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Development of a Novel metallo-lipid microparticle Delivery System for a Leishmania mexicana DNA Vaccine Candidate

Joanna B. Valencia

University of Texas at El Paso, berepoppy03@Yahoo.com

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DEVELOPMENT OF A NOVEL METALLO-LIPID MICROPARTICLE DELIVERY SYSTEM FOR A
Leishmania mexicana DNA VACCINE CANDIDATE

JOANNA B. VALENCIA

Department of Public Health Sciences

APPROVED:

Rodrigo X. Armijos, M.D., ScD., Chair

M. Margaret Weigel, Ph.D.

Lorraine Torres, Ed.D.

Patricia D. Witherspoon, Ph.D.
Dean of Graduate School

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DEVELOPMENT OF A NOVEL METALLO –LIPID MICROPARTICLE DELIVERY SYSTEM FOR A
Leishmania mexicana DNA VACCINE CANDIDATE

BY

Joanna B. Valencia, B.S.

THESIS

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ABSTRACT

Background and Significance.

The leishmaniasis are an important group of neglected tropical parasitic diseases caused by parasitic protozoa of the genus *Leishmania*. International health authorities estimate that 350 million people around the world are at risk for contracting leishmaniasis. Two million new cases occur each year and 12 million people are presently infected worldwide. Leishmaniasis is endemic throughout the Americas region including all of Central America, most of South America, Mexico and southern Texas. *Leishmania* is also considered a threat to the military readiness of U.S. troops deployed to the Middle East or Latin America.

First- and second-line treatments for leishmaniasis are highly toxic. Drug resistance is on the increase. Elimination of the sandfly vector and mammalian reservoirs has not proven practical. For this reason, the World Health Organization has designated the development of an effective leishmaniasis vaccine as a major global public health priority.

Leishmanization is the oldest form of vaccination against leishmaniasis which had been in practice for hundreds of years. This practice was replaced later by an attenuated *Leishmania major* vaccine, but severe side effects and complications had limited its use. Research and technology have advanced into third generation vaccines, DNA vaccines. DNA vaccines are stable at high temperatures and inexpensive to produce. However, they possess lower than desired immunogenicity because of the degradation of DNA by DNase before they reach the target cells. Biopterin transporter (Bt), Intracellular adhesive molecule (ICAM), open reading frames (ORFF), and Amastin are important molecules found in *Leishmania* amastigote or promastigote stages. Previous studies have shown that Bt and ORFF can produce partial

protection against experimental leishmaniasis. Therefore, the development of a method to protect DNA until its delivery to the target cell, i.e., the *Leishmania* parasite, would be an important scientific advance. Encapsulating DNA within a degradable delivery system such as in nanoparticles could theoretically help to improve the delivery of leishmaniasis DNA vaccines to target cells since degradation would be reduced.

Objectives.

The two major objectives of the Phase 0 experimental study were to: (1) describe the immune response and side effects induced by a DNA vaccine candidate encapsulated within metallo-lipid microparticles and (2) define protection generated by a DNA vaccine candidate metallo-lipid microparticle against infection caused by *Leishmania mexicana* in murine models.

Hypothesis.

It is hypothesized that the use of a DNA delivery system incorporating copper or zinc metallo-lipid microparticles will increase the immunogenicity of the DNA leishmaniasis vaccine candidate by decreasing the amount of degradation that occurs prior to the delivery to the target cells.

Methodology.

The experimental study was conducted using 6-week old female BALB/c mice. The mice were randomized to one of seven groups. Group 1 (n=3) received pVAX-bt-icam-I(5µg) + pVAX-orff-amastin(5µg)+ metallo-lipid Zinc 32ng (ML-Zn). Group 2 (n=3) received pVAX-bt-icam-I(5µg) + pVAX-orff-amastin(5µg)+ metallo-lipid Copper 32ng (ML-Cu). Group 3 (n=4) received pVAX-bt-icam-I(5µg) + pVAX-orff-amastin(5µg)+PBS. Group 4 (n=6) received pVAX-bt-icam-I(50µg) +

pVAX-orff-amastin(50µg). Group 5 (n=4) received metallo-lipid Copper (ML-Cu). One control group, Group 6 (n=6), received 100µL of PBS and the other, Group 7 (n=7), received only PVax.

Six weeks after the first vaccination, the mice were challenged with *Leishmania mexicana* (10^6) in their right hind footpad. Footpad thickness was measured weekly for eight weeks. The mice also were examined for potential adverse effects. Two aliquots of lymph nodes and spleen cells from the sacrificed mice were used to analyze intracellular cytokine expression by flow cytometry. Gene expression was analyzed using Quantitative Real Time Polymerase Chain Reaction with TaqMan Probes (Interleukin-2, Interleukin-4, Interleukin-10, and Tumor Necrosis Factor- α , Interferon- γ). Tissue samples from hind foot pad lesions were processed and plated to determine the amount of parasites present per gram of tissue.

Results.

The study results suggested that a partial protective effect occurred in the pVAX-ORFF-Amastin (5µg) + pVAX-Bt-ICAM (5µg) + ML-Cu and pVAX-ORFF-Amastin + pVAX-Bt-ICAM (100µg) experimental groups. These groups had smaller mean footpad lesions compared to the control group mice. In addition, the pVAX-ORFF-Amastin (5µg) + pVAX-Bt-ICAM (5µg) + ML-Cu group had a Th1 cytokine profile (high IFN- γ , and low IL-4 and IL-10 with values of 27.5, 0, and 0.57 χ -fold more RNA copies than the calibrator sample, respectively. This type of pattern has been reported as associated with immune protection against *Leishmania* infection.

Discussion.

The results of this study suggest that pVAX-ORFF-Amastin (5µg)+ pVAX-Bt-ICAM(5µg)+ML-Cu is immunogenic and provides partial protection in the BALB/c mouse model.

Further experimentation is needed to increase the immunogenicity of this candidate vaccine such as increasing the concentration of the plasmid or ML-Cu microparticle.

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

1.1 Epidemiology of Leishmaniasis

The leishmaniasis are a group of vector-borne parasitic disease caused by protozoan flagellates of the genus *Leishmania* (Chakravarty and Sundar, 2010; Dedet, 2002).

Leishmaniasis is widely distributed around the world. It currently threatens 350 million people in 88 countries in five continents (Reithinger, 2007). Out of the 88 countries, 16 are industrialized and 72 are developing countries. Thirteen of these are ranked among the poorest in the globe (Shaw, 2002). Leishmaniasis can be found in Europe, Africa, Asia, South America, Central America, and North America (Shaw, 2002). It is estimated that there are two million new cases each year and it is responsible for 70,000 deaths per year (Murray et al., 2005). There are currently 12 million people infected in the world (Reithinger, 2007).

1.2 *Leishmania* Species

There are approximately 25 different *Leishmania* species. These include Old World and New World species that differ from each other regarding their molecular and biochemistry characteristics (Reithinger et al., 2007). The *Leishmania* species found in the Old World are *Leishmania donovani* (Africa, Asia), *Leishmania major* (Middle East, Asia, and Africa) *Leishmania infantum* (Europe), and *Leishmania tropica and aethiopica* (Asia, Middle East, and Africa) (Killick-Kendrick, 2002). These species cause cutaneous, visceral, and mucocutaneous leishmaniasis (Killick-Kendrick, 2002). The *Leishmania braziliensis* complex (*L. braziliensis*, *L. panamensis*, *L. guyanensis*, and *L. peruviana*) cause cutaneous and mucocutaneous leishmaniasis (Shaw, 2002). The *Leishmania mexicana* complex (*L. mexicana*, *L. amazonensis*, and *L. venezuelensis*) is the causative agent of cutaneous and diffuse leishmaniasis.

The *L. Mexicana* complex has a wide distribution. *L. mexicana* is found from northern Brazil to South-Central U. S. in Texas and Oklahoma (Bailey & Lockwood, 2007).

Leishmania is present in the New World from Brazil up through Central America, Mexico, and South Central United States (CDC, 2008). In 2006, there were 62000 leishmaniasis cases reported in the Americas region (PAHO, 2007). More than 5000 of these were visceral leishmaniasis cases (PAHO, 2007). In 1903 there were 30 indigenous cases reported in the southern part of Texas (Wright et al., 2008) and subsequently eight local cases of cutaneous leishmaniasis were reported from 1970-1989 (Furner, 1990). From 2005-2007 there were nine cases reported in the northern part of Texas around the Dallas-Fort Worth metroplex area (Wright et al., 2008). Leishmaniasis is not considered to be endemic in Texas. However, these findings do illustrate that both the vector and reservoir species are spreading into the United States (Wright et al., 2008).

1.3 Clinical Manifestations

Leishmania parasites can cause four different clinical manifestations: cutaneous leishmaniasis, mucocutaneous leishmaniasis, visceral leishmaniasis and diffuse cutaneous leishmaniasis (Desjeux, 2004). Clinical severity primarily depends upon host immunity and the parasite species involved (Murray et al., 2005).

Cutaneous leishmaniasis is predominantly caused by *L. major* in the Old World and by many several species in the New World including *L. mexicana* being one of the most prevalent (Desjeux P. , 1996). Cutaneous leishmaniasis is characterized by self-healing lesions at the site of the sandfly bite (Pearson & Sousa, 1996). This clinical manifestation is limited to skin lesions which, rarely penetrate into the subcutaneous tissues. Occasionally, it may affect deeper tissue

such as mucus membranes, depending on the *Leishmania* species (Bailey & Lockwood, 2007). Incubation periods range from a few days to months. The lesions vary from ulcerating to smooth nodules, tend to be circular or oval, and are usually not larger than 10 cm in diameter (Bailey & Lockwood, 2007). Typically, the sandfly bite will evolve into a nodule that will enlarge and ulcerate which takes about one to three months (Murray et al., 2005). Active lesions and permanent scar formation on exposed body parts such as hands and face are associated with social stigma (Bern et al., 2008). Social stigmatization from this disease may cause anxiety, depression symptoms, decreased body satisfaction and an overall diminished quality of life (Yanik et al., 2004).

Mucocutaneous leishmaniasis is caused by *L. brasiliensis*, *L. panamensis*, and *L. guayanensis* (Shaw, 2002). This infection is due to the migration of the parasite via blood or lymph to the mucosal membranes such as the mouth, nasal passages, pharynx, and rarely the genitals (Osorio et al., 1998). Clinical progression to this mucosal disease appears in 1-10% of patients (David & Craft, 2009). When left untreated, these lesions can result in facial deformities and is potentially life threatening (Bern et al., 2008; David and Craft, 2009).

Visceral leishmaniasis is caused by *L. donovani*, *L. infantum*, and *L. chagasi* (Pearson & Sousa, 1996). Persons with this clinical form usually have symptoms such as fever, malaise, weight loss (Pearson & Sousa, 1996). The incubation period may range from 10 days to 34 months, but is typically 3-8 months (Pearson & Sousa, 1996). The parasite attacks the liver which causes hepatomegaly and splenomegaly (Pearson & Sousa, 1996). If left untreated, infected individuals may develop diarrhea, tuberculosis, measles or other secondary infections

in the late stages due to immunosuppression (Pearson & Sousa, 1996). These are the most frequent causes of death in individuals with active late-stage visceral disease.

The least common clinical manifestation is diffuse cutaneous leishmaniasis. There have been documented cases in Latin America, the United States of America, and Africa (Convit et al., 1972). Diffuse cutaneous leishmaniasis is predominantly caused by *L. mexicana* and rarely by *L. amazonensis* (Shaw, 2002). Parasite filled nodules appear throughout the individual's body (Sinha et al., 2008). Inflammation of the lymph nodes in the neck, shoulder-blade and groin areas are common in patients with this form (Convit et al., 1972). This clinical manifestation appears to be more frequent among individuals with defective cell-mediated immune responses (Desjeux, 2004). Relapse is inevitable (Desjeux P. , 2004).

1.4 Leishmaniasis Risk Factors

Leishmaniasis in the New World is considered an occupational disease due to the risk associated with agriculture, mining, and oil industry work in endemic areas where the vector is present (Desjeux, 1996). Other risk factors are migration, urbanization, co-morbidities including HIV/AIDS, immunosuppression, and malnutrition can also affect the infection (Desjeux P. , 2001; Sinha et al., 2005). Tourists and military personnel may also be infected when traveling to endemic regions such as the Middle East and Latin America (Cardo, 2006). One risk factor in endemic regions is blood contamination there have been a few leishmaniasis cases described after organ transplant or blood transfusion (Cardo, 2006).

1.5 *Leishmania* Life Cycle

Leishmania parasites have a dimorphic life cycle which includes a promastigote and an amastigote stage (Dumonteil et al., 2003). Figure 1 shows, the female sandfly becomes

infected once it takes a blood meal from an infected mammal reservoir or human host (CDC, 2008). Female sand flies need protein with high biological value for the maturation of their fertilized eggs which, they obtain from blood meals. Approximately eight hours after the blood meal reaches the gut, *Leishmania* amastigotes transform into promastigotes (Olivier et al., 2005). The promastigotes subsequently migrate up the alimentary tract of the sandfly where they multiply by binary fission and live extracellularly (Mosser & Brittingham, 1997). Four to five days later, the promastigotes travel up to the esophagus to the salivary glands of the sandfly where they accumulate until the next blood meal (Sanguenza et al., 1993). Once the proboscis pierces the skin during the sandfly's next feeding, the saliva containing anti-coagulant and the promastigotes are introduced into the mammalian host (Pearson & Sousa, 1996). Once inside the host, the promastigotes are engulfed by macrophages where they rapidly revert to amastigotes, the obligate intracellular stage (Olivier et al., 2005).

