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In-vivo Delivery of DNA Vaccines Using Metallo-lipid Nanoparticles

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IN-VIVO DELIVERY OF DNA VACCINES
USING METALLO-LIPID NANOPARTICLES

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IN-VIVO DELIVERY OF DNA VACCINES
USING METALLO-LIPID NANOPARTICLES

by

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THESIS

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Abstract

There has been a rapidly growing area of research in the design and synthesis of molecules that self-organize in water to form functional nanosystems and due to the high interest in the area metal ligand complexes were tested as drug delivery systems with a *Leishmania* vaccine. Herein, we present the design, synthesis and functional activity of Cu(II) and Zn(II) complexes that self-assemble in water to form spherical nanoscale structures that exhibit an affinity to bind DNA and deliver it into eukaryotic cells with a high percent efficiency *in-vitro*. In order to assess the effectiveness of these nanoparticles to deliver DNA vaccines *in-vivo*, we investigated the ability of the Cu(II) and Zn(II) complexes to bind and deliver a gene vaccine against *Leishmania mexicana* challenged with *Leishmania major*, into mice models. Comparison of the efficacy of these molecules will be discussed in regards to preventing murine leishmaniasis infection.

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Chapter 1. Introduction.

1.1 MOTIVATION

Leishmaniasis is a tropical parasitic disease caused by a number of different parasite species belonging to the genus *Leishmania*. It is a public health problem of global importance. Worldwide, there are approximately 12 million people affected, 1.5-2 million new cases each year, and 350 million at-risk for developing leishmaniasis. It is endemic in 88 countries on five continents including 22 countries in the New World and 66 in the Old World. The incidence of leishmaniasis is increasing in many regions of the world as the result of decreased immunocompetence associated with HIV-AIDS, deforestation of primary rainforests, increased overseas travel and military deployment to endemic areas. Leishmaniasis is considered a threat to the military readiness of coalition forces serving in the Middle East. For example, in 2005 CDC has reported over 600 confirmed cases of the cutaneous form of leishmaniasis (CL) in U.S. soldiers deployed to Afghanistan, Iraq, and Kuwait, with many more cases suspected.

The complex epidemiological characteristics of the disease and its transmission have limited the success of disease control efforts in endemic countries. In addition, the conventional treatment of CL with meglumine antimoniate, an antimony-based drug, is associated with cardiovascular-, hepato- and reno-toxic side-effects. The development of a vaccine candidate that elicits a strong and long lasting CD4⁺ and CD8⁺ cytotoxic T lymphocytes against *Leishmania* could represent a significant advance. Prior authors have reported varying levels of protection against leishmaniasis using a whole killed parasite approach^{6,7} or recombinant antigen vaccines. More recently, investigators have begun to focus on the development of DNA vaccines for leishmaniasis to prevent the disease. Finding a candidate vaccine for leishmaniasis is an ambitious goal but many will benefit from it if accomplished.

1.2 OBJECTIVES

The literature suggests that weak immunogenicity is the main obstacle for developing an effective DNA vaccine. Prior studies indicate that the immune response produced by naked DNA is insufficient to protect against the disease. Various attempts have been made to overcome low DNA vaccine immunogenicity (efficacy). Studies in our lab have showed that metallo-liposomes (ML) have low toxicity and bind DNA more efficiently and therefore have been utilized as DNA delivery systems for the vaccine.

I hypothesize that encapsulating DNA vaccine candidate within metallo-lipid micelles helps to avoid DNA degradation from surrounding DNases prior to reaching target cells. The first objective was to determine the efficacy of p-Vax-*BT-ICAM*, and/or p-Vax-*ORFF-Amastin* plus metallo-liposomes in preventing murine leishmaniasis infection. The second objective was to determine if metallo-liposomes possess adjuvant properties that enhance the innate immune response.

1.3 BACKGROUND

Gene therapy is increasingly growing in its efficiency especially when presented with the task of constructing methods for the delivery of DNA into mammalian cells. Viral vectors are most commonly used in gene therapy but unfortunately pose many problems such as producing undesired side effects, targeting the wrong cells in the body, and/or not guaranteeing that no genes will be affected that are already present in the genome. Currently, viruses have been genetically altered to encapsulate and deliver the desired genes to human cells in a pathogenic manner. Other vectors employ the non-viral method, which consists of transfections and gene expression. The use of naked DNA is one of the simplest methods of non-viral transfection in

gene therapy. Naked DNA does not contain histones and therefore does not contain chromatin, making the unwound DNA in chromosomes very long. In DNA vaccines or gene immunization the term “naked DNA” is described as DNA that is free of any agents, allowing for transfection capabilities. Transfection differs from transformation in that the DNA is simply expressed and not necessarily incorporated in the cell’s genome; the DNA is only introduced into cells using a vector or another method of delivery. Transfections allow the uptake of foreign material to enter the cell by opening the transient pores in the cell’s membrane and allowing genetic material, proteins, or antibodies to enter.

DNA vaccines have been applied to numerous viral, bacterial, and parasitic models of disease. The DNA vaccination technique is utilized to protect an organism against disease by injecting it with genetically engineered DNA to produce an immunological response. It is termed genetically engineered because it employs techniques of molecular cloning and transformation to alter the structure and characteristics of genes directly. DNA vaccines have yet to be perfected but do have many advantages including their ability to induce a much broader range of immune responses. Vaccines are characterized under generations; DNA vaccines are labeled as third generation and consist of small, circular fragments of bacterial DNA called a plasmid that is manipulated and genetically engineered to exhibit antigenic properties from a protein belonging to a microorganism. When the vaccine is injected into a body’s cells the host cells proceed to “read” the DNA and convert it to pathogenic proteins. Once the host cell has processed the information, the proteins are recognized as being foreign and that in turn triggers immune responses by activating the immune system.

Two types of immune responses exist, one being humoral immune responses and the second being cellular immune responses. In a humoral immune response, the secretion of

antibodies produced by B lymphocyte cells mediates immunity. The secreted antibodies bind to the surface of antigens caused by a virus or bacteria and signals for their destruction. On the other hand, a cellular immune response does not involve antibodies but the activation of macrophages, natural killer cells (NK), antigen-specific cytotoxic T-lymphocyte cells, and the release of cytokines in response to the antigen. The activation of antigen-specific cytotoxic T-lymphocytes induces apoptosis in the cell's body displaying epitopes of foreign antigen on their surface, such as cells with intracellular bacteria. The activation of macrophages and natural killer cells enable them to destroy the intracellular pathogens and stimulate the cells to secrete the cytokines that influence the function of other immune cells. Cell-mediated immunity targets those microorganisms that are able to survive in phagocytes and participate in the defense from protozoans.

Phagocytic cells play a crucial role in the immune system by ingesting and destroying microorganisms.¹⁹ A variety of different phagocytic cells exist such as blood polymorphonuclear leucocytes, (neutrophils or granulocytes), which are able to respond to sites of infections by migrating to the area. Monocytes are also found in the blood stream and become macrophages once they leave the circulation and penetrate tissues. Some pathogenic microorganisms are able to survive and replicate even after being ingested by macrophages, which hinders the treatment. Defense mechanisms and antibiotics are unable to reach these pathogenic microorganisms due to their intracellular location.^{13,29}

Some pathologies that are caused by intracellular microorganisms include Leishmaniasis, tuberculosis or histoplasmosis, among others. These intracellular microorganisms cause more complications such as opportunistic infections especially in immunosuppressed patients. These patients are more susceptible since the compromised immune system presents more of an

opportunity for the pathogen to infect.⁴⁸ Aminoglycosides, fluoroquinolones, beta-lactams, and macrolides are among the most commonly used antibiotics in treatments of this type. These antibiotics have been characterized as having different abilities to penetrate phagocytic cells, possibly limiting their efficacy to treat intracellular infections.

The process of phagocytosis involves the ingestion of microorganisms by the cell followed by their destruction and elimination. This process is similar to those seen performed by macrophages and neutrophils. The phagocyte begins the process by forming a vesicle or phagosome once it uptakes the microorganism with the aid of surface receptors that are occasionally mediated by antibodies. The vesicle then fuses with the lysosomes, giving rise to a phagolysosome. Macrophages produce bacterial agents such as cationic proteins and nitric oxide to assist in the process; neutrophils produce derivatives of oxygen and halogen ions.^{27,59}

Germ at times have the ability to bypass immune mechanisms by preventing fusion of the phagosome and lysosome or by withstanding microbiocidal mechanisms in the phagolysosome. These germs are able to survive in cellular compartments or in the cytoplasm if they are able to cross the membrane successfully.^{2,59} The intracellular location serves as a protective barrier from host defense mechanisms, such as antibodies and their activity.^{13,29} Because they can prevent the action of antibiotics by penetrating in cells and exiting the host cell when levels of the antibiotic are low enough, it makes it quite difficult to treat these infections and causes further relapse in patients.^{13,43}

Phagocytic cells also have the capability of releasing drugs by way of chemotactic mechanisms from the blood or tissues to the site of infection. It is important to recognize that the antibiotic must have the capacity to become concentrated within the phagocytic cells in order to combat against the extracellular microorganisms located at the site of inflammation or

infection.^{22,25} The majority of antibiotics administered to the organism remain in the extracellular space when they are needed to reach inside cells. Because the desired concentration is unable to penetrate inside the cells, efficacy against intracellularly located pathogens *in-vitro* show little or no antibacterial activity *in-vivo* even when high concentrations of the antibiotic drug is present.⁵⁶ Fluoroquinolones, beta-lactams and macrolides are able to penetrate cells through diffusion or in the case of aminoglycosides by receptor-mediated uptake.¹³

Carrier systems must also possess certain characteristics especially if being utilized for delivery of antibiotics to phagocytic cells to increase cellular penetration for the treatment of intracellular infections. Not only should carrier systems remain stable under the *in-vivo* conditions in which they are to be used but also must be quickly recognize and withdraw from the circulation by the phagocytic cells of the reticuloendothelial system (RES) where the pathogen is present in order to reach the desired concentration levels in the target cells. In order to achieve therapeutic levels at the infection site over a duration of time, they must sustain the drug for continuous release. They must decrease the possibility of side effects by preventing the drug from manifesting its toxicological actions until the RES has been reached. Premature degradation and immunological reactions should also be prevented while at the same time maintaining drug retention in tissues. Most importantly, they must maintain the therapeutic efficacy of the drug while decreasing its toxicity.

1.3.1 *Leishmania major*

Drug delivery systems have been employed in many intracellular pathogenic diseases but have yet to be successful in treating the infections due to poor efficiency in the drug delivery systems and high toxicity levels of the treatment itself. *Leishmania major*, an intracellular

parasite continues to pose challenges in its treatment. *Leishmania major* is a protozoan parasite from the family Trypanosomatidae which causes large skin lesions that take long periods of time to heal. This parasite is of increasing interest because it is slowly moving northward from South America into the United States. According to previous studies, it is estimated that *Leishmania* infects approximately 5 million people worldwide and 350 million people live in areas where leishmaniasis is endemic.⁴⁹

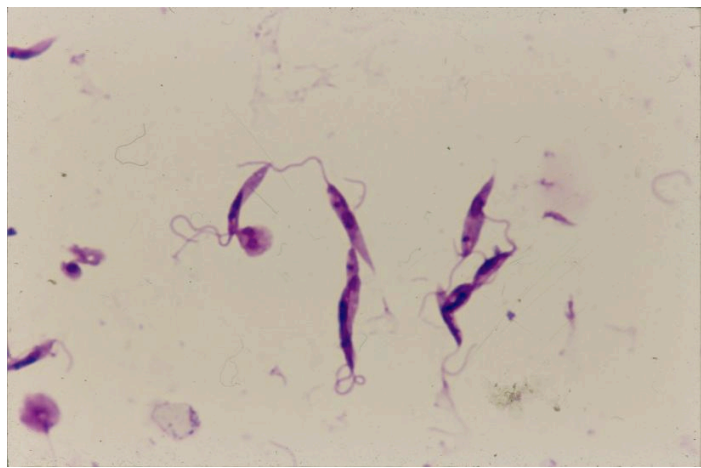


Figure 1.1. Microscopic view of *Leishmania* parasite.⁴¹

Not all of the single celled parasites infect humans; the word *Leishmania* refers to a genus of parasites that includes many different species, some of which target rodents. Leishmaniasis is the name of the actual disease that is caused by these parasites; there are many types of species that have been identified. *Leishmania major* causes cutaneous lesions and is the least lethal species that is gradually entering the United States, specifically Texas, from Central and South America. Cutaneous leishmaniasis of the Old World (OWCL) is normally caused by three species of leishmania: *L. tropica*, *L. major*, and *L. aethiopica*. Cutaneous leishmaniasis of the New World (NWCL) is caused by numerous species and subspecies of *Leishmania*. The

clinical features are similar to those of Old World cutaneous leishmaniasis but the lesions tend to be more severe and chronic.

Three forms of leishmaniasis have been classified – cutaneous, mucocutaneous, and visceral. Cutaneous leishmaniasis is a skin disease where the *Leishmania* parasite replicates itself in the skin causing an ulcer that does not heal or takes months to do so. It is quite common for a bacterial infection to follow after contact with the parasite. Mucocutaneous leishmaniasis targets the mouth, nasal passages, pharynx and genitals. If left untreated the lesions in the mucous membranes can result in deformities and may lead to serious complications that may result in death. The most serious form of the disease is visceral leishmaniasis, which causes severe damage to the immune system by proliferating in internal organs and tissues. Victims that do not receive treatment for the disease die within three years of the initial onset of symptoms.

1.3.2 Parasitic Life Cycle

All species of leishmaniasis follow the same parasitic life cycle and alternate between two distinct lifestages, the promastigote and the amastigote. *Leishmania* parasites are transmitted to humans by the bite of an infected sand fly. The cycle varies in duration from 4 to 18 days according to the species of the infection and begins when motile promastigotes are transmitted by *Phlebotomus* (OWCL) and *Lutzomyia* (NWCL) sandfly vectors to the mammalian host. In order for the cycle to successfully progress forward, cool and low temperatures are required, since high temperatures tend to shorten it.⁷⁰ During the initial bite, the sand fly feasts on human cells that contain the oval, microscopic, protozoan Leishman-Donovan bodies (or amastigotes). The amastigotes enter the fly gut where they mature into the promastigotes, and attach to the gut wall. Promastigotes are quickly engulfed by phagocytic cells at the site of

infection, but only differentiate into the obligate intracellular amastigote in response to the acidic environment of the macrophage phagolysosome.^{4,26} Promastigotes are significantly larger than amastigotes with a long whip-like flagellum attached at one end.

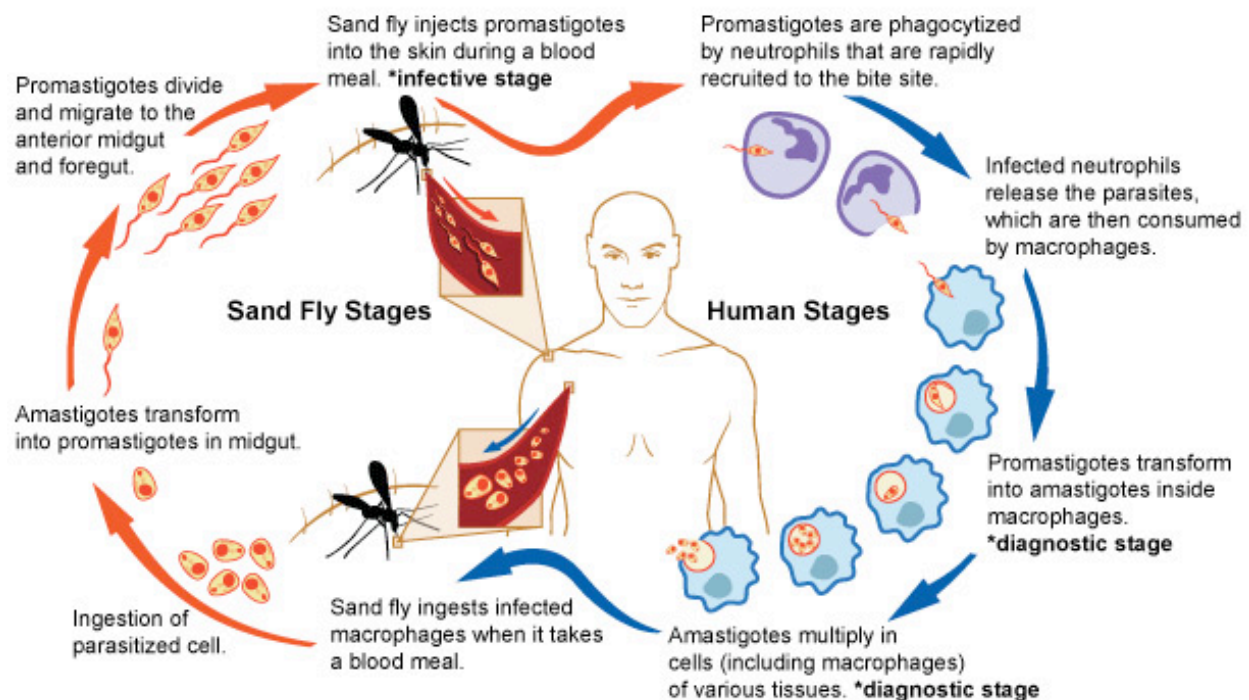


Figure 1.2. *Leishmania* parasitic life cycle.⁵¹

The promastigotes multiply by way of binary fission, which allows their nuclear material to divide in two, soon multiplying in large numbers in the fly's gut. Before moving to another host, the promastigotes are expelled by the sand fly as they become lodged in its esophagus where it then releases them into its victim during a meal. They begin to invade host cells, often macrophages, where they transform to amastigotes and begin the process once more. When human macrophages become packed with amastigotes they rupture and are destroyed, releasing themselves into new cells and tissues causing lesions. Leishmanial infections all share a

common histological feature – the early accumulation of mononuclear phagocytic cells (or hyperplasia) in the site of infection.

