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IMMUNOGENICITY OF CYSTEINE PROTEASE *LEISHMANIA MARTINIQUENSIS* DNA BASED VACCINE IN MICE

THUNTAWAT AUNGULDEE

Graduate School Srinakharinwirot University

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IMMUNOGENICITY OF CYSTEINE PROTEASE *LEISHMANIA MARTINIQUENSIS* DNA BASED VACCINE IN MICE

THUNTAWAT AUNGULDEE

A Dissertation Submitted in partial Fulfillment of Requirements

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IMMUNOGENICITY OF CYSTEINE PROTEASE *LEISHMANIA MARTINIQUENSIS* DNA BASED VACCINE IN MICE

BY

THUNTAWAT AUNGULDEE

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES AT SRINAKHARINWIROT UNIVERSITY

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Title	IMMUNOGENICITY OF CYSTEINE PROTEASE *LEISHMANIA MARTINIQUENSIS* DNA BASED VACCINE IN MICE
Author	THUNTAWAT AUNGULDEE
Degree	DOCTOR OF PHILOSOPHY
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A DNA vaccine was developed containing a partial consensus cysteine protease B (*cpb*) gene (pcDNA_*cpb*) derived from autochthonous *L. martiniquensis* strains in Thailand. BALB/c mice were intramuscularly immunized three times in two-week intervals with humanized codon *cpb* plasmid DNA (pcDNA_*cpb*) or in combination with monoolein (MO) as adjuvant (pcDNA_*cpb*-MO). The analysis of immune response demonstrated that pcDNA_*cpb* and pcDNA_*cpb*-MO could stimulate immune responses. In addition, 3 weeks after *L. martiniquensis* promastigote challenge, vaccinated mice sera showed significantly increased production of Th1 response; IgG_{2a} and IFN-g and reduced IL-10 levels than the vehicle control mice sera. IFN-g/IL10 ratio was significantly higher in both pcDNA_*cpb* and pcDNA_*cpb*-MO with significant differences from control groups indicating potentially *protective* immune responses in mice. Mice were potentially protected after three weeks of being challenged with *L. martiniquensis* promastigote , which was demonstrated by a significantly lower parasite burden in the livers and spleens of mice quantitated by qPCR and anti-LPG binding to amastigotes using an immunohistochemical method. Monoolein, as an adjuvant, produced a booster effect to enhance mice Th1 protective immunity. This is the first report of the potential protection of partial cysteine protease B DNA vaccine of *L. martiniquensis* in a mouse model.

Keyword : Leishmania, L. martiniquensis, DNA vaccine, Cysteine protease, Monoolein, Immunization

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THUNTAWAT AUNGULDEE

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

O

CHAPTER 1
INTRODUCTION

Leishmaniasis is a disease caused by the protozoa genus *Leishmania* which causes three different clinical disease symptoms known as cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis [1-7]. The most serious infection is visceral leishmaniasis that is globally widespread in more than 80 countries including Thailand[8-15]. Untreated visceral leishmaniasis is normally mortal. Treatment with chemotherapy is prolonged, costly, and toxic[16-18]. Thus, finding other approaches for treatment or prevention of visceral leishmaniasis are the points of interest such as vaccination. Currently, there are various strategies of vaccine development including live-attenuated, protein, and DNA vaccines which present either prophylactic or therapeutic vaccines. However, the first [19] and second generations *(* recombinant protein, subunit vaccines, no risk of infection*)* are difficult in terms of quality control, the antigen in subunit vaccines must be purified proteins, peptides, or polysaccharide. These antigens alone have weak immunogenicity so in order to enhanced immunity of the subunit vaccine, a potent adjuvant or a well-defined delivery system is required.[20] The new non-infectious vaccine strategy as DNA vaccine could overcome the problems, in case of safety (no risk for infection), low cost of production, simplicity of construction, stability for storage, handling and induction both of humoral and cellular immunity[21, 22]. The successful DNA vaccine studies have been reported in mice, non-human primates and clinical trials in humans. However, DNA vaccine immunization still presented low immunogenicity, therefore various approaches have been used to improve the competency of DNA vaccines such as the structural design and the vaccine improvement with adjuvant [23].

Monoolein is a glycolipid non-ionic molecule which widely used as a drug delivery method [24]. The plasmid DNA vaccine was encapsulated with monoolein as glycolipid structure. With this structure and suitable preparation condition, the vaccine will be able to bind on the surface of the cell and enter into the cytoplasm of target cells [1, 25-27].

The current efforts to develop the candidate vaccines focus on the critical regions of antigens that elicit an immune response. Cysteine proteases are the enzymes that play roles in parasite development and involve in pathogenesis[28]. Currently, cysteine proteases are used as the candidate antigens which have been reported that it could enhance the immune response in both murine and canine models[29, 30].

In Thailand, leishmaniasis was previously reported as imported cases in Thai workers who returned home from the Middle East countries[14]; however, autochthonous leishmaniasis has been currently recognized as an emerging infectious disease caused by *Leishmania siamensis* and *Leishmania martiniquensis*. The predominant case reports were VL caused by *L. martiniquensis*[15]. There are indigenous sandfly vectors located in north and south of Thailand, so the disease surveillance is monitoring.

In this study, the DNA vaccine consists of *L. martiniquensis (* isolated in Thailand*)* nucleotide sequence using humanized codon which is inserted into a mammalian expression vector. The constructed vaccine was used to immunized mice intramuscularly. Monoolein, an adjuvant was added into the DNA vaccine formulation to test whether it can potentiate the immune response of mice against the constructed DNA vaccine. The immunogenicity and protection of the leishmanial cysteine protease B DNA vaccine and monoolein formulated DNA vaccine in mice was assessed by immune profiles (IgG_1, IgG_{2a}, IFN-γ, and IL10) and parasite burden in the liver tissues of mice.

CHAPTER 2
LITERATURE REVIEW

Leishmania sp. are intracellular protozoan parasites that are transmitted via a sandfly vector during blood meal sucking into mammalian host phagocytes for intracellular replication. The main hosts of these protozoa are vertebrates, including humans, native dogs and cats[31]. *Leishmania* may cause asymptomatic infection or localized skin lesions or progress into a serious visceral form of the disease that increases the mortality rate in patients. Nowadays, leishmaniasis is classified into visceral leishmaniasis (1), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL).

2.1 The *Leishmania* parasite

Leishmania is an intracellular protozoan parasite, belonging to the family Trypanosomatidae, Order Kinetoplastida, genus *Leishmania* as shown in Table1. *Leishmania* is normally classified into two core clusters; the Old-World species occurring in Europe, Africa, Asia, and the New World species occurring in South America (CFSPH, 2009). Approximately, more than 30 species have been reported, and more than 20 species of these protozoa are pathogenic in mammals including humans and dogs[32]. *Leishmania* sp. has two main morphological forms, amastigote and promastigote, which are propagated in both vertebrate and invertebrate hosts, respectively [33]. After entering the mammalian host through sandfly bites, promastigotes are phagocytosed by the vertebrate macrophages and then transform into amastigote form, spherical without flagellum and multiply by binary fission in macrophage cells. The infected macrophage lyses and the released amastigotes enter the new macrophages and the multiplication begins again[34]. In cases of VL, the reticuloendothelial organs containing macrophages and phagocytes are mostly infected, for example the lymph nodes, spleen, liver, and bone marrow [35].

Table 1 The Taxonomic classification of Leishmania.

Kingdom	Protozoa
Phylum	Protista
Sub-phylum	Mastigophora
Class	Zoomastigophora
Order	Kinetoplastida
Suborder	Trypanosoma
Genus	Leishmania

2.2 Epidemiology and Transmission of Leishmaniasis

The leishmaniases are endemic in a hundred countries, mainly in Africa, Asia, and Latin America, as well as more than 350 million people, are at risk of the infection[36]. More than 12 million people are currently infected with Leishmania, which is commonly associated with poverty and inadequate socioeconomic conditions, such as starving and immunosuppressed which are generally increased risk of getting an infection. The most frequent clinical forms of the disease are CL which 0.7 to 1.2 million new cases occur annually while VL, the most severe form there are 200,000 to 400,000 new cases arise annually [36]. Human CL and VL show a wider geographic distribution (Figure 1, 2). Leishmaniasis is widely distributed around the world. They range over in tropical rainforest zones including America, Africa and extend to temperate regions including South America, Southern Europe, Asia including Thailand. Geographical distribution of the diseases depends on species of sand-fly biological vectors, the ecological niche and the internal developmental factor of the parasite[37]. The *Leishmania* parasites that cause diseases in humans are *L. tropica*, *L. major*, *L. aethiopica* which are classified as the Old World leishmaniasis or oriental sore while *L. braziliensis* and *L. peruviana* cause the New World CL and *L. donovani* cause VL or kala-azar[38]. The burden of VL is still unidentified because numerous cases are not detected[39]. However, it has been estimated that there are approximately half a million new cases of VL annually worldwide, with more than 50,000 associated deaths. More

than 90% of VL cases occur in six countries, *i.e.*, India, Nepal, Bangladesh, Sudan, Ethiopia and Brazil [40]. *L. infantum* (or synonym: *L. chagasi*) is the species that mainly cause VL which present in infants, young children and immunosuppressed individuals in Southern Europe and the Mediterranean Basin[41]. Dogs are an important reservoir host of *L. infantum* in Europe. During the last two decades, the emergence of resistance to pentavalent antimonial drug causes a huge impact on the epidemiology of leishmaniasis[42].

In Thailand, the first autochthonous VL was reported in a 3-year-old girl at Surat Thani Province in 1996 and followed in an HIV patient at Nan Province in 2005 however, the species that caused VL was not identified. In 2008, using PCR-based assay, VL caused by a new species was reported in immunocompetent patients at Phangnga Province [8, 43]. Later, the second, third and fourth cases of VL were reported in HIV-infected patients at Chiangrai, Chantaburi and Trang Provinces in 2007, 2009 and 2012, respectively. Species identification of a novel causative agent was reported as *Leishmania siamensis* [8]. However, the first isolation of another species of *Leishmania, L. martiniquensis*, which was previously described in Martinique Island[44], was reported in an HIV-infected Thai patient in Northern Thailand[15]. Moreover, molecular studies confirmed several of asymptomatic infections of *L. martiniquensis* and *L. siamensis* in HIV-infected people, which is a high-risk group (personal communication, Leelayoova et al). So far, sporadic cases of VL were reported in six southern provinces, one eastern province, and four northern provinces. This brings the serious concern of this emerging disease in Thailand where we have both infected persons and potentially transmittable vector, sandflies.