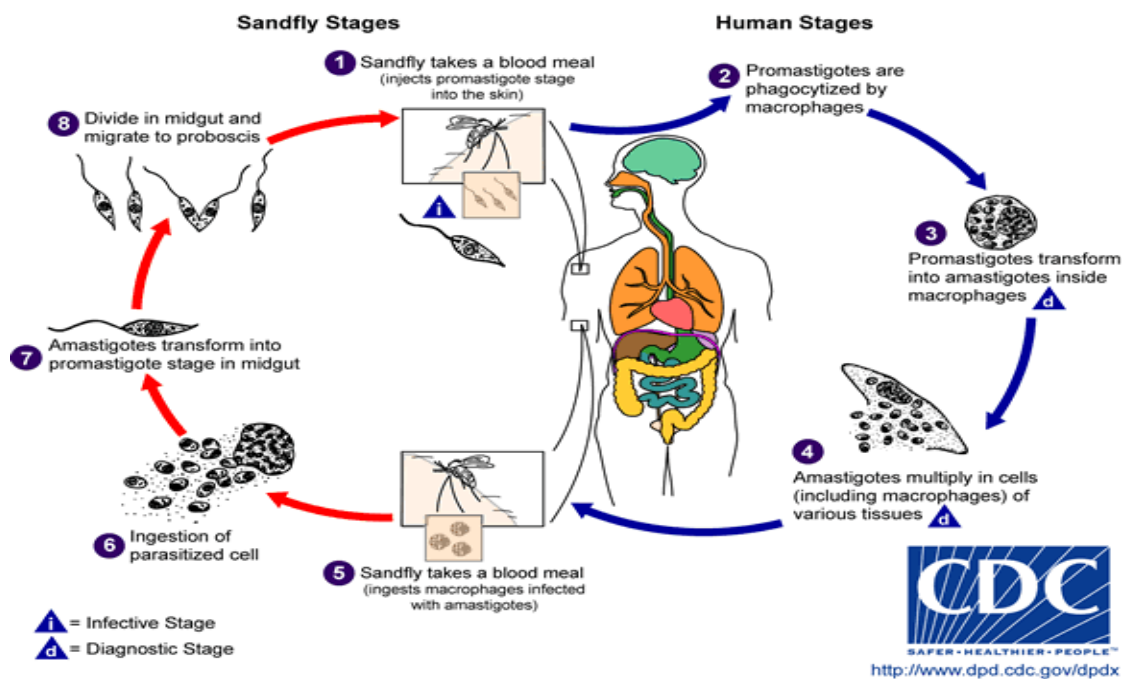


Figure 1. (Leishmaniasis, 2009)

1.6 *Leishmania* Morphology

The amastigote stage occurs in the host where the parasite is able to survive and multiply inside macrophages. The amastigotes are usually two to five micrometers in length (Garnham, 1971). Amastigotes tend to have a spherical shape; they multiply by binary fission, and have an internalized flagellum (Garnham, 1971). Promastigotes are elongated in shape, 20µm in length, and have an external flagellum. These live inside the sandfly vector (Olivier et al., 2005). Promastigotes also multiply by binary fission but division starts in the anterior portion of the parasite (Garnham, 1971). The promastigote stage may be further divided into the procyclic and infective metacyclic stages (Olivier et al., 2005). Pro-cyclic promastigotes multiply in the gut. In contrast, the meta-cyclic promastigotes can be found in the mouthparts and are infective (Olivier et al., 2005).

1.7 *Leishmania* Vector

The sandfly corresponding to Suborder Nematocera: Order Diptera: Family Psychodidae: Sub-Family Phlebotominae is the *Leishmania* vector (Killick-Kendrick, 2002). The female sandfly is the vector for all *Leishmania* species approximately 30 species of sand flies are documented as *Leishmania* vectors (Desjeux P. , 1996). The Old World (Europe, Asia, and Africa) genera is *Phlebotomus* is divided into 12 subgenera. The New World (North America and South America) genera is *Lutzomyia* which is divided into 25 subgenera. All of these are proven vector species that transmit *Leishmania* are in these two genera (Killick-Kendrick, 2002). *Lutzomyia diabolica* and *Lutzomyia anthophora* have been identified as vectors for *L. mexicana*, and can be found in Mexico and the United States (Gonzalez et al., 2010).

1.8 *Leishmania* Reservoir

Reservoirs for *Leishmania* include rodents, sloths, primates, and carnivores (Saliba & Oumeish, 1999). *Leishmania major*'s main reservoir is the great gerbil, *Rhombomys opimus*, in Central Asia (Saliba & Oumeish, 1999). There are many species of rodents that serve as reservoir for *L. mexicana*. One of the primary hosts found in Mexico and Belize is *Ototylomys sp* (Saliba & Oumeish, 1999). Secondary hosts belong to the genera *Heteromys*, *Nyctomys*, *Oryzomys*, and *Sigmodon* (Saliba & Oumeish, 1999). The wood rat, *Neotoma micropus*, is reported as a reservoir found in Texas (Saliba & Oumeish, 1999). *Neotoma mexicana* is found in northern parts of Mexico and in the United States and is also believed to be a reservoir host for *L. mexicana* (Gonzalez et al., 2010).

1.9 Leishmaniasis Diagnosis

Definitive diagnosis procedures use tissue samples which, are either acquired by scraping or biopsy taken from the edge of the lesion which do not exacerbate the ulcerated lesion (Bailey & Lockwood, 2007). The tissue samples can be stained with Giemsa at a pH of 6.8 in order to look for *Leishmania* amastigotes (Roussel et al., 2006; Desjeux, 1996). Polymerase chain reaction (PCR) tests can also be performed on the DNA obtained from tissue samples and have shown high specificity and high sensitivity which enables a scientist to do a species specific diagnosis in a few hours (Berman, 1997).

1.10 *Leishmania* Treatment

The first-line treatment for leishmaniasis recommended by the World Health Organization (WHO) is pentavalent antimony. This drug is administered by injection via parenteral, intramuscularly or intravenously (Reithinger et al., 2007). This treatment has

potentially toxic side effects including musculoskeletal pain, renal failure, hepatotoxicity and cardiotoxicity. It is not recommended for pregnant and breastfeeding women, very young children, and individuals with some chronic illnesses (Reithinger et al., 2007). Misuse of first-line antileishmanial drugs has led to parasite resistance (Chakravarty & Sundar, 2010; Berman, 1997).

An alternative injectable treatment is amphotericin B which is administered by i.m. injection (Reithinger et al., 2007). The drug a fungizone with toxic side effects, is usually given to patients who do not respond to the pentavalent antimony treatment (Berman, 1997). Pentamidine is also administered by injection via parenteral and is prescribed at 2mg/kg on alternate days for seven doses, which also has toxic side effects (Soto-Mancipe et al., 1993; Lai A Fat et al., 2002). Other treatments are topical creams such as paromomycin which is prescribed for cutaneous leishmaniasis (Croft & Yardley, 2002). An application of twice daily on the lesion from 10 to 20 days and was seen to be effective towards cutaneous leishmaniasis (Croft & Yardley, 2002). Alternative oral treatments are miltefosine and ketoconazole which are prescribed for treating Old World visceral and cutaneous leishmaniasis (Saenz et al., 1990; Soto et al., 2004). Miltefosine is an oral treatment prescribed as 2.5 mg/kg for duration of 28 days and has limited toxic-side effects, but less effective for New World cutaneous leishmaniasis (Soto et al., 2004). The available first-line and second-line drugs for leishmaniasis have severe toxic side effects; thus, the prevention through the use of a vaccine would be the best strategy.

1.11 *Leishmania* Molecules

Leishmania parasites appear to possess several different survival mechanisms. The metacyclic promastigotes have long branched lipophosphoglycans (LPG) on their surface which seems to prevent the attachment of C5b-C9 and avoids complement driven lysis (Beverley & Turco, 1998; Olivier et al., 2005). Another surface molecule present on the amastigote surface is a glycoprotein (Gp63) that has been shown to help in amastigote survival (Olivier et al., 2005). The most abundant surface molecule on the promastigote stage is glycosylinositol phospholipid (GIPL). This molecule has a short length and a long half-life which has been suggested as capable of defending parasites against proteolytic damage (Olivier et al., 2005).

Other important molecules found in *Leishmania* species are Biopterin Transporter (BT), intracellular adhesive molecule (ICAM), open reading frames (ORFF), and amastin. The biopterin transporter gene is responsible for encoding the transport of biopterin an essential nutrient into the parasite (Landfear, 2002). This gene also has shown partial protection against leishmaniasis when used as a vaccine (Papadopoulou, 2002). ICAM was first described in *L. amazonensis* (Chiang et al., 2002). It is present on the parasite surface in the promastigote stage as well as in the nucleus (Chiang et al., 2001). The ORFF gene was first described in *L. donovani* by Myller (Myller et al., 1994). This molecule has protein coding functions which confer an advantage in parasitic growth and survival (Ghosh et al., 1999). Amastin is present during the intracellular amastigote stage of the parasite life cycle (Rochette, 2005). It plays a role in proton or ion traffic across the cellular membrane in order to adjust cytoplasmic pH (Wu, 2000).

1.12 *Leishmania* Immunology

Both Th1 and Th2 immune responses are activated during *Leishmania* sp. Infection. Each of these stimulate the production of different cytokines (Heinzel, 1991). Immunity against leishmaniasis occurs through the stimulation of Th1 cells to produce lymphokines including interferon-gamma (IFN- γ), interleukin-12 (IL-12), and tumor necrosis factor-alpha (TNF- α) (Scott, 1988, Liew, 1990). These activate macrophages to destroy intracellular amastigotes (Scott, 1988, Liew, 1990). Interleukin-2 (IL-2), produced by Th1 cells, causes macrophage activation indirectly by stimulating the secretion of IFN- γ (Murray, 1993). This autocrine also makes Th1 immune response more efficient. The suppressive Th2 immune response produces IL-4, IL-5, IL-6, and IL-10 which downregulate Th1 immunity. Macrophages are thus unable to effectively kill the intracellular amastigotes; this allows *Leishmania* survive and replicate within phagolysosomes of resident macrophage resulting in disease chronicity (Liew, 1989).

1.13 Experimental Leishmaniasis Vaccines

The evidence indicates that once an individual has been infected with pathogenic species of *Leishmania* they develop resistance to subsequent infection (Reithinger et al., 2007). This suggests anti-*Leishmania* vaccines would be a feasible prevention method (Reithinger et al., 2007). There are no current approved vaccines against any form of leishmaniasis for human use. However, a number of different experimental vaccines are being tested (Handman, 2001). Leishmanization is the oldest form of vaccination against leishmaniasis (Dunning, 2009). It produces strong subsequent immunity to the disease (Dunning, 2009). Leishmanization involves inoculating virulent *Leishmania* from a cutaneous lesion into a less visible area of the body in order to prevent multiple lesions to the face or other exposed regions of the body

(Dunning, 2009). Large scale vaccination trials using attenuated *L. major* were conducted in the 1970s and 1980s in Israel, Iran and the Soviet Union where a higher than expected number of complications such as psoriasis, immunosuppression and large persistent lesions were observed in inoculated patients (Dunning, 2009). This experience caused a search for new strategies using whole parasites for vaccine trials against leishmaniasis (Dunning, 2009). A live vaccine is still in use in Uzbekistan which is a mixture of *L. major* and killed parasite which decreases its virulence (Noazin et al., 2008). A clinical trial done in Colombia against American cutaneous leishmaniasis with a killed whole-cell *L. amazonensis* candidate vaccine did not offer any protection (Velez et al., 2005). A cocktail of killed *L. braziliensis*, *L. guyanensis*, and *L. amazonensis* vaccine with BCG adjuvant was used in a study in Ecuador against cutaneous leishmaniasis and produced a 72.9% protective efficacy (Armijos et al., 1998).

There is also a fucose mannose ligand (FML) vaccine that induced protection for up to three and a half years against canine visceral leishmaniasis in Brazil (Borja-Cabrera et al., 2004). A protein purified vaccine, dp72, isolated from *Leishmania donovani* promastigotes showed partial protection in BALB/c mice when infected with *L. donovani* and *L. major* (Rachamim & Jaffe, 1993). Loss of potency and stability during transportation for second generation vaccines has lead research into third generation vaccines (DNA vaccines) which are more stable (Palatnik-de-Sousa, 2008).

1.14 DNA Vaccines

DNA vaccines are relatively simple and inexpensive to produce (Sasaki et al., 2003). Genes are inserted into a plasmid and once injected are read by the target cells, and in turn, produces proteins. These proteins are foreign to the body and will be destroyed by

macrophages and natural killer cells (Xiang et al., 2010). They will be recognized by T-cells which will then produce memory T-cells (Xiang et al., 2010). These will be able to identify the pathogen if the individual's immune system is attacked by it (Xiang et al., 2010). Such vaccine candidate can effectively engage both major histocompatibility class (MHC) I and MHC II pathways that activate both CD8+ and CD4+ T cells (Khamesipour et al., 2006). DNA vaccines are also stable at high temperatures and safer as they do not contain virulent *Leishmania* pathogens (Dumonteil, 2007).

DNA vaccine research has been tested against *Leishmania*. Anti-*L. mexicana* DNA vaccines that encode for GP63, CPb, LACK and GP46, present in *L. amazonensis*, were used and evaluated in BALB/c mice (Dumonteil, 2003). Dumonteil and his colleagues reported partial protection against infection with each separate vaccine (Dumonteil, 2003). The protection was further increased when a mixture of the GP63, CPb, and GP46 vaccines were administered to the mice suggesting that these three antigens would be good candidates in DNA vaccines against *L. mexicana* (Dumonteil, 2003). Substantial protection was seen against *L. donovani* with a DNA vaccine using ORFF as an antigen (Sukumaran et al., 2003). Another study that used PSA-2 as an antigen in a DNA vaccine against *L. major* also showed significant protection against infection; but, the antibody levels were miniscule (Dumonteil, 2003).