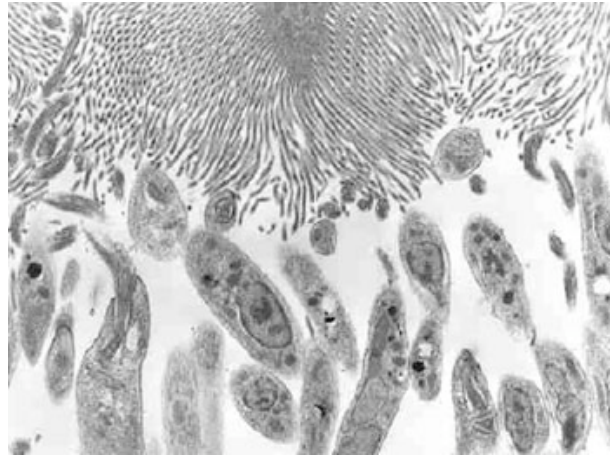


Figure 1.3. *Leishmania* parasites in sandfly midgut.⁴⁴

The balance between parasite multiplication, the degenerative changes, and immune response of the patient all impact and determine the clinical form of the disease. The chronic symptom of the disease is due to the ability of the amastigotes to evade host macrophage cells and persist for long periods of time in the host.³ Host complement receptors CR1 and CR3, assist in the entry of *Leishmania* into macrophage cells without triggering oxidative burst.

The host-parasite relationship was not fully understood for many years but now it is known that within the host macrophages, leishmanial amastigotes are safely protected from antibodies and any substance that may be near the target tissue and that may be harmful to the parasite.⁷⁰ It is therefore crucial for the microorganism to become incorporated in the cells in order to ensure parasitic survival. Parasite infested phagosomes fusing with lysosomes do not seem to harm the parasites that eventually multiply in the phagolysosomes. Studies done *in-vitro* have shown to induce an increase in oxygen uptake when there is phagocytosis of *Leishmania* in

macrophages. Following the process of oxygen uptake, oxygen metabolites such as oxygen peroxide are generated. Generally promastigotes are unable to withstand these compounds and are destroyed in macrophages, however, in intracellular infections of this kind they are able to go on and replicate themselves within the macrophage.

When the parasite has successfully invaded the tissues and has begun the process of destruction at the site, oedema in the superficial dermis and damage to collagen and elastin is usually seen, followed by fibrosis.⁷⁰ In severe cases, there is necrosis of collagen or epidermis. Capillaries at the site may show endothelial swelling or proliferation, and often there is vasculitis leading to inflammation of the wall of blood vessels including veins, arteries, and capillaries due to leukocyte migration and resultant damage.

1.3.3 Available Treatments

There is no known prophylactic vaccine available for leishmaniasis but several pentavalent antimonials are available: sodium stibogluconate (Pentostam ®, GSK, United Kingdom) and its generic forms, used in field conditions, and meglumine antimoniate (Glucantime ®, Aventis, France). Old World localized cutaneous leishmaniasis requires local infiltration with pentavalent antimony. The World Health Organization (WHO) recommends an injection of 1-3ml under the edges of the lesion and the entire lesion until the surface has blanched; injections should be administered every 5-7 days, for a total of 2-5 times.¹¹ It was reported in 1999 that intra-lesional antimonials partially or completely cured 72-97% of the lesions caused by *L. major*. In a separate study done in Tunisia adverse events were observed in 5%, which included bacterial super-infections; it was seen most commonly in patients with cutaneous leishmaniasis in the face or limbs.⁴⁷ Other intra-lesional injections of hypertonic

sodium chloride solution of zinc sulphate have shown to be effective as well as local sodium stibogluconate in some Iraqi patients.⁶⁴

Another treatment option is the use of paromomycin (aminosidine) which is an ointment used in amoebiasis, intestinal helminthiasis or visceral leishmaniasis. It belongs to the aminoglycoside family of antibiotics that is virtually identical to neomycin. Both compounds contain a 2-deoxystreptamine attached to three aminosugars, the difference lies in the substituents. Paromomycin has a CH₂OH substituent on one of the aminosugars, whereas neomycin has a CH₂NH₂ substituent.⁵⁷ Even though these two compounds are nearly identical, only paromomycin has antiprotozoal activity. Topical treatments available for cutaneous leishmaniasis include a 15% paromomycin sulphate dissolved in a soft white paraffin base, either with 12% methyl-benzothonium chloride or with 10% urea.¹⁰ A separate study showed that *L. mexicana* infected mice were treated with paromomycin sulphate 15% + 12% methyl-benzothonium chloride and proved to be effective but unfortunately failed to produce results in 68% of 53 Belizean patients infected with the same species.⁶⁸ A similar study was conducted in Guatemala where 76 patients infected with *L. braziliensis* (75%) and *L. mexicana* (25%) were treated with 15% paromomycin sulphate plus 12% methyl-benzothonium chloride for 20 days resulting in an initial cure rate of about 91% but after a one year follow up study was conducted, their progress declined to 86%.⁵

Another method used to treat cutaneous *Leishmania* is through an FDA approved device called ThermoMed ® which targets the lesions directly by delivering localized radiofrequency-generated heat through prongs that are placed onto the infected site. Most sessions require heating at 50 °C for 30s and local anesthetic due to the painful procedure. Guatemalan patients

with *L. mexicana* induced cutaneous leishmaniasis experienced similar effective results when using meglumine antimoniate as with the localized controlled heat.⁵²

The first major attempt at liposomal therapy involved the encapsulation of a pentavalent antimony. Alving et al. conducted the study and composed the liposome utilizing dicetyl phosphate, cholesterol, and dipalmitoylphosphatidylcholine.⁵⁷ The preparation of the liposome involved adding the drug to lipid solubilized in chloroform, followed by the evaporation of the chloroform. The formulation of the liposome was not considered for clinical use due to its high toxicity; dicetyl phosphate is not a compatible bodily constituent and may be toxic once administered and also, there is no guarantee that the evaporation process of chloroform will remove it entirely.⁵⁷

The second major attempt was directed towards developing a liposomal delivery system that was less toxic but just as effective at combating the infection. Lopez-Berestein synthesized a liposome composed of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol (7:3) containing amphotericin B (10% by weight).⁵⁷ The researchers' main concern was for the liposome to be maintained in circulation once in the body and avoiding the removal of it by the kidneys. It was hoped that it would bound to free fungi and to fungi within the host cells and in the process would decrease the amphotericin B resulting in an increase in activity.⁵⁷ Clinical studies showed that patients were able to tolerate up to 5 mg of Amb in the form of liposome/kg/d, the normal maximal dose was actually determined to be 1 mg Amb (free)/kg/d. Clinical studies of the liposomal preparations are currently underway and are available for trial as anti-leishmanial agents. The safest and best preparation of the liposomes involved determining which method would lead to the least accumulation of the drug in the kidneys, liver, spleen, and bone marrow.

1.3.4 Vaccine Candidate

At the present the only known and effective immunizing intervention for preventing cutaneous leishmaniasis caused by *L. major* in humans is Leishmanization (LZ).⁵⁴ LZ is accomplished by injecting the live virulent parasite in healthy individuals. LZ is no longer being practiced in countries such as Iran and Israel since the 1970s and 1980s.

In Brazil, investigators prepared killed parasites for trials in 1939 and was followed by the evaluation of the polyvalent vaccine of 18 strains of *Leishmania* in the 1940s. This progress was a stepping-stone for Mayrink and coworkers in the 1970s when they developed a pentavalent vaccine.⁵⁴ The efficacy of the vaccine could not be assessed since no cases occurred in the study after vaccination in either the vaccine or placebo group. Mayrink and coworkers continued their studies in 1981 and 1983 when they administered controlled trials of the pentavalent vaccine using different doses in Brazilian army personnel.⁵⁴ Unfortunately, the trial results did not show a significant difference between the vaccine and the placebo arms.

There has been only one leishmaniasis vaccine in which efficacy was observed in the entire vaccinee's cohort and was conducted by Armijos et al. in Ecuador.^{6,7,54} Safety, immunogenicity and efficacy of two intradermal doses of a locally prepared trivalent vaccine were assessed against two doses of Bacille Calmette-Guérin (BCG) adjuvant. Promastigotes of *L. braziliensis*, *L. guyanensis*, and *L. amazonensis* were originally collected from the lesions of patients living in the study area and were mixed with BCG (prepared from a strain of the attenuated (weakened) live bovine tuberculosis bacillus, *Mycobacterium bovis*, that has lost its virulence in humans by being specially cultured in an artificial medium for years) to construct the vaccine.^{6,7,54} The vaccine was shown to be safe after a 12-month follow-up with a 2.1% incidence of CL in the vaccine arm vs. 7.6% in the control arm, resulting in an efficacy of 73%.

Patients were followed up for another 4 years and although the protective efficacy was still significant between the 13th and 18th month, it was not after the 19th month follow-up. Total incidence from the 19th to the 60th month was not significantly different between the vaccine and control arm subjects who were followed up.^{6,7,54} The loss of the statistical significance is thought to be due to the reduction in the number of new cases in the control group and not due to an increase in the number of cases in the vaccine arm.^{6,7,54}

In Iran, one injection of a vaccine was compared with one injection of BCG alone in a randomized, double-blind, controlled field efficacy trial of autoclaved *L. major* (ALM) + BCG against CL due to *L. major*.⁵⁴ Volunteers ranged in age from 5 to 72 years and were healthy, leishman skin test (LST) negative residents. The leishman skin test measures delayed type hypersensitivity reactions to an intradermal injection of a suspension of killed promastigotes. After 2 years of follow-up there was no significant difference in the leishmaniasis incidence rates in the two arms and infection rates among LST converted individuals (both in the vaccine and the control arms) were lower than in those whose LST had not converted (7.3% vs. 11.3% in the vaccine arm and 3.4% vs. 10% in the control arm).⁵⁴

Protective efficacy has not been seen in many clinical trials of first generation leishmaniasis vaccines except for those constructed by Armijos et al.^{6,7,54} Even though efficacy has only been shown to be present in one vaccine, prophylactic findings discussed above show very encouraging results. This study is unlike any other attempted before in that previous vaccines used BCG as the adjuvant and this investigation is the first to implement metal ligand complexes to improve DNA delivery.

Chapter 2. Metallo-Lipid Nanoparticles for Drug Delivery Systems.

2.1 DRUG DELIVERY SYSTEMS FOR INTRACELLULAR INFECTIONS

Because one of the biggest obstacles in producing a treatment for intracellular infections is determining an efficient and effective method for the delivery of the drug to the targeted site, all options must be presented that may serve as possible candidates as a delivery system. Many of the recently explored techniques include the use of micelles, nanoemulsions, nanoparticles, and liposomes. While they may all have their advantages and distinct capabilities, one method has yet to be proven effective, at least for the treatment of leishmaniasis. Micelles, nanoemulsions, nanoparticles, and liposomes have been continuously investigated for their drug delivering capabilities.⁴³ As stated by Hussein,³⁰ targeted drug delivery is essential to modern medicine in which specifically designed and effective drugs are employed to work on selected tissues, cells, and cellular structures. In the end, it would be ideal to localize the delivery of the drug to a specific organ instead of systemically injecting and exposing the entire body to the potentially toxic drug and at the same time use less of the costly drug.

2.1.1 Micelles as Drug Delivery Systems

Micelles have recently played a dynamic role in chemotherapy treatments and have been found to have applications as drug delivery systems. A micelle is an aggregate of surfactant molecules dispersed in a liquid colloid. Micelles take on various shapes and are of different sizes. A normal phase micelle, which is a typical oil in water micelle, forms an aggregate with the hydrophilic “head” regions that are in contact with the solvent in aqueous solution, sequestering the hydrophobic tail regions in the micelle center. Others have the head groups at

the center with the tail extending outward, these are referred to as inverse micelles. Micelles, for the most part, are spherical in shape although some are ellipsoids, cylinders, and bilayers. Surfactant concentration, pH, temperature, as well as ionic strength determine the shape that the micelle will exhibit.

Polymeric micelles have been the focus as drug delivery vectors. They are formed by a spontaneous assembly from amphiphilic polymers in selective solvent to decrease their free energy and are of great interest since they are thought to water-solubilize hydrophobic drug molecules. Their structure and 10-100 nm size allows them to be sterilized by simple filtration and avoid mechanical clearance by filtration in the spleen.¹⁶ Because they are smaller than 200 nm they are ideal particles for systemic administration for a long-circulation drug carrier, and therefore are difficult to be recognized and internalized by the reticuloendothelial system (RES) because of their hydrophilic shell. This is of extreme importance since the RES consists of mostly phagocytic, monocytic, and macrophage cells located in the reticular connective tissues which help initiate specific immune mechanisms in the body by phagocytosing cellular debris and pathogens. Micelles also have the capability of storing indissolvable drugs to increase their solubility. When referring to chemotherapy applications, micelles with small size and long circulation are easily accumulated at the tumor site due to their enhanced permeability and retention (EPR). When utilizing micelles for therapeutic use, researchers suggest that administration of drugs should be combined to increase efficiency of the treatment instead of encapsulating one single drug.

Chemical conjugation is another method of incorporating drugs into micelles. Copolymer-drug conjugates are the result of covalent coupling of the drug to the hydrophobic block of copolymers.^{73,74,75} Conjugates in the form of micelles have been found to circulate the

blood much longer than when introduced in free drug form. Studies performed by Kwon et al. showed that lower levels of conjugate were detected in major organs, whereas a much higher conjugate level was found at the site of the target, in this case being the tumor.¹²

Unfortunately, most micellar carriers exhibit a rapid clearance from circulation after administration by way of endocytosis, phagocytosis, and liver uptake.⁶⁸ A method by which this can be resolved is by incorporating an exterior layer of poly ethylene oxide which results in the modification of the micelle surface, preventing them from being cleared from circulation as easily. Micellar drug carriers are able to remain longer in the blood circulation because they go undetected and are protected due to water association with the PEO chains, leading to steric repulsion of proteins and a decrease in protein adsorption on the surface of the drug vehicles. Other researchers have found that an increase was seen in the concentration of encapsulated drug in the blood, leading to an increase in drug concentration in tumors when PEO chains were incorporated in the design of the drug delivery systems.⁶⁸

2.1.2 Nanoemulsions as Drug Delivery Systems

Emulsions are a second drug delivery system candidate that are defined as a mixture of two immiscible (unblendable) substances where one substance, referred to as the dispersed phase, is dispersed in the other, and is referred to as the continuous phase. In emulsions a surfactant stabilizes the system to prevent the dispersed phase from coalescing in a macroscopic phase.⁶¹ In drug delivery systems, the drug being delivered is transported or is itself the non-aqueous liquid phase of the emulsion and many times, the continuous phase is the aqueous phase. In many cases, it is common to see hydrocarbons or fluoro-carbon liquids acting as the dispersed phase, carrying a hydrophobic drug that will be transferred to the target cells and

tissues by a disruption or the dissolving of the nanoemulsion carriers, causing a quick release for the drug transfer. Therapeutics can be slowly delivered by diffusion while utilizing a more stable emulsion.