2.3 The Leishmania biological vectors, sandfly

Leishmania parasites are transmitted by a diminutive insect vector, known as the phlebotomine sandfly. Like mosquitoes, only the female sandfly is a hematophagous insect for egg production. The phlebotomine is a subfamily of the family Psychodidae. The common name is sandfly. The phlebotomine includes many genera of blood-feeding (hematophagous) flies, including the primary vectors of leishmaniasis,

bartonellosis and pappataci fever. Phlebotomus and Lutzomyia are the most common genus of normally sandfly which are biological vectors [45]. Naive sandfly developed with Leishmania amastigotes during a blood drain from an infected mammalian host. The infected blood is coated in a film-like structure, known as the peritrophic membrane composed of chitin and proteins. Within about 12 to 18 hours, the amastigotes go through a morphological transformation into procyclic promastigotes, depend on environmental conditions, for example, the decrease in temperature and increase of pH. The procyclic promastigotes attach to the microvillar surface of the midgut, all through the binding of lipophosphoglycan (LPG), Leishmania surface molecule, and reproduce as the blood meal persists to be digested[46]. The procyclic promastigotes also transform during 48 to 72 hours into the intermediate forms, named as nectomonad [47] (Figure 3). The nectomonads are developed the peritrophic membrane, which protects them from the hydrolytic enzymes from the [46], Next, the activity of Leishmania chitinase are induced nectomonads turn into cryptomonads, which are normally produced a mucin-like glycoprotein, known as promastigote secretory gel (PSG)[48]. The last step is leptomonads reproduce and subsequently differentiate into metacyclic promastigotes in a process known as metacyclogenesis. Nowadays 500 species of sandfly genus phlebotomine have been reported, but only 30 species are biological vectors of leishmaniasis. The phlebotomine sandfly is found in tropical and temperate regions and transmit old world leishmaniasis. Phlebotomine females suck blood from various mammals and also reptiles and birds. One time of blood meal can provide the production of about 100 eggs. Females lay their eggs in moist soil fulfill with organic compound[49]. The New World leishmaniasis is transmitted by sandflies in the genus Lutzomyia which commonly live in dark areas such as caves. In the Old-World sandflies in the genus, Phlebotomus spread leishmaniasis. The female sandfly lays its eggs in the burrows of certain rodents, in the bark of old trees, in house walls, in animal shelters, in household rubbish, by which in such environments that the larvae will find warm and humid ecological niches including organic matter, which are the important factors for their development[49].

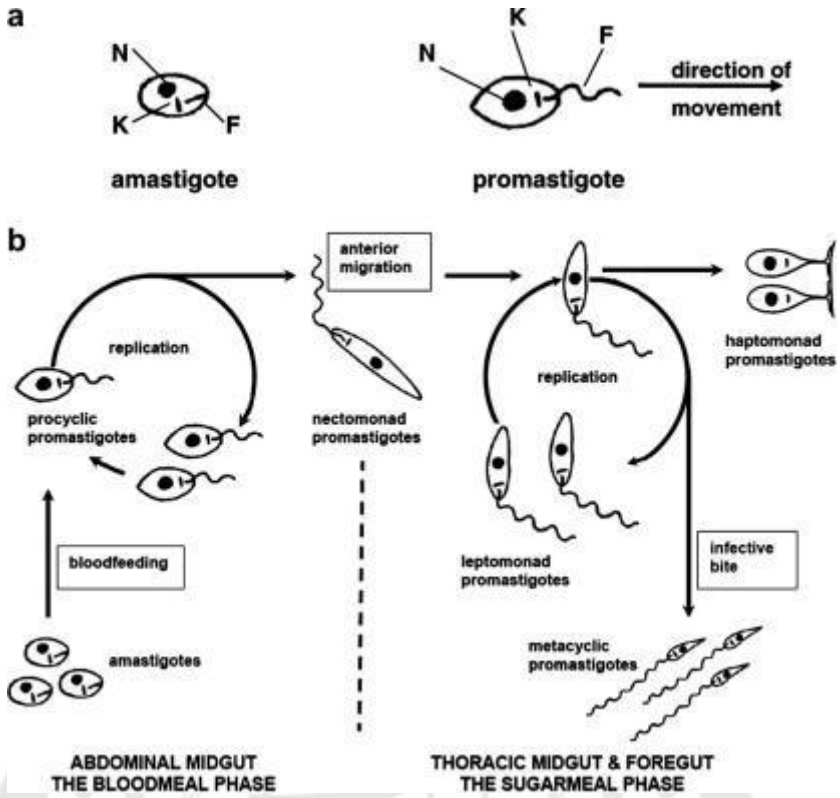

Figure 1 The Leishmania life cycle within the sandfly vector.

Source:

https://www.researchgate.net/publication/6316043_Transmission_of_Leishmania_metacyclic_promastigotes_by_phlebotomine_sand_flies_Int_J_Parasitol/figures?lo=1

2.4 Life Cycle of Leishmania

The life cycle of *Leishmania* consists of two forms; the promastigote which develops and lives extracellularly in the mid-gut of sandfly vector and the amastigote which multiplies intracellularly in the reticuloendothelial cells of the host [50] (Figure 4). Although different species of *Leishmania* infect humans, they all share the same pattern of life cycle. The infected sandfly bites the mammal hosts and transmits the *Leishmania* promastigotes directly from the proboscis or by vomiting them during blood-feeding[51, 52]. Shortly, promastigotes are engulfed by macrophages, then transform into amastigotes and multiply. The female sandfly picks up amastigotes from infected macrophages of mammalian hosts during sucking blood meal. The amastigotes are

released in the mid-gut of the sandfly and then transformed to the promastigotes and start growing and differentiating actively through the intermediate stages; pro-cyclic, nectomonad and hepatomonad forms, before developing into infectious metacyclic promastigotes. After that, parasite flagellum extends from flagella pocket located in anterior pole and pulls the parasite forwards. When mature, metacyclic promastigotes migrate to the proboscis of sandfly and are ready to be transmitted to the new mammalian host. The metacyclic promastigotes enter the skin of the vertebrate host when the infected sandfly takes its next blood meal and the life cycle begins again[53] (Figure 2).

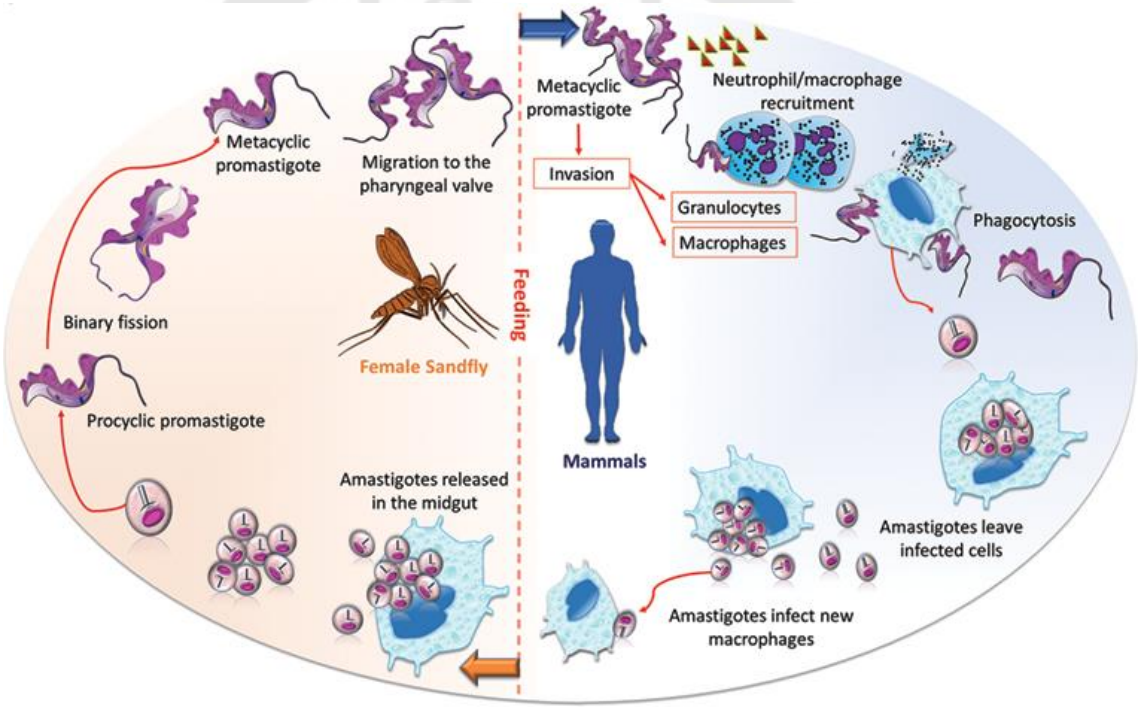

Figure 2 The Life Cycle of *Leishmania* Species

Source: https://www.intechopen.com/books/the-epidemiology-and-ecology-of-leishmaniasis/visceral-leishmaniasis-and-natural-infection-rates-of-leishmania-in-lutzomyia-longipalpis-in-latin-a

2.5 Leishmania pathogenesis

Up to the present time, more than 20 *Leishmania* species have been recognized as the causative agents of leishmaniasis. Currently, about 12 million people are infected, while 350 million people in 88 countries from all over the world are at risk to develop one kind of disease associated with *Leishmania* parasite[54-56]. Leishmaniasis may cause asymptomatic infection or localized skin lesions, and progress into a serious visceral form of the disease that increases the mortality rate in patients. Therefore, leishmaniasis is classified into cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (1) (Table 2). The multiplication of amastigotes in macrophage has occurred during the infection and then these intracellular amastigotes spread through the mucosal epithelium or internal organs including the spleen and liver. The inflammatory response of the host immune response to leishmaniasis infection caused pathogenesis in humans and dog[57].