1.15 Nanocarriers for Vaccine Delivery

DNA and recombinant protein vaccines are quite effective but lose immunogenicity due in part to the presence of DNase (Shahiwala et al., 2007). In order to increase immunogenicity there is a need for safe adjuvants and/or delivery systems that can be used for DNA vaccines (Shahiwala et al., 2007). An effective nanocarrier needs to protect the DNA in the vaccine from

extracellular and intracellular degradation and accommodate large plasmids (Xiang et al., 2010). Different nanocarrier delivery systems have been designed in order to provide effective immunization by enhancing an antibody response at the cellular level (Shahiwala et al., 2007). Liposomes, dendrimers, micro and nano particles, emulsions, micelles, and immunostimulating complex (ISCOMs) have been used as delivery systems against pathogens (Xiang et al., 2010).

Liposomes are organized phospholipid vesicles that have been used to encapsulate protein and DNA vaccines and increase the immune response (Shahiwala et al., 2007). The mechanism by which liposomes enhance the adjuvant properties is not well understood but their interaction with macrophage and dendritic cells after the activation of the complement system could be a major factor (Shahiwala et al., 2007). There is a developed liposomal vaccine against Tetanus toxoid that was found to be effective in inducing mucosal immunity (Tafaghodi et al., 2006).

Nanoparticle complexes are used to target dendritic uptake and have been shown to improve immunogenicity (Xiang et al., 2010). A study using micro-particles and nanoparticles encapsulated with *Toxoplasma gondii* tachyzoites used as an intranasal vaccine showed increased levels and higher mucosal and systemic immunity (Stanley et al., 2004).

Dendrimers are synthetic polymers that have a branched shape (Boas Heegaard, 2004). These have been shown to increase immune response against influenza (Boas Heegaard, 2004). Small & amounts of dendrimers have been shown to be effective adjuvants for influenza and do not cause toxic complexes (Shahiwala et al., 2007). These polymers have also been reported as effective in increasing an immune response against renal cell cancers (Shahiwala et al., 2007). Immunostimulating complexes (ISCOM) are open complexes that usually have a diameter of 30-

80 nm and are made up of phospholipids, cholesterol, immunogen, and saponin (Sun et al., 2009). When ISCOMs are used as vaccine adjuvants they produce a broad immune response such as high T cell response and increased antibody levels (Sun et al., 2009). These studies suggest that nanocarriers can be effective in increasing immunity against pathogens.

1.16 Study Rationale

There has yet to be an effective preventative vaccine against cutaneous leishmaniasis. Most of the experimental naked DNA vaccines have shown poor immunogenicity mainly because they undergo massive degradation immediately after being inoculated into experimental animals and before reaching target cells. Therefore, the development of an efficient system for DNA delivery is needed. A metallo-lipid microparticle delivery system may be an effective alternative in increasing DNA vaccine immunogenicity because it encapsulates the DNA and protects it from DNase until it reaches the target cells in the mouse.

CHAPTER 2: STUDY AIMS AND HYPOTHESIS

2.1 Hypothesis

It is hypothesized that the use of a DNA delivery system incorporating copper or zinc metallo-lipid microparticles will increase the immunogenicity of the DNA leishmaniasis vaccine candidate by decreasing the amount of degradation that occurs prior to the delivery to the target cells.

2.2 Study Objective

The overall objective of the proposed experimental study is to determine the efficacy of pVAX-Bt-ICAM and pVAX-orff-Amastin plus metallo-lipid microparticles in preventing murine leishmaniasis caused by *Leishmania mexicana*.

The specific aims of the study are:

1. To describe the immune response and side effects induced by a DNA vaccine candidate encapsulated within metallo-lipid microparticles.
2. To define protection generated by a DNA vaccine candidate metallo-lipid microparticle against infection caused by *Leishmania mexicana* in a susceptible BALB/c murine model.

CHAPTER 3: METHODS AND MATERIALS

3.1 Study Design

The study design was an experimental Phase 0 study.

3.2 Study Approvals

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

3.3 Study Funding Source

UTEP ORSP URI Program. January 2008.

3.4 Metallo-Lipid Carrier

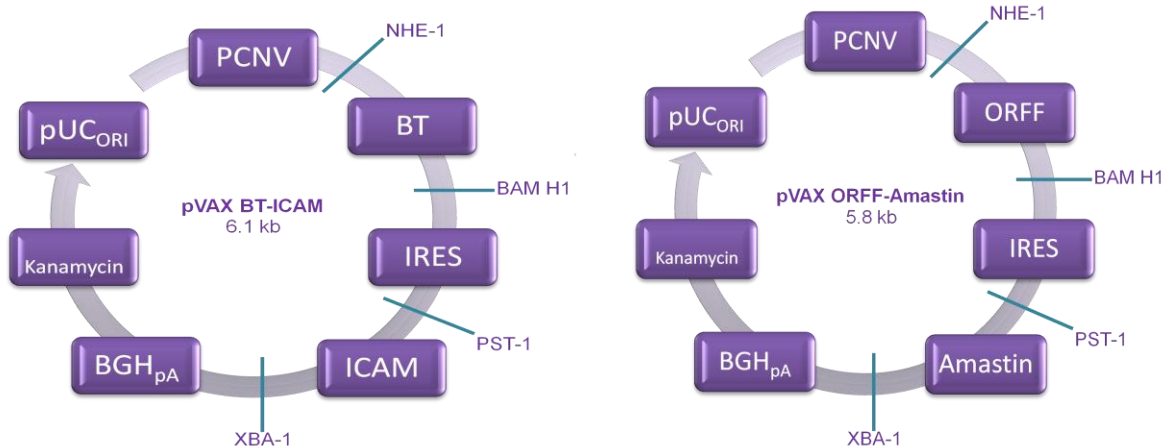
The zinc and copper lipids were synthesized as follows: Compound 1,4,7-tricyclontriazacyclononane was reacted with 1-bromododecane. Then a substitution reaction using tetrahydrofuran solvent was placed in the reaction and left in reflux for 48 hours. The solvent was subsequently purified using column chromatography with 90% chloroform and 10% methanol. The reaction was conducted in acetonitrile with two equivalents of the functionalized 1,4,7-tricyclontriazacyclononane and one equivalent of $\text{Cu}(\text{OTf})_2$. The reaction 1,4,7-tricyclontriazacyclononane was added by drops to $\text{Cu}(\text{OTf})_2$ for one hour. This reaction was carried out in room temperature in an inert atmosphere consisting of nitrogen or argon. In order to transform the complex into the liposomes, a solution of 1mM concentration in water along with the complex was sonicated with 100 μL of chloroform to generate emulsification. Finally, the sonicated solution was rotovaped to get rid of the chloroform.

3.5 Cytotoxicity Assay

The copper and zinc metallo-lipids were tested using U-937 (ATCC) cells provided by the UTEP Human Immunology and Nutrition Research Laboratory. The cells were cultured in RPMI-1640 medium (Sigma-Aldrich) containing 2mM glutamine, 200 µg ml⁻¹ penicillin, 10% fetal bovine serum and 100µg ml⁻¹ streptomycin and maintained at 37 degrees in 5% humidity CO₂ atmosphere. In order to estimate 50% lethal dose (LD₅₀) the [³H]-thymidine DNA labeling micromethod was used. The cells were counted and plated twice in triplicate at a concentration of 2.5 x 10⁵ cells /well. Each metallo-lipid was dissolved in PBS and a different concentration from 2-256 nM/well of the lipids was added to the cells. The cells were incubated for 48 hours during which the last 18 hours were pulsed with 1 µCi of [³H]-thymidine (GE Healthcare Life Sciences: Amersham) and then were harvested on glassfibre filters. The [³H]-thymidine incorporation was determined by liquid scintillation with a beta-liquid scintillation counter (Perkin Elmer Tri-carb2900tr).

3.6 Vaccine Design

The experimental leishmaniasis vaccine used was comprised of the antigens Biopterin Transporter (BT), Intracellular Adhesive Molecule (ICAM), Amastin, and Open Reading Frames (ORFF).



pVAX-BT-ICAM vaccine

pVAX-orff-Amastin vaccine

Figure 2. Bicistronic Plasmids for the Experimental Leishmaniasis Vaccine.

The CMV served as the promoter region for BT, ORFF and the IRES cassette assured the adequate expression of ICAM and Amastin. The restriction sites used to insert the *Leishmania* gene segments and IRES cassette are NEH-I, BAM H1, PST-1 and XBA-1. BGH_{pA} acted as the stop codon and pUC_{ORI} acted as the start codon. Kanamycin resistant gene is used for plasmid selection in *E. coli*.

3.7 Study Protocol

A total of 33 six week old female BALB/c mice purchased from Jackson Laboratory were used and maintained under pathogen-free conditions. *Leishmania mexicana* LV4 strain was provided by Dr. Eric Dumonteil from the Universidad Autonoma de Yucatan-Merida. The LV4 strain was isolated from a human cutaneous leishmaniasis case from Belize.

3.8 Immunization Protocol

As shown in Table 1. The mice were randomized to one of seven experimental groups. Mice in the seven study groups were injected with a volume of 100 µl via intra-muscular in the

hind leg quadriceps using a one mL BD syringe with the cocktail vaccine. All of the mice were injected three times with the vaccine assigned to each group as illustrated in Table 1.

Table 1. Immunization Groups

Group (No. of mice)	Plasmid	Metallo-lipid
1 (n=3)	pVAX-bt-icam-I(5µg) + pVAX-orff-amastin(5µg)	ML-Zn (32ng)
2 (n=3)	pVAX-bt-icam-I(5µg) + pVAX-orff-amastin(5µg)	ML-Cu (32ng)
3 (n=4)	pVAX-bt-icam-I (5µg) + pVAX-orff-amastin +PBS (5µg)	-
4 (n=6)	pVAX-bt-icam-I (50µg) +pVAX-orff-amastin (50µg)	-
5 (n=4)	-	ML-Cu (32ng)
6 (n=6)	- (PBS 100µl)	-
7 (n=7)	-(pVAX 100µl)	-

3.9 Challenge Infection and Lesion Measurement

After the third immunization, the mice were challenged in the right hind footpad with *Leishmania mexicana* promastigotes (10×10^6) in the infective meta-cyclic stage. Mouse footpads were measured for thickness with a digital caliper weekly for two months. Both hind legs were measured in order to compare swelling size in the inoculated right hind leg and the left hind leg without infection.

3.10 Adverse Vaccination Side Effects

During the eight week time frame after the mice were challenged with the parasite any detectable side effects were recorded. Mice were observed and checked for visible weight

change, posture, loss of fur on their backs, and lethargic behavior. Food and water intake were also observed during this time frame.

3.11 Immunogenicity Determination

Mice were euthanized CO₂ inhalation in order to compare the cellular immune responses of the different experimental groups. The mice were dissected and the spleen and hind leg lymph nodes were extracted. The spleen and lymph nodes were immediately processed by grinding the tissue and cleaning the cells before plating them in a 96-well plate. In order to clean the spleen cells, the homogenized tissue was transferred to a 50mL conical tube where 5mL of 1X RBC Lysis buffer from eBiosciences was added and incubated at room temperature for four minutes. To stop the reaction, 20-30mL of 1X PBS was added and then the cells were centrifuged for 10 minutes at 1500 RPM in a refrigerated centrifuge (Beckman Coulter Allegra X-12R). The supernatant was removed and one milliliter of fresh media, RPMI media with 35mL of FBS and 500 µl of streptomycin, was added. The processing of the lymph node cells was performed as follows. Tissues were homogenized and transferred to a 15 mL conical tube in order to remove any solids from the tube. The homogenized tissue was centrifuged for one minute. The supernatant was removed and fresh media was added to the pellet. The lymph node and spleen cells were counted using a hemacytometer in order to assure sufficient (10×10^6) cells per well.

3.12 Quantitative Real Time PCR (QRT-PCR) Procedure

The rest of the cells which were not used for the 96-well plates were processed in order to extract RNA with the Ambion Ribo Pure Isolation of High Quality Total RNA kit (Applied Biosystems). The RNA concentration was measured using a NanoDrop 1000 V3.5.2 (Nanodrop

Technologies). After measuring the concentration, the RNA was processed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Quantitative Real Time Polymerase Chain Reaction (QRT-PCR) was used to quantify selected cytokines (IL-2, INF- γ , IL-4, TNF- α , and IL-10) using TaqMan probes (Applied Biosystems). Beta-actin was used as the reference gene. Complementary DNA (cDNA) was plated into a PCR plate. Each well in the plate had 500ng of RNA, 1 μ L TaqMan probe, 10 μ L TaqMan Universal Master Mix. The addition of nuclease free water brought the total volume in each well to 20 μ L. For the analysis the PCR plate was placed in an iCycler iQTM Optical Module thermal cycler (BioRad).

Table 2.

<i>QRT-PCR Thermal Cycler Program</i>			
	Cycle 1 (1 cycle)	Cycle 2 (45 cycles)	
	<i>I</i>	<i>I</i>	<i>II</i>
<i>Temperature</i>	50°C	95°C	60°C
<i>Time</i>	3 minutes	10 seconds	60 seconds

CHAPTER 4: STATISTICAL ANALYSIS

Data are presented as mean \pm SEM. Data obtained from the QRT-PCR was analyzed by normalizing relative quantification using β -actin as the reference gene and the $2^{-\Delta\Delta C_T}$ (Livak) Method which produces normalized expression ratios. Descriptive analysis was used for the footpad swelling for the eight week time frame and for the QRT-PCR results.

