad thickness when compared to all experimental groups, this was correlated with the cytokine profiles obtained. Group 1 (pVAX-BT-ICAM) showed higher levels of IFN-γ in the lymph node cells. As it was previously mentioned, IFN-γ is critical for the activation of macrophages and it is responsible for the killing of intracellular amastigotes (Bogdan & Rollinghoff, 1998). However, for Group 1, TNF-α and IL-2 levels were low. Researchers have agreed that the three Th1 cytokines (IL-2, TNF-α, and IFN-γ) must be activated in order to successfully resist the progression of the disease (Kemp et al., 1998). Hence, the vaccine was not able to slow the progression of the disease. For Group 2 (pVAX-ORFF-Amastin), IL-4 was activated at a higher level than any of the Th1-related cytokines, meaning this vaccine candidate did not prevent the progression of the disease.

The negative control, Group 7 (PBS), showed the natural progression of cutaneous leishmaniasis. As previously mentioned, BALB/c mice are susceptible to infection with *L. mexicana.* Therefore, mice receiving PBS were expected to have an increased Th2 immune response (Padigel et al., 2003). For the other negative control, Group 8 (pVAX), TNF-α and IL-2 levels were high. However, IFN-γ levels which are critical for the killing of the *Leishmania* parasite were low. Therefore, the progression of the disease was not stopped.

5.1 Conclusion

In conclusion, only vaccine formulation given to Group 3 (pVAX-BT-ICAM-I plus pVAX-ORFF-Amastin) provided partial protection in BALB/c mice. At the end of the eighth week, this vaccine candidate was able to decrease parasite burden better than single bicistronic (pVAX-BT-ICAM or pVAX-ORFF-Amastin) vaccine candidates. Only Group 3 (pVAX-BT-ICAM-I + pVAX-ORFF-Amastin) had a predominant Th1 immune response by showing higher levels of IFN-γ, in addition to increased levels of TNF-α, and IL-2. However, as previously mentioned, this protective cytokine profile was reduced by the presence of IL-4 which correlates to a partial protection observed after the eight-week follow-up. Hence, in order to successfully attack *L. mexicana*, the vaccine must be composed of several antigens. Further studies need to be conducted to support these findings as well as to evaluate the efficacy of this vaccine in other *Leishmania* species that cause CL clinical form.

5.2 Strengths and Limitations

One of the strengths was that experimental and the control groups lived under the same environmental conditions. Also, mice were randomly assigned into the different experimental group and weekly mice's footpad measurements were group blinded. Some of the limitations include using a small sample size. However, a large sample size does not guarantee an optimal trial protocol, a broad application of the trial results, or the validity of conclusions reached (Institute of Medicine (US) Roundtable on Value & Science-Driven Health Care, 2010). In addition, frozen cDNA was at 20^{o}C instead of 80^{o}C temperature for several hours due to technical problems with the freezer. Therefore, the reading values in the QRT-PCR could have been affected. Finally, Mean $2^{-\Delta\Delta}$CT (following the Livak Method) was calculated by hand and this could have introduced error in the analyses.

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TABLES AND FIGURES

Table 1. Immunization protocol for experimental and control groups

Experimental Group	Experimental Condition	Mice (n)
1	pVAX-BT-ICAM (100 μg)	4
2	pVAX-ORFF-Amastin (100 μg)	4
3	pVAX-BT-ICAM -I (100 μg) + pVAX-ORFF-Amastin (100 μg)	6
4	PBS (100 μl) – Negative control	8
5	pVAX (100 μg) – Negative control	7

Table 2. Thermal cycler program for cDNA synthesis

	Cycle 1 (1 cycle)		*Cycle 2 (1 cycle)*
	I	I	II
Temperature	25°C	37°C	85°C
Time	10 min	120 sec	5 minutes

Table 3. QRT-PCR thermal cycler program.

	Cycle 1 (1 cycle)	*Cycle 2 (50 cycles)*	
	I	I	II
Temperature	50°C	95°C	60°C
Time	3 min	10 sec	60 sec

Table 4. Descriptive statistics for the footpad inflammatory outcome.

Group (No. Mice)	Plasmid	Mean	S.E.M
1 (n = 4)	pVAX-BT-ICAM	2.29	0.12
2 (n = 4)	pVAX-ORFF-Amastin	1.87	0.12
3 (n = 6)	pVAX-BT-ICAM-I plus pVAX-ORFF-Amastin	1.35	0.10
4 (n = 8)	PBS (Negative control)	1.81	0.10
5 (n = 7)	pVAX (Negative control)	1.84	0.09