2.5.1 Cutaneous and Mucocutaneous leishmaniasis

CL is the leishmaniasis of the skin which is caused by parasites *L. vianna brasiliensis* and *L. mexicana* complex in the Central and Southern America (New World leishmaniasis) and *L. major* and *L. tropica* are observed in the Middle East, Africa, Southern and Eastern Mediterranean regions (Old World leishmaniasis). It is mainly a zoonotic disease by which small rodents and canine are reservoir hosts. Moreover, the anthroponotic infection can occur in the case of *L. tropica*. An occurrence of 1-1.5 million new cases per year has been reported by WHO[58]. The disease mostly affects the skin area which is prone to insect bite such as the face, arms, legs, however, it may cause sub-clinical symptoms. The first symptom appears as papules that progress to nodules and later to lesions after the first week of infection (Figure 5). These lesions heal spontaneously in a few months and resulting in scars. The complications may be occurred by dissemination of the parasites from the skin to the nasopharyngeal mucosa and cause progressive destruction of mucosal tissue leads to mucocutaneous leishmaniasis (MCL) which is also named as espundia. MCL is traditionally referring to metastatic sequelae of the New World cutaneous infection mainly caused by *L. braziliensis* and *L. panamensis*[54, 59-61].

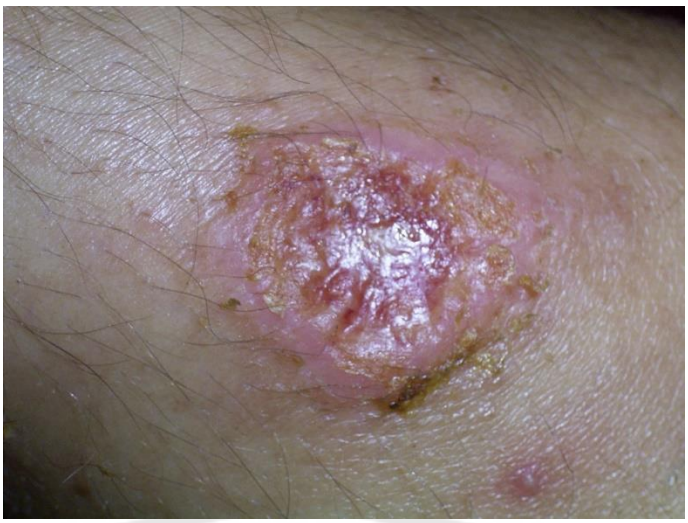

Figure 3 cutaneous leishmania on the skin of a patient

Source: http://www.who.int/leishmaniasis/cutaneous_leishmaniasis/en/

2.5.2 Visceral leishmaniasis

The most severe form leishmaniasisis is VL, which is called “kala-azar” in India, Latin America, and Asia. Five hundred thousand of new cases and 50,000 deaths have been reported each year[60)52)]. VL is mainly zoonotic, by which dogs are being the main reservoir host, however, for *L. donovani* is anthroponotic. In many cases, infection remains asymptomatic, however, it can develop to acute or chronic infection. The incubation period of the first exposure is between two and six months. The symptoms are fever, fatigue, weight loss, weakness and finally hepato-splenomegaly *(*Figure 6*)* and anemia which is caused by bone marrow suppression. Untreated patients may die from secondary co-infections such as the patient who was infected with the HIV or has massive internal bleedings or anemia[62-66].

Post kala-azar dermal leishmaniasis (PKDL) is a complication of VL which develops in various periods after VL recovery, for example, 0-6 months (Sudan) or more than 2 years (India). It is characterized by a maculopapular, nodular or papular rash that spreads from face to the whole body, depends on the grade of disease[67]. These rashes harbor high numbers of infective parasites which are serving as a reservoir site for the anthroponotic cycle.

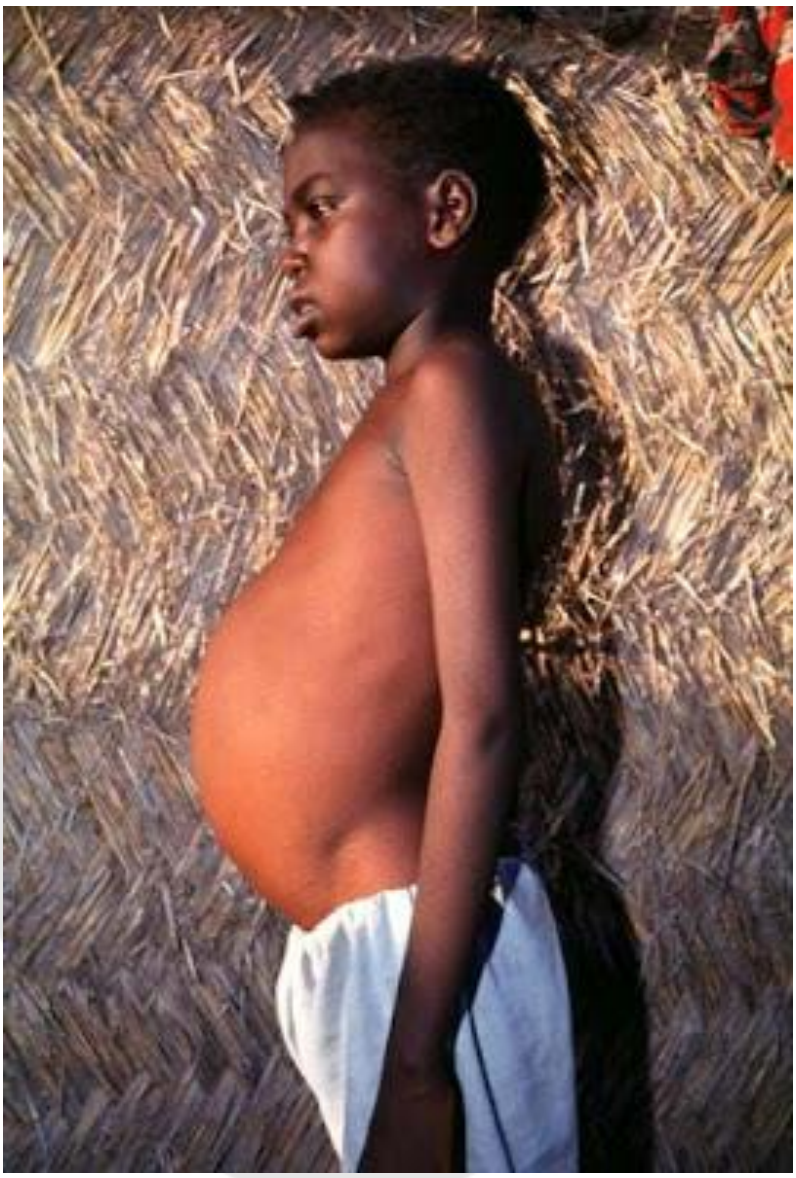

Figure 4 VL with splenomegaly in a patient

Source:http://profilaxia-pragmatica.blogspot.com/2009/08/leishmania-visceral.html

Table 2 Human-infective Leishmania species and their clinical forms CL, localized cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; MCL mucocutaneous leishmaniasis; VL, visceral leishmaniasis.

	New world species
CL	L. venezuelensis, L. mexicana, L. amazonensis, L. infantum, L. panamensis, L. peruviana, L. lainsoni
VL	L. infantum
MCL	L. braziliensis, L. guyanensis, L. panamensis
	Old world species
CL	L. major, L. tropica, L. aetheopica, L. infantum
VL	L. donovani, L. infantum, L. martiniquensis
MCL	L. major, L. tropica

2.6 The Development of Promastigote Virulence in the SandFly

The promastigotes in sand-fly migrate from mid-gut to the proboscis and differentiate to metacyclic promastigotes which are potent to be the infective stage. BALB/c mice injected with metacyclic *L. major* and *L. chagasi* promastigotes develop dangerous lesions and increase more parasites than using the stationary phase parasites of *in vitro* cultivation, by which the original parasites were obtained from infected visceral organs of patients [68, 69]. GP63 of *Leishmania* species is a virulent factor which can cleave C3 during complement classical pathway, instead of stimulation the complement fixation of host cell process, it converts C3 to cleavage C3b, which play a critical role to enhance opsonization using CR1 and CR3 receptor of macrophages [70]. The previous study showed that the complement in the serum inhibits the increasing of parasite numbers in the liver and spleen [69, 71, 72]. The previous experiment showed C3 complement in human serum bound to major surface protease *(* surface metalloprotease*)* of parasite named GP63 which is expressed higher in metacyclic than

promastigotes[70]. Moreover, the phosphatidylinositol lipid-anchored glycoconjugate lipophosphoglycan (LPG), comprises phosphorylated disaccharide backbone and the modified oligosaccharide side chains, is a major promastigote surface binding factor which plays critical pleiotropic roles in parasite survival and infectivity in both the sandfly vector and the mammalian host[73, 74]. The previous study showed in *L. major* that, the multiplication of disaccharide backbone and the number of terminal arabinose side chains increases dramatically the inhibition of phagosome activation in macrophage[75]. Thus, promastigotes from different growth phases cannot be killed in phagolysosomes in macrophages.