2.1.3 Nanoparticles as Drug Delivery Systems

Nanoparticles differ from emulsions in that they consist of a solid core or form a macroscopic solid at room temperature and may not necessarily be spherical in shape. A vast array of solid nanoparticles are employed and used in drug delivery.⁵⁸ The advantage of polymeric nanoparticles is that they consist of non-soluble polymers that allow them to release drugs as they degrade or can release the drug from the polymeric core by diffusion. Once the nanoparticle has been formed, covalently crosslinking the polymer will sustain its structure so that it does not dissolve in water or blood and proceed into forming hydrogel nanoparticles that are soluble or amphiphilic polymers that are capable of carrying the drug. A dendrimer is another type of polymeric drug carrier that allows for drugs to be attached to its highly branched polymer arms or that sequesters them and releases them by diffusion.

Other nanoparticles that are non-polymeric have also been researched as drug delivery systems. Nanotubes and fullerenes consisting of graphitic carbon can carry drugs within their volume or if attached to their tubular or spherical cage-like surface.³⁷ Other examples include aggregates of hydrophobic proteins or drugs that have the potential to partially crystallize into nanoparticles. Nanoparticles that consist of a solid lipid core that can carry a hydrophobic drug stabilized by an external monolayer of steric or charged surfactant are referred to as solid lipid nanoparticles.

Compared to liposomes, nanoparticles have greater encapsulation efficiency, greater stability in the presence of serum and during storage, as well as the ability to achieve sustained release.^{9,55} Along with their advantages, they can also affect biological behaviors at the cellular, subcellular, and protein level. Because they are so small in size, they can easily be distributed throughout the body, reaching target tissues and sites in the cell that are of delicate nature such as the nucleus or mitochondria, leading to genetic damage and mutations.^{53,58} Nanometer size polymers are not ideal as nanoparticles because they are taken up by cells and are degraded inside them, with the possibility of causing cytotoxic effects.^{50,58}

As mentioned above, hybrids between polymeric microparticles and liposomes, are known as solid lipid nanoparticles (SLN), and have the advantages that fatty emulsions and polymeric nanoparticles exhibit.²¹ Because they consist of a solid matrix composed of lipids stabilized in the form of aqueous suspensions by a surfactant covering, they have proven to be adequate for the encapsulation of lipophilic drugs. In a study, it was shown that SLNs are less toxic than polymeric microparticles when analyzed in a culture of human granulocytes.⁵⁰ They too have shown to have the ability to degrade faster and have better control when releasing a drug at the site of infection.^{18,28,35,39}

2.1.4 Liposomes as Drug Delivery Systems

Liposomes are classified by their phospholipid bilayers that surround an aqueous compartment, they are simply defined as microscopic vesicles. Liposomes are classified as either single-layer vesicles (SLV) or multilayer vesicles (MLV). Their overall structure is composed of phospholipids, cholesterol, and may contain other lipids and proteins. They are widely known for their ability to encapsulate hydrophilic drugs in aqueous phase due to their

range in size from 25nm to several microns. Hydrophobic drugs are able to be bound to the lipid bilayer for delivery as well. Some of their advantages include their low toxicity and their ability to biodegrade.

Liposome toxicity varies depending on cell types. For example, liposomes composed with cationic lipids were shown to be toxic to phagocytic macrophages and monocyte-like cells, but not to nonphagocytic T lymphocytes.²¹ Other different liposomal formulations led to symptoms of neurotoxicity in experimental animals.^{1,72} It is therefore extremely important to take into account the choice of the main lipid and the charge component to minimize the toxicity as much as possible.

Liposomes have the ability to quickly be removed from circulation by monocytes and macrophages and accumulate in the major organs, this in turn is an important attribute when working with intracellular infections. A mechanism exists describing the steps taken during the penetration of liposomes by phagocytic cells. The first step in the process requires a stable adsorption on the cell membrane, followed by the vesicle being internalized through an energy dependent mechanism and the fusion of the endocytic vesicles with the lysosomes. Before the drug can be released, the liposome must be degraded by lysosomal enzymes.^{2,33} This proves to be an ideal treatment when germs from infections are present in the lysosome but is not a useful treatment if they are residing in the cytoplasm. When the germs are located in the cytoplasm, pH sensitive liposomes are used, liposomes of this nature will destabilize the lipid bilayer due to the decrease in pH in the endosome, allowing fusion of the liposome with the endosomal membrane, thus releasing the drug in the area.^{15,45,67}

In order for liposomes to successfully reach the site of infection and release the drug a number of conditions must first be met. They must bind to and be phagocytosed by

macrophages and most importantly maintain the encapsulation of the drug as well as prevent its degradation before reaching the target tissue. Once these conditions have been met, liposomes must make their way to the organ where the infection is localized.² The infection site is abundant with macrophages and in order for liposomes to penetrate into these macrophages their size, composition, cholesterol content, and surface properties of the lipid bilayer must be taken into consideration.

Liposome nanoparticles have been documented as promising candidates for having adjuvant properties and being carrier systems for antigen delivery.⁷⁰ Liposome based adjuvants can be manipulated to increase immune responses by modifying chemical derivatization of the lipid, conjugation of ligands or polymers on the surface of the liposome, or by implementing other modulator molecules to the interior compartments of the liposomes.⁷⁰

2.2 COPPER AND ZINC LIPID NANOPARTICLES

Recently, metal-mediated supramolecular complexes have been synthesized that are able to successfully deliver large fragments of DNA into eukaryotic cells. As cited from Arroyo, the molecular design consists of the formation of amphiphilic Cu(II) complexes that self-assemble into metallo-liposomes in water and condense DNA plasmids into deliverable structures programmed to react with intracellular components via redox- and ligand-exchanged reactions. Marine bacteria and other organisms consist of natural coordination-capable lipids allowing them to self-assemble into micelles in water and upon binding to transition metal ion, forming metallo-liposomes as they undergo phase transitions. These siderophores have been utilized to study electron-transfer reactions across membranes.

Prior studies have shown that these synthetic amphiphiles with coordination-capable head-groups are able to self-assemble via coordination interaction (first generation assembly)

and by hydrophobic-hydrophilic interactions of the amphiphiles (second generation assembly). These molecules have been referred to as *coordination amphiphiles*. These molecules were designed to self-organize via coordination and hydrogen bonding interactions into molecular ensembles ranging in sizes between 10 nm or smaller in aprotic solvents. Coordination amphiphiles that have the capability of forming liposomes or vesicles result in nanospheres composed of amphiphilic bilayers that are able to separate from the bulk aqueous phase into an aqueous internal compartment that range in size between 50-500 nm.

2.2.1 Ligand Design

When selecting a ligand, it was extremely important that it possess the capability of being functionalized with hydrophobic alkyl chains while sustaining its ability to produce coordination interactions with transition metals. 1,4,7-triazacyclononane (TACN) was chosen due to its three secondary amines capable of being modified with hydrophobic groups and because of its Lewis base character.

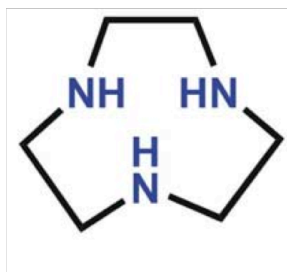


Figure 2.1. Chemical structure of the ligand 1,4,7-triazacyclononane (TACN).

2.2.2 Synthesis of alkyl modified triazacyclononane ligands with coordination complexes

In recent studies, lipids forming bis-complexes with Cu(II) ions, namely $[\text{Cu(II)}(\text{L}_{\text{dt}})_2](\text{OTf})_2$ and $[\text{Cu(II)}\text{L}_{\text{ot}}_2](\text{OTf})_2$, where L_{dt} = 1-dodecyl-1,4,7-triazacyclonone, L_{ot} =

1-octadecyl-1,4,7-triazacyclononane, and OTf = trifluoromethanesulfonate have been synthesized having coordination capabilities. The ligands were prepared from corresponding nucleophilic addition of one equivalent of 1-bromoalkane to 1,4,7-triazacyclononane (L_{tacn}) in dry THF over sodium hydride at 60 °C for 12 h. After flash column purification, they were reacted with half equivalents of $\text{Cu}(\text{OTf})_2$ in acetonitrile at room temperature to form corresponding amphiphilic Cu-complexes which were isolated by fractional crystallization with diethyl ether at -40 °C. The amphiphilic complexes self-assembled into metallo-liposomes in water and did exhibit an affinity to bind the PO_4 groups of DNA via electrostatic and hydrogen-bonding interactions.

A tridentate metal binding domain and a hydrocarbon ($\text{C}_{12}\text{H}_{37}$) group in the lipid ligand 1-dodecyl-1,4,7-triazacyclononane (L_{ot}), allowed them to have amphiphilic properties. Upon reactions with divalent transition metal salts, they formed bis octahedral complexes. In particular, the Cu(II) bis-complex of L_{ot} forms $[\text{Cu}(L_{\text{ot}})_2]^{2+}$ (1d), self-assembled into metallo-liposomes with bilayer composition in aqueous media. Due to the cationic nature of the surface of 1d and the hydrogen bonding motifs available, these metallo-liposomes had an affinity to bind DNA strands. AFM experiments revealed that the most favorable interaction of 1d with DNA was the formation of a surface complex that appeared to have a tetrahedral structure.

The alkyl modified triazacyclononane ligand was previously documented by Arroyo⁸ and was used when reproducing the synthesis. Alkyl substituted triazacyclononane ligand containing 12 carbons in the alkyl chain were synthesized by the nucleophilic addition of one equivalent of 1-bromododecane to 1,4,7-triazacyclononane (TACN) in dry THF over sodium hydride at 60° for 24 hours. Flash column using a gradient of chloroform-methanol was used to purify the product.

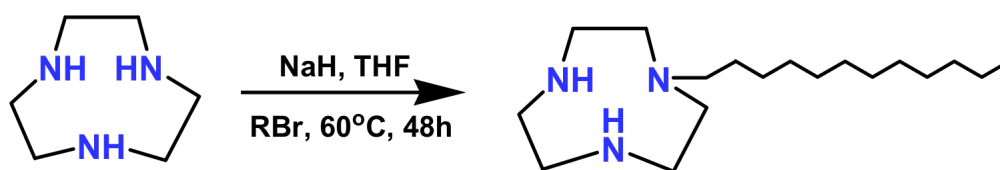


Figure 2.2 Synthesis of an alkylated derivated of TACN.

Synthesis of the amphiphilic coordination complexes followed, by reacting the alkyl monosubstituted triazacyclononane ligand at room temperature for 24 h with one half of equivalent of Cu(II)/Zn(II) triflate in acetonitrile in order to create a coordination amphiphile with a complete coordination sphere surrounding the metal. Copper and zinc were ideal for the synthesis due to their biocompatibility and coordination capabilities. The copper synthesis is not shown or illustrated but same procedures were followed.

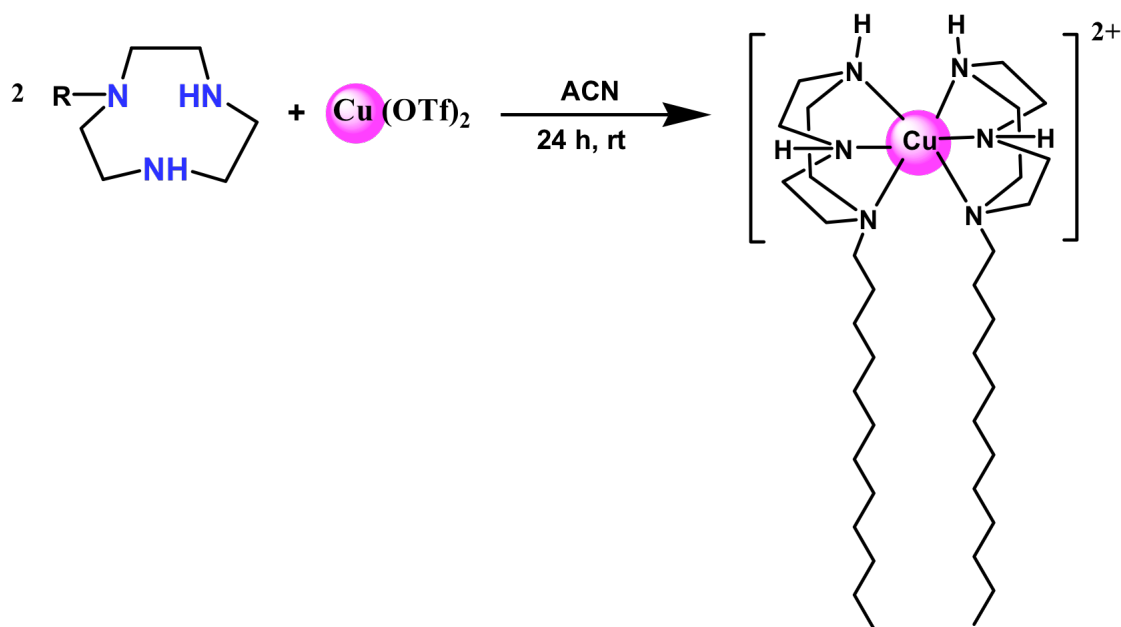


Figure 2.3. Synthesis of copper coordination amphiphile.

2.2.3 Immunological Applications

As mentioned previously, DNA delivery systems have their share of disadvantages and limitations due to their dependency on electrostatic interactions between positively charged structures and the negatively charged DNA. Successfully transfecting mammalian cells with exogenous DNA or RNA is an ambitious goal especially when considering the lack of treatment options existing for *Leishmania*.

The copper and zinc amphiphiles that have been synthesized are hypothesized to bind to phosphate groups in DNA via electrostatic interactions and hydrogen bonding due to their head groups. It has been documented by Arroyo that the distance between two adjacent phosphate groups is the same as the phosphate analogues (OTf) in the structure and therefore it is thought that it was possible for the copper/zinc complex to bind to DNA using a hydrogen bond between N-H...O where the N-H is the amine group of the ligand and the oxygen is part of the phosphate group in the backbone of the DNA. Due to these findings, we have high expectations that the head group will interact strongly with the DNA and therefore serve as an effective DNA delivery system.

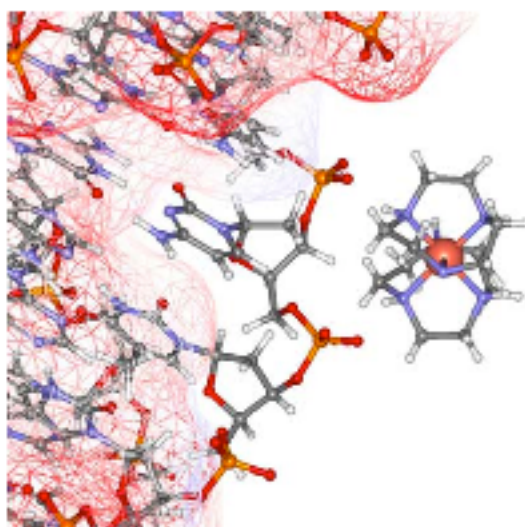


Figure 2.4. Electrostatic and hydrogen bond of the Cu lipid complex with DNA.

In order to ensure that there was encapsulation of the DNA, an agarose gel was treated with SYBR green. The first well served as the control and was clearly showed that no DNA was encapsulated due to the bands seen. The second and third well contained low volumes of the Zn lipid-complex with 500 ng of DNA and it was determined that a higher volume was needed for full encapsulation. The last two wells showed full encapsulation of DNA with higher volumes of the Zn lipid complex. Same results were seen when the Cu lipid complex was tested for encapsulation efficiency. For full encapsulation of the pVax-BT-ICAM plasmid, 65 μ l of the Cu lipid complex was utilized and 40 μ l of the Zn lipid complex. The pVax-ORFF plasmid required 60 μ l of the Cu lipid complex and 40 μ l of the Zn lipid complex for full encapsulation of DNA.

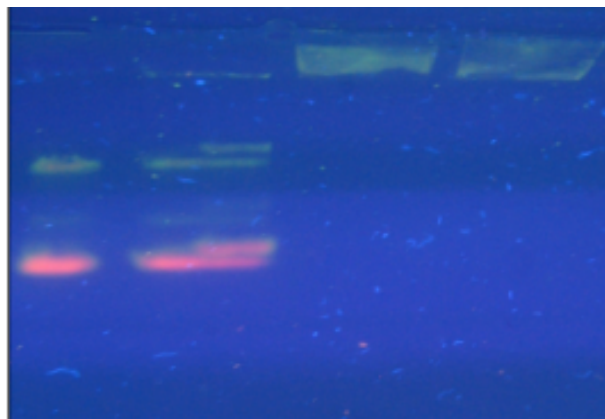


Figure 2.5. DNA encapsulation with Zn lipid-complex.