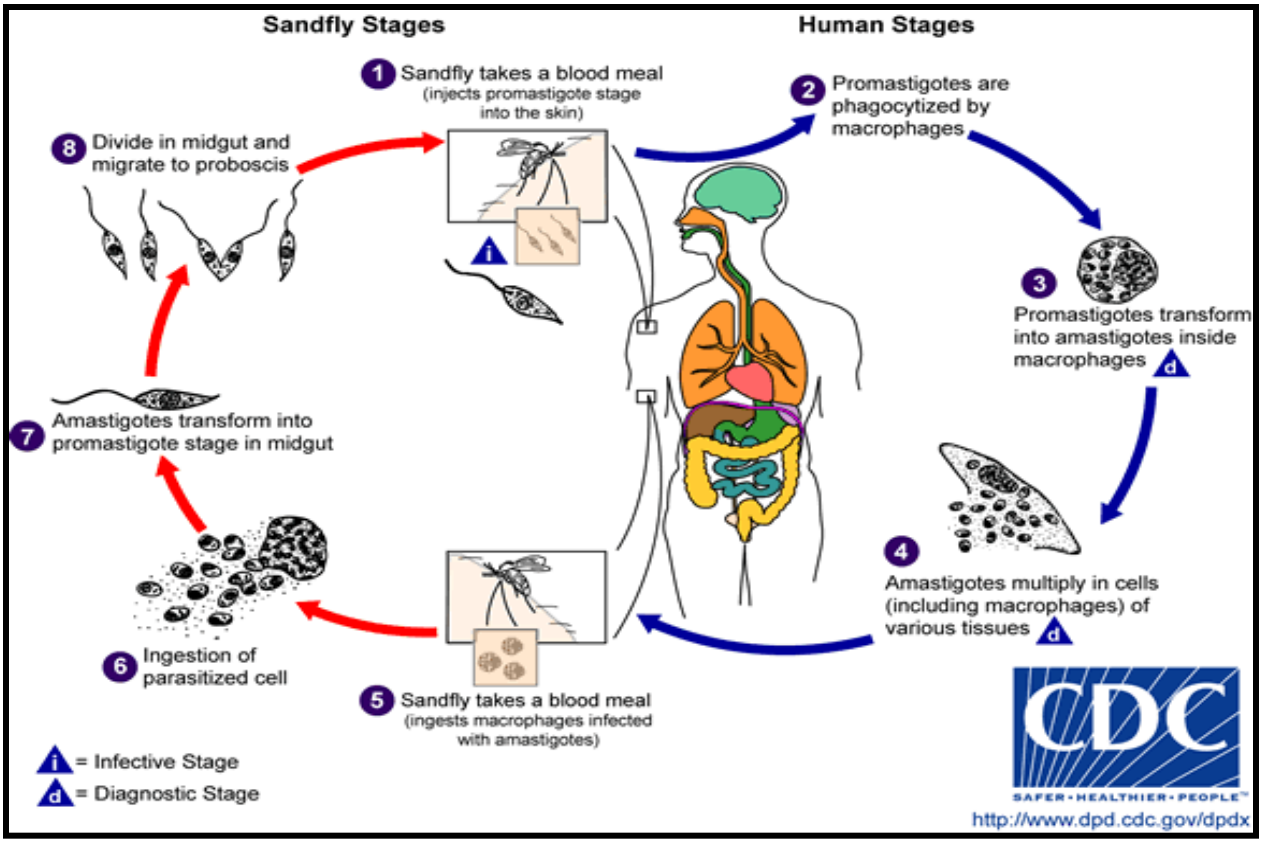

Figure 1. Life cycle for cutaneous leishmaniasis (Centers for Disease Control (CDC), 2009). All species of cutaneous leishmaniasis follow the same parasitic life cycle, alternating between the promastigote and amastigote life stages. Infection begins with an infected female sandfly bite to the mammal host. The sandfly becomes infected when feeding on the blood of an infected individual or an animal reservoir host.