2.7 Treatment

Nowadays, there are several treatments for leishmaniasis, but the method and efficacy depend on several factors such as parasite species, the clinical symptoms of the disease and the host immune profiles. Nowadays, chemotherapy is the traditional treatment to treat leishmaniasis[76]. The drugs used for CL is generally used to increase of healing wound and prevent the parasite distribution to other tissues [77, 78].The treatment of VL is always recommended because of the severity of the disease. Pentavalent antimonial compounds or stibogluconate (Pentostam, Glaxo-Smith-Kline) or meglumine antimonite (Glucantime) are the first-line drugs that are recommended by the World Health Organization (WHO)[79, 80]. Pentavalent antimonial has been used for more than 50 years in leishmaniasis based on WHO guidelines [77, 78, 81, 82]. Additional common drugs are amphotericin B, pentamidine, imidazoles, allopurinol (Zyloric), miltefosine (Impavido or Miltex) and paromomycin. However, several difficulties endure in the treatment of leishmaniasis. Most treatments require times (weeks to months) and there is a high rate of significant adverse drug reactions. Moreover, the cost of treatment is high because many patients suffer from drug toxicity and may require secondary treatment[83]. These factors interrupt patients from continuing and completing their therapies. Most importantly, these drugs cannot treat all patients because of the occurrence of drug-resistant parasite strains or immunosuppression in patients [82-85]. Moreover, the other oral drugs such as imidazoles, allopurinol, miltefosine are also toxic

[82, 84]. Additionally, the occurrence of drug-resistant parasites in the patients who are co-infections, especially with HIV patients causes a decrease in choosing the drugs for treatments. While all current drugs used are targeting directly to the parasite, the novel option for leishmaniasis treatment by which targeting the parasite and also modulating the host immune response or in combination are the targets of interest.

2.8 Immune response in leishmaniasis

2.8.1 Innate immunity

After tissue damage to the microvasculature of the epidermis and dermis caused by sandfly during *Leishmania* injection induced a robust local inflammatory response, with the coordinated recruitment of non-specific immune response. The pathogenesis at the site of inoculation during infection with *Leishmania* spp. that cause CL and VL, the early local inflammatory response during VL is still unclear.

2.8.1.1 The complement system

The metacyclic promastigotes are primarily exposed to the non-specific immune proteins, including soluble serum proteins of the complement system, for example, the C3b that cleaved from C3, is one of the most powerful opsonin by binding to the surface of extracellular microorganisms and the complement receptors. Neutrophils and macrophages are also phagocytic cells which are activated by using CR1 and CR3, to promote their phagocytic uptake and pathogen clearance. Nevertheless, the mechanisms that evade the lysis process while developing opsonic complement factors for receptor-mediated entry into their target host cells. The major surface protein of *Leishmania*, metalloprotease glycoprotein gp63, increases C3b cleavage to a proteolytically inactive fragment, iC3b, which is still able to opsonize but cannot trigger the oxidative burst which releases reactive oxygen species (ROS) [71]. Moreover, LPG of metacyclic promastigotes also acts as a barrier preventing the insertion of the C5b-C9 membrane attack complex (MAC) into the *Leishmania* surface membrane to prevent pore formation[86].

2.8.1.2 Leishmania-neutrophil and macrophage interactions.

The neutrophil is polymorphonuclear granulocytes mainly in an innate immune cell that are engaged to the site of infection, following a few hours after parasite infection[87]. Neutrophils circulate via blood vessels and induced inflammation response (Figure 5). The chemotaxis which is released by neutrophil induced by the presence of and *Leishmania* derived factors from sand fly [87, 88]. Furthermore the chemoattraction of innate immune response with complement C3 [89], CXCL1 (14) and CXCL2 (MIP-2) from epithelial cells, keratinocytes, fibroblasts, and also neutrophils, which are homolog with human IL-8 was related to the migration of *L. major* in mice models[90]. It suggests that the production of chemokines by way of the *L. major* regulate migration of cells under innate immune response. The promastigotes are phagocytosed by neutrophils approximately 80-90%, although only 10 to 20% are phagocytosed by monocytes or macrophages[87, 91, 92]. The *Leishmania* are survived within the intracellular environment of the neutrophil; also, Leishmania can escape the adaptive immune response, as other immune cells are not capable while it's inside the neutrophils[93] *(*Figure 5 *)*.

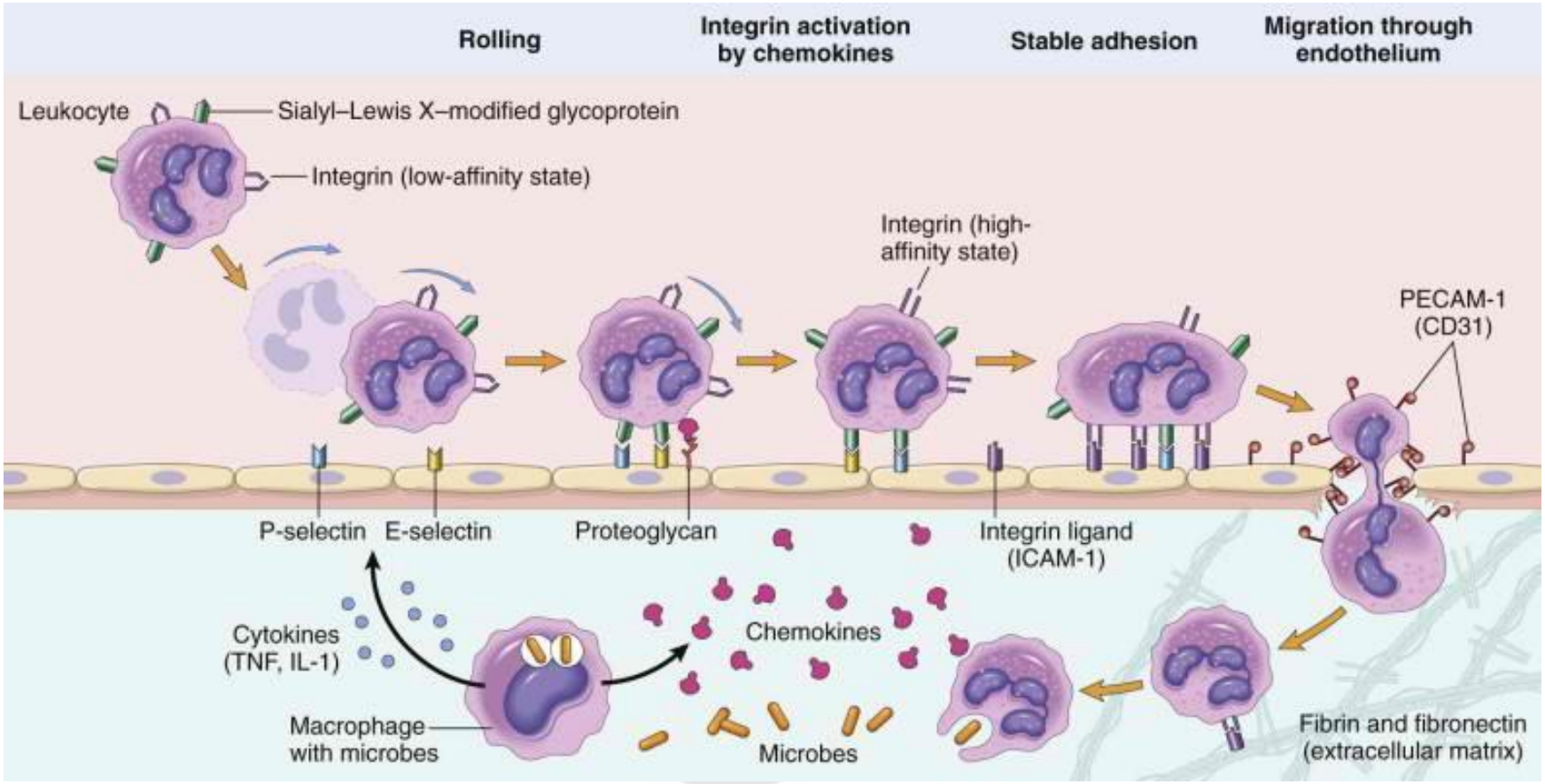

Figure 5 Neutrophil migration from the blood vessels to the site of infection.

Macrophages are the major host cell type which protected from *Leishmania* infection. The infection with *Leishmania*, neutrophils secrete significantly higher levels of the chemokines CCL3 (MIP-1α) and CCL4 (MIP-1β) [(94)], which trigger monocytes and macrophages to migrate to the site of infection approximately two or three days post-infection. The presence of growth factor and cytokine promoted monocytes differentiation into either macrophages or dendritic cells *(*DC*)* at the site of infection. The infection process of the parasite into macrophage is still unclear. There are many ideas about the process of macrophage infection as following; parasites released from the neutrophils and taken up directly by macrophages; or that macrophage clearance of apoptotic neutrophils sheltering parasites leads to the indirect uptake of Leishmania in a process [(94)] or that parasites escape from the apoptotic neutrophils and are taken up directly by macrophages together with the apoptotic cells in a process[(87)]. The infection of *Leishmania* parasites into macrophages is manipulating the modulation of the macrophage intracellular signaling pathways by Leishmania to provide them insensitive activation, or to downregulate or inhibit the antimicrobial effector functions, for example, ROS and nitric oxide (NO), which role as potent effectors against both extra- and intracellular Leishmania infection. Moreover, the production of IFN-γ by NK and Th1 cells predominantly stimulates the robust expression of the nitric oxide synthase (*iNOS*) of macrophages. Leishmania can inhibit macrophage activation and suppress *iNOS*-mediated generation of nitric oxide by handing the immunomodulatory protein[(95)].

2.8.2 Adaptive immune response

2.8.2.1 T Cell

Naïve T cells grow from stem cells of the bone marrow and mature in the thymus. In this mature stage T cells are either $CD4^+$ T helper cells or $CD8^+$ which are classified into two different class requirements on activation stimuli. The effector $CD8^+$ T cells used specific interaction with MHC class I molecule-antigen complexes. These CTL can selectively destroy cells that show the specific antigen on the cell surface by MHC class I molecules but do not co-stimulate through CD80/86 molecules. For a

previous study of T cell differentiation towards Th_1 and Th_2 cells was known. These subclasses require different factors and perform various effector mechanisms ranging from acute inflammation (Th17) to immune silencing (Treg)[96]. The effector functions activated Th0 cells which differentiated for the Th1 phenotype in secondary lymphoid organs and migrate to the site of infection. Consequently, they express chemokine receptors (CXCR3 and CCR) to migrate towards increasing levels of secreted chemokines that are related to the site of inflammatory infection[97]. Moreover, Th_1 cells are normally express the adhesion molecules, for example, E- and P-selectin ligands to vesicle walls closely inflammatory tissues. So, Th_1 cells can directly interrelate with antigen-presenting cells. Next, the production of IFN-γ is mainly the effector mechanism of T cell. This cytokine activates macrophage which can kill intracellular pathogens[98]. Additionally, phagocytosis of *Leishmania* sp. by macrophage is considerably enhanced and supported by the complement pathway by binding and opsonizing antibodies. The production of the respective antibody is induced and enhanced by IFN-γ production and direct interaction between Th_1 cells and B cells and therefore activation of the respective B cells. Moreover, Th_1 cells activate neutrophils by secretion of TNF and lymphotoxin (LT). This leads to enhance microbial killing at the site of inflammation. In summary, Th_1 immune responses support cell-mediated clearance of intracellular and extracellular pathogens.