CHAPTER 5: RESULTS

5.1 Metallo-Lipid Synthesis and Transfection.

The copper and zinc metallo-lipid microparticles were first synthesized (Fig.3). In order to ensure that the plasmid DNA vaccine was encapsulated within the microparticles, a gel electrophoresis was conducted. As seen in figure 4 the first well is a control, the second and third wells show the DNA with a low volume of the zinc microparticle and the last four wells show the plasmid DNA vaccine with higher volumes of the zinc microparticle. Full encapsulation of the plasmid DNA vaccine was visualized with 65 μ l of the copper microparticle and 40 μ l of the zinc microparticle.

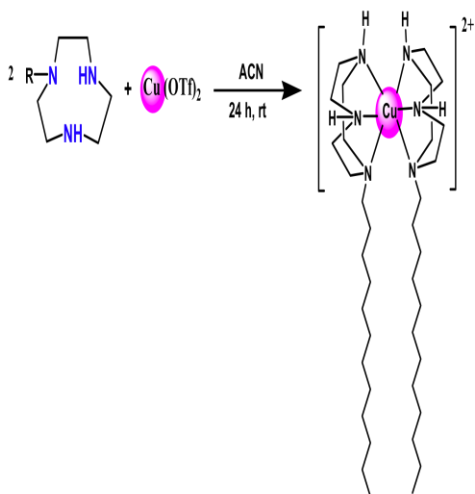


Figure 3. Metallo-lipid- Cu^{2+}

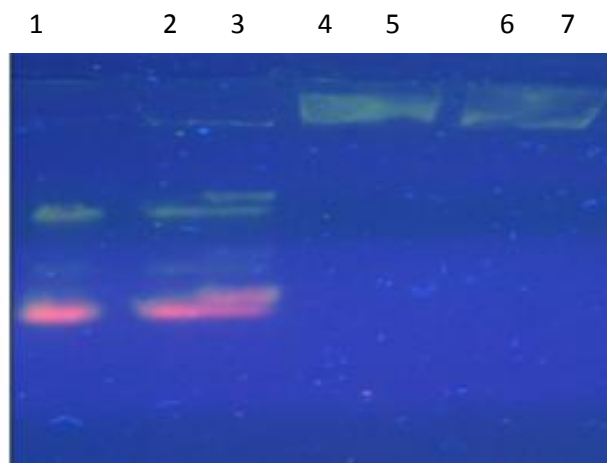


Figure 4. Gel electrophoresis

In order to visualize gene expression T291 cells were transfected with pEGFPN1, containing a green fluorescent protein gene plus the metallo-lipids- Cu^{2+} and Zn^{2+} . As seen in figures 5A and 5B the transfected cells expressed the green fluorescent protein at a transfection efficiency of 39%.

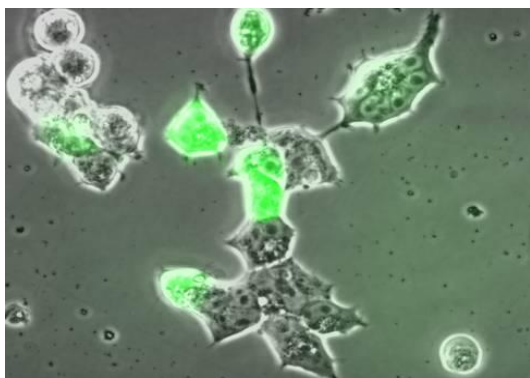


Figure 5A. T291 cells transfected with pEGFPN1 plus metallo-liposome- Zn^{2+}

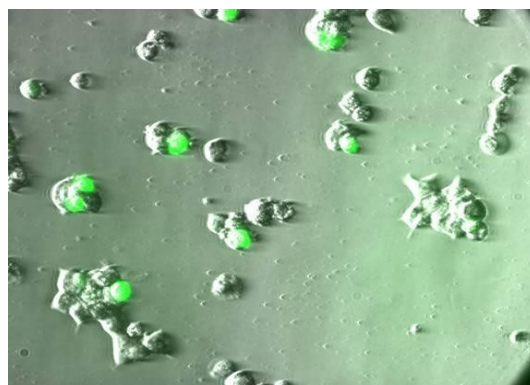


Figure 5B. T291 cells transfected with pEGFPN1 plus metallo-liposome- Cu^{2+}

5.2 Cytotoxicity Assay.

Copper and zinc metallo-lipids cytotoxicity was evaluated using U-937 cells. The LD_{50} was shown to be 24mM/24nM for both the copper and zinc metallo-lipids. As is illustrated in Figure 6 A&B, copper and zinc appear to be relatively non-toxic. Based on these experiments it was decided they were safe to use as transporters at concentrations below 32ng.

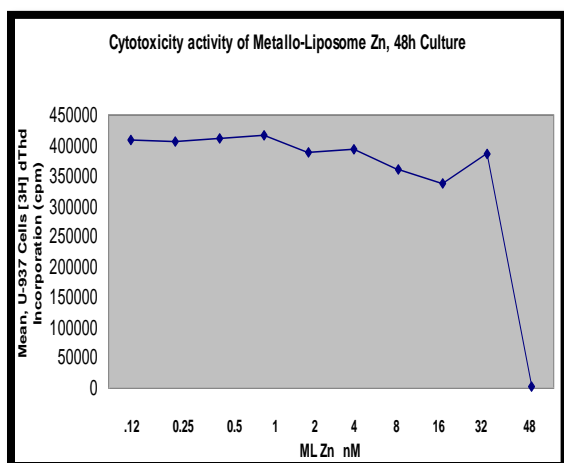


Figure 6 A. Cytotoxicity Assay for Zn.

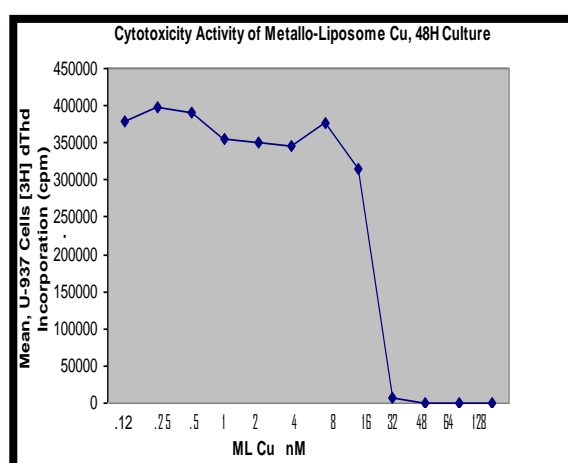


Figure 6 B. Cytotoxicity Assay for Cu.

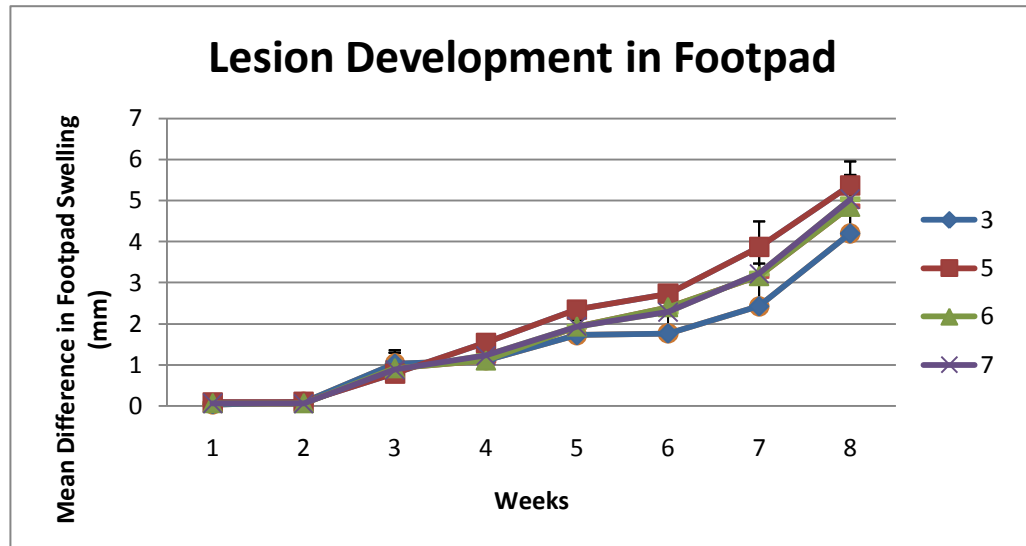


Figure 7A. Mean difference in lesion development between groups 3, 5, 6, and 7

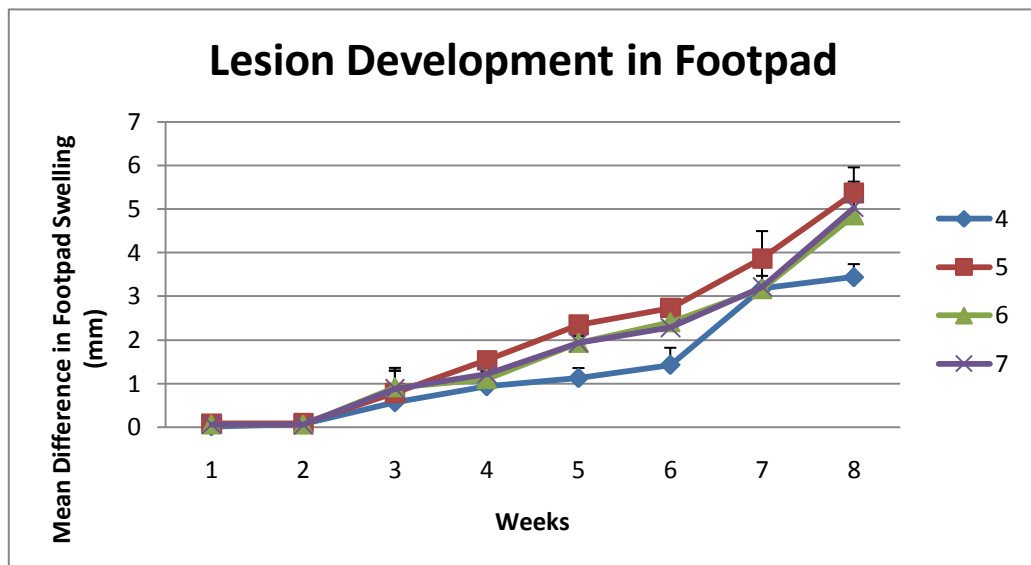


Figure 7B. Mean difference in lesion development between groups 4, 5, 6, and 7

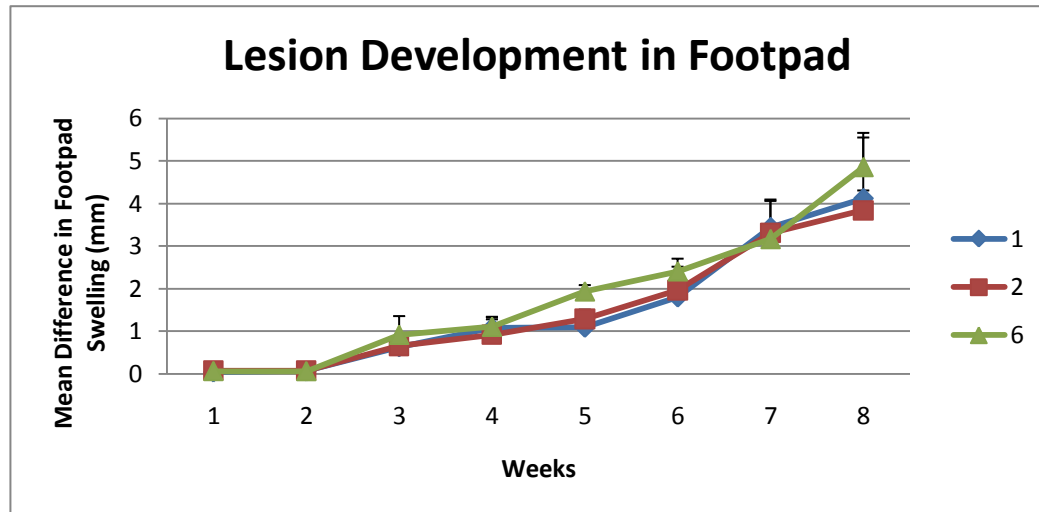


Figure 7C. Mean difference in lesion development between groups 1, 2, and 6

5.3 Protection Against Infection.

Evaluations were carried out in order to determine whether the DNA vaccine was able to induce protection against infection with *L. mexicana*. Immunized mice were challenged with an injection of 10^6 *L. mexicana* metacyclic promastigotes in the right hind footpad, and both infected and contra lateral footpads were measured for a total of eight weeks. At eight weeks group 3 (pVAX-Bt-ICAM [5µg]+pVAX-ORFF-Amastin[5µg]+PBS) and group 4 (pVAX-Bt-ICAM[50µg]+pVAX-ORFF-Amastin[50µg]) had the smallest mean difference footpad swelling between the infected and contra lateral footpad (Fig. 7, A-B). As Figure 7B shows group 5 (Copper [32ng]) had the largest mean difference between hind footpads at eight weeks after parasite challenge. Group 1 (pVAX-Bt-ICAM[5µg]+pVAX-ORFF-Amastin[5µg]+ML-Zn[32ng]) and group 2 (pVAX-Bt-ICAM[5µg]+ pVAX-ORFF-Amastin[5µg]+ML-Cu[32ng]) were both similar in mean difference of footpad swelling at the end of the eight weeks (Figure 7C). Also shown in Figure 7A and 7B, the control groups 5 (Copper [32ng]), 7 (pVAX) and 6 (PBS) had the highest mean difference in footpad swelling as was expected.