In previous studies conducted by Arroyo,⁸ the material was tested for its ability to transfect eukaryotic cells with DNA plasmids containing the reporter GFP gene, which encodes for the Green Fluorescent Protein, an excellent indicator for gene expression. Originally the highest transfection value observed was 4%. However, efficiency was improved to 39 % by changing the length of the hydrocarbon groups.

Under the experimental conditions discussed above, the lipid metal complex, Cu(II) bis-complex of L_{ot} forms $[Cu(L_{ot})_2]^{2+}$, which contained two n-dodecane groups, exhibited comparable transfection efficiency to the well-established commercial transfection agent lipofectamine (45%), Figures 2.6 and 2.7.

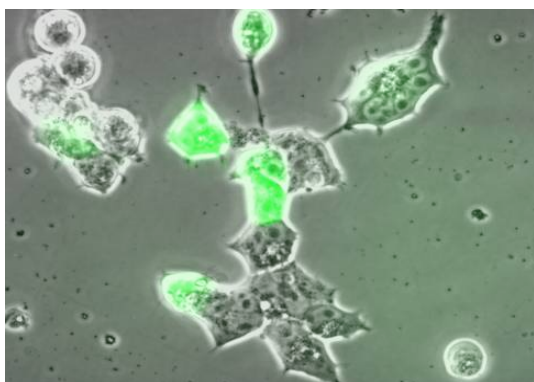


Figure 2.6. T291 cell transfected with pEGFPN1 plus metallo-liposome- Zn^{2+} . Expression of green fluorescent protein was visualized.

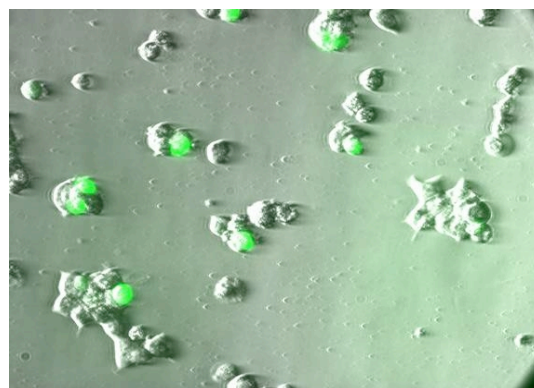


Figure 2.7. T291 cells transfected with pEGFP-N1 plus metallo-liposome- Cu^{2+} . Expression of green fluorescent protein was visualized.

2.2.4. Cytotoxicity Assay Study

U-937 cells were cultured in a 96-well microtitre culture plate using a cell suspension adjusted to 2.5×10^5 cells/well. The metal ligand complexes were dissolved in PBS, which was diluted to produce concentrations of 2, 4, 8, 16, 32, 64, 128, and 256 nM/well. Each experiment included the different ligand concentrations, the solvent, an untreated control, and positive control (pentamidine). These were added to their respective wells. Fresh culture media with the ligand complex was replaced after 48 h of culture. After 54 h $1 \mu\text{Ci}$ of $[^3\text{H}]$ thymidine was added to each well; 18 h later $[^3\text{H}]$ thymidine incorporation was measured. All cultures were performed twice in triplicate. LD 50 was determined to be 24 mM/24nM for both Cu and Zn complexes.

In summary, it was shown that transfection efficiency was comparable to the commercially available Lipofectin when tested on COS1 cells. Encapsulation efficiency was shown to for both Cu and Zn lipid complexes and may prove to be an effective DNA delivery system.

Chapter 3. DNA Vaccine for *Leishmania major*

The use of bioprotein transporter (*BT*), cell adhesion molecule-like (*ICAM-1*), *ORFF*, and *Amastin* *Leishmania* antigens as a prophylactic DNA vaccine constitute a novel approach for the prevention of cutaneous leishmaniasis (CL) caused by *Leishmania major* using a *Leishmania mexicana* antigen. The *BT* molecule of *Leishmania* was first described in *Leishmania donovani* by Lemley and associates.⁴² Recombinant *BT* previously was found to confer partial protection when used as vaccine candidate for preventing visceral *leishmaniasis* in a murine model. This is the first attempt to use *BT* as a vaccine candidate to prevent CL in a mouse model.

To date, the *ICAM* molecule is the only of its kind found in *Leishmania*. It was first described in *L. amazonensis* by Chiang.⁵⁴ *ICAM-1* is located in the parasite nucleus and on its surface. Previous studies characterized this molecule as a *Leishmania* ligand, which interacts with receptors on phagocytic cells.⁵⁴ Our study also is the first to use *ICAM-1* as a vaccine to prevent CL in a mouse model. The *ORFF* molecule was first described by Myler and colleagues, who suggested it had protein coding functions.⁵⁴ Tewary showed that this molecule has been shown to confer partial protection against murine visceral leishmaniasis.⁵⁴ Finally, *Amastin*, recently described by Almeida and associates belongs to an extended family of external surface proteins on *Leishmania*.⁵⁴ To date, no attempts have been made to utilize this molecule as a CL vaccine candidate.

3.1 DNA PLASMID ANTIGENS

The *L. major* cocktail vaccine utilized for the inoculation of mice in this research consists of two antigens (*BT* and *ICAM*) that will be expressed for the same plasmid. Five separate

constituents construct the vaccine – *BT*, *ICAM*, *ORFF*, *Amastin*, and pVAX. The reason that so many genes were utilized in the vaccine was due to the size of the parasite. Because, they are large in size, minimal protection would exist from the use of only one gene and in turn would make the parasite more viable and adaptable. If only one molecule was targeted in a parasite, the parasite would continue replicating due to its inefficiency.

BT (biopterin transporter) is a gene responsible for encoding the transport of biopterin. It is present in the promastigote and amastigote stages in the parasitic life cycle.²⁴ The biopterin transporter 1 (BT1), initially known as ORFG, is amplified in all strains of *Leishmania*. BT1 is characterized as a membrane transporter that is found in Trypanosomatids. Research has found that biopterin may be a potential growth promoter for *Leishmania* cells.²⁴ The exact role of BT1 in *Leishmania* is unresolved but it is thought that pteridines must be acquired from a host in order to uptake biopterin and therefore ensure the survival of the parasite.⁴²

ORFF is one of two molecules that is responsible for adhering to the parasitic cells and is thought to also be part of the amplification unit of the parasite.³¹ It is present in the promastigote and amastigote stages of the *Leishmania* life cycle. The *ORFF* gene is localized 365-bp upstream of the BT1 and is approximately 1086 nucleotides in size. The gene contains a S-adenosyl methionine (SAM)-dependent methyltransferase motif.^{31,66} Like BT1, *ORFF* has been shown to amplify all strains of *Leishmania* resulting in LD1 amplification and therefore an advantage for parasitic survival.

ICAM is also a gene present in the promastigote and amastigote stages in the parasitic life cycle and is located in the nucleus.²⁴ In order for a vaccine to combat a parasite, the survival mechanism of that parasite must be hindered. *ICAM* interacts with specific receptors in macrophages that target the mechanism of survival and is responsible for adhering to parasitic

cells. Microorganisms avoid phagocytosis, but in the case of *Leishmania*, it induces the process since it requires for it to be engulfed by the macrophages in order to survive. Unlike *T. cruzi* that is able to survive within the cytoplasm of a cell, *Leishmania* is unable to do so and therefore it is crucial that it be phagocytosed. *Leishmania* contains enzymes that allow it to resist immunological attacks and because it is unable to synthesize DNA bases, it requires a host in order to obtain these bases to multiply and duplicate.

Amastin is specifically expressed in the intracellular amastigote stage of the parasite. The *Leishmania Amastin* gene homologs contain four predicted transmembrane helices and are localized within the plasma membrane of the parasites.²⁴ As transmembrane proteins, *Amastins* have the capability of catalyzing the specific transport of ions, metals, nutrients or metabolites across the membrane barriers. Once in the phagolysosomes, most of the *Amastin* homologs are expressed once the parasite is fully differentiated into its intracellular amastigote form.²⁴ Being able to survive inside the acidic environment of phagosomes requires the efficient transport of water soluble molecules for parasite pH homeostasis and growth, and *Amastins* may play a role in these proteins.

PVAX is an unstable plasmid. Because it is unstable, it is only temporarily present in the cells. When constructing a vaccine, it is important and crucial to avoid incorporating foreign DNA in another being. In the case of this vaccine, the genes are only being expressed temporarily until the body is able to generate memory cells. Those cells will in turn fight the parasite after a sufficient amount of memory cells are produced. PVAX is the only plasmid approved by the FDA for human vaccinations due to its unstable properties that allow for its easy removal from the body.

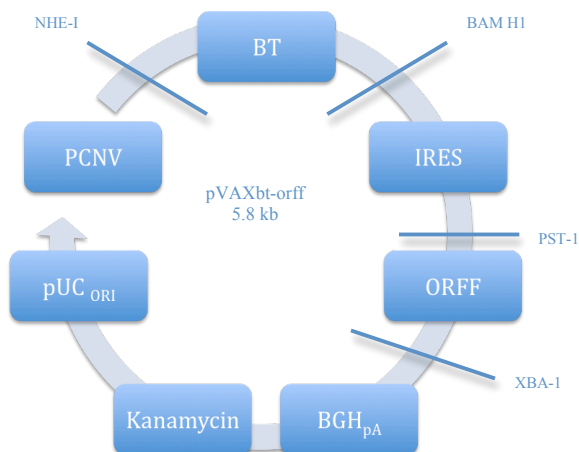


Figure 3.1. pVAXBT-ICAM plasmid.

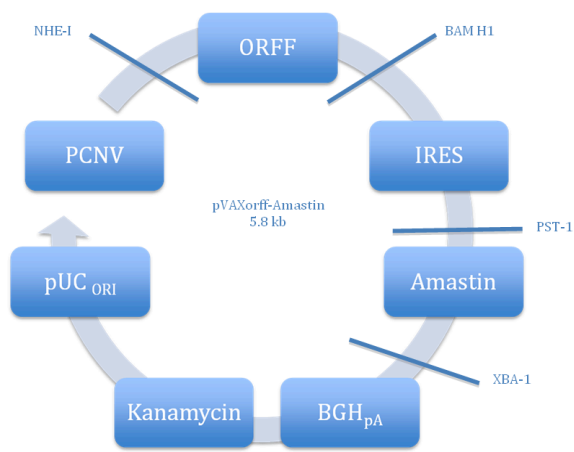


Figure 3.2. pVAXORFF-Amastin plasmid.

The above diagram represents the cocktail vaccine comprised of the antigens (*BT*, *ICAM*, *Amastin*, and *ORFF*) where PCNV serves as the promoter for *BT*, *ORFF*, *Amastin* and *ICAM* and is responsible to assure that *BT* and *ORFF* is expressed and read in ribosomes; IRES reassures expression of *ICAM* and *Amastin*. NEH-I, BAM H1, PST-1 and XBA-1 are restrictive enzymes present in the plasmid. BGH_{pA} acts as the stop codon, whereas pUC_{ORI} 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.

3.1.1 Transfection of COS1, Hela, and Macrophage Cell Lines

COS1 cells were transfected with the DNA plasmids in order to determine if the antigens were being expressed to promote antibody activity. This was determined with the use of a fluorescent tag. Cells were seeded on cover slips in 6-well plates at 2×10^5 cells/well in 3 ml of DMEM and incubated at 37 °C in 6% CO₂ until reaching 50-60% confluency. A final volume of 300 µl and 10 µl of plasmid DNA was mixed with 30 µl of Lipofectin (Invitrogen). Staining was

performed by incubation in 1:100 diluted rabbit anti-*BT* or anti-*ICAM-I* antibody for 2 h at room temperature. Cells were washed 4x with PBS and incubated for 30 min with goat anti-rabbit Ig-fluorescein isothiocyanate conjugate.

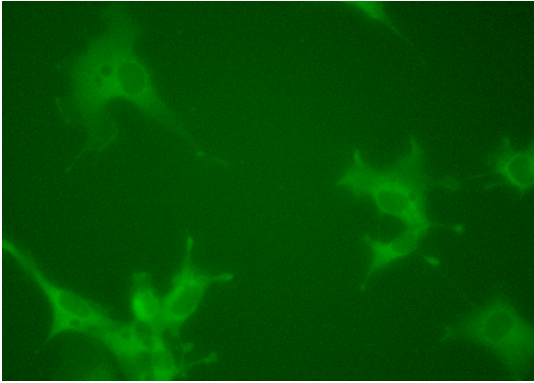


Figure 3.3. COS1 cells transfected with pVAXBT-*ICAM* and incubated with anti-*BT*.

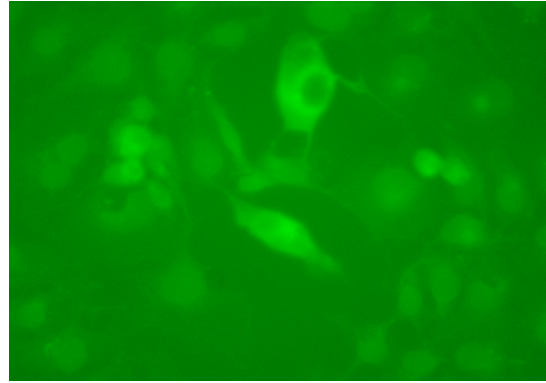


Figure 3.4. COS1 cells transfected with pVAXBT-*ICAM* and incubated with anti-*ICAM*.

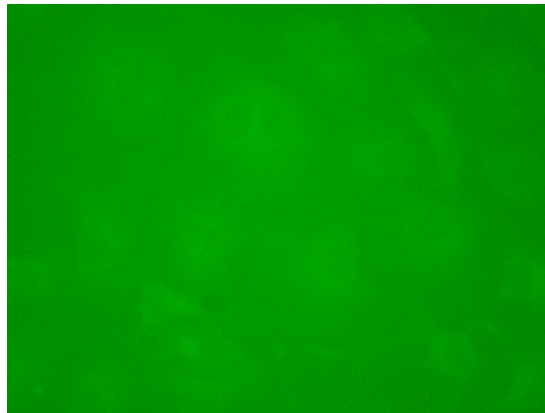


Figure 3.5. COS1 cells transfected with pVAXBT-*ICAM* and incubated with anti-*ICAM*, control.

Macrophages and Hela cell lines were transfected with mice serum and anti-antibody. Cells were cultured on a 24-well plate at 10^6 cells/well with DMEM and incubated at 37 °C in 6% CO₂ until reaching 50-60% confluency. Cells were fixed with fixation solution and

incubated for 10 minutes. Cells were washed and incubated for 1 hour with mice serum. Cells were washed once more before incubation with anti-antibody for 40 minutes.

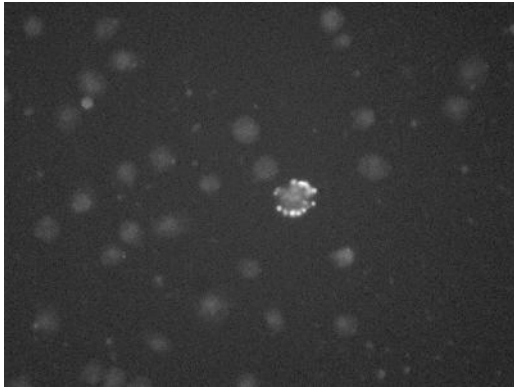


Figure 3.6. Macrophages transfected with *BT/ORFF* + Cu complex

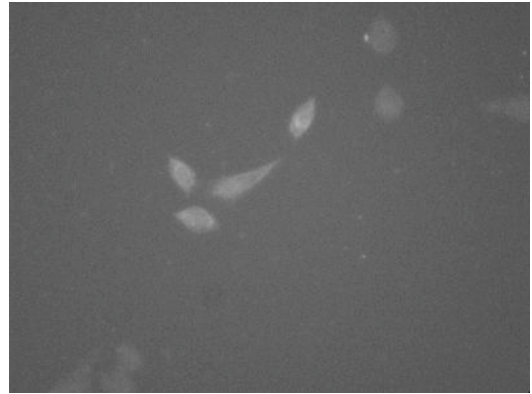


Figure 3.7. Macrophages transfected with *BT/ORFF* + Cu complex

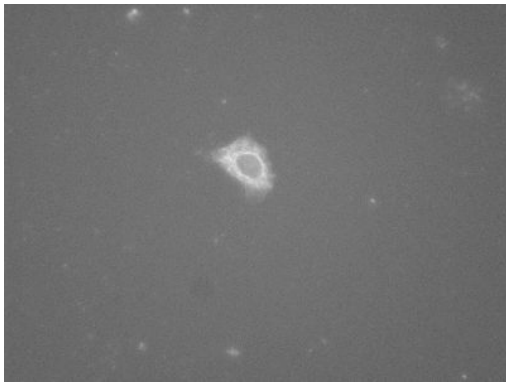


Figure 3.8. HeLa cells transfected with *BT/ORFF* + Cu complex

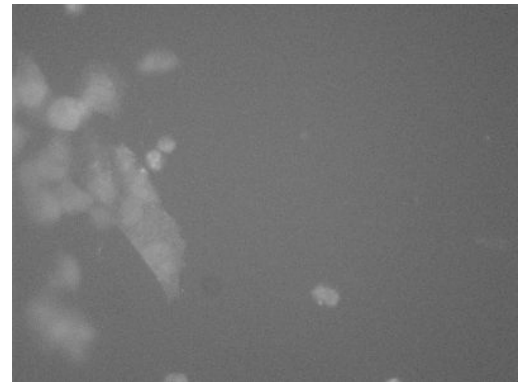


Figure 3.9. HeLa cells, control.