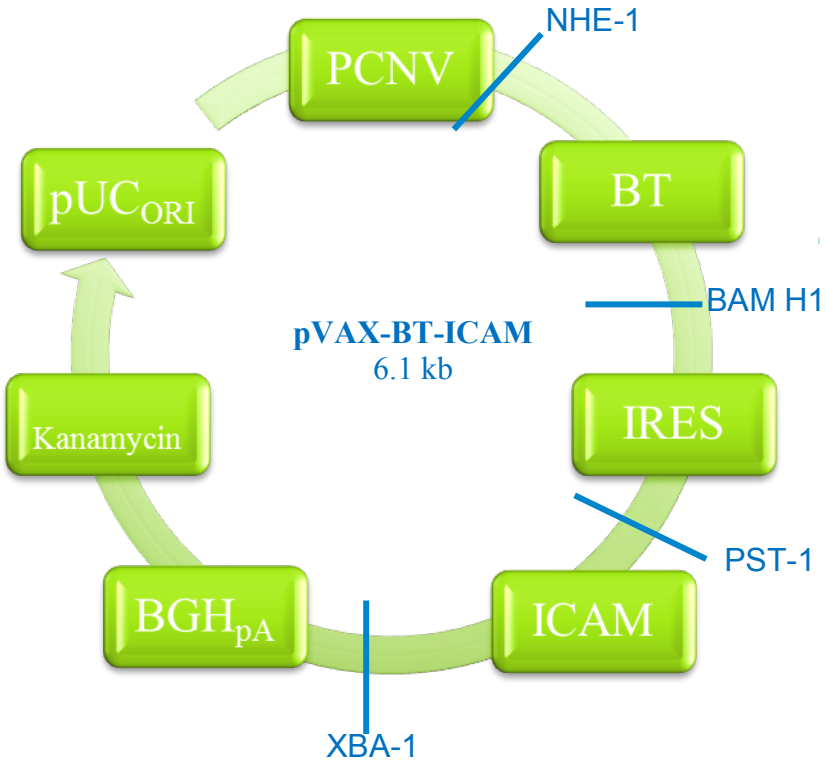

Figure 2. Schematic representation of pVAX backbone which encodes L. mexicana antigens BT and ICAM. PCNV serves as the promoter for BT and ICAM, also assures that BT and ICAM are expressed and read in ribosomes present in *Leishmania* life cycle. IRES reassure expression of ICAM. NEH-I, BAM H1, PST-1 and XBA-1 are restrictive enzymes present in the plasmid. BGHpA acts as the stop codon, whereas pUCORI is the start codon. Kanamycin is present in order to segregate those cells that are able to become resistant to the antibiotic from those that are not.

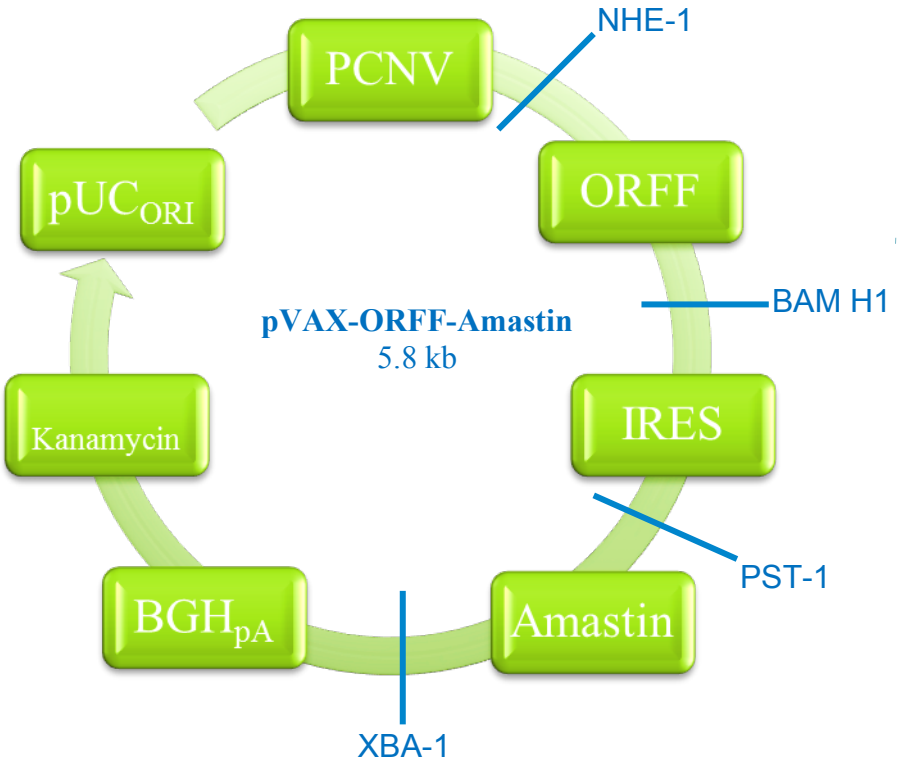

Figure 3. Schematic representation of pVAX backbone which encodes L. mexicana antigens ORFF and Amastin. PCNV serves as the promoter for ORFF and Amastin, also assures that ORFF and Amastin are expressed and read in ribosomes present in *Leishmania* life cycle. IRES reassure expression of Amastin. NEH-I, BAM H1, PST-1 and XBA-1 are restrictive enzymes present in the plasmid. BGHpA acts as the stop codon, whereas pUCORI is the start codon. Kanamycin is present in order to segregate those cells that are able to become resistant to the antibiotic from those that are not.