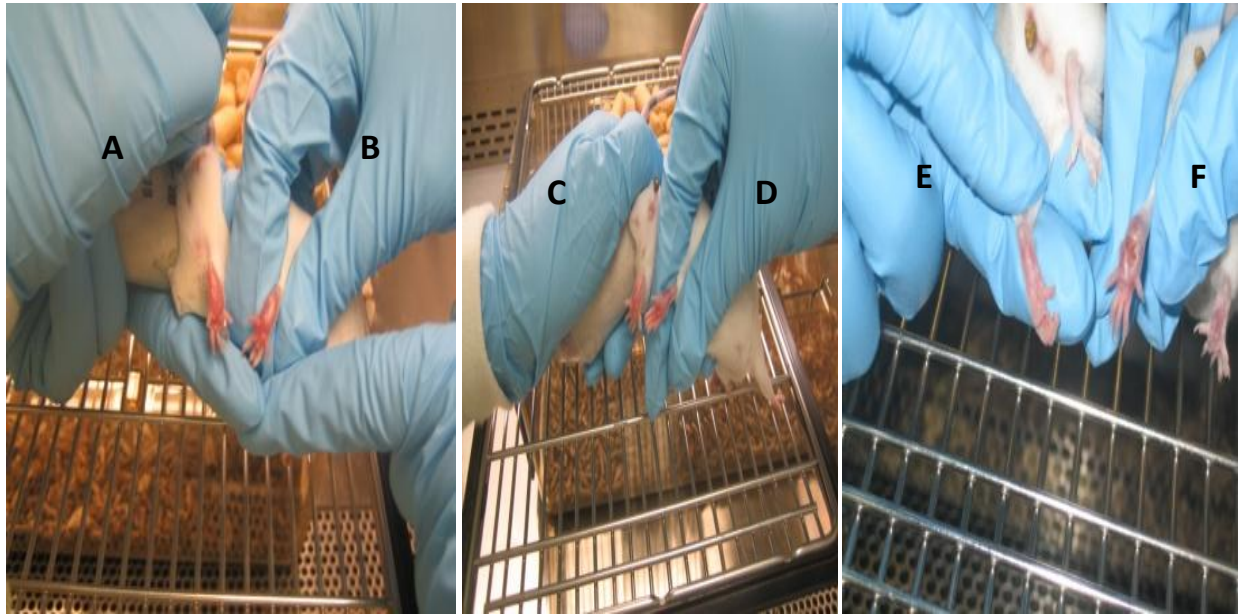


Figure 7D. **A** (pVAX-Bt-ICAM-I + pVAX-ORFF-AMASTIN (10µg) +ML-Zn) **B** (pVAX-Bt-ICAM-I + pVAX-ORFF-AMASTIN (10µg)+ ML-Cu)

Figure 7E. **C** (pVAX-Bt-ICAM-I + pVAX-ORFF-AMASTIN (10µg)+PBS) **D** (ML-Cu)

Figure 7F. **E** (pVAX-Bt-ICAM-I + pVAX-ORFF-AMASTIN (100µg)) **F** PBS

Partial protection is seen in the mice footpads in figure 7F where group 4 [E] (pVAX-Bt-ICAM-I + pVAX-orff-AMASTIN (100µg) does not have a lesion as compared to control group 6 that has a lesion.

5.4 Quantitative Real Time-Polymerase Chain Reaction.

We evaluated the spleen and lymph node cells with select cytokines, IL-2, IL-4, IFN-γ, TNF-α, and IL-10, by quantifying and analyzing them through the use of TaqMan probes and used β-actin as the reference gene.

5.4.1 Lymph Node Cells.

Group 1 [pVAX-Bt-ICAM (5µg) + pVAX-ORFF-Amastin (5µg) + ML-Zn (32ng)] shows a mixed Th₁ and Th₂ immune response with elevated levels of IFN-γ and IL-10 suggesting disease

progression (Figure 8A). Figure 8B illustrates group 2 ([pVAX-Bt-ICAM (5μg) + pVAX-ORFF-Amastin (5μg) + ML-Cu (32ng)] having Th₁ immune response with high levels of IFN-γ. Group 3 [pVAX-Bt-ICAM (5μg) + pVAX-ORFF-Amastin (5μg) + PBS] showed a mixed Th₁ and Th₂ immune response with elevated levels of IL-4, IL-10, and IFN-γ (Fig. 8C). Group 4 [pVAX-Bt-ICAM (50μg) + pVAX-ORFF-Amastin (50μg)] which is shown in Figure 8D, suggests a mixed Th₁ and Th₂ immune response. Groups 5[copper (32ng)], 6(PBS), and 7(pVAX) all illustrated a mixed Th₁ and Th₂ immune response as is illustrated in (Fig. 8, E-G).

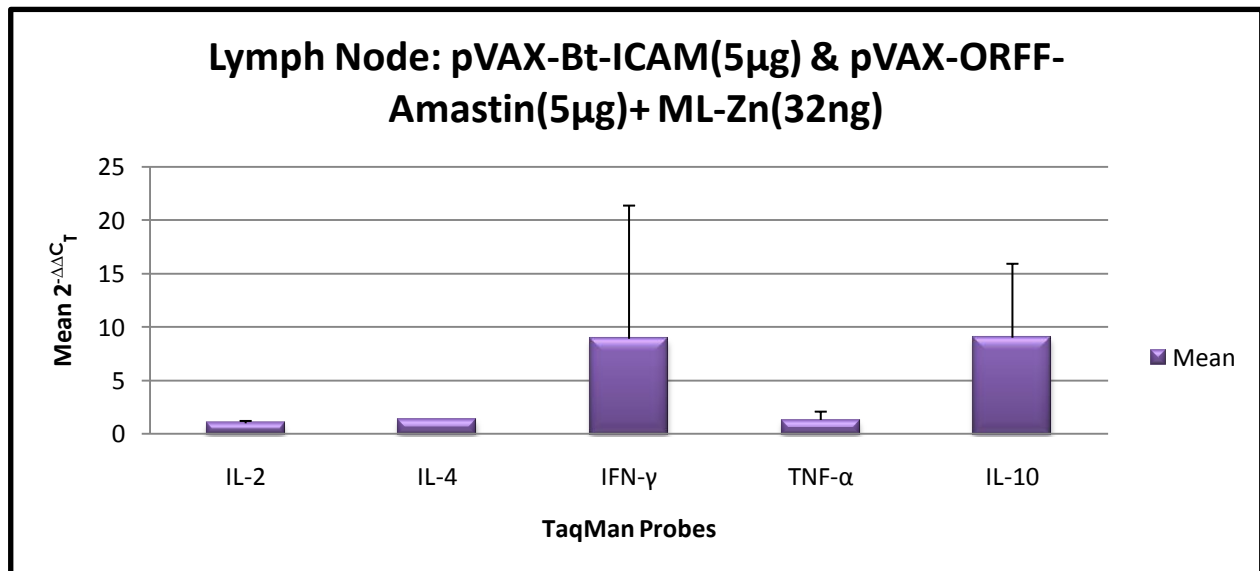


Figure 8A.

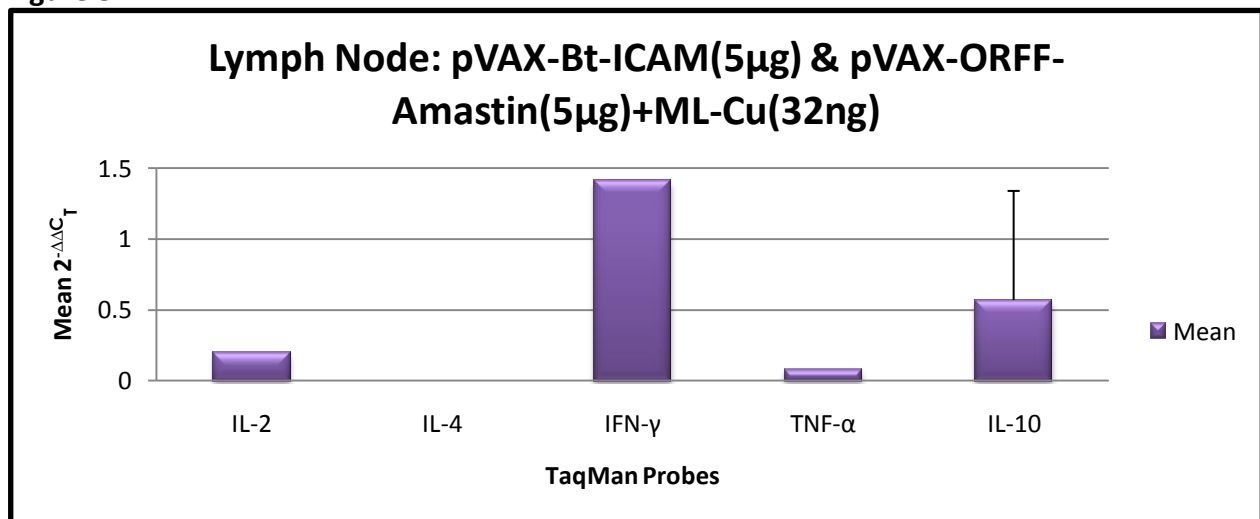


Figure 8B.

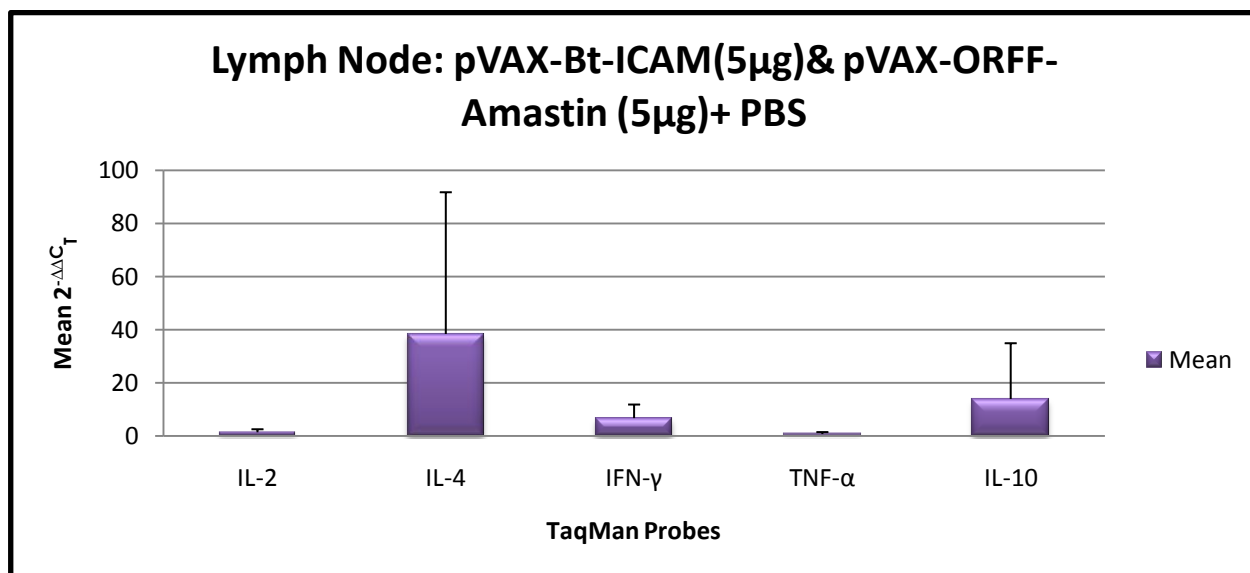


Figure 8C.

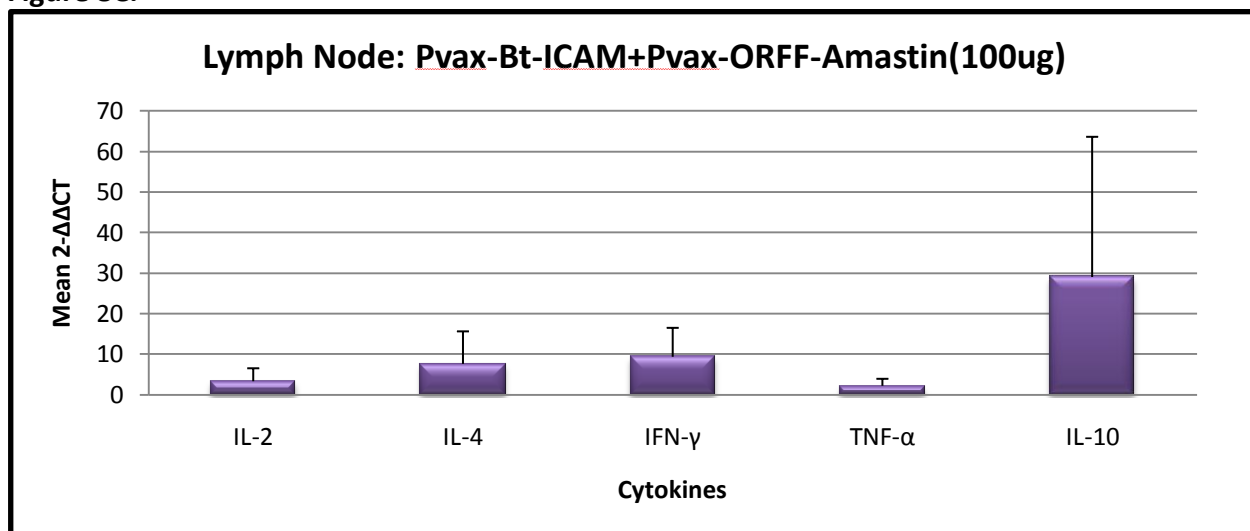


Figure 8D.