3.2 PARASITE

The *Leishmania major* promastigotes used in the study were maintained by successive culture in RPMI 1040 media supplemented with 10% fetal bovine serum.

3.3 DNA LEISHMANIA VACCINE CANDIDATE

The proposed study is a logical follow-up of research conducted in Ecuador, which tested the effectiveness of killed, whole-parasite vaccine candidates. The current study protocol was approved by the UTEP Institutional Biosafety Committee recombinant DNA Protocol (IBC) on March 14, 2005 (protocol # 2005-02). The *BT*, *ICAM*, *ORFF*, and *Amastin* genes, shown to be immunogenic in other *Leishmania* species, were PCR-amplified from the *L. mexicana* genome and were inserted into the cloning site of pVAX1[®] (Invitrogen, cat No: V26020). A eukaryotic expression vector, pVAX1[®] was specifically designed for DNA vaccine development following FDA recommendations for plasmids used in DNA vaccines for preventable infectious diseases. It is a 3 kbp plasmid which has CMV based promoter, bovine growth hormone polyadenylation sequence and kanamycin resistance gene for selecting in *E. coli*. Therefore, it offers only those sequences required for expression in order to minimize the possibility of chromosomal integration and was recently shown to be an adequate carrier for non-viral vaccines. The novel pVAX-*BT-ICAM* or pVAX-*ORFF-Amastin* products, containing the *BT* and *ICAM-1* or *ORFF* and *Amastin* reading frames are under the control of CMV promoter and IRES enhancer element to yield the expression vector. It was inserted downstream of a kozak consensus sequence and in frame with an initiation codon. The DNA was purified by ion exchange chromatography with a Promega maxi kit.

3.4 IMMUNIZATION

All groups of BALB/c female mice were injected a maximum volume of 100 µl via an intra-muscular route in the hind leg quadriceps using a one ml syringe with a 30 gauge, ½-inch

needle (BD Ultra-Fine needle TM). The experimental groups were immunized with 100 µg pVax-*BT-ICAM-I* (group 1A), 100 µg pVax-*ORFF-Amastin* (group 2A), 100 µg pVax-*BT-ICAM-I*/pVax-*ORFF-Amastin* (group 3A), 5 µg pVax-*BT-ICAM* plus 5 µg pVax-*ORFF-Amastin* plus 32 ng ML-Zn²⁺ (group 4A), 5 µg pVax-*BT-ICAM* plus 5 µg pVax-*ORFF-Amastin* plus 32 ng ML-Cu²⁺ (group 5B), 5 µg pVax-*BT-ICAM* plus 5 µg pVax-*ORFF-Amastin* (group 6A), PBS (group 7A), and 32 ng ML-Zn²⁺ (group 9) respectively.

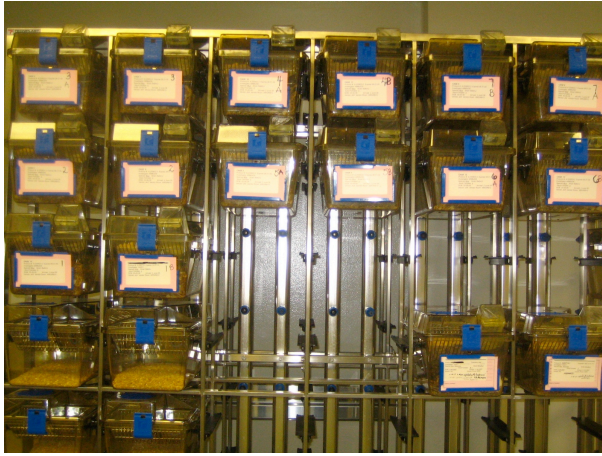


Figure 3.10. BALB/c female mice groups.

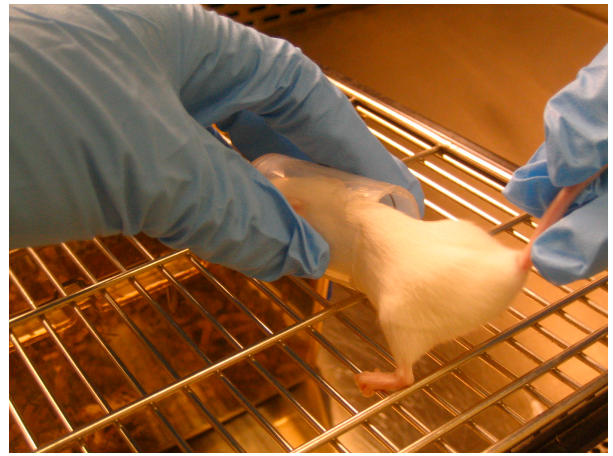


Figure 3.11. Female BALB/c mouse entering trap for immunization.

3.5 INFECTION CHALLENGE AND LESION MEASUREMENT

Three weeks after the third immunization, the mice were challenged subcutaneously in the right hind footpad with 40 µl of 5×10^4 live stationary phase *L. major* promastigotes. The development of footpads lesions (i.e., swelling) at the infection site were measured every week using a digital caliper. The measurements were expressed as the difference between the thickness of the infected versus contra-lateral non-infected footpad on the same experimental animal. As weeks progressed, lesion measurements increased in inflammatory responses. Group 3A, which were treated with the cocktail vaccine, induced the most protection when compared to individual

plasmid molecules. *BT* and *ORFF* plasmids were shown to be less effective when individually used.

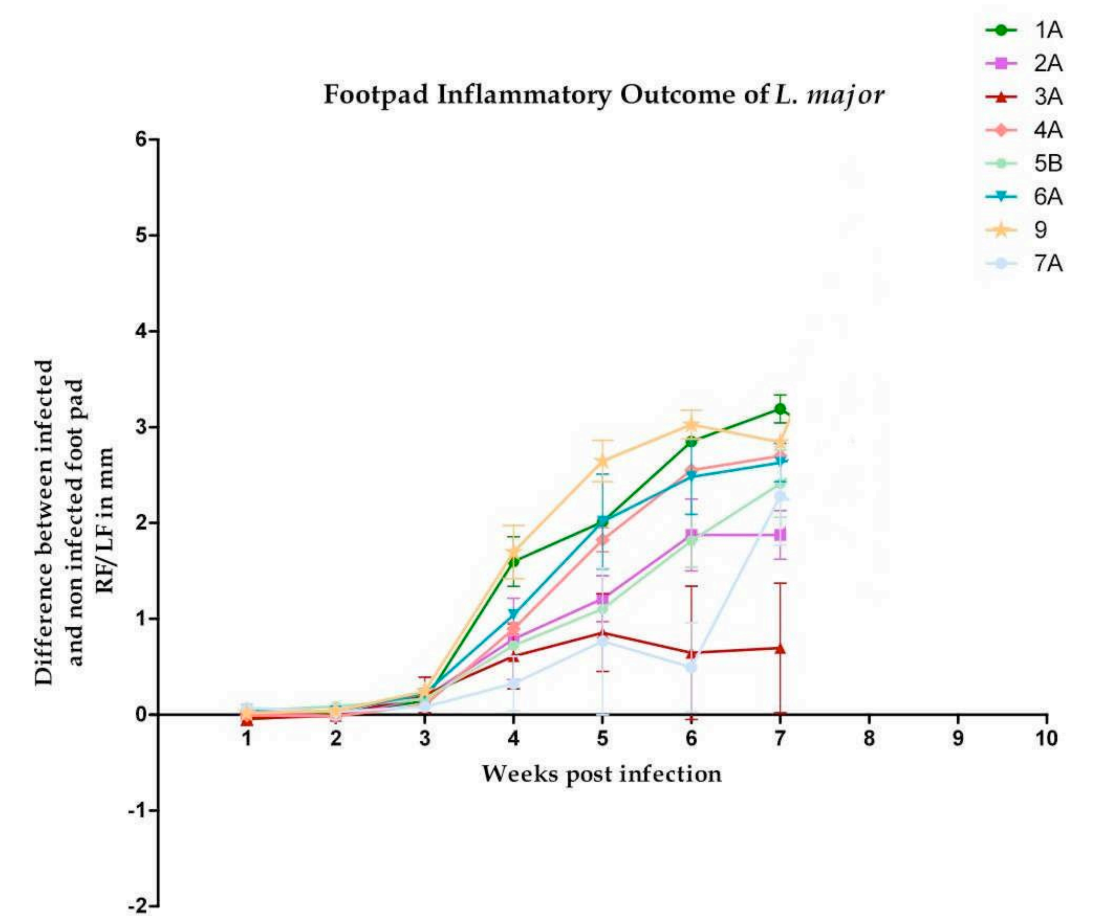


Figure 3.12. Footpad lesion inflammatory measurements taken throughout an 7 week period after mice were challenged subcutaneously with *L. major* parasite.



Figure 3.13. Zn control (group 9) and PBS control (group 7A) footpad lesion comparison at week 6.



Figure 3.14. PBS control (group 7A) and cocktail vaccine footpad lesion comparison (group 3A) at week 6.

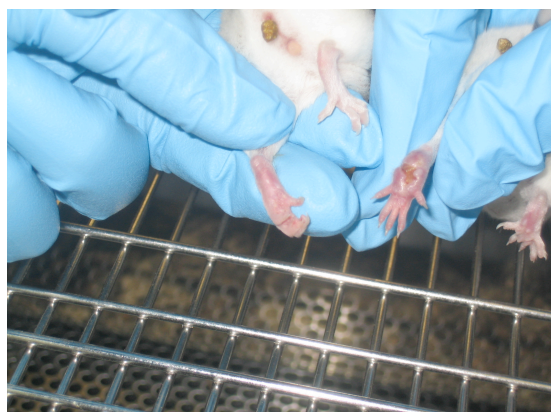


Figure 3.15. Cu lipid complex + cocktail vaccine (group 5B) and Zn control (group 9) footpad lesion comparison at week 6.

3.6 IMMUNOLOGICAL MEASUREMENTS

Previous studies have documented the importance of a strong cellular immune response in reducing tissue parasite burden and subsequent clinical pathology in humans and mice. Three weeks after the final immunization and at the end of 8th week post infection challenge, in order to compare cellular (Th1) immune responses four mice per experimental group were sacrificed via

CO₂ inhalation. These animals were used as source of tissue for post-mortem immunological, and parasitological studies.

Multiparameter flow cytometry. 1.5×10^6 leukocytes from spleen or lymph node cells were incubated with 10 µg/ml leishmania antigen and 2 µl/ml antibody to CD28 for 2 h at 37 °C. Brefeldin A was added at final concentration of 10 µg/ml and cells were incubated for 4 hours. To define T cell phenotype and intracellular cytokine staining, cells were incubated with the viability dye ViViD, followed by staining for, CD4, IFN-γ, IL-2, and TNF-α using the BD Cytotfix/Cytoperm kit. Finally the cells were suspended in BD stabilizing fixative. A more detailed description of the procedure is found in the Appendix portion.

Using the multiparameter flow cytometer and with assistance from Dr. Kristine Garza and Ph.D. student Oscar Ramirez from the Biology department, quantification of cytokines was determined. T cell percentage for both spleen and lymph nodes increased when comparing mice groups 1A to 9. T lymphocytes are a major source of cytokines and bear antigen specific receptors on their cell surface that allow for the recognition of foreign pathogens. CD4 and CD8 are two main subsets of T lymphocytes that assist in differentiating the presence of cell surface molecules. For this study CD4 or T helper cells were the focus of the Th1 immune responses. CD4 or T helper cells have no cytotoxic or phagocytic capabilities and cannot kill infected cells or remove pathogens but are able to manage the immune responses by signaling other cells to perform those tasks. It is therefore assumed that as footpad lesions became more inflamed due to parasitic exposure, an increase in T cells were produced in response to the infection.

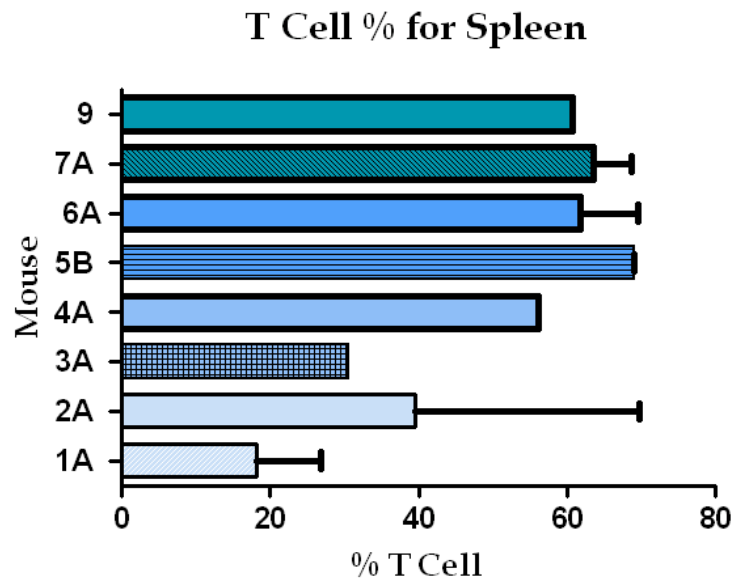


Figure 3.16. % T cells for spleen samples in mice groups 1A–9.

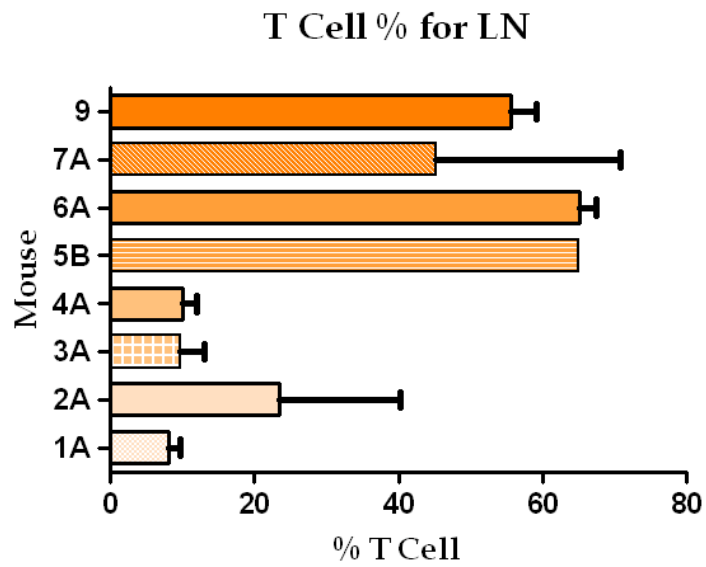


Figure 3.17. % T cells for lymph node samples in mice groups 1A–9.

Individual cytokines were quantified from the percentage of T cells that were detected by the flow cytometer. Scattered and fluorescent light is picked up by the detectors and by analyzing fluctuations in brightness at each detector, information about physical and chemical structures were able to be determined for each individual particle. INF- γ , IL-2, and TNF- α were measured and compared based on the treatment the mice received per group. Lymph node and spleen cells were analyzed because lymph nodes are known to act as filters and trap particles while becoming inflamed or enlarged when infection occurs. Lymph nodes are a good source of lymphocytes that also help fight off infection and help in preventing infection by trapping disease-causing germs. The spleen also contains high amounts of lymphocytes and macrophages that engulf and destroy bacteria and in turn remove matter from the blood.