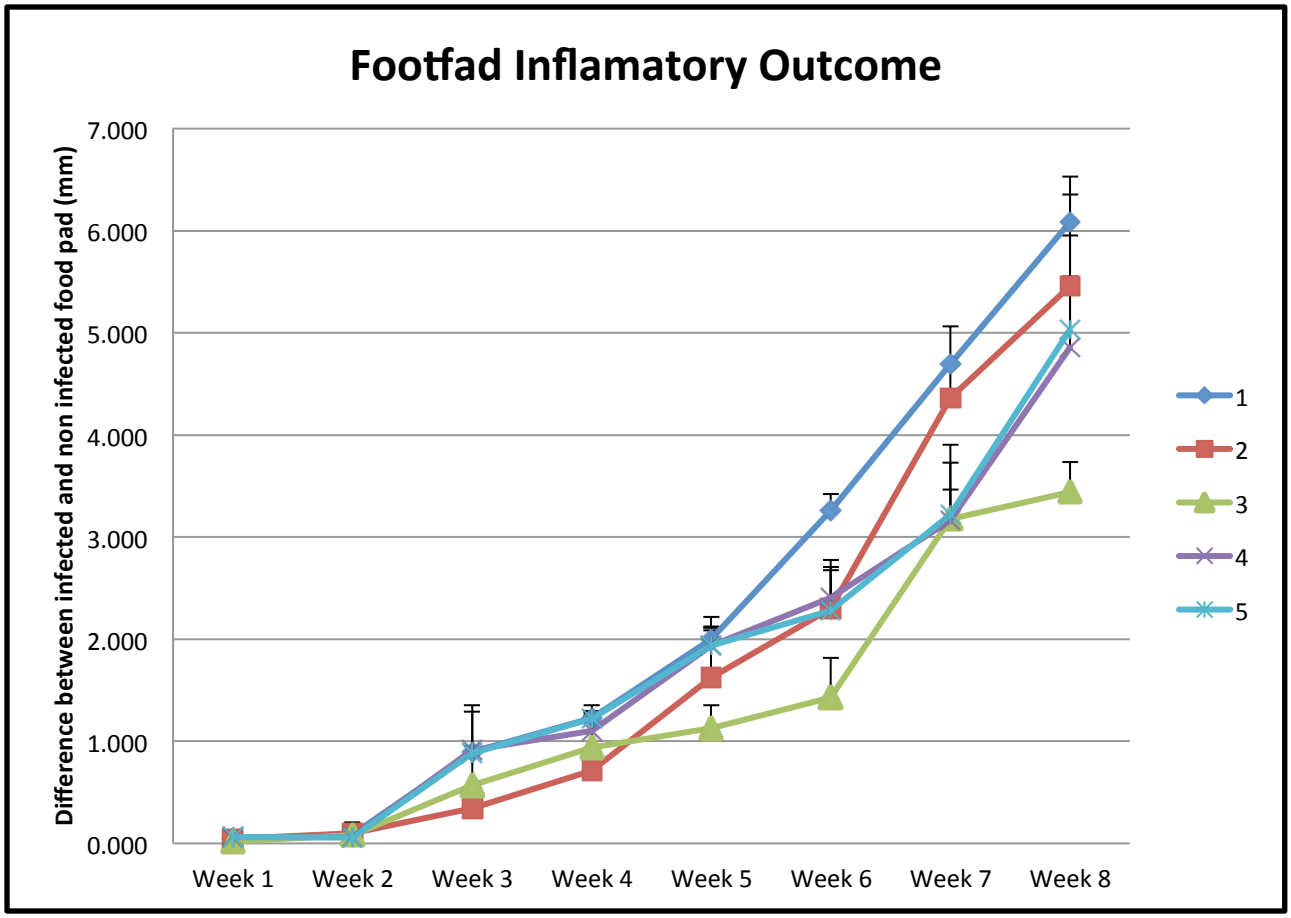

Figure 4. Level of protection of BALB/c mice vaccinates with pVAX-BT-ICAM (1), pVAX-ORFF-Amastin (2), and pVAX-BT-ICAM plus pVAX-ORFF-Amastin (3) vs. Control groups; PBS (4), and pVAX (5) over the eight weeks.