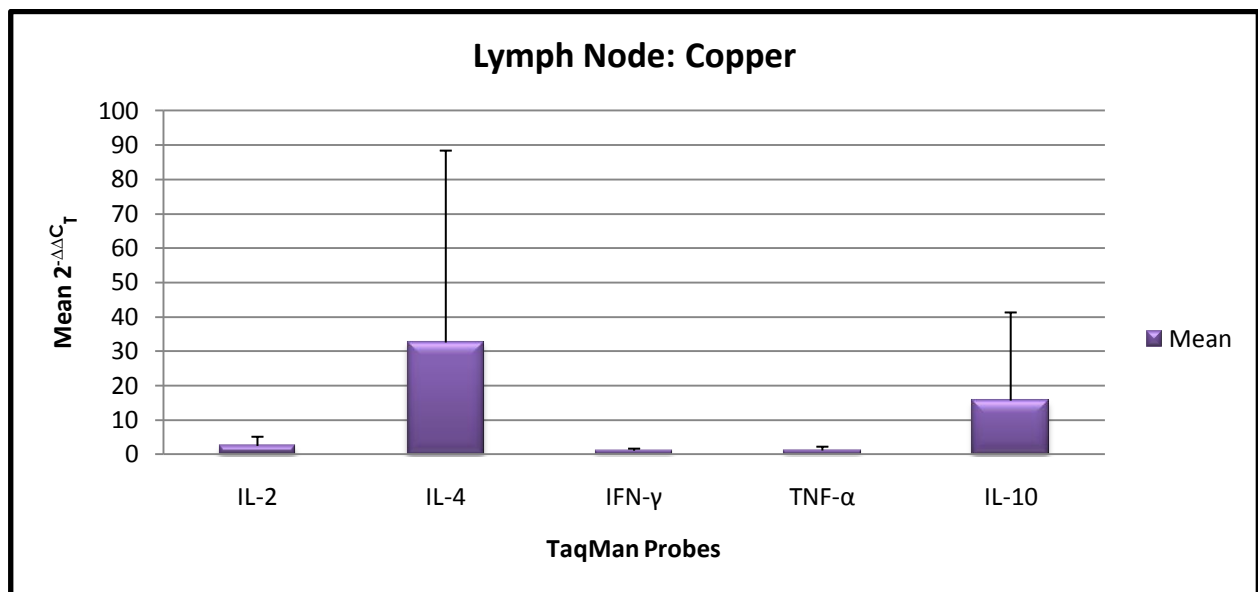


Figure 8E.

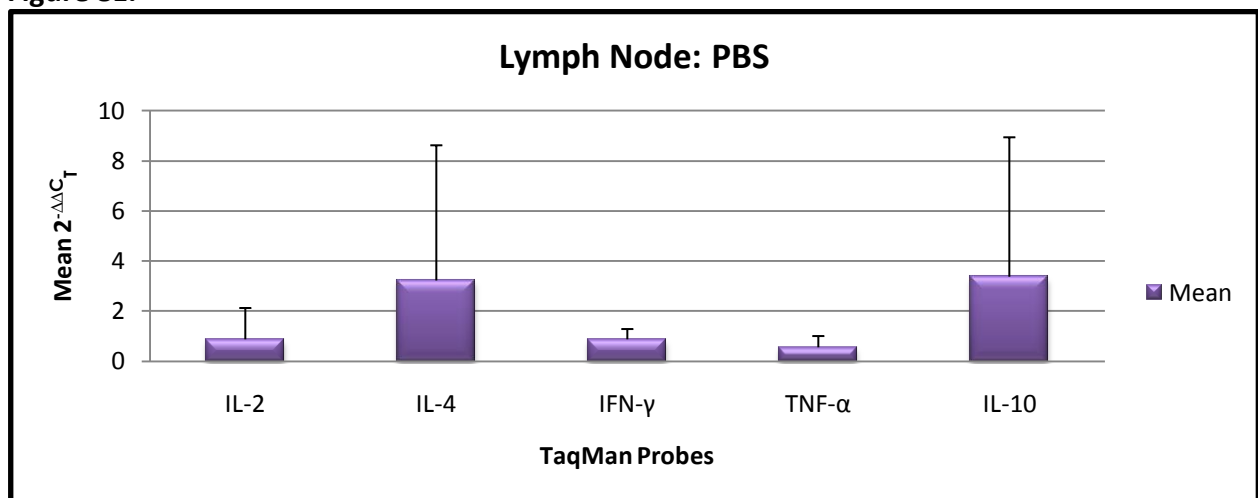


Figure 8F.

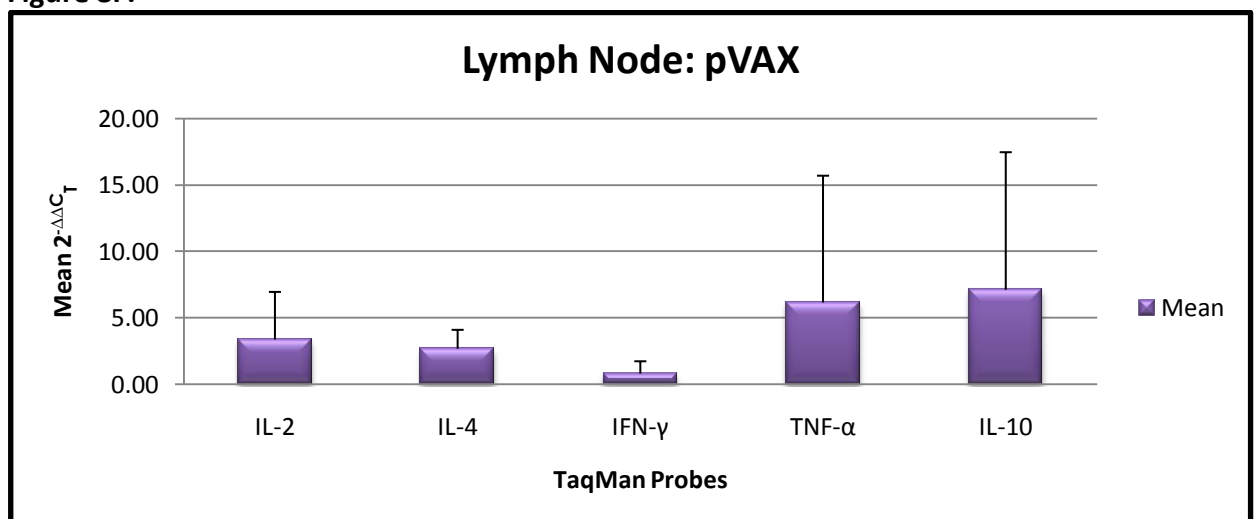


Figure 8G.

5.4.2 Spleen Cells.

As Figure 9A illustrates group 1 [pVAX-Bt-ICAM (5µg) + pVAX-ORFF-Amastin (5µg) + ML-Zn (32ng)] has a slightly mixed Th₁ and Th₂ immune response with Th₂ being predominant with higher levels of IL-4, IL-10 in comparison to IFN-γ and TNF-α levels. Group 2 [pVAX-Bt-ICAM (5µg) + pVAX-ORFF-Amastin (5µg) + ML-Cu (32ng)] displayed a typical Th₁ immune response as is shown in Figure 9B. Group 3 [pVAX-Bt-ICAM (5µg) + pVAX-ORFF-Amastin (5µg) + PBS] displayed a mixed Th₁ and Th₂ immune response with elevated levels of IL-2, IL-4, IFN-γ, and IL-10 (Fig. 9C). Group 4 [pVAX-Bt-ICAM (50µg) + pVAX-ORFF-Amastin (50µg)] in Figure 10D illustrates a mixed Th₁ and Th₂ immune response with elevated levels of IL-2, IFN-γ and IL-10. Groups 5[copper (32ng)], 6 (PBS), and 7 (pVAX) all have a mixed Th₁ and Th₂ immune response with high levels of IL-10 which suggests disease progression (Figures 9, E-G).

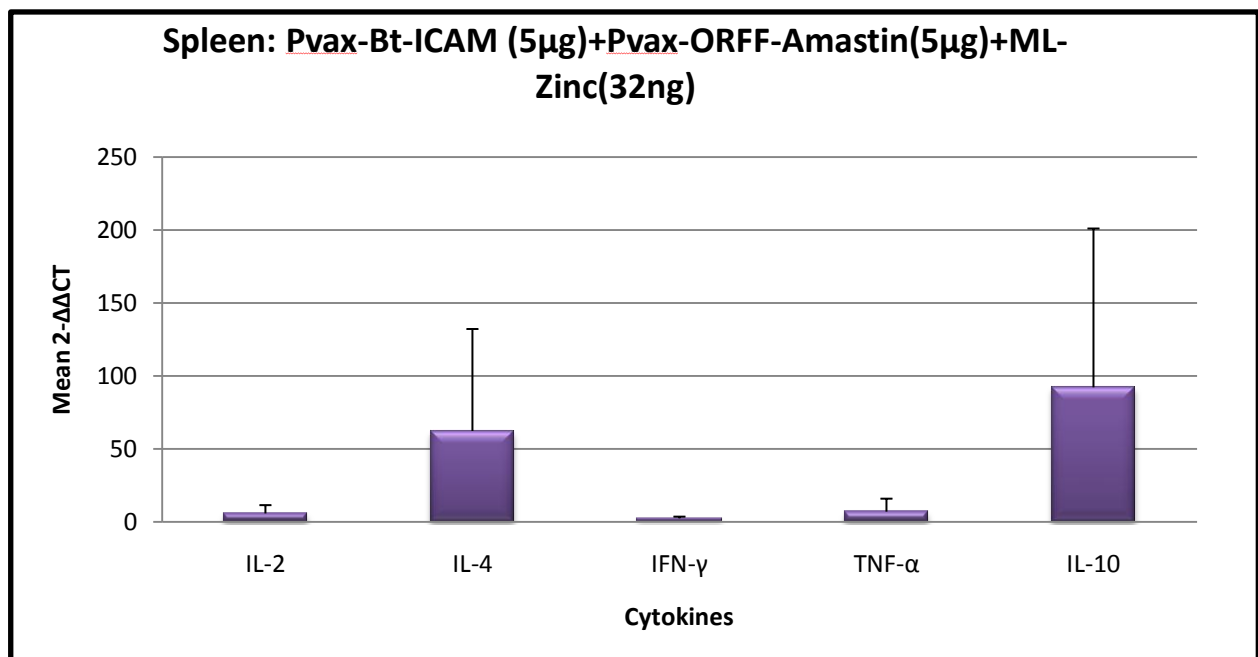


Figure 9A.

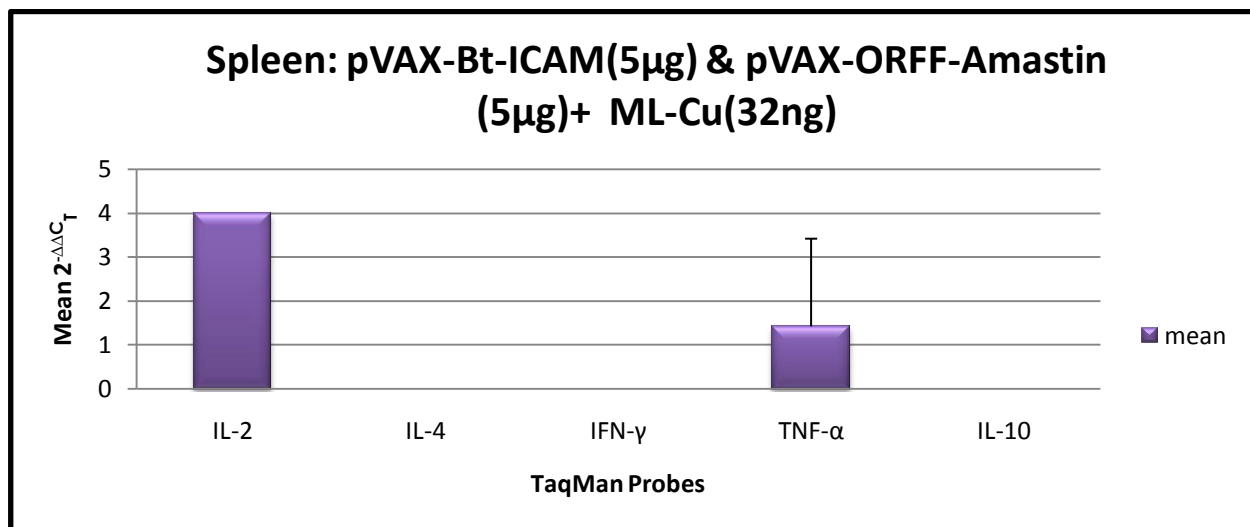


Figure 9B.

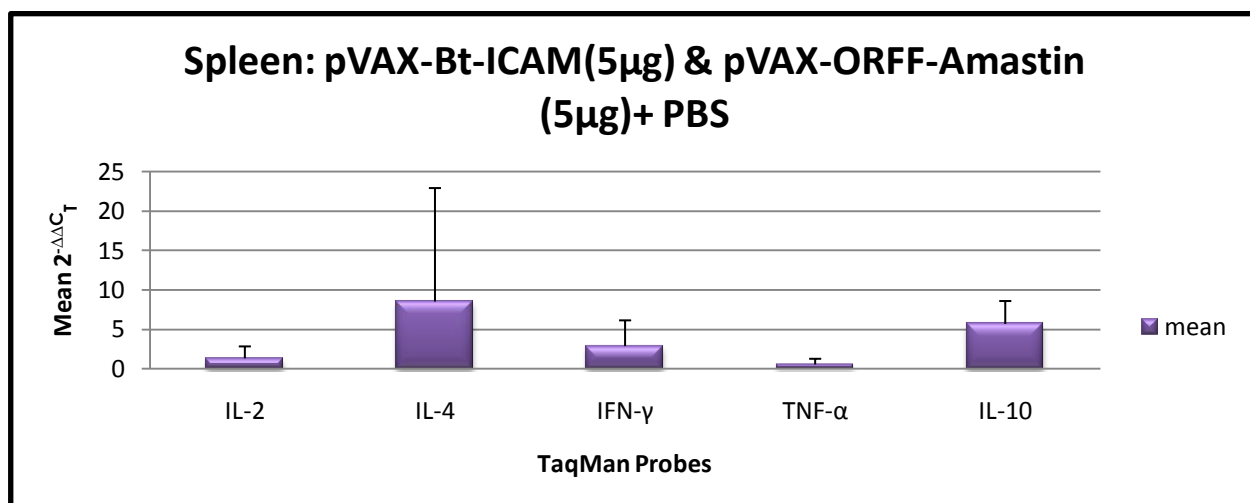


Figure 9C.