Cytokine quantification is graphed separately based on spleen and lymph node samples. Th1 type cytokines are responsible for producing inflammatory responses that kill intracellular parasites and for perpetuating autoimmune responses. High percentages of tumor necrosis factor alpha (TNF- α) cytokines were seen in all groups due to natural killer cells being produced after the parasite antigen involved since the early defense of *Leishmania* infection. TNF- α plays a crucial role in the systemic inflammation that stimulates the acute phase reaction and is able to induce apoptotic cell death to induce inflammation. This type of cytokine is produced mostly in macrophages but also present in other types of cells. Interleukin-2 (IL-2) percentages are significantly lower than TNF due to IL-2 being immediately used up by cells when exposed to an infection. IL-2 stimulates the immune system and can lead to large increases in the number of CD4 (T-helper) cells. IL-2 cytokines are able to differentiate between self and non-self foreign materials and participate in the maintenance of regulatory T cells that maintain immune system homeostasis. Interferon gamma (IFN- γ) is present when excessive pro-inflammatory responses

lead to uncontrolled tissue damage. Interferon gamma attempts to counteract this mechanism by activating macrophages, which also leads to the activation of natural killer cells (NK), antigen-specific cytotoxic T lymphocytes and the release of cytokines in response to the antigen.

Cellular immunity protects the body against intracellular pathogens such as *Leishmania* by activating antigen-specific cytotoxic T lymphocytes that are able to induce apoptosis of cells.

Mean intensity is also graphed as fluorescence emitted by cytokines and is a measurement of the amount of cytokines that were produced.

As expected, mice treated with the cocktail vaccine produced a higher amount of cytokines as opposed to those treated with the Cu/Zn lipid complex. The mean intensity studies showed that the amount of cytokines produced by T cells was large compared to those with the plasmids alone or with the controls. It is hypothesized that higher Th2 responses will be seen for mice treated with lipid complex.

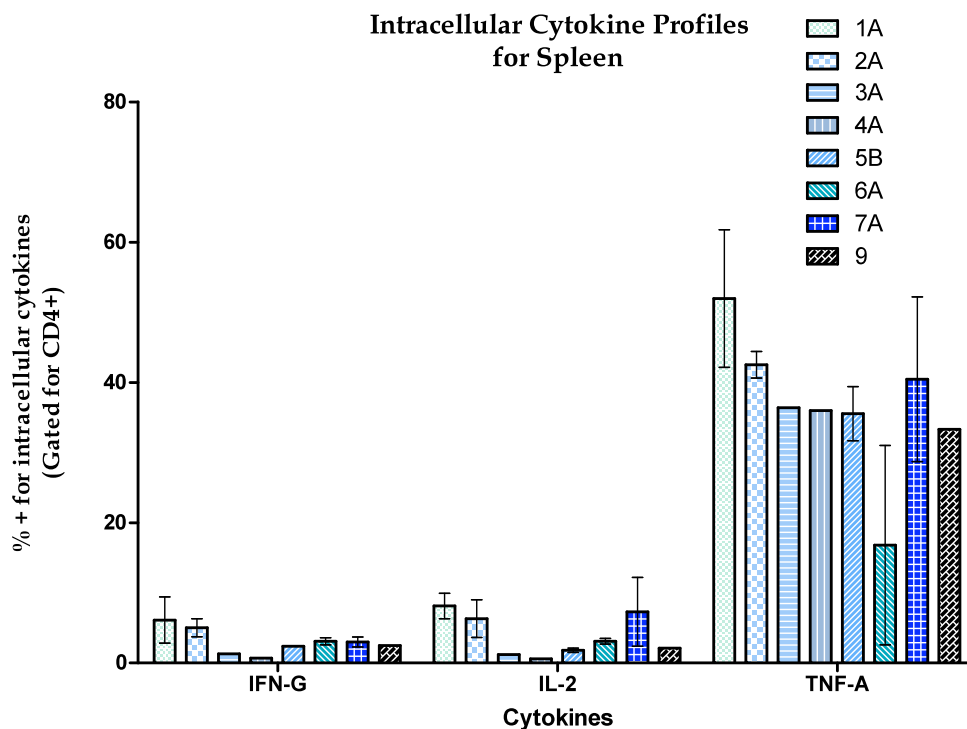


Figure 3.18. Cytokine quantification for spleen samples.

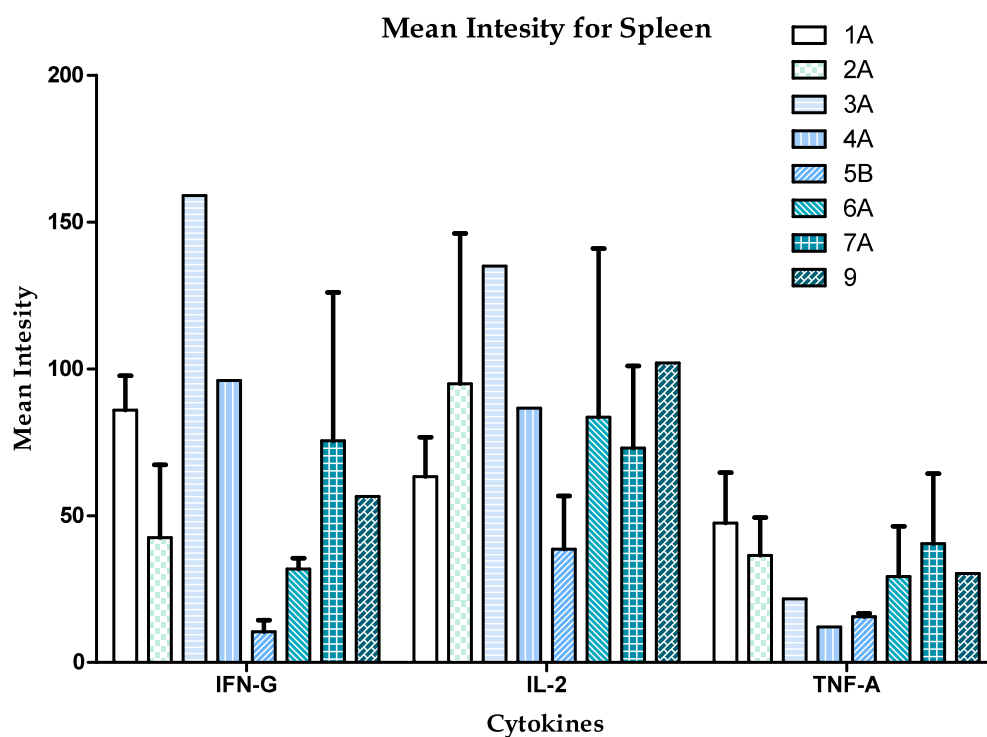


Figure 3.19. Mean intensity for spleen samples.

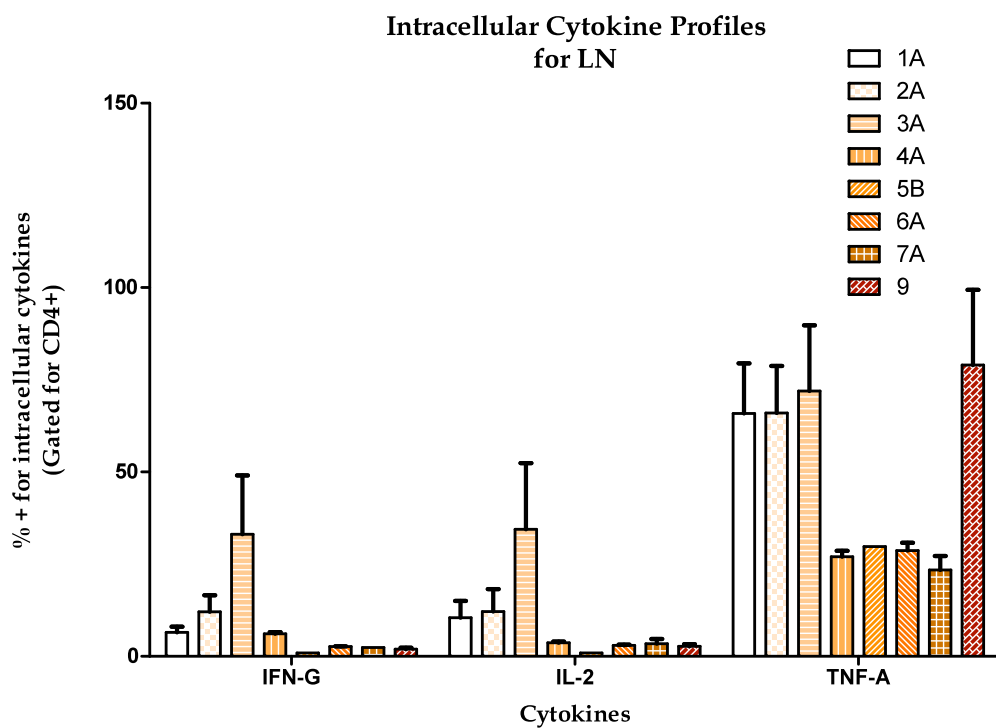


Figure 3.20. Cytokine quantification for lymph node samples.

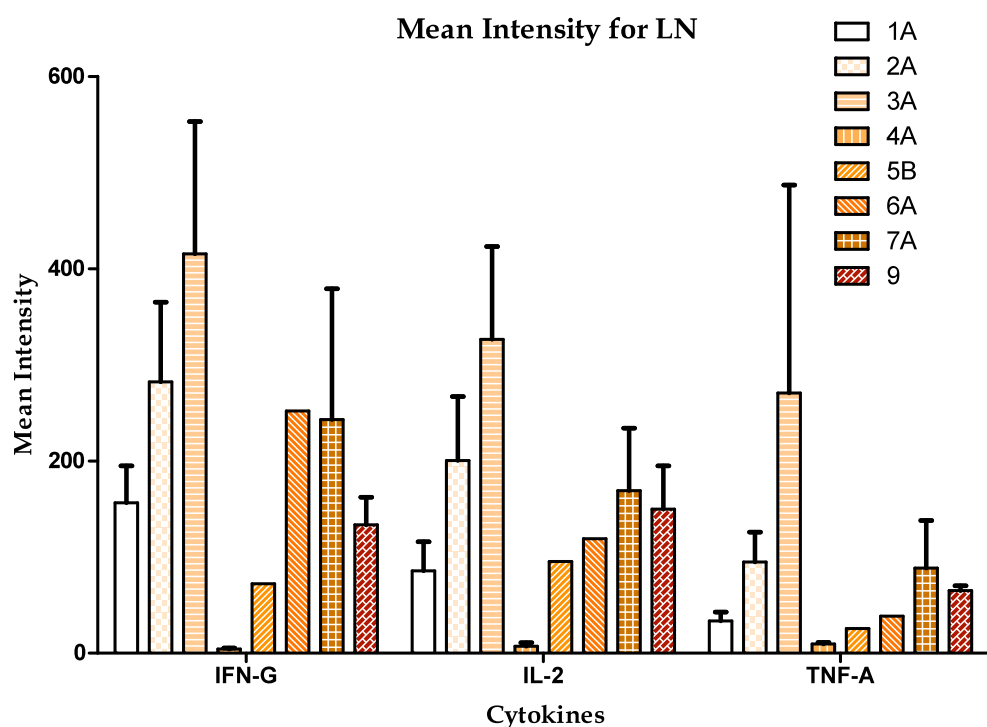


Figure 3.21. Mean Intensity for lymph node samples.

In summary, Zn and Cu lipid complexes showed to have transfection capabilities in Hela, COS1, and macrophages cell lines. Footpad lesion measurements revealed that the cocktail vaccine resulted in a higher protection than mice groups treated with the metal lipid complexes or with individual plasmids. Flow cytometric analysis confirmed that higher quantities of cytokines were produced in mice groups treated with the cocktail vaccine and much lower amounts for those treated with the lipid metal complexes. Overall, the cocktail vaccine showed to have protective properties than any other treatment.

Conclusions

The design and synthesis of molecules that self-organize in water to form functional nanosystems were tested as drug delivery systems with a *Leishmania* vaccine. The design, synthesis and functional activity of Cu(II) and Zn(II) complexes achieved the self-assemble in water to form spherical nanoscale structures and were able to exhibit an affinity to bind DNA when tested in agarose gels but were not as effective in the delivery of eukaryotic cells. We investigated the ability of the Cu(II) and Zn(II) complexes to bind and deliver a gene vaccine against *Leishmania mexicana* challenged with *Leishmania major*, into mice models and flow cytometric analysis revealed the quantity of cytokines produced by T cells. The cocktail vaccine showed a significant amount of protection when compared to the individual plasmid components and the metallo-lipid nanoparticle treated mice.

We have accomplished our objectives to determine the efficacy of p-Vax-BT-ICAM, and/or p-Vax-ORFF-Amastin plus metallo-liposomes in preventing murine leishmaniasis infection and determine if metallo-liposomes possess adjuvant properties that enhance the innate immune response. Future studies will analyze Th2 immune response with these metallo-lipid nanoparticles as well as be tested with mice models challenged against *Leishmania mexicana*.

References

- 1 Adams, D. H., Joyce, G., Richardson, V. J., Ryman, B. E., Wisniewski, H. M., Liposome toxicity in the mouse central nervous system, *J. Neurol. Sci.* 31 (2) (1977) 173 – 179.
- 2 Ahsan, F., Rivas, L.P., Khan, M. A., Torres Suarez, A. I., Targeting to macrophages: role of physicochemical properties of particulate carriers – liposomes and microspheres – on the phagocytosis by macrophages, *J. Control. Release* 79 (1-3) (2002) 29-40.
- 3 Aebischer, T. 1994. Recurrent cutaneous *leishmaniasis*: A role for persistent parasites. *Parasitology Today* 10, 25-28.
- 4 Alexander, J., and Russell, D. G. 1992. The interaction of *Leishmania* species with macrophages. *Advances in Parasitology* 31, 75-254.
- 5 Arana, B. A., Mendoza, C. E., Rizzo, N. R., Kroeger, A. Randomized, controlled, double-blind trial of topical treatment of cutaneous leishmaniasis with paromomycin plus methylbenzethonium chloride ointment in Guatemala. *Am. J. Trop. Med. Hyg.* 2001; 65: 466-70.
- 6 Armijos R. X., Weigel, M. M., Hidalgo, A., et al. Safety, immunogenicity, and efficacy of an autoclaved *Leishmania amazonensis* vaccine plus BCG adjuvant against New World Cutaneous Leishmaniasis. *Vaccine* 2004; 22 (9-10): 1320-1326.
- 7 Armijos R. X., Weigel, M. M., Romero, L., et al. Field trial of a vaccine against New World cutaneous leishmaniasis in an at-Risk child population: How long does treatment last? *J Infect Diseases* 2003; 187: 1959-1961.
- 8 Arroyo, I. Z. Metal-Mediated Molecular Materials at the Nano- and Mesoscale. University of Texas at El Paso, 2007.

- 9 Barratt, G., Colloidal drug carriers: achievements and perspectives, *Cell. Mol. Life Sci.* 60 (1) (2003) 21-37.
- 10 Berman, J. D., Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin Infect Dis* 1997; 24: 684-703.
- 11 Blum, J., Desjeux, P., Schwartz, E., Beck, B., Hatz C. Treatment of cutaneous leishmaniasis among travellers. *J Antimicrob Chemother* 2004; 53: 158-66.
- 12 Briones, E., Colino, C. I., Lanao, J. M. Delivery Systems to increase the selectivity of antibiotics in phagocytic cells. *Journal of Controlled Release* 125 (200*) 210-227.
- 13 Carryn, S., Chanteux, H., Sera, C., Mingeot-Leclercq, M. P., Van Bambeke, F., Tulkens, P. M., Intracellular pharmacodynamics of antibiotics, *Infect. Dis. Clin. North. Am.* 17 (3) (2003) 615-634.
- 14 Chung, J. E., Yokoyama, M., Yamato, M. Aohagi, T., Sakurai, Y., Okano, T., Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly (*n*-isopropylacrylamide) and poly (butylmethacrylate), *J. Control. Release* 62 (1999) 115-127.
- 15 Cordeiro, C., Wiseman, D. J., Lutwyche, P., Uh, M., Evans, J. C., Finlay, B. B., Webb, M. S., Antibacterial efficacy of gentamicin encapsulated in pH-sensitive liposomes against an in vivo *Salmonella enterica* serovar typhimurium intracellular infection model, *Antimicrob. Agents Chemother.* 44 (3) (2000) 533-539.
- 16 Djordjevic J., Barch, M., Uhrich, K. E., Polymeric micelles based on amphiphilic scorpion-like macromolecules: novel carriers for water-insoluble drugs. *Pharm Res* 2005; 22:24-32.