The Th_2 cells migrate to the site of infection and increasing levels of chemokines. However, these chemokines are dissimilar from Th_1 chemokine. The Th_2 release cytokines, for example, IL-4, IL-10, IL-5, and IL-13. IL-4 promotes activated B cells to generate IgE that in turn can attach to *Leishmania* sp. and activating stimulus for mast cell degranulation. The expression of IFN-γ can suppress IL-10 which impede macrophage activation. In generally, IL-10 is immune silencing cytokine. However, in Th_2 related immune responses, an alternative introduction pathway of macrophage activation can be triggered[99].

Mosmann and his-coworkers demonstrated a simple model of intracellular pathogen susceptibility and/or resistance in mice, which influence by

Th_1/Th_2 type responses.[100, 101]. In leishmaniasis, the control of disease had been shown by increasing Th_1 cytokines type response including IL-12, IFN-γ, TNF α, IL-2[102]. However, there is some evidence of *L. major* and *L. donovani* infection that typical induced Th_2 cytokines for example; IL-4 and IL-13 which can enhance macrophages and dendritic cell activities by inducing IL-12 production which involved in the differentiation of naïve T cell into Th_1 cells [103-122]. Furthermore, IFN-γ, and IL-4 producing T cells have been found in asymptomatic and cured patients. The protection is decreased when there is an increased frequency of Th_2 cytokine-producing T cells or the ratio of IFN-γ /IL-4.[123]. Additionally, Th_2 cytokine, IL-10 is the agonist against Th_1 cytokine has been shown to promote parasite burden and increase number in resistant mice. Interleukin-10 also known as an anti-inflammatory property and is produced by several cell types including macrophages, dendritic cells, B cells, T cells, and epithelial cells. From the previous study, it has been found that the pathogenesis of VL is mediated by inhibition or downregulation of IL-12 and MHC-II, thus promoting parasite replication and disease progression [124].

Immune response against VL are complicated and not-well understood. BALB/c mice infected with *L. donovani* or *L. infantum* are often used to study the immune progression of VL. After infection, the parasites progress during the first two weeks. Later the parasite growth is controlled by host immunity. To study the mechanism of VL pathogenesis, a hamster model is used. In the hamster, after challenging with *L. donovani* or *L. infantum, the* pathogenesis of disease pattern is like those of humans and dogs. However, the availability of immunological tools for hamsters is very limited and the usefulness of the model is restricted. IFN-γ and IL-2 act as important factors in the development of protective responses in BALB/c model. The level of Th_2 cytokine, for example, IL-10 was found to be related to pathogenesis in the BALB/c model as well as in humans [125, 126]. In hamsters, the total absence of specific T-cell responses after the infection has been reported by several investigators [127, 128]. T-cells, as well as macrophages, have been implicated to mediate this immune

suppression apparent. The overall concept of the immune response against leishmaniasis is demonstrated in Figure 8.

2.8.2.2 Interferon Gamma

IFN-γ is a pro-inflammatory cytokine involved in host defense against infection. Invading pathogens soon encounter macrophages. IFN-γ which is originally called macrophage-activating factor, is one of the most important cytokine that activates macrophages[129]. The main sources of IFN-γ producing cells are $CD4^+$ Th1 lymphocytes, $CD8^+$ (CTLs), NK and natural killer T (NKT) cells. Nevertheless, it had been reported that B cells, mast cells, macrophages and professional antigen-presenting cells (APCs) are also IFN-γ expressing cells[130, 131].

2.8.2.3 Interleukin-10

IL-10 is a product of T-helper-type 2 (Th2) cells that inhibits the cytokine production of Th1 cells[132]. IL-10 is generated by several types of cells, for example B cells, macrophages, dendritic cells (DCs) and several subsets of T cells, including Tregs, Th2 cells, and Th17 cells[133]. Murai M and his co-worker showed that IL-10 provided Fork head box P3 (FoxP3) expression and suppressive function in mice models[134]. IL-10 performs as an in direct immunosuppressive activity, through the effects on APCs such as monocytes, DCs, and macrophages. In addition, IL-10 also downregulates major histocompatibility molecule (MHC) and B7 co-stimulatory molecule expression on APCs.

2.8.3 The immunity of organs against *Leishmania* species infection.

The splenomegaly and hepatomegaly are the main clinical symptom of VL infection. Nowadays, it has standard criteria between immune responses that induce long-term protection and effectively regulate parasite growth, parasite persistence, and correlated disease. Therefore, some micro variations in spleen and hepatic tissue affect the ability to engender effective immune responses and parasite control in organs.

The liver is the main target organ of VL infection. The liver is auto-regenerated in 2–3 months after infection of *Leishmania* species[135]. These correlated through the development of granuloma formation by a Th_1 immune response both in

humans and canine[136, 137]. The inflammatory granulomas around infected liver recruit macrophages, initiate T-cell-response and also TLR7, TLR8, TLR9, IL-1, and IL-18 through the signaling pathway[138]. A granuloma formation enhanced *iNOS* production by macrophages[135, 139], which is controlled by numerous pro-inflammatory (Th_1) cytokines, for example, IL-12 and IFN-γ. The study in mice model of *L. donovani* infection, infected Kupffer cells (liver macrophages) have a different transcriptomic network profile compared to non-infected Kupffer cells isolated from the same mouse[140]. Moreover, the hepatic cell infected with *L. donovani* in both of *in vitro* and *in vivo* generate immunoregulatory cytokines that provoke $CD4^+$ T cells and turns into Treg which leads to parasite persistence[141]. VL leads to hepatic dysfunction and increased serum concentrations of numerous liver-specific enzymes, and fluctuations in the cholesterol biosynthesis[142, 143]. The total parasite load in the host cell *(*parasite burden*)* indicated virulence of the disease and the pathology is reversed when hepatic levels of *miR*-122 are restored with increased serum cholesterol and reduction of liver parasite burden[144]. The granuloma is a formation of a various type of immune cell for example; macrophage and monocyte which caused inflammation during the infection. The histological immune response was classified into 4 stages as following; lack of granuloma *(93)*, immature granuloma *(*IG*)*, mature granuloma *(*MG*)* and involuting granuloma *(*IVG*)*. The infected liver with a lack of inflammatory response and mononuclear cell is assigned as lack of granuloma *(93)* stage. IG was categorized by the existence of amastigote, infected Kupffer cells and mononuclear cell- with or without inflammatory response. MG was observed as the amastigotes infected into macrophages and epithelial cells that encircled as a layer together with an inflammatory response and the IVG is the MG stage that lack of amastigotes inside the granuloma[136].

In conclusion, the granuloma formation, mostly display signs of macrophage activation and parasite destruction. Moreover, the granuloma inflammation correlated with the secretion of cytokine by infected macrophages, with the target of stimulating the production of nitric oxide and superoxide toxic against *Leishmania* species. Even Though, Th1-like cytokine for example; IFN-γ are produced by various

cell types and alternatively activated macrophages are sparse in the *L. donovani* granuloma, the Th2-associated cytokines IL-4 and IL-13 are nevertheless necessary for granuloma formation[145].

The spleen also becomes chronically infected by mechanisms that are unclear understood. The spleen develops enlarged and splenomegaly for up to 15% of the bodyweight of infected mice in a 6-8 week post-infection[135]. The spleen composed of red (RP) and white pulp (WP) and marginal zone (MZ). The splenic RP contains macrophages that recycle iron blood from elderly red blood cells. The WP is composed of T-cell and B-cell follicles which are used for generating antigen-specific immune responses[146]. The vascular disruption, DCs fails to migrate to the T cell area, causing in a reduces priming of T cells[147]. Thus, vascular remodeling and splenomegaly associated with VL induced pathology in the spleen. The persistence of parasites in the spleen is correlated with changes in the splenic lymphoid and simultaneous T cell apoptosis[135, 148].