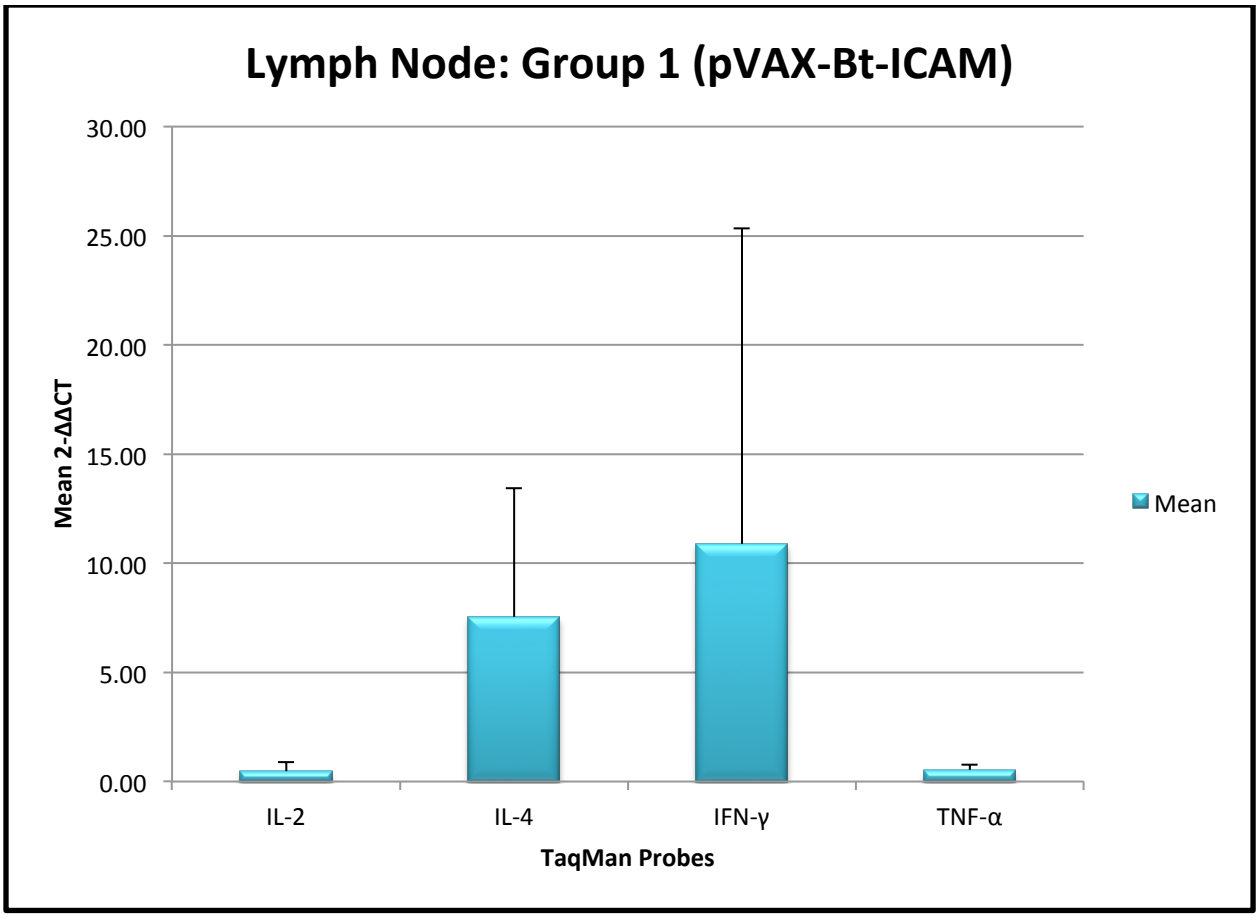

Figure 5. Real Time PCR results. Mean $2^{-\Delta\Delta C}{}_T$ for the lymph node cells using pVAX-BT-ICAM. Only IFN-γ was activated at a higher level.

IL-2 ($\bar{x}$ =0.47mm ± 0.41)
IL-4 ($\bar{x}$ =7.54mm ± 5.90)
IFN-γ ($\bar{x}$ =10.89mm ± 14.46)
TNF-α ($\bar{x}$ =0.53mm ± 0.25)