- 17 Dorn, K., Hoerpel, G., Ringsdorf, H., eds. Polymeric anti-tumor agents on a molecular and cellular level. Bioactive polymer systems, ed. C. G. Gebeliein and J. C. E. Carraher. 1985, Plenum: New York. 531-585.
- 18 Emi, C., Suard, C., Freitas, S., Dreher, D., Merkle, H. P., Walter, E., Evaluation of cationic solid lipid microparticles as synthetic carriers for the targeted delivery of macromolecules to phagocytic antigen-presenting cells, *Biomaterials* 23 (23) (2002) 4667-4676.
- 19 Ernst, J. D., Stendahl, O., Phagocytosis of Bacteria and Bacterial Pathogenicity, Cambridge University Press, Cambridge, 2006.
- 20 Fillion, M. C., Phillips, N. C., Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells, *Biochim. Biophys. Acta.* 1329 (2) (1997) 345-356.
- 21 Freitas, C., Muller, R. H., Spray-drying of solid lipid nanoparticles (SLNTM), *Eur. J. Pharm. Biopharm.* 46 (1998) 145-151.
- 22 Gladue, R. P., Bright, G. M., Isaacson, R. E., Newborg, M. F., *In vitro* and *in vivo* uptake of azithromycin (CP-62,993) by phagocytic cells: possible mechanism of delivery and release at sites of infection, *Antimicrob. Agents Chemother.* 33 (3) (1989) 277-282.
- 23 Gladue, R. P., Snider, M. E., Intracellular accumulation of azithromycin by cultured human fibroblasts, *Antimicrob. Agents Chemother.* 34 (6) (1990) 1056-1060.
- 24 Ghosh, A., Madhubala, R., Myler, P. J., Stuart, K. D., *Leishmania donovani*: characterization and expression of ORFF, a gene amplified from the LD1 locus, *Expt Parasitol* 1999; 93: 225-34.

- 25 Hand, W. L., Interactions between antimicrobial agents, phagocytic cells and bacteria, Curr. Med. Chem. – Anti-Infec. Agents 2 (1) (2003) 73-82 (10).
- 26 Handman, E. 1999. Cell biology of *Leishmania*. *Advances in Parasitology* 38, 1-39.
- 27 Hazenbos, W. L. W., Brown, E. J., Ernst, J. D., Stendahl, O. (Eds.), Phagocytosis receptors and biology, in: Phagocytosis of Bacteria and Bacterial Pathogenicity, Cambridge University Press, Cambridge, 2006, pp. 4-53.
- 28 Hoarau, D., Delmas, P., David, S., Roux, E., Leroux J. C. Novel long-circulating lipid nanocapsules, Pharm. Res. 21 (10) (2004) 1783-1789.
- 29 Holmes, B., Quie, P. G., Winhorst, D. B., Pollara, B., Good, R. A., Protection of phagocytized bacteria from the killing action of antibiotics, Nature 210 (41) (1966) 1131-1132.
- 30 Husseini, G. A., Pitt, W. G., Micelles and nanoparticles for ultrasonic drug and gene delivery. *Advanced Drug Delivery Reviews*, Vol. 60, Issue 10, 30 June 2008, 1137-1152.
- 31 Jain, M., Madhubala, R., Characterization and localization of ORFF gene from the LD1 locus of *Leishmania donovani*. *Gene* (416) 1-2 (2008) 1-10.
- 32 Kabanov, A. V., Batrakova, E. V., Alakhov, V. Y., Pluronic block copolymers as novel polymer therapeutics for drug and gene delivery. *J Control Rel* 2002; 82: 189-212.
- 33 Karlowsky, J. A., Zhanel, G. G., Concepts on the use of liposomal antimicrobial agents: applications for aminoglycosides, *Clin. Infect. Dis.* 15 (4) (1992) 654-667.
- 34 Kataoka, K., Kwon, G. S., Yokoyama, M., Okano, T., Sakurai, Y., Block copolymermicelles as vehicles for drug delivery, *J. Control. Release* 24 (1993) 119-132.

- 35 Kayser, O., Olbrich, C., Croft, S. L., Kiderlen, A. F., Formulation and biopharmaceutical issues in the development of drug delivery systems for antiparasitic drugs, *Parasitol. REs.* 90 (2) (2003) S63-S70.
- 36 Klemperer, M. S., Ristuccia, A. M., Cunha, B. A. (Eds.), White cells, in antimicrobial therapy, Raven Press, New York, 1984, pp. 513-517.
- 37 Kohli, P., Martin, C. R., Smart nanotubes for biotechnology, *Curr. Pharma. Biotechnol.* 6 (2005) 35-47.
- 38 Kwon G. S., Yokoyama M., Okano, T., Sakurai, Y., Kataoka, K., Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly (ethylene oxide-co-aspartate) block copolymer-adriamycin conjugates, 6th International Symposium on Recent Advantages in Drug Delivery Systems, 1993.
- 39 Lamprecht, A., Bouligand, Y., Benoit, J. P., New lipid nanocapsules exhibit sustained release properties for amiodarone, *J. Control. Release* 84 (1-2) (2002) 59-68.
- 40 Leishmania mexicana – Parasite, One of a Group of Parasites That Cause Leishmaniasis, Rosemary Drisdelle, Sep 19, 2007.

http://humaninfections.suite101.com/article.cfm/leishmania_mexicana_parasite
- 41 Leishmaniasis in U.S. Soldiers

<http://images.google.com/imgres?imgurl=http://bp2.blogger.com/>
- 42 Lemley, C., Yan, S., Dole, V. S., Madhubala, R., Cunningham M. L., Beverly, S. M., Myler, P. J., Stuart, K. D. The *Leishmania donovani* LD1 locus gene ORFG encodes a bioppterin transporter (BT1). *Molecular and Biochemical Parasitology*, 104 (1) (1999) 93-105.

- 43 Liu, P., Wang, B., Qiao, J., Multi-anticancer drugs encapsulated in the micelle: A novel chemotherapy to cancer. *Medical Hypotheses*, Vol. 71, Issue 3, September 2008, 379-381.
- 44 Liverpool School of Tropical Medicine
<http://www.liv.ac.uk/lstm/groups/vectorstaffprofiles/images/RodDillon1.jpg>
- 45 Lutwyche, P. Cordeiro, C., Wiseman, D. J., St-Louis, M., Uh, M., Hope, M. J., Webb, M. S., Finlay, B. B., Intracellular delivery and antibacterial activity of gentamicin encapsulate in pH-sensitive liposomes, *Antimicrob. Agents Chemother.* 42 (10) (1998)
- 46 Mäder, K., Solid lipid nanoparticles as drug carriers in: V. P. Torchilin, (Ed.) *Nanoparticulates as Drug Carriers*, Imperial College Press, London, 2006, pp. 187-213.
- 47 Masmoudi, A., Maalej, N., Boudaya, S., Turki, H., Zahaf, A., Adverse effects of intralesional Glucantime ® in the treatment of cutaneous leishmaniasis. *Med Mal Infect* 2006; 36: 226-8.
- 48 Mehta, R. T., Liposome encapsulation of clofazimine reduces toxicity *in vitro* and *in vivo* and improves therapeutic efficacy in the beige mouse model of disseminated *Mycobacterium avium-M. intracellulare* complex infection, *Antimicrob. Agents Chemother.* 40 (8) (1996) 1893-1902.
- 49 Modabber, F. 1993. Leishmaniasis. In “Tropical Disease Research Progress 1999-1992,” pp. 77-87. WHO, Paris [World Health Organization].
- 50 Müller, R. H., Maaben, S., Weyhers, H., Specht, F., Lucks, J. S., Cytotoxicity of magnetite-loaded polylactide, polyactide/glycolide particles and solid lipid nanoparticles, *Int. J. Pharm.* 138 (1996) 85-94.
- 51 National Institute of Allergy and Infectious Diseases, National Institutes of Health

www3.niaid.nih.gov/.../0/leishDiagram1.jpg

- 52 Navin, T. R., Arana, B. A., Arana, F. E., De Merida, A. M., Castillo, A. L., Pozuelos, J. L., Placebo-controlled clinical trial of meglumin antimoniate (glucantime) vs. localized controlled heat in the treatment of cutaneous leishmaniasis in Guatemala. *Am. J. Trop. Med. Hyg.* 1990; 42: 43-50.
- 53 Nel, A., Xia, T., Madler, L., Li, N., Toxic potential of materials at the nanolevel, *Science* 311 (5761) (2006) 622-627.
- 54 Noazin, S., Modabber, F., Khamesipour, A., Smith, P. G., Moultron, L. H., Nasser, K., Sharifi, I., Khalil, A. G., Bernal, I. D. V., Antunes, C. M. F., Kieny, M. P., Tanner, M. First generation leishmaniasis vaccine: A review of field efficacy trials. *Vaccine, JVAC*-8565; 2008 (Article in Press).
- 55 Prior, S., Gamazo, C., Irache, J. M., Merkle, H. P., Gander, B., Gentamicin encapsulation in PLA/PLGA microspheres in view of treating *Brucella* infections, *Int. J. Pharm.* 196 (1) (2000) 115-125.
- 56 Prokesch, R. C., Hand, W. I., Antibiotic entry into human polymorphonuclear leukocytes, *Antimicrob. Agents Chemother.* 21 (3) (1982) 373-380.
- 57 Raoult, D., *Antimicrobial Agents and Intracellular Pathogens*, CRC Press, Inc. 1993.
- 58 Rawat, M., Singh, D., Saraf, S., Nanocarriers: promising vehicle for bioactive drugs, *Biol. Pharm. Bull.* 29 (2006) 1790-1798.
- 59 Reinoso, R. F., Lanao, J. M., Lorente, F., Lopez, F. G., Dominguez-Gil, A., Uptake of antibiotics by phagocytic cells: study methods and therapeutic implications, *Rev. Esp. Quimioterap.* 8 (1995) 100-108.

- 60 Roy, G., Kündig, C., Olivier, M., Papadopoulou, B., Quellette, M. Adaptation of Leishmania Cells to in Vitro Culture Results in a More Efficient Reduction and Transport of Biopterin. *Experimental Parasitology*, 97 (3) (2001) 161-168.
- 61 Sarker, D. K., Engineering of nanoemulsions for drug delivery. *Curr. Drug Deliv.* 2 (2005) 297-310.
- 62 Seddon, J. M., Templer, R. H., *Polymorphism of Lipid-Water Systems*, from the Handbook of Biological Physics, Vol. 1, ed. R. Lipowsky, and E. Sackmann. (c) 1995, Elsevier Science B.V.
- 63 Sharquie, K. E., A new intralesional therapy of cutaneous leishmaniasis with hypertonic sodium chloride solution. *J Dermatol* 1995; 22: 737-7.
- 64 Sharquie, K. E., Najim, R. A., Farjou I. B., A comparative controlled trial of intralesionally administered zinc sulphate, hypertonic sodium chloride and pentavalent antimony compound against acute leishmaniasis. *Clin Exp Dermatol* 1997; 22: 169-73.
- 65 Smistad, G., Jacobsen, J., Sande, S. A., Multivariate toxicity screening of liposomal formulations on a human buccal cell line, *Int. J. Pharm.* 330 (1-2) (2007) 14-22.
- 66 Sunkin, M. S., McDough, P., Cunningham, M. L., Beverly, S. M., Stuart, K. D., Myler, P. J., 2001. Conservation of the LD1 region in Leishmania includes DNA implicated in LD1 amplification. *Mol. Biochem. Parasitol.* 113, 315-321.
- 67 Szoka Jr., F. C., The future of liposomal drug delivery, *Biotechnol. Appl. Biochem.* 12 (5) (1990) 496-500.
- 68 Uhrich, K. E., Cannizzaro, S. M., Langer, R. S., Shakesheff, K. M., Polymeric systems for controlled drug release, *Chem. Rev.* 99 (1999) 3181-3198.

- 69 Weinrauch, L., Cawich, F., Craig, P., Sosa, J. X., El-On, J. Tropical treatment of New World cutaneous leishmaniasis in Belize: a clinical study. *J. Am. Acad. Dermatol.* 1993; 29: 443-6.
- 70 World Health Organization. The leishmaniasis. WHO technical report series, vol. 701. Geneva: WHO; 1984.
- 71 Yan, W., Huang, L. Recent Advances in Liposome-Based Nanoparticles for Antigen Delivery. *Polymer Reviews*, 47: 329-344, 2007.
- 72 Yanez, A. M., Wallace, M., Ho, R., Shen, D., Yaksh, T. L., Touch-evoked agitation produced by spinally administered phospholipids emulsion and liposomes in rats. Structure-activity relation, *Anesthesiology* 82 (5) (1995) 1189-1198.
- 73 Yokoyama, M., Kwon, G. S., Naito, M., Okano, T., Sakurai, Y., Seto, T., Kataoka, K., Preparation of micelle-forming polymer-drug conjugates, *Bioconj. Chem.* 3 (1992) 295-301.
- 74 Yokoyama, M., Okano, T., Sakurai, Y., Fukushima, S., Okamoto, K., Kataoka, K., Selective delivery of adriamycin to a solid tumor using a polymeric micelle carrier system, *J. Drug Target.* 7 (1999) 171-186.
- 75 Yokoyama, M., Sugiyama, T., Okano, T., Sakurai, Y., Naito, M., Kataoka, K., Analysis of micelle formation of an adriamycin-conjugated poly (ethylene) glycol-poly (aspartic acid) block copolymer by gel permeation chromatography. *Pharma. Res.* 10 (1993) 895-899.

Appendix

A.1. SUPPORTING INFORMATION FOR CHAPTER 2

Synthesis of 1-dodecyl-1,4,7-triazacyclononane (TACN-C12). 0.1g of 1,4,7-triazacyclononane, 0.19 g of 1-bromododecane, 0.02 g of NaH, and 1.6 equivalents of NaOH were used for the reaction. 300 ml of THF were degassed and distilled by employing an Argon environment for a few minutes. THF was then left heating at 60°C and left stirring simultaneously. The first 50 ml of solution were disposed from a round bottom flask and first 200 ml of THF was collected and was degassed with Argon for a few minutes. 1,4,7-triazacyclononane was dissolved in dried THF in a round bottom flask and was left stirring and heating at 60°C for the entire reaction. NaH was added and 1-bromododecane was dissolved using 5 ml of THF in a 10 ml separatory funnel. This was added drop-wise to the 1,4,7-triazacyclononane and THF solution; this approximately took 3-5 hours. The reaction was left running for 24 hours and tracked by thin layer chromatography. TLCs were performed in order to assure that the complex was pure. Ultimately, a yellow viscous oil was the result after completion of the reaction. Chloroform was added in small amounts in order to weigh vial containing the complex and was evaporated; weight was approximately 0.0167 g. Copper triflate/zinc triflate was added in a 2:1 ratio. 0.0097 g of Cu/Zn(OTf)_2 was suspended in 5 ml of acetonitrile in a vial. 5 ml of acetonitrile was also added to the ligand in a vial and transferred to a round bottom flask. $\text{Cu(OTf)}_2/\text{Zn(OTf)}_2$ was added drop-wise to the ligand under Argon conditions for approximately 5 hours. The solution was transferred to a vial and all solvent was evaporated. Once the Cu/Zn complexes were successfully synthesized they were suspended in water for a 1.5 mM solution in water. Metal ligand complexes were rotovaped for 15 minutes in

a 10 ml round bottom flask and 80 μ l of chloroform with 500 μ l of water was added. The solution was sonicated until it appeared as a milky white solution and rotovaped once more for 15 minutes. That solution was transferred to a vial and brought to a final weight of 0.5 g. 400 μ l of the 1.5 μ M solution was transferred to 600 μ l of water and kept in a separate eppendorf tube. The desired amount of DNA (μ g) was placed in a second eppendorf tube and brought to a final volume of 1 ml by adding water. The two eppendorfs were combined and mixed and were incubated for 15 minutes prior to immunization. This synthesis was good for 20 doses of 100 μ l each.

A.2. SUPPORTING INFORMATION FOR CHAPTER 3

A.2.1. Preparation of DNA Plasmids

LB Broth. LB broth was prepared by bringing a 500 ml container to 400 ml with DI water. 5g of NaCl, 2.5 g of yeast, and 5 g of tryptone were mixed in the container and dissolved. The container was brought to 500 ml of DI water and pH was measured (pH must be approximately 7.4). Broth was autoclaved for 30 minutes, left to cool at room temperature.