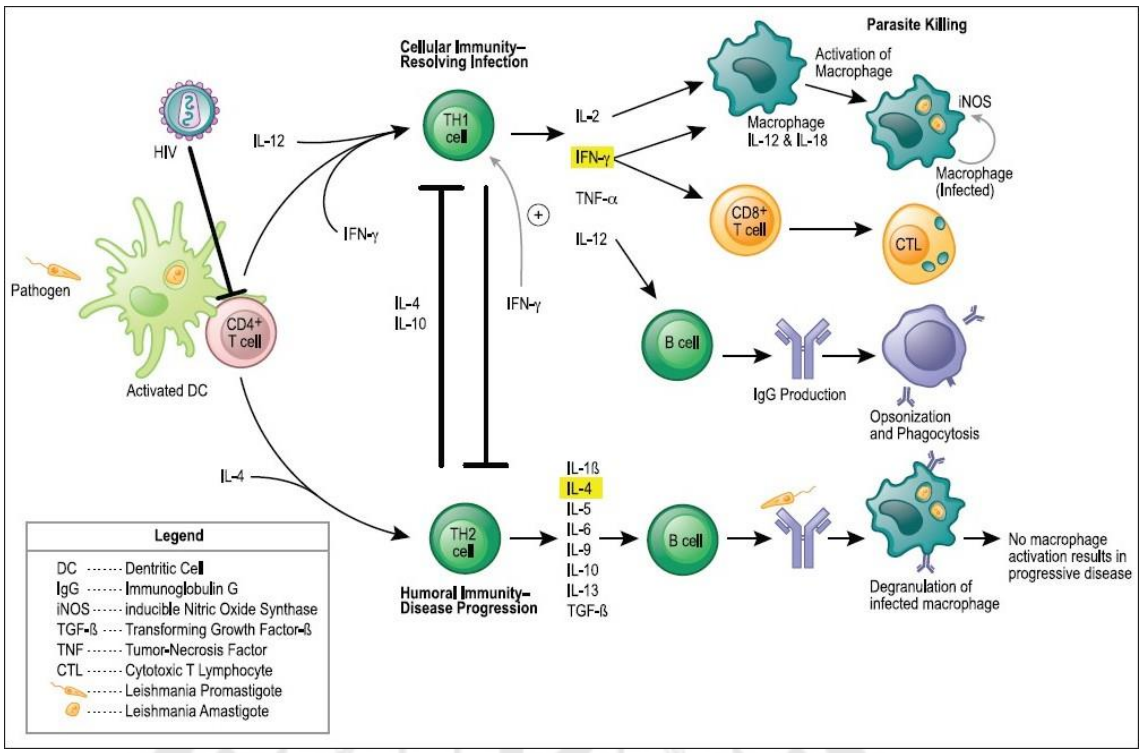

Figure 6

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http://www.jgid.org/articles/2010/2/3/images/JGlobalInfectDis_2010_2_3_248_68528_f1.jpg

2.9 The pipeline of leishmania vaccines

An ideal vaccine against VL needs to be safe and effective, especially in respect of the main vaccination target including human populations in developing and under-developing countries. It should be capable of enhancing both $CD4^+$ and $CD8^+$ T cell response and inhibit IL-10 and needed to induce a lasting protective immunity. Broad protection against species causing CL and VL would be preferable. Moreover, the vaccine formulation needs to be able to storage at room temperature. Recently, the fatal rate of VL is increasing and uncontrollable because of drug resistance, insufficient treatment and/or increasing evidence of the disease in immunocompromised hosts. The toxicity of drug use is the main obstacle for treatment. Thus, the current efforts approach to develop novel drugs or drug delivery systems and vaccine target therapy. However,

the cost of drugs and long-term therapy are noteworthy limited the efficacy of treatment. Therefore, the development of vaccines had been performed. At present, there are various strategies of vaccine development such as live-attenuated, protein purification, and DNA vaccines, which implicated either prophylactic or therapeutic vaccines [149, 150]. To construct an effective vaccine against VL, various parasite antigens have been tested for their potential to induce anti-leishmanial immune responses. *Leishmania* surface protein gp63 and *Leishmania* homolog of receptors for activated C kinase (LACK) have been extensively studied in a wide range of adjuvant and delivery systems. Other antigens include lipophospoglycan (LPG), hydrophilic acylated surface protein B1 (HASPB1), promastigote surface antigen 2 (PSA-2), kinetoplastid membrane protein (KMP-11), thiol-specific-antioxidant (151) and the poly-antigen Leish-111f were also used as antigens for vaccine development [152-155]. In an attempt to tackle *leishmania* infection at an early stage, salivary proteins of the sandfly have also been evaluated for vaccination. Studies in hamsters and dogs showed promising results with the induction of type I responses and significant IFN-γ and IL-12 production [156, 157].

2.9.1 Live attenuated vaccines

The pipeline of vaccine can be classified into three generations depend on type of vaccines, as follows. The first generations of anti-leishmanial vaccines were heat-killed and live attenuated *leishmania.* The heat-killed or autoclaved *Leishmania* parasites *(L. major* or *L. donovani)* combined with adjuvant *Bacillus Calmette-Guerin (*BCG*)* at the final concentration 1 mg per dose was used to immunize in human volunteers. Those combinations significantly induced the activation of $CD4^+$ and $CD8^+$ T cells.[158, 159]. Additionally, the combination of heat-killed *L. major* and *Mycobacterium vaccae* induced cytokine responses including interferon-gamma and interleukin-4 [160]. Subsequently, those combinations were used as chemotherapy, which induced a slow rate of parasite re-infection [161]. The prophylactic vaccine had also been studied by using radioactive *L. donovani* attenuated immunized three times intramuscularly *(*IM*)* in BALB/c mice. The results showed increasing in $CD4^+$ T cells, inducible nitric oxide synthase *(iNOS)* immune response and protected the mice from VL challenge [162].

Although the first generation of the vaccine showed good immune responses against leishmanial infections, there was the limitation of heat-killed or live attenuated vaccine in that it may cause some toxicity to the host. Thus, the purified recombinant subunit protein has arisen as a new strategy.

2.9.2 Subunit vaccines

Purified recombinant subunit proteins, either alone or combined with several adjuvants or with different delivery methods such as bacteria or viral vector have also been studied in preclinical trials. The immunization with Leishmune® *(*licensed by Brazil*)* in dogs, showed the protection against canine VL and also inhibited the transmission of protozoa from canine towards humans[163]. Fucose Mannose Ligand *(*FML*)*, saponin, and glycoside adjuvant vaccine were another vaccine candidate in canine. These candidate vaccines increased activation of both $CD8^+$ T cells and innate immunity including neutrophils and macrophages [164]. *L. donovani* p45 recombinant protein named as "rLDp45" were used to immunize three times subcutaneously *(*50 µg/dose*)* in mice. It could induce the cytokine profile, for example, interferon-gamma, interleukin-12 *(*IL-12*)*, tumor-necrotic factor-alpha and reduced parasite load in the spleen and liver [165]. An antigenic specific for amastigote *(*A2*)* antigen was studied by formulation with various adjuvants such as saponin and alum [166]. The results showed that the vaccine candidates induced both $CD4^+$ and $CD8^+$ T cells responses. Additionally, the combination of 25 µg of *Leishmania* polyprotein LEISH-F1 *(* Leish111F*)* antigen formulated with 25 µg monophosphoryl lipid A (MPL-SE*)* adjuvant was used to immunize three times in humans and evaluated in phase II clinical trials. The immunogenicity and safety of this vaccine have been reported in healthy, VL and CL volunteers [167-169]. However, subunit vaccine candidates showed some limitation, since different strains of the parasites may induce host immune responses differently and may lead to uncontrollable parasite replication. Therefore, the DNA vaccine is a trend for leishmaniasis prevention. Subunit vaccine antigen candidates and the results of the previous studies are shown in Table 3.

Table 3 Subunit vaccine candidates for leishmaniasis

Antigen	Adjuvant	Mice models	Challenge	Result
Gp46/M-2[170]	P. acnes	BALB/c	L. amazonensis	Partially protection
PSA-2[171]	P. acnes	C3H	L. major	Partially protection
rLcr1[172]	FCA	BALB/c	L. chagasi	Partially protection
P-8[173]	P. acnes	BALB/c	L. amazonensis	Partially protection
P-4[173]	P. acnes	BALB/c	L. amazonensis	Partially protection
rGp63[174]	none	CBA	L. major	Partially protection
FML[175]	saponin	BALB/c	L. donovani	Partially protection
rLACK[176]	IL-12	BALB/c	L. donovani	Partially protection
rLACK[177]	IL-12	BALB/c	L. amazonensis	none
rLeish-111f[178]	MPL-SE	BALB/c	L. major	none
rCP[179]	IL-12	BALB/c	L. mexicana	Partially protection
rA2[177]	IL-12	BALB/c	L. amazonensis	Partially protection

Abbreviations: Gp46/M-2: purified membrane protein of *L. amazonensis*; rPSA-2: purified membrane protein of *L. major*; rLcr1: unidentified recombinant protein of *L. chagasi*; P-8 and P-4: purified amastigote specific antigens of *L. pifanoi*; rGP63: recombinant major surface antigen of *L. major*; FML: fucose mannose ligand, glycoprotein complex purified from *L. donovani*; rLACK: recombinant *L. donovani* homolog of receptors for activated C kinase; rLeish-111f: recombinant fusion protein of TSA, LmSTI1 and LeIF proteins of *L. major*; rCP: recombinant cysteine peptidase B of *L. mexicana*; rA2: amastigote specific antigen of *L. donovani*; FCA: Freund's complete adjuvant; rIL-12: recombinant murine IL-12; MPL-SE: monophosphoryl lipid A plus squalene.

2.9.3 DNA vaccine

The third generation of leishmanial vaccine is a DNA vaccine that was used to prevent leishmaniasis. In principle, the gene of interests was inserted into expression vectors, result in constructed plasmid or DNA vaccine. The DNA vaccine was transported to host cells for protein expression and these proteins will induce an immune response against those protein expression antigens[23] *(* Figure 9*)* . The advantage of DNA vaccine are safety, stability, easy to produce in either small or large scales, long-time storage at room temperature and inexpensive [151, 180-186]. The concept of DNA vaccine is shown in Figure 7.

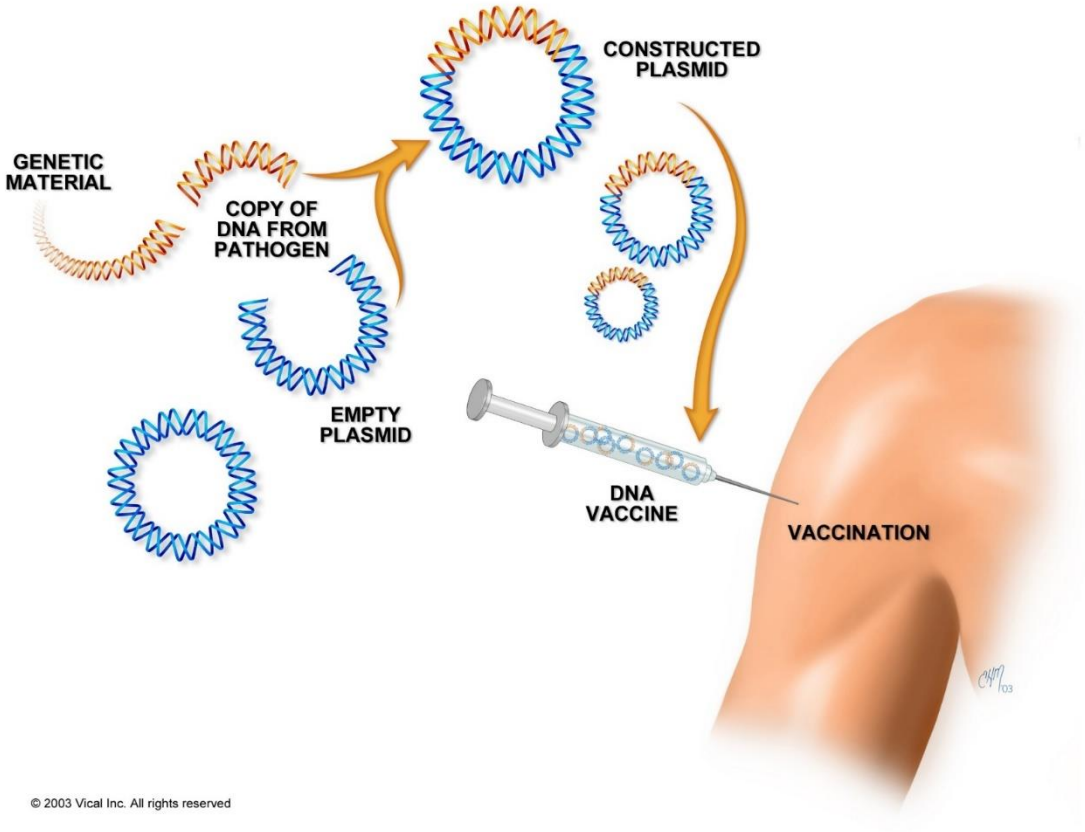

Figure 7 The concept of DNA vaccines

Source: http://www.vical.com/technology/dna-technology/

Nowadays, the various antigenic epitopes of *Leishmania* have been used to develop DNA vaccine, for example, kinetoplastid membrane protein 11 (KMP-11) DNA vaccine which was used three times to immunize intramuscularly (100 µg/dose) in hamsters. It could induce $CD8^+$ and $CD4^+$ T cells against *L. donovani* in both antimony or antimony resistant hamsters and showed high efficiency to protect the parasite challenge compared with an unvaccinated group. Moreover, KMP-11 was confirmed by *Basu, 2007* and his coworkers that KMP-11 expression peptide was able to generate immune responses including *iNOS* and $CD8^+$ T cell responses[187]. LJM19 protein known as *Lutzomyia longipalpis* (sandfly) salivary proteins was also used as the candidate antigen. LJM19 gene was sub-cloned to VR2001 mammalian expression vector, 20 µg of candidate vaccine were used to immunize three times in hamsters. The results showed the potency of vaccine that could induce IFN-gamma and reduced interleukin-10 and partially protected from VL in hamsters [188]. Subsequently, *Da Silva.,* et al*. 2011* used the combination of 100 µg KMP-11 and 10 µg LJM19 vaccine to immunize intradermally three times in hamsters. The results showed that IFN-gamma/IL-10 and IFN-gamma /TGF-beta ratio in the spleen of immunized mice were higher than those in the control. Also, the number of parasites in the liver and spleen were reduced after 2-5 months challenging[189].

The strategy of prime/boost vaccination was also important. The prime/boot strategy selectively increases memory T cells specific to the candidate antigens. Both homologous (prime and boost with DNA) and heterologous immunization (prime with DNA, boosting with recombinant proteins) induced high efficiency of cellular and humoral immune responses [190, 191] The prime-boost immunization strategy was also evaluated by priming 100 µg with leishmanial activate kinase (LACK) DNA vaccine intraperitoneally (IP) and boosting with LACK recombinant vaccinia virus vector. The results showed that it could protect BALB/c mice from *Leishmania* infection. However, when priming-boosting those vaccines in canines only partial protection were reported [189]. *Pereira* and his co-workers, in 2015 showed the potential heterogeneous prime-boost DNA vaccination strategy. An antigenic DNA/protein vaccine candidate from *L.*

infantum was primed in hamsters three times intramuscularly with 100 µg pcDNA-LiPO and boosted intradermally with 10 µg rLiPO protein. The results showed that heterologous prime-boost induced IFN-gamma and TGF-beta after immunization. Interestingly, the vaccinated mice were protected from *L. infantum* challenge [192]. Additionally, the intramuscular immunization with 100 µg of *L. donovani* ribosomal P1 using pcDNA mammalian expression vector in mice inhibited parasite metastasis to the spleen and enhanced $CD4^+$ T cells activation, and the mortality rate in vaccinated mice was decreased[193].

The current efforts to develop DNA vaccines focus on the critical regions of proteins that elicit an immune response. Cysteine proteases are one of the candidate antigens which have been reported that it could enhance immune responses in both murine and canines models [194, 195]. The combination of cysteine proteinase A and cysteine proteinase B antigens heterologous prime-boost was used, the results successfully demonstrated good protection after *leishmania* parasite challenge in both mice and canines [196]. The DNA vaccine delivery method by using nanoparticles has been evaluated by the Sanofi Pasteur company[197]. The nanoparticles were combined with cysteine proteinase type I, II and III pcDNA mammalian expression vector and used as antigens *(*50 µg per dose per each construct*)* to immunize mice. The successful induction of IFN-gamma and protection after challenge with *L. major* was shown [29].Also, the heterologous prime/boost DNA vaccine with CPI and CPII of *L. infantum* in mice provoked IgG_{2a} antibodies, signifying the generation of Th1 immune response[198]. Cysteine proteases are a group of enzymes that present in several organisms for example bacteria, protozoa, insects, mammals, plants, and viruses. These enzymes are correlated with the development of many diseases for example arthritis and cancer [199]. The role of cysteine proteases is occurred within the endocytic pathway (lysosomes and endosomes) and can transport to the nucleus, secretory vesicles, mitochondria, and extracellular or pericellular sites[200]. Even though endocytic pathways have been characterized for most cysteine proteases, the whole mechanisms of cysteine proteases transport to the mitochondria, nucleus, and secretory vesicles are still unclear [200].

Cysteine proteases are also enzymes that are in the members of super papain family and most papain-like cysteine proteases are produced as proenzymes that contain a pre-domain and a matured domain. In some parasites, cysteine proteases also contain a long carboxy-terminal extension (CTE) [201]. The exact function of the CTE is still unclear; however, CTE is thought to be highly immunogenic and enhance the host immune response and act as the proteolytic enzymes which play roles in physiological processes. However, these enzymes can encompass the pathogenesis effect of protozoa infection [202, 203].

Cysteine proteases are normally classified into three types as follows, Cysteine protease *(*CPA, CPII*)*, Cysteine protease b *(*CPB, CPI*)* and Cysteine protease c *(* CPC, CPIII*)* . CPA and CPB are cathepsin L-like while CPC is cathepsin B-like. Cathepsin B-like and cathepsin L-like enzymes have key structural differences correlated with unique substrate binding and proteolytic pathways[204]. CPA is expressed at a higher-level during amastigote developmental stage and can be found in the stationary phase of promastigotes while CPB is normally expressed superfluous in the development stage of amastigotes. Moreover, the expression of CPC associated with the survival of *Leishmania mexicana* in the host cell[205]. Thus, it can be concluded that cysteine proteases are necessary for the *leishmania* life cycle. From the previous study, the heterologous prime/boost with CPI and CPII of *L. infantum* in mice provoked IgG_{2a} antibodies, signifying the generation of Th1 immune response[198].

However, there are limitations to the DNA vaccine platform. It has been recognized that the DNA vaccine has driven significantly weaker immune responses in mice models and humans. It may be due to the less immunogenic vaccine compared with other candidate vaccines such as viral vectors. Therefore, DNA vaccine technology currently conveys the effort to optimize the antigen expression and vaccine immunogenicity. Nowadays, various ways were used for the improvement of DNA vaccine efficacy as shown in Figure 10. The strategies were used to improve DNA vaccines are as follows. 1) In order to increase the interested antigen expression gene(s), the DNA was optimized by modifying the nucleotide sequence in order to avoid

and enhance protein production such as insert Kozak sequence *(* Shine-Dalgarno sequence*)* that used for ribosomal binding regions to increase proteins expression 2) The DNA delivery transfer method improvement, such as gene gun, electroporation, intramuscular injection in order to increase the efficiency to transfer the DNA plasmid into muscular cells. 3) Modifying immune adjuvants, such as the Interleukin-2 nucleotide sequence can be added in the sequence as an immune adjuvant to enhance the immune response. 4) Other methods such as encapsulation DNA with adjuvant, this method is used to protect for extracellular degradation and its help DNA vaccine easily transfect directly to the cytosol of muscular cells [(206)]. The currently DNA vaccine candidates are shown in Table 4.

To enhance the immunogenicity of some purified antigens which were not good immunogens, the addition of adjuvant is required to induce its efficacy. Alum and squalene are the only two adjuvants approved for human vaccines. Although they are potent inducers of humoral immunity, their induction of Th_1 responses which is required for protection against VL is poor. The combination of IL-12 and antigen-induced a strong Th_1 immune response. However, IL-12 is not recommended for human vaccination as it may promote immune disorders. Also, IL-12 cannot induce immunological memory, as seen in mice immunized with LACK *(Leishmania* homolog of receptors for activated C-kinase*)* [(207)]. The adjuvant using in DNA vaccine nowadays, such as, naked DNA or recombinant vaccinia virus, which acts as an adjuvant by activation of the innate immune response via non-methylated CPG sequences. Thus, the DNA vaccine confers protection by a prolonged intracellular expression of the vaccinated antigen.

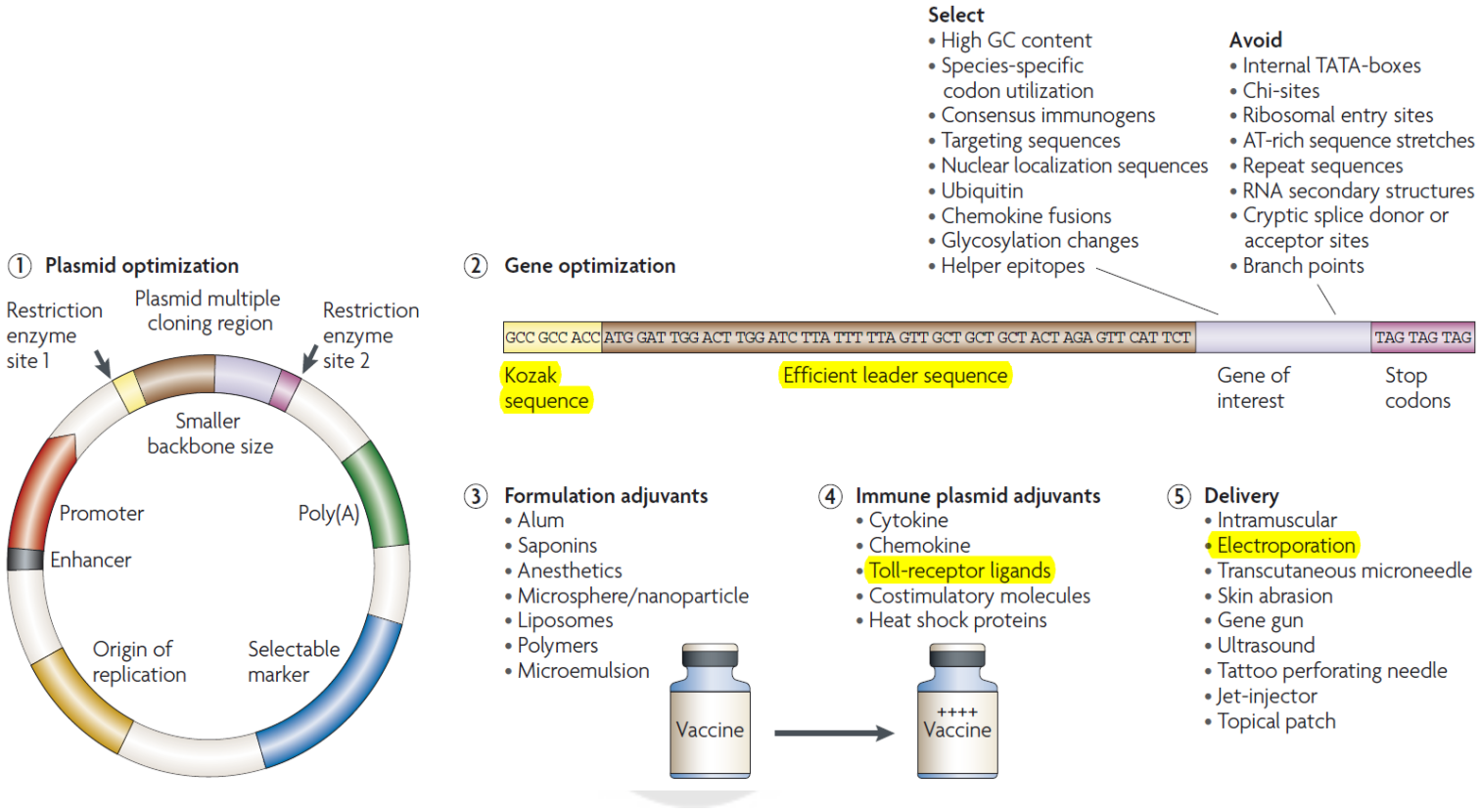

Figure 8 Novel strategies to improve the DNA vaccines

Source: http://www.nature.com/nrg/journal/v9/n10/fig_tab/nrg2432_F1.htm

Monoolein *(*MO*)* is a lipids surfactant and polymer molecules that have both polar and non-polar known as amphiphilic *(*Figure 11*)*. Monoolein is easily very soluble in oil, petroleum ether, chloroform and even warm alcohol [24]. The hydrophobic effect drives amphiphilic molecules into polar solvents to freely self-assemble. The content of water or other solvent molecules changes the self-assembled structures. MO is non-toxic and biodegradable. A general development of phases, working from gradient amphiphile concentration is a discontinuous cubic phase (micellar cubic phase), hexagonal phase (hexagonal columnar phase) (middle phase), lamellar phase, a bi-continuous cubic phase, reverse hexagonal columnar phase inverse cubic phase (Inverse micellar phase). At very low amphiphile concentration, the molecules will be dispersed randomly without any order. At slightly higher (but still low) concentration, amphiphilic molecules will spontaneously assemble into micelles or vesicles [208]. Monoolein was also used as adjuvant, Carneiro et al. 2016 used cell wall surface proteins *(*CWSP*)* of *Candida albicans* formulated with the combination of monoolein and dioctadecyldimethylammonium bromide. The monoolein adjuvant enhanced the immunogenicity against *Candida albicans* in mice models. So, monoolein could be a candidate liposome adjuvant used for vaccination in mice models[209].

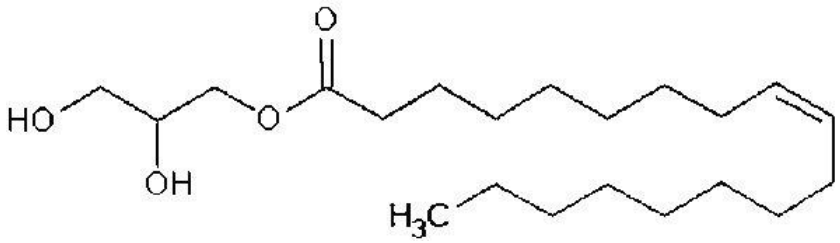

Figure 9 The diagram shown the chemical structure of monoolein

Source:

https://www.researchgate.net/publication/49707624_Monoolein_A_magic_lipid/figures

Table 4 The candidate DNA vaccines were used to study in mice.

Antigen	Mice model	Challenge	Result
Gp46[210]	BALB/c	L. amazonensis	Partially protection
Gp63[211]	BALB/c	L. major	Partially protection
LACK[212]	BALB/c	L. major	Partially protection
PSA-2[213]	BALB/c	L. major	Partially protection
PSA-2[214]	BALB/c	L. major	none
CP[195, 198]	CBA	L. major	Partially protection
ORFF[215]	BALB/c	L. donovani	Partially protection

CHAPTER 3
MATERIAL AND METHOD

3. 1 Design of cysteine protease B DNA sequence and construction.

The cysteine protease b (*cpb)* consensus sequence was generated using BioEdit Sequence Alignment Editor Program, Version 7.0.5.3 from 12 *cpb* nucleotide sequences of autochthonous *L. martiniquensis* strains isolated from Thai patients with VL in Thailand, by three of which were from GenBank (accession number MH752382.1 to MH752384.1) and nine nucleotide sequences were from other VL Thai isolates. The *cpb* consensus sequence was transformed to amino acid sequence (http://www.kazusa.or.jp/codon/) to forecast antigenic amino acid epitopes. The designed humanized chimeric DNA sequences (565 bp) were synthesized by GenScript Company, USA, in addition to the Kozak consensus sequence, initiation translation region and the 6-His tag at the end of *cpb*. The *EcoR*I and *Xho* I recognition nucleotide sequences were added at 5' and 3' end, respectively, as the cloning sites *(*Figure 12*)*. The synthesized *cpb* DNA sequence was inserted in the pVax1™ vector (Invitrogen, ThermoFisher, USA) *(*Figure 13*)*, downstream of Kozak consensus sequence under the control of the CMV promoter. The DNA construct was designated as pcDNA_*cpb (*Figure 14*)* and transformed to competent *E. coli* cells *(*DH5-α strain). The pcDNA_*cpb* clones were extracted using the Endo-Free Plasmid Giga Kit (Qiagen, USA). The *cpb* DNA insert sequence was confirmed in size by gel electrophoresis and the sequences by nucleotide sequence analyzer (Pacific Science, Thailand) using pCMVF upstream primers containing 5'- TAGGCGTGTACGGTGGGAGGAGGTC-3' and pCMVR while the downstream primers contained 3'CTACTCAGACAATGCGATGC-5'.

(----------Humanized cysteine protease B sequence---------)

Figure 10 Design of pcDNA_cpb DNA vaccine constructs

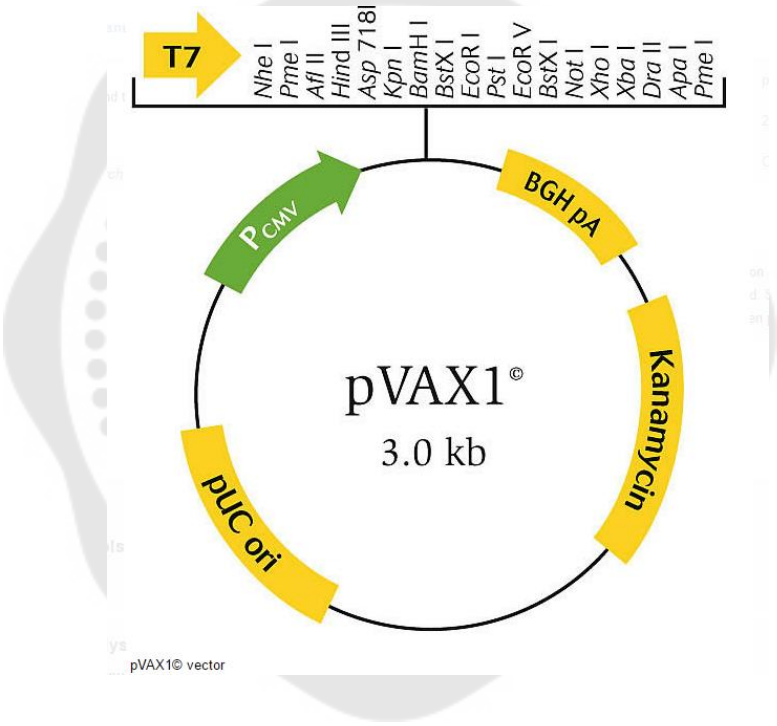

Figure 11 Diagram of the pVax1 mammalian expression vector (Invitrogen, ThermoFisher, USA)