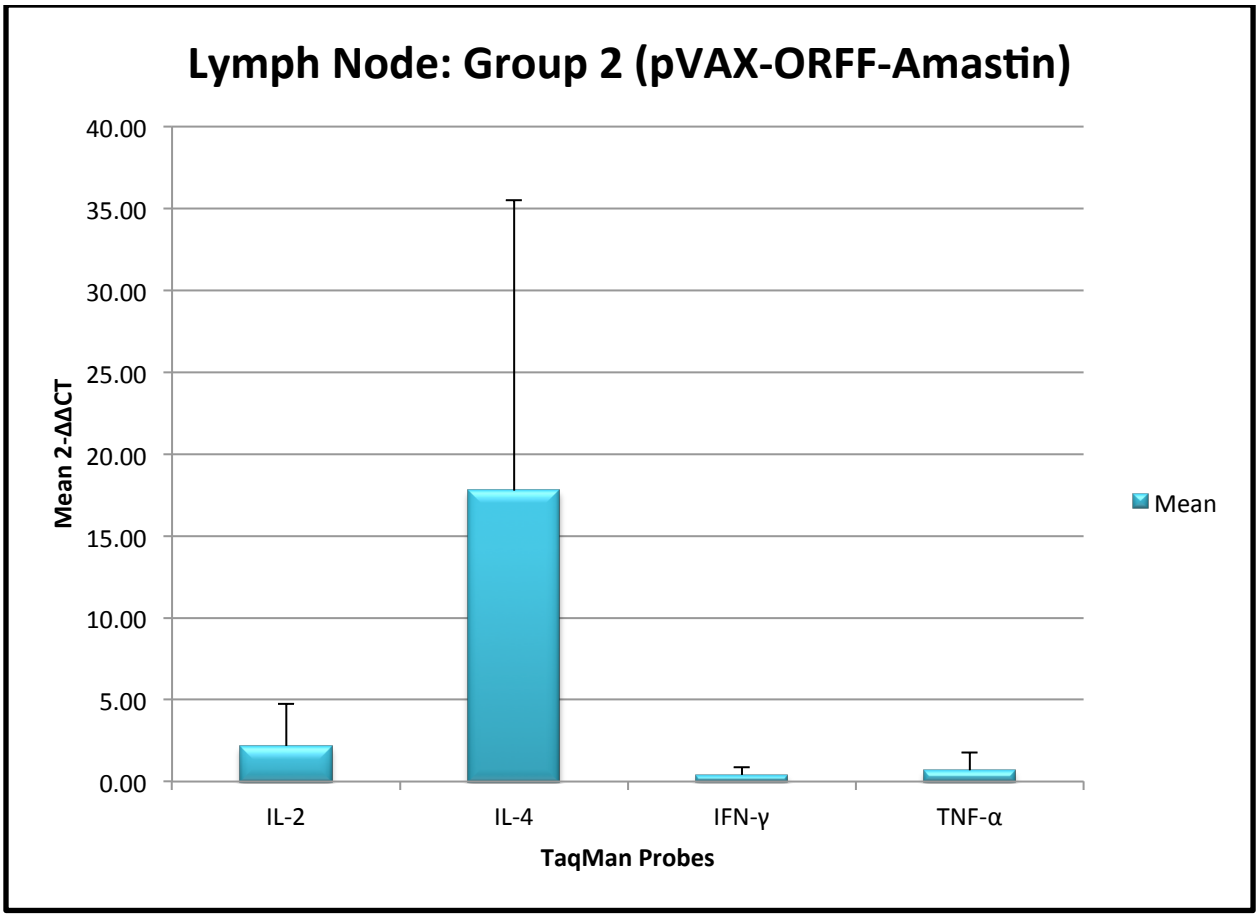

Figure 6. Real Time PCR results. Mean $2^{-\Delta\Delta C}{}_T$ for the lymph node cells using pVAX-ORFF-Amastin. The vaccine did induced protection in BALB/ mice.

IL-2 ($\bar{x}$ =2.18mm ± 2.58)
IL-4 ($\bar{x}$ =17.77mm ± 17.75)
IFN-γ ($\bar{x}$ =0.39mm ± 0.47)
TNF-α ($\bar{x}$ =0.69mm ± 1.08)

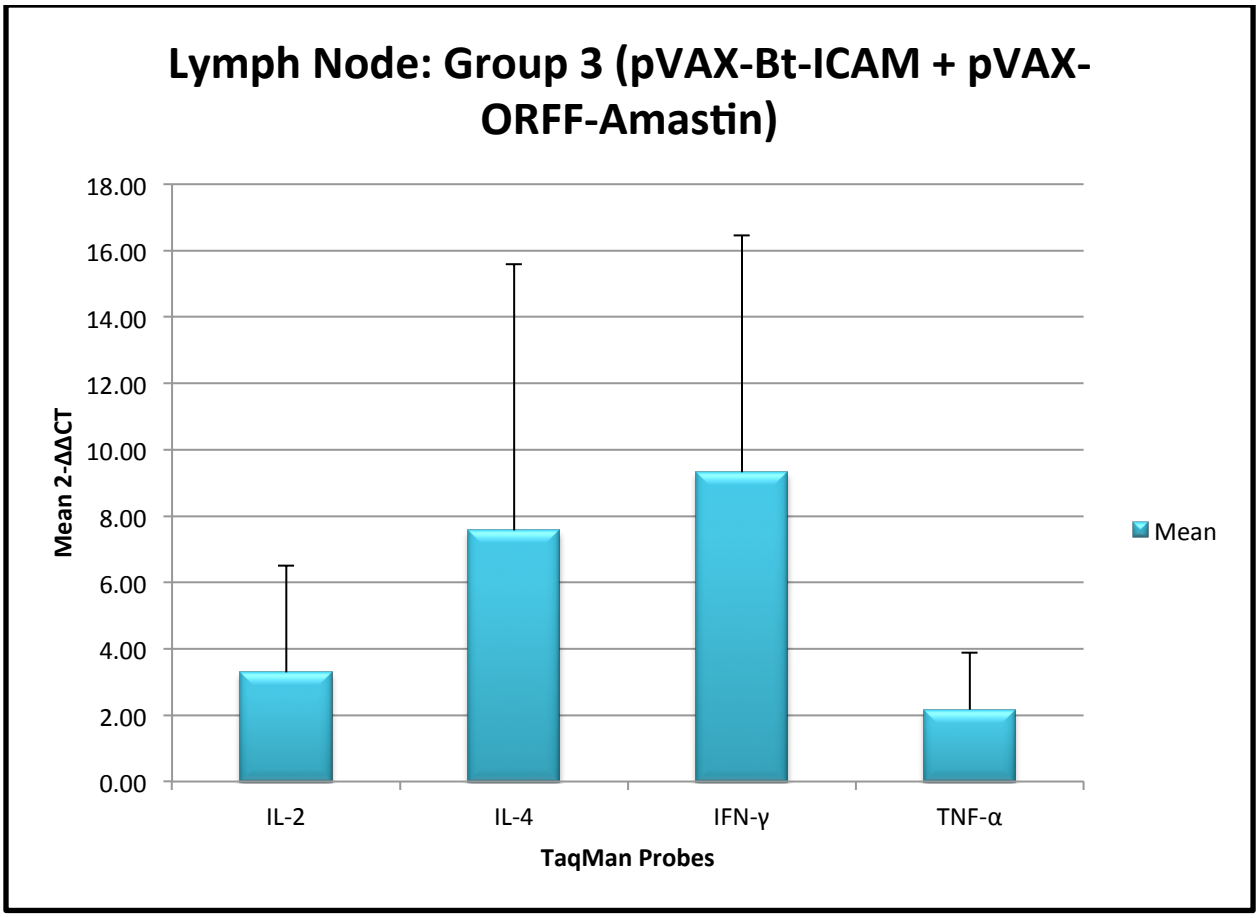

Figure 7. Real Time PCR results. Mean $2^{-\Delta\Delta C}{}_T$ for the lymph node cells using pVAX-BT-ICAM + pVAX-ORFF-Amastin. The vaccine induced partial protection in BALB/c mice.