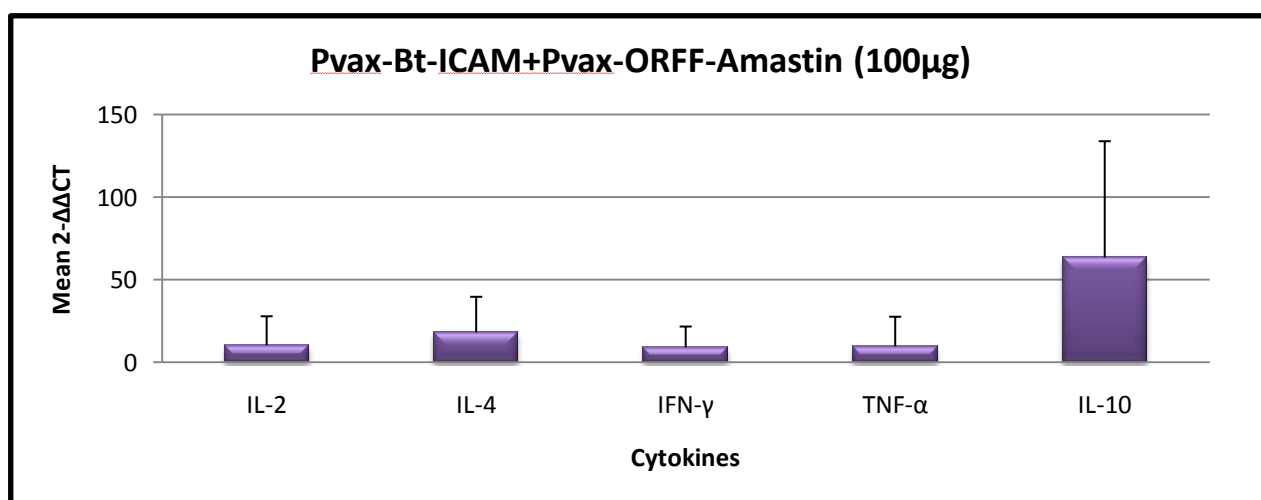


Figure 9D.

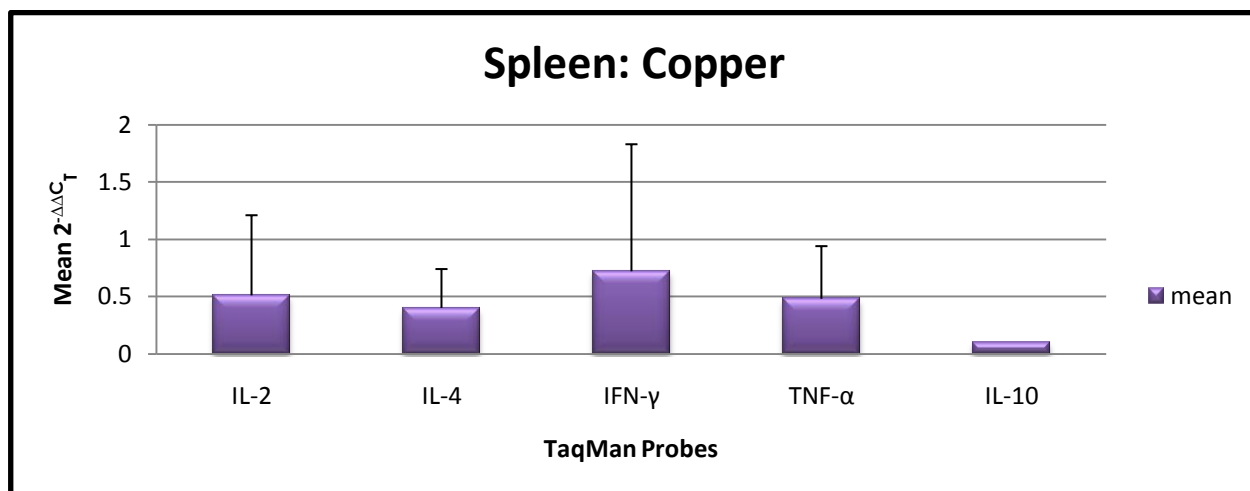


Figure 9E.

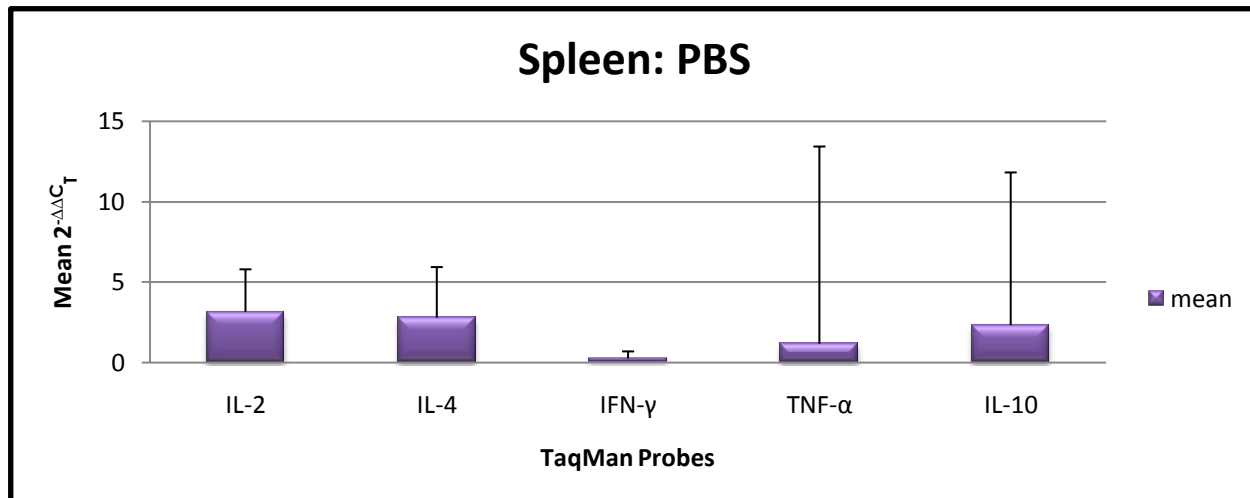


Figure 9F.

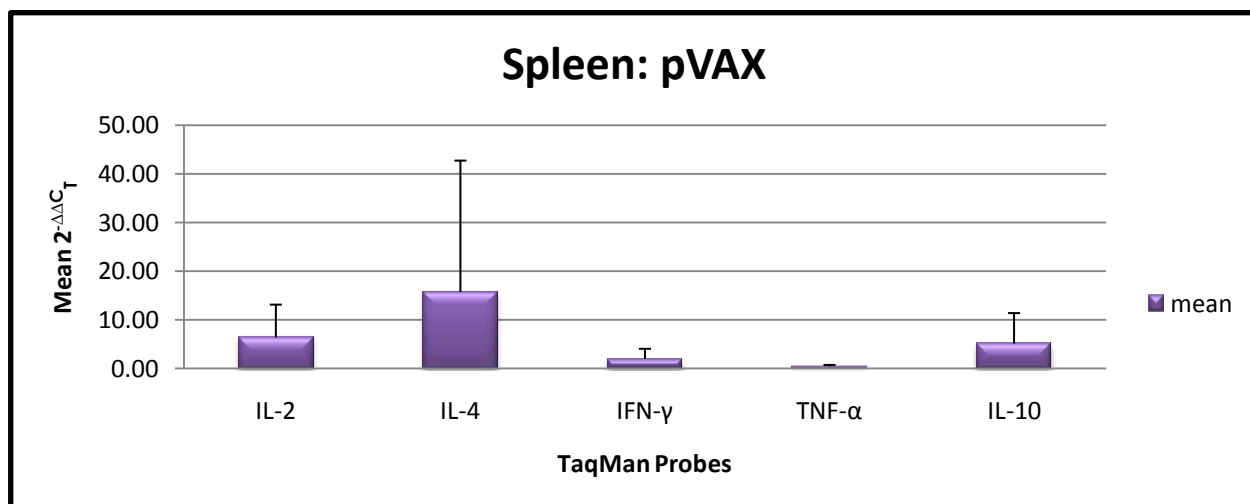


Figure 9G.

5.5 Limitations

Sample size per group was very small; therefore, when running statistical analysis power was not high enough to have statistical significance. Multiple samples were not read during the QRT-PCR due to low cDNA concentrations.

CHAPTER 6: Discussion

This Phase 0 study evaluated the immune response and protection induced by the DNA vaccine (pVAX-Bt-ICAM+ pVAX-ORFF-Amastin) against *L. mexicana* infection with Cu(II) and Zn(II) microparticle delivery systems. This is the first study to incorporate these four genes, found on promastigotes and amastigotes, into bicistronic plasmids in a vaccine against *L. mexicana*. A major finding of the present study is that pVAX-Bt-ICAM (5µg) + pVAX-ORFF-Amastin (5µg) + ML-Cu displayed immunogenicity and provided partial protection in BALB/c mice. This was illustrated with the Th₁ (increased levels of IFN-γ and TNF-α) immune response in the spleen and a lower mean difference in footpad thickness than our control groups (Group 5 Copper, Group 6 PBS, Group 7 Pvac). Elevated levels of IFN-γ promote inflammation and activate macrophages which in turn kill *Leishmania* through the use of nitric oxide. Studies have shown that the induction of a Th₁ immune response against leishmania correlates with protection in vaccinated mice (Handman, 1995). Moreover other studies have shown that weak Th₁ immune responses are sufficient to show protection against *L. major* (Sjolander et al., 1998; Uzonna et al., 2004). As previously mentioned before Group 4 (pVAX-Bt-ICAM+ pVAX-ORFF-Amastin (100µg)) displayed a mixed Th₁ and Th₂ immune response which shows that at the beginning of the trial it had a high Th₁ immune response and at some point during the trial the immune response decreased thus increasing levels of IL-10 were present due to disease progression. Group 3 pVAX-Bt-ICAM+pVAX-ORFF-Amastin(10µg) displayed a prominent Th₂ immune response in both spleen and lymph node cells; however, the mean difference for Group 3 and 4 in footpad thickness was the lowest out of the seven groups. This clinically displays partial protection as is seen in Figure 7E suggesting that the DNA vaccine is

immunogenic and provides partial protection in murine models. From the footpad thickness results the RT-PCR results had been expected to show intracellular cytokines to display a prominent Th₁ immune response which would correlate with the protection against *L.mexicana* but rather a mixed Th₁/Th₂ and a prominent Th₂ (increased levels of IL-4 and IL-10) immune response was illustrated. The prominent Th₂ immune response shows that the infection is at systemic level as opposed to a mixed Th₁/Th₂ response which exhibits the infection at a localized level. Elevated levels of IL-4 promote a Th₂ immune response which has no microbicidal effect against leishmania, thus promoting disease progression. High levels of IL-10 down regulate Th1 cytokines which inactivates macrophages. As was seen in this study there were mice groups with elevated levels of IL-10 which, indicate disease progression which is due in part to the inactivation of macrophages and Th₁ by IL-10. Disease progression was also due to the BALB/c mice because of their susceptibility to leishmaniasis. Additionally other studies have shown that production of IL-10 impedes IFN-γ from activating macrophages; therefore, preventing them from killing the *Leishmania* parasites (Kane and Mosser, 2001). Group 6 (PBS) illustrated the natural evolution of a cutaneous leishmaniasis infection. This particular strain of mice, BALB/c, are also extremely susceptible to leishmaniasis therefore a Th₂ immune response increases as is seen in Group 6.

6.1 Conclusion

This Phase 0 study suggests that the DNA vaccine pVAX-Bt-ICAM + pVAX-ORFF-Amastin at a concentration of 100μg and when combined with the ML-Cu (32ng) show a partial protection against *L. mexicana* in murine models. Further research is needed to increase the

immunogenicity of this DNA vaccine. Finding the optimal concentration of ML-Cu could improve immunogenicity of the vaccine.

6.2 Recommendations

Having the same number of mice in each group will help during the statistical analysis. When extracting the RNA the researcher should be extremely careful and precise in order to obtain high RNA yields, subsequently increasing cDNA yields for the QRT-PCR experiment.