Kanamycin Antibiotic. Kanamycin antibiotic aliquots were prepared by placing 0.25 g of kanamycin into 10 ml of DI water. Contents were stirred and filtered using a syringe and stored in eppendorf tubes where they were stored until use in a 4°C freezer.

Bacterial Growth. 500 µl of kanamycin antibiotic were added to a 500 ml glass container containing LB broth and swirled. 100 µl of desired bacterial plasmid was added, capped loosely and placed in a 37°C incubator overnight.

A.2.2. Purification of DNA

Wizard Plus Maxipreps DNA Purification System. Maxipreps were performed in order to isolate the plasmid DNA from the bacteria. In order to precipitate both DNA and proteins, the bacteria was lysed under alkaline conditions. Preparation of the clear lysate was accomplished by pelting cells at 5,000 x g for 10 minutes at room temperature. Pellet was suspended in 10 ml Cell Resuspension Solution. 10 ml Cell Lysis Solution was added and inverted to mix. 10 ml of Neutralization Solution was added and inverted to mix. Lysate was centrifuged at 14,000 x g for 15 minutes. Supernatant was filtered into a graduated cylinder and volume was measured and liquid was transferred to centrifuge bottle. 0.5 volume isopropanol

was added and inverted to mix. Contents were centrifuged at 14,000 x g for 15 minutes and pellet was resuspended in 2 ml of TE buffer. When purifying plasmid DNA, resin was resuspended and 10 ml of resin were added to DNA and swirled. Maxicolumn was attached to vacuum manifold and resin/DNA mixture was transferred to Maxicolumn. Vacuum was applied, releasing all liquid from the column. Washing was accomplished by adding 25 ml of Column Wash Solution containing ethanol. Vacuum was applied, pulling liquid through column. Vacuum was released and 5 ml of 80% ethanol was added. Vacuum was again applied and continued for 1 minute after liquid passed through Maxicolumn. Maxicolumn was transferred to a 50 ml centrifuge tube. It was centrifuged at 1,300 x g for 5 minutes, using a swinging bucket rotor. Maxicolumn was placed on manifold and vacuum was applied for a final time for 5 minutes. Maxicolumn was placed in Reservoir tube and 1.5 ml of preheated water was added. After 1 minute, it was centrifuged at 1,300 x g for 5 minutes to elute DNA. Eluted DNA was filtered and centrifuged filtrate at 14,000 x g for 1 minute. Supernatant containing DNA was transferred to a new centrifuge tube and stored at -20°C or below.

A. 2.3. cDNA Synthesis

cDNA Synthesis Directly from Cells Using SuperScript™ III CellDirect cDNA Synthesis System. The SuperScript™ III CellsDirect cDNA Synthesis System is an optimized kit for synthesizing first-strand cDNA directly from mammalian cell lysate without first isolating the RNA. Lysis and reverse transcription were performed in the same tube, and the resulting first-strand cDNA was ready to use in cloning and PCR.

In traditional RT-PCR, RNA is first isolated from cells in a time-consuming procedure that can lead to a loss of material. Using the SuperScript™ III CellsDirect cDNA Synthesis

System, the cells are lysed and the cDNA is generated from the lysate in a single tube with minimal handling and no sample loss. DNase I is added to eliminate genomic DNA prior to first-strand synthesis.

Cells were grown in tissue culture wells with Cu/Zn complexes along with combinations of the four antigens that compose *Leishmania* and the following procedure was used to lyse the cells. Media was aspirated from each well and washed with 1X cold PBS. PBS was aspirated and Resuspension Buffer/Lysis Enhancer solution was added to each well. The buffer should fully cover the cells in the well. The plates were incubated on ice for up to 10 minutes and during this time, the plate was periodically tapped and cells were checked under a microscope every 2-3 minutes to see whether they had detached or burst. After 10 minutes, the cells were pipetted up and down to dislodge the remaining attached cells. Cells were counted using a hemacytometer and 10 μ l of cell suspension was transferred to 0.2-ml thin-walled PCR tubes. 1 μ l of RNaseOUTTM was added to the PCR tubes. The tubes were transferred to the thermal cycler preheated to 75°C and incubated for 10 minutes. After incubation, the tubes were spun briefly to collect the condensation. The cell lysate was then treated with DNase I to degrade any contaminating DNA by placing each tube on ice and adding 5 μ l DNase I and 1.6 μ l 10X DNase I Buffer. All contents were mixed gently and pipetted up and down and spun briefly. Tubes were incubated for 5 minutes at room temperature, spun briefly, and 1.2 μ l of 25 mM EDTA was pipetted to each while on ice. Contents were again mixed, pipetted up and down, and spun briefly followed by another incubation period of 5 minutes at 70°C. Tubes were spun and first strand of cDNA synthesis was begun. Each tube was pipetted with 2 μ l of Oligo(dT)₂₀ (50 μ M) and 1 μ l of 10 mM dNTP mix. They were again mixed gently and pipetted up and down followed by a quick spin. Tubes were incubated at 70°C for 5 minutes and spun to collect the

condensation. They were then placed on ice for 2 minutes and were pipetted with 6 µl of 5X RT Buffer, 1 µl of RNaseOUT™, 1 µl of SuperScript™ III RT, and 1 µl of 0.1 M DTT. Tubes were mixed and pipetted up and down and spun to collect contents and were transferred to the thermal cycler preheated to 50°C. They were incubated for 50 minutes at this temperature and switched to 85°C for 5 minutes to inactivate the reaction. 1 µl of RNase H was pipetted to each tube and incubated at 37°C for 20 minutes. The reaction was chilled on ice and the single stranded cDNA was stored at -20°C.

A. 2.4. Polymerase Chain Reaction (PCR)

PCR. PCR was utilized in order to exponentially amplify the DNA template. Four flasks of cells were infected with *Leishmania* and were trypsinized and their DNA was purified using protocol: *DNA Purification from Cultured Cells Using the Gentra Puregene Cell Kit*. This was used as the positive control when running the gel to assure that the DNA was present and being expressed. The objective was to amplify 4 genes (*ICAM-1*, *BT*, *ORFF*, and *Amastin*, all of which are from the *Leishmania* genome). In order to copy, primers were utilized, two primers one on each end of the original strand of DNA (one in the 3' end and the other at the 5' end). The DNA was denatured in order to separate the double helical strand; the chain was then copied. In an agarose gel, the four genes were amplified, all in their own specific size. The DNA ladder was placed in the gel in order to determine the different DNA sizes and the number of base pairs. Materials utilized for PCR included 1 µl *Leishmania* DNA (served as the template with a concentration of 677.9 ng/µl), 12.5 µl master mix (comprised of Mg, DNTP, DNA polymersase), 1 µl reverse primer, 1 µl forward primer, and nuclease free water. If the amount of primers was decreased, nuclease free water was increased to compensate for the loss such as

when running *ORFF* (0.7 µl of primers were used). All contents were combined in PCR 100 µl tubes, vortexed, and centrifuged for a few seconds so that all contents collected at the bottom of tube. Genes ran under the following conditions in a thermal cycler:

BT

95 C for 30s
 95 C for 30s
 60 C for 30s
 72 C for 1m 50s
 72 C for 10m

} 35X

ORFF

95 C for 30s
 95 C for 30s
 56 C for 30s
 72 C for 1m

} 35X, used 0.7 µl primers

ICAM

95 C for 30s
 95 C for 30s
 60 C for 30s
 72 C for 1:45

} 35X

Amastin

95 C for 30s
 95 C for 30s
 60 C for 30s
 72 C for 1m

} 35X

PCR tubes were quickly removed and place in ice after completion of cycles. 2 µl of dye was pipetted into each sample, vortexed, and centrifuged and loaded into an agarose gel.

Agarose Gel Preparation. 0.504 g of agarose were weighed and added to 84 ml of TAE 1X buffer in an Erlenmeyer flask. Contents were heated until agarose was fully dissolved and 2 µl of Ethidium Bromide was pipetted, and swirled in flask. Gel was poured in gel electrophoresis apparatus and left to solidify.

A. 2.5. Protein Determination

Bicinchoninic Acid Protein Assay Kit. Protein determination is one of the most common operations performed in biochemical research. The principle of the bicinchoninic acid (BCA) assay is similar to the Lowry procedure. In that both rely on the formation of a Cu^{2+} protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+} . The

amount of reduction is proportional to the protein present. It has been shown that cysteine, cystine, tryptophan, tyrosine, and the peptide bond are able to reduce Cu^{2+} to Cu^{1+} . BCA forms a purple-blue complex with Cu^{1+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins.

The BCA working reagent was prepared combining 25 ml of Bicinchoninic Acid solution and 500 μl of Cu(II) Sulfate solution. Dilutions were prepared in test tubes and numbered accordingly – (1) 100 μl DI water, (2) 80 μl DI water + 20 μl Protein Standard, (3) 60 μl DI water + 40 μl Protein Standard, (4) 40 μl DI water + 60 μl Protein Standard, (5) 20 μl DI water + 80 μl Protein Standard, (6) 100 μl Protein Standard, (7) 80 μl DI water + 20 μl *L. mexicana* antigen, (8) 40 μl DI water + 60 μl *L. mexicana* antigen, (9) 100 μl *L. mexicana* antigen, (10) 80 μl DI water + 20 μl *L. major* antigen, (11) 40 μl DI water + 60 μl *L. major* antigen, and (12) 100 μl *L. major* antigen. All samples were incubated at 37°C for 30 minutes and allowed to cool at room temperature. All samples were then transferred to a cuvet and their absorbance was measure in order to calculate concentration.

A. 2.6. Mice Protocols

Intramuscular Injection of Mouse. A 22- and 30-G needle syringe was used for immunizations. Animal was placed in a trap and restrained; needle was inserted in the heavy musculature of the lower posterior leg. Syringe was aspirated briefly before injecting to prevent intravenous or intra-arterial injection. Moderate pressure was placed when injecting to avoid tissue damage.

Euthanasia. Methods of euthanasia should be painless, quick, easy to perform, safe for

those performing the procedure, efficient, economic and minimize distress. Methods should not result in gross histological or histochemical changes that would adversely affect research results. Carbon dioxide asphyxiation was used in the study and utilized in all rodents. Animals were placed in CO₂ chamber and lid was placed. A hose connected to a CO₂ tank was fed through an opening in the chamber and slowly turned on. Animal was left in chamber for approximately a minute and death was verified by lack of cardiac pulse and dilated pupils.

A. 2.7. Flow Cytometry

BD Cytofix/Cytoperm Kit. Lymph nodes and spleen were grinded with RPMI media containing Fetal Bovine Serum and Streptomycin. Spleen cells were transferred to a 15 ml conical tube in order to remove tissue solids. 5 ml of Lysis Buffer was added at room temperature and left incubating for 4 minutes with occasional shaking. The reaction was terminated by diluting the Lysis Buffer with 30 ml of 1X PBS and centrifuged for 10 minutes. Supernatant was removed and 1 ml of fresh media was added. Lymph nodes were also transferred to a 15 ml conical tube in order to remove tissue solids. Conical tube was centrifuged for 2 minutes and 1 ml of fresh media was added. Both spleen and lymph node cells were counted and plated in a 96 well plate with 10⁶ cells/well. Cells were incubated with 0.5 µl of CD28 and 1 µl of *Leishmania major* antigen/well for 2 h at 37°C. 0.2 µl of BD GolgiPlug was added per well, mixed, and incubated for 4 h. The plate was centrifuged and BD GolgiPlug containing media was removed. 50 µl of Staining Buffer and 1.25 µl CD4 were added per well and incubated for 30 minutes at 4°C. Cells were centrifuged for 10 minutes, washed twice with Staining Buffer and centrifuged. Cells were resuspended and 100 µl of Fixation/Permeabilization solution was added per well and left incubating for 20 minutes at 4°C.

Cells were centrifuged for 10 minutes, washed twice in 1xBD Perm/Wash buffer and 200 µl of 1xBD Perm/Wash buffer was added in addition to 2 µl of TNF- α -FITC, 2 µl of IL-2-PE, and 1 µl of IFN- γ per respective well. Plate was left incubating for 30 minutes at 4°C. Cells were centrifuged for 10 minutes and cells were washed once with 1xBD Perm/Wash buffer. 0.3 µl of Texas Red were added to wells containing TFN and left incubating for 30 minutes at 4°C. Cells were centrifuged for 10 minutes, washed twice with 1xBD Perm/Wash buffer and resuspended in 200 µl of Staining Buffer prior to flow cytometric analysis. If analysis did not follow immediately, cells were resuspended in 4% paraformaldehyde, covered with aluminum foil to avoid direct light and left in 4°C.

A. 2.8. Culturing Macrophages and Footpad Lesions

Macrophages. Macrophages were collected from rodents in 50 ml conical tube. Cells were centrifuged for 10 minutes at room temperature. Media was removed leaving only 1 ml of media with cells. Cells were counted and 10^6 cells/well were plated in a 24 well plate. 500 µl of RPMI media containing Fetal Bovine Serum and Streptomycin was added to each well. Cells were left to incubate overnight to assure proper adhesion and washed every two days. Washing was performed by removing media from wells and replaced with RPMI media (without FBS and Streptomycin). Cells were resuspended with 1.5 ml of RPMI media containing FBS and Streptomycin.

Mice Footpad Lesions. Eppendorfs containing 1 ml RMPI media (containing FBS and Streptomycin) were previously weighed. Footpad lesions were grinded and placed in eppendorf and weighed once more to calculate weight of tissue. Cells received 10 µl of additional

Streptomycin and 150 µl of media containing cells were plated per well. All wells received 150 µl media. Dilutions were made beginning with 300 µl in first well, reduced to 150 µl to second well and so on and so forth. Plate was incubated at room temperature for one week.

A. 2.9. RNA/cDNA

RNA Homogenization/Extraction/Purification. Excess cells from lymph nodes and spleen was utilized. Cells were homogenized in 1ml TRI Reagent per 10^6 suspension culture cells. Homogenate was incubated for 5 minutes at room temperature. Cells were centrifuged 12,000 x g for 10 minutes at 4°C and supernatant was transferred to a new tube. 1 ml of homogenate was transferred to a labeled 1.5 ml microcentrifuge tube. 200 µl of chloroform was added per tube and vortexed at maximum speed for 15 seconds. Tubes were incubated at room temperature for 5 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. 400 µl of top layer was transferred to a new 1.5 ml microcentrifuge tube. 200 µl of 100% ethanol was added and vortexed for 5 seconds. Sample was passed through a filter cartridge and centrifuged at 12,000 x g for 30 seconds at room temperature. Flow through was discarded and filter was returned to cartridge. 500 µl of Wash Solution was added to filter cartridge-collection tube assembly. Assembly was centrifuged for 30 seconds at room temperature and flow through was once more discarded and returned to original collection tube. Wash was done once more followed by centrifugation for 30 seconds at room temperature. Filter cartridge was placed in a new collection tube and 50 µl of Elution Buffer was added to filter column. Incubation at room temperature for 2 minutes followed. Filter column was centrifuged for 30 seconds to elute the RNA from the filter.

cDNA Reverse Transcription Reaction. 10 µl of 2X RT master mix was pipetted into each individual tube. 10 µl of RNA sample was added into each tube and pipetted up and down. Tubes were centrifuged briefly to spin down contents and to eliminate air bubbles. Tubes were placed on ice until loading into thermal heater.

Curriculum Vitae

Clarissa Sara Gomez received her Bachelor of Science degree from the University of Texas at El Paso in May 2006. She had previously worked with Dr. Elizabeth Gardner (now at the University of Alabama at Birmingham) and received her first publication entitled *Particle-Particle Interactions Between Layered Double Hydroxide Nanoparticles*. Her undergraduate research included studying the changes in chemical properties of nanoscale particles including quantum size effect, changes in the cell parameters and lattice symmetry, and surface and interface effects.

As a graduate student she was given the opportunity to gain a vast amount of teaching experience. She was a recipient of the NSF GK-12 Fellowship from 2006-2008 where she served as a science mentor for 6th and 7th grade students from Ross and Henderson Middle School. She was given the privilege of introducing chemistry and biology concepts in the classroom and created science modules mirroring her own research, as well as other hands on activities that mimicked graduate research. In the department of chemistry she was under the supervision of Dr. Juan C. Noveron where she collaborated with Dr. Rodrigo X. Armijos from the department of public health. She has been accepted into the Interdisciplinary Health Sciences program in the College of Health Sciences and has chosen to work with Dr. Armijos where she will continue her research in the area of infectious diseases. She has presented her work in national conferences such as the Society for the Advancement of Chicanos and Native Americans in Science (SACNAS) and the US-Mexico Health Research Symposium.

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