IL-2 ($\bar{x}$ =3.29 ± 3.20)
IL-4 ($\bar{x}$ =7.57mm ± 8.02)
IFN-γ ($\bar{x}$ =9.32mm ± 7.14)
TNF-α ($\bar{x}$ =2.16mm ± 1.73)

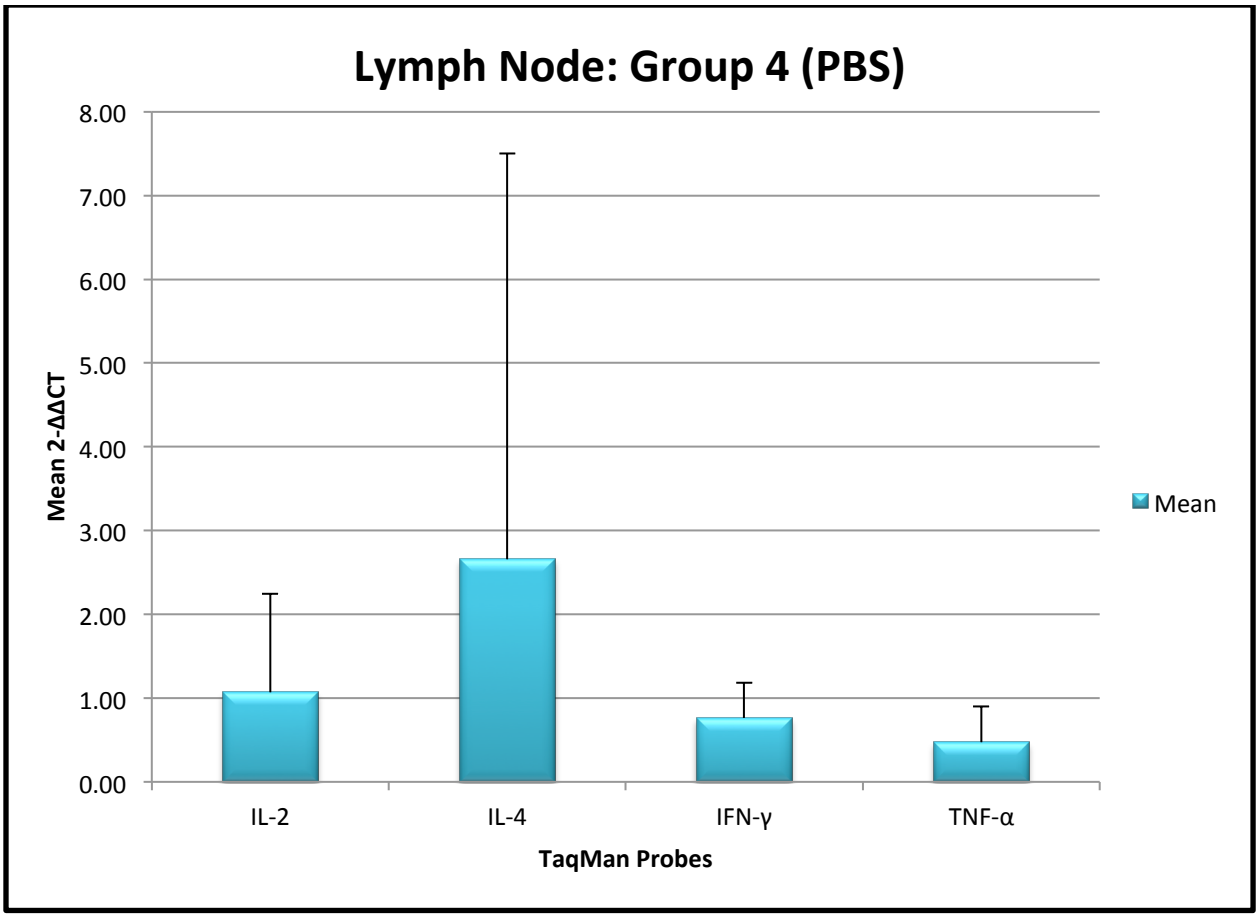

Figure 8. Real Time PCR results. Mean $2^{-\Delta\Delta C}{}_T$ for the lymph node cells using PBS. The control did not induced protection in BALB/c mice.

IL-2 ($\bar{x}$ =1.07mm ± 1.17)
IL-4 ($\bar{x}$ =2.66mm ± 4.84)
IFN-γ ($\bar{x}$ =0.76mm ± 0.43)
TNF-α ($\bar{x}$ =0.47mm ± 0.43)

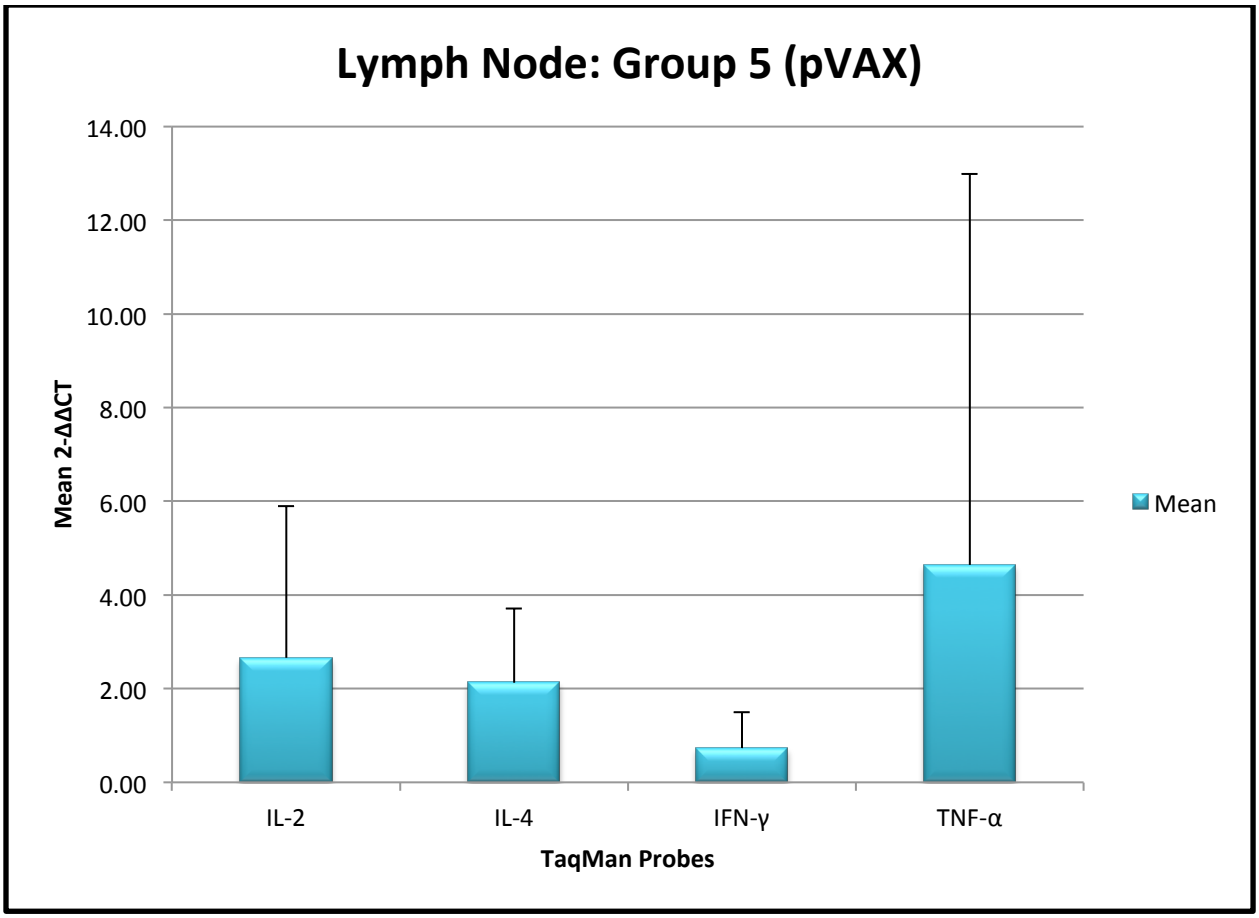

Figure 9. Real Time PCR results. Mean $2^{-\Delta\Delta C}{}_{T}$ for the lymph node cells using pVAX. The control did not induced protection in BALB/c mice.

IL-2 ($\bar{x}$ =2.66mm ± 3.24)
IL-4 ($\bar{x}$ =2.13mm ± 1.59)
IFN-γ ($\bar{x}$ =0.73mm ± 0.77)
TNF-α ($\bar{x}$ =4.64mm ± 8.34)

CURRICULUM VITAE

Rosina Rodarte was born in El Paso, Texas, the first daughter of Ismael Rodarte Rodriguez and Silvia Alarcon Encinas. She graduated from the University of Texas at El Paso in fall of 2008 obtaining the degree of Bachelor of Science in Chemistry with a minor in Biology. After completion of her bachelor's degree, Rosina pursued the Master of Public Health at the University of Texas at El Paso. In the summer of 2009, Rosina had the opportunity to do her practicum at the Pan American Health Organization U.S.-Mexico Regional Border Office. She was able to participate in the project screening blood samples from Chihuahua blood banks trying to identify Chagas disease. Later, she joined the Pan American Health Organization U.S.-Mexico Regional Border Office as a Consultant and Project Assistant. In 2009, she attended the World Congress on Public Health where she presented a poster on research for her thesis.