REFERENCES

- Armijos, R., Weigel, M., Aviles, H., Maldonado, R., & Racines, J. (1998). Field Trial of a Vaccine against New World Cutaneous Leishmaniasis in an At-Risk Child Population: Safety, Immunogenicity, and Efficacy during the First 12 Months of Follow-Up. *The Journal of Infectious Diseases* , 1352-1357.
- Bailey, M. S., & Lockwood, D. N. (2007). Cutaneous leishmaniasis. *Clinics in Dermatology* , 203-211.
- Berman, J. D. (1997). Human Leishmaniasis: Clinical, Diagnostic, and Chemotherapeutic Developments in the Last 10 Years. *Clinical Infectious Diseases* , 684-703.
- Bern, C., Maguire, J. H., & Alvar, J. (2008). Complexities of Assessing the Disease Burden Attributable to Leishmaniasis. *PLoS Neglected Tropical Diseases* , 1-8.
- Beverley, S. M., & Turco, S. J. (1998). Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoans parasite Leishmania. *Trends in Microbiology* , 35-40.
- Boas, U., & Heegaard, P. (2004). Dendrimers in drug research. *Chemical Society Review* , 43-63.
- Borja-Cabrera, G., Mendes, A., Souza, E., Okada, L., Trivellato, F., Kawasaki, J., et al. (2004). Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. *Vaccine* , 2234-2243.
- Cardo, L. J. (2006). Leishmania: risk to the blood supply. *Transfusion* , 1641-1645.
- Casas, J. A., Dachs, J. N., & Bambas, A. (2001). Health disparities in Latin America and the Caribbean: The Role of Social and Economic Determinants. *Equity and Health* , 22-29.
- Chakravarty, J., & Sundar, S. (2010). Drug Resistance in Leishmaniasis. *Journal of Global Infectious Diseases* , 167-176.
- Chiang, S., Chang, S., & Lee, S. (2002). ICAM-L Gene is Conserved Only in Leishmania Species in the Family of Kinetoplastida. *Molecular and Biochemical Parasitology* , 47-50.
- Chiang, S.-C., Ali, V., Huang, A.-L., Kuan-Yu, C., & Lee, S. T. (2001). Molecular, cellular and functional characterizations of a novel ICAM-like molecule of the immunoglobulin superfamily from Leishmania mexicana amazonensis. *Molecular and Biochemical Parasitology* , 263-275.
- Convit, J., Pinardi, M., & Rondon, A. (1972). Diffuse Cutaneous Leishmaniasis: a Disease Due to an Immunological Defect of the Host. *Transactions of the Royal Society of Tropical Medicine and Hygiene* , 603-610.
- Croft, S. L., & Yardley, V. (2002). Chemotherapy of Leishmaniasis. *Current Pharmaceutical Design* , 319-342.
- David, C. V., & Craft, N. (2009). Cutaneous and mucocutaneous leishmaniasis. *Dermatologic Therapy* , 491-502.

Dedet, J.-P. (2002). Current Status of Epidemiology of Leishmaniasis. In *World Class Parasite: Volume 4 Leishmania* (pp. 1-10). Boston: Kluwer Academic Publishers.

Desjeux, P. (1996). Leishmaniasis Public Health Aspects and Control. *Clinics in Dermatology* , 417-423.

Desjeux, P. (2004). Leishmaniasis: current situation and new perspectives. *Comparative Immunology, Microbiology and Infectious Diseases* , 305-318.

Desjeux, P. (2001). The increase in risk factors for leishmaniasis worldwide. *Transaction of the Royal Society of Tropical Medicine and Hygiene* , 239-243.

Dumonteil, E. (2007). DNA Vaccines against Protozoan Parasites: Advances and Challenges. *Journal of Biomedicine and Biotechnology* , 1-11.

Dumonteil, E. e. (2003). DNA vaccines induce partial protection against *Leishmania mexicana*. *Vaccine* , 2161-2168.

Dunning, N. (2009). *Leishmania* vaccine: from leishmanization to the era of DNA technology. *Bioscience Horizon* , 73-82.

Furner, B. B. (1990). Cutaneous leishmaniasis in Texas: Report of a case and review of the literature. *Journal of the American Academy of Dermatology* , 368-371.

Garnham, P. (1971). The Genus *Leishmania*. *Bull. World Health Organization* , 477-489.

Ghosh, A., Madhubala, R., Myler, P. J., & Stuart, K. D. (1999). *Leishmania donovani*: Characterization and Expression of ORFF a Gene Amplified from the LDI Locus. *Experimental Parasitology* , 225-230.

Gonzalez, C., Wang, O., Strutz, S. E., Gonzalez-Salazar, C., Sanchez-Cordero, V., & Sarkar, S. (2010). Climate Change and Risk of Leishmaniasis in North America: Predictions from Ecological Niche Models of Vector and Reservoir Species. *PLoS Neglected Tropical Diseases* , 1-16.

Handman, E. (2001). Leishmaniasis: Current Status of Vaccine Development. *Clinical Microbiology Reviews* , 229-243.

Jain, M., & Madhubala, R. (2008). Characterization and localization of ORFF gene from the LD1 locus of *Leishmania donovani*. *Gene* , 1-10.

Kane, M., & Mosser, D. (2001). The Role of IL-10 in Promoting Disease Progression in Leishmaniasis. *The Journal of Immunology* , 1141-1147.

Kassi, M., Kassi, M., Afghan, A., Rehman, R., & Kasi, P. (2008). Marring Leishmaniasis: The Stigmatization and the Impact of Cutaneous Leishmaniasis in Pakistan and Afghanistan. *PLoS Neglected Tropical Disease* , 1-3.

Kendrick-Killick, R. (2002). Phlebotomine Sand Flies: Biology and Control. In *World Class Parasites: Volume 4 Leishmania* (pp. 33-43). Boston: Kluwer Academic Publishers.

- Khamesipour, A., Rafati, S., Davoudi, N., Maboudi, F., & Modabber, F. (2006). Leishmaniasis vaccine candidates for development: A global view. *Indian Journal of Med Res* , 423-438.
- Kuzmenok, O. I., Chiang, S.-C., Lin, Y.-C., & Lee, S. t. (2005). Retardation of cell cycle progression of macrophages from G1 to S phase by ICAM-L from Leishmania. *International Journal of Parasitology* , 1547-1555.
- Lai A Fat, E. J., Vrede, M. A., Soetosenojo, R. M., & Lai A Fat, R. F. (2002). Pentamidine, the drug of choice for the treatment of cutaneous leishmaniasis in Surinam. *International Journal of Dermatology* , 796-800.
- Landfear, S. M. (2002). Membrane Transport and Metabolism in Leishmania Parasites. In *World Class Parasites: Volume 4 Leishmania* (pp. 75-87). Boston: Kluwer Academic Publishers.
- Leishmania Infection*. (2008, September 22). Retrieved September 29, 2009, from Center for Disease Control and Prevention: http://www.cdc.gov/ncidod/dpd/parasites/leishmania/factsht_leishmania.htm
- Leishmaniasis*. (2009, July 20). Retrieved December 2009, from Laboratory Identification of Parasites of Public Health Concern CDC: <http://www.dpd.cdc.gov/DPDx/html/Leishmaniasis.htm>
- Leishmaniasis: 2007 Update*. (2007). Retrieved October 2009, from Pan American Health Organization: <http://www.paho.org/English/AD/DPC/CD/leish-2007.pdf>
- Mosser, D., & Brittingham, A. (1997). Leishmania, macrophages and complement: a tale of subversion and exploitation. *Parasitology* , S9-S23.
- Murray, H. W., Berman, J. D., Davies, C. R., & Saravia, N. G. (2005). Advances in leishmaniasis. *The Lancet* , 1561-1577.
- Noazin, S., Modabber, F., Khamesipour, A., Smith, P., Moulton, L., Nasser, K., et al. (2008). First generation leishmaniasis vaccines: A review of field efficacy trials. *Vaccine* , 6759-6767.
- Olivier, M., Gregory, D. J., & Forget, G. (2005). Subversion Mechanisms by Which Leishmania Parasites Can Escape the Host Immune Response: a Signaling Point of View. *Clinical Microbiology Reviews* , 293-305.
- Osorio, L. E., Castillo, C. M., & Ochoa, M. T. (1998). Mucosal Leishmaniasis due to Leishmania (Viannia) Panamensis in Colombia: Clinical Characteristics. *American Journal of Tropical Medicine and Hygiene* , 49-52.
- Palatnik-de-Sousa, C. (2008). Vaccines for leishmaniasis in fore coming 25 years. *Vaccine* , 1709-1724.
- Papadopolou, B. e. (2002). Reduced Infectivity of a Leishmania donovani Biopterin Transporter Genetic Mutant and Its Use as an Attenuated Strain for Vaccination. *Infection and Immunity* , 62-68.
- Parham, P. (2005). *The Immune System*. New York: Garland Science Publishing.

- Pearson, R. D., & Sousa, A. d. (1996). Clinical Spectrum of Leishmaniasis. *Clinical Infectious Diseases* , 1-13.
- Rachamim, N., & Jaffe, C. (1993). Pure Protein from *Leishmania donovani* Protects Mice against Both Cutaneous and Visceral Leishmaniasis. *The Journal of Immunology* , 2322-2331.
- Reithinger, R., Dujardin, J.-C., Louzir, H., Pirmez, C., Alexander, B., & Brooker, S. (2007). Cutaneous leishmaniasis. *Lancet infectious Diseases* , 581-596.
- Rochette, A. e. (2005). Characterization and developmental gene regulation of a large gene family encoding amastin surface proteins in *Leishmania* spp. *Molecular & Biochemical Parasitology* , 205-220.
- Roussel, M., Nacher, M., Fremont, G., Rotureau, B., Clyti, E., Sainte-Marie, D., et al. (2006). Comparison between one and two injections of pentamidine isethionate, at 7 mg/kg in each injection, in the treatment of cutaneous leishmaniasis in French Guiana. *Annals of Tropical Medicine & Parasitology* , 307-314.
- Saenz, R. E., Paz, H., & Berman, J. D. (1990). Efficacy of Ketoconazole Against *Leishmania braziliensis panamensis* Cutaneous Leishmaniasis. *The American Journal of Medicine* , 147-155.
- Saliba, E. K., & Oumeish, O. Y. (1999). Reservoir Hosts of Cutaneous Leishmaniasis. *Clinics in Dermatology* , 275-277.
- Sanguenza, O. P., Sanguenza, J. M., Stiller, M. J., & Sanguenza, P. (1993). Mucocutaneous leishmaniasis: A clinicopathologic classification. *Journal of the American Academy of Dermatology* , 927-932.
- Sasaki, S., Takeshita, F., Xin, K., Ishii, N., & Okuda, K. (2003). Adjuvant formulations and delivery systems for DNA vaccines. *Methods* , 243-254.
- Shahiwala, A., Vyas, T. K., & Amiji, M. M. (2007). Nanocarriers for Systemic and Mucosal Vaccine Delivery. *Recent Patents on Drug Delivery and Formulation* , 1-9.
- Shaw, J. J. (2002). New World Leishmaniasis: The Ecology of Leishmaniasis and the Diversity of Leishmanial species in Central and South America. In *World Class Parasites: Volume 4 Leishmania* (pp. 11-31). boston: Kluwer Academic Publishers.
- Sinha, P., Pandey, K., & S, B. (2005). Diagnosis & management of leishmania/HIV co-infection. *Indian J Med Res* , 407-414.
- Sinha, S., Fernandez, G., Kapila, R., Lambert, C., & Schwartz, R. (2008). Diffuse cutaneous leishmaniasis associated with the immune reconstitution inflammatory syndrome. *International Journal of Dermatology* , 1263-1270.
- Soto, J., Arana, B., Toledo, J., Rizzo, N., Vega, J., Diaz, A., et al. (2004). Miltefosine for New World Cutaneous Leishmaniasis. *Clinical Infectious Diseases* , 1266-1272.

- Soto-Mancipe, J., Grogl, M., & Berman, J. D. (1993). Evaluation of Pentamidine for the Treatment of Cutaneous Leishmaniasis in Colombia. *Clinical Infectious Diseases* , 417-425.
- Stanley, A. B. (2004). Intranasal immunization with Toxoplasma gondii tachyzoite antigen encapsulated into PLG microsphere induces humoral and cell mediated immunity in sheep. *Vaccine* , 3929-3941.
- Sukumaran, B., Tewary, P., Saxena, S., & Madhubala, R. (2003). Vaccination with DNA encoding ORFF antigen confers protective immunity in mice infected with Leishmania donovani. *Vaccine* , 1292-1299.
- Sun, H., Xie, Y., & Ye, Y. (2009). ISCOMs and ISCOMATRIX. *Vaccine* , 4388-4401.
- Tafaghodi, M., Jaafari, M., & Tabassi, S. (2006). Nasal immunization studies using liposomes loaded with tetanus toxoid and CpG-ODN. *European Journal of Pharmaceutics and Biopharmaceutics* , 138-145.
- Velez, I., Gilchrist, K., Arbelaez, M., Rojas, C., Puerta, J., Antunes, C., et al. (2005). Failure of a killed Leishmania amazonensis vaccine against American cutaneous leishmaniasis in Colombia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* , 593-598.
- Wright, N. A., Davis, L. E., Aftergut, K. S., Parrish, C. A., & Cockerell, C. J. (2008). Cutaneous leishmaniasis in Texas: A northern spread of endemic areas. *Journal of American Academy of Dermatology* , 650-652.
- Wu, Y. e. (2000). A new developmentally regulated gene family in Leishmania amastigotes encoding a homolog of amastin surface proteins. *Molecular and Biochemical Parasitology* , 345-357.
- Xiang S., S. C. (2010). Delivery of DNA vaccine: an overview on the use of biodegradable polymeric and magnetic nanoparticles. *Nanomedicine and Nanobiotechnology* , 205-218.
- Xu, D., & Liew, F. (1994). Genetic vaccination against leishmaniasis. *Vaccine* , 1534-1536.
- Yanik, M., Gurel, M., Simsek, Z., & Kati, M. (2004). The Psychological Impact of Cutaneous Leishmaniasis. *Clinical Dermatology* , 464-467.

CURRICULUM VITAE

Joanna Berenice Valencia was born in El Paso, Texas. The eldest daughter of Jose Luis Valencia and Refugio Silvia Valencia, she graduated from Baylor University in Waco, Texas in the spring of 2007 earning a bachelor's degree in biology with a minor in chemistry. In the fall of 2008 she began her graduate career pursuing a Master of Public Health at the University of Texas at El Paso. She attended the SACNAS Conference in 2009 where she did an oral presentation on her research for her thesis. She was treasurer for the Students for Public Health organization. She intends to continue her graduate career by pursuing a doctorate degree in Public Health in the near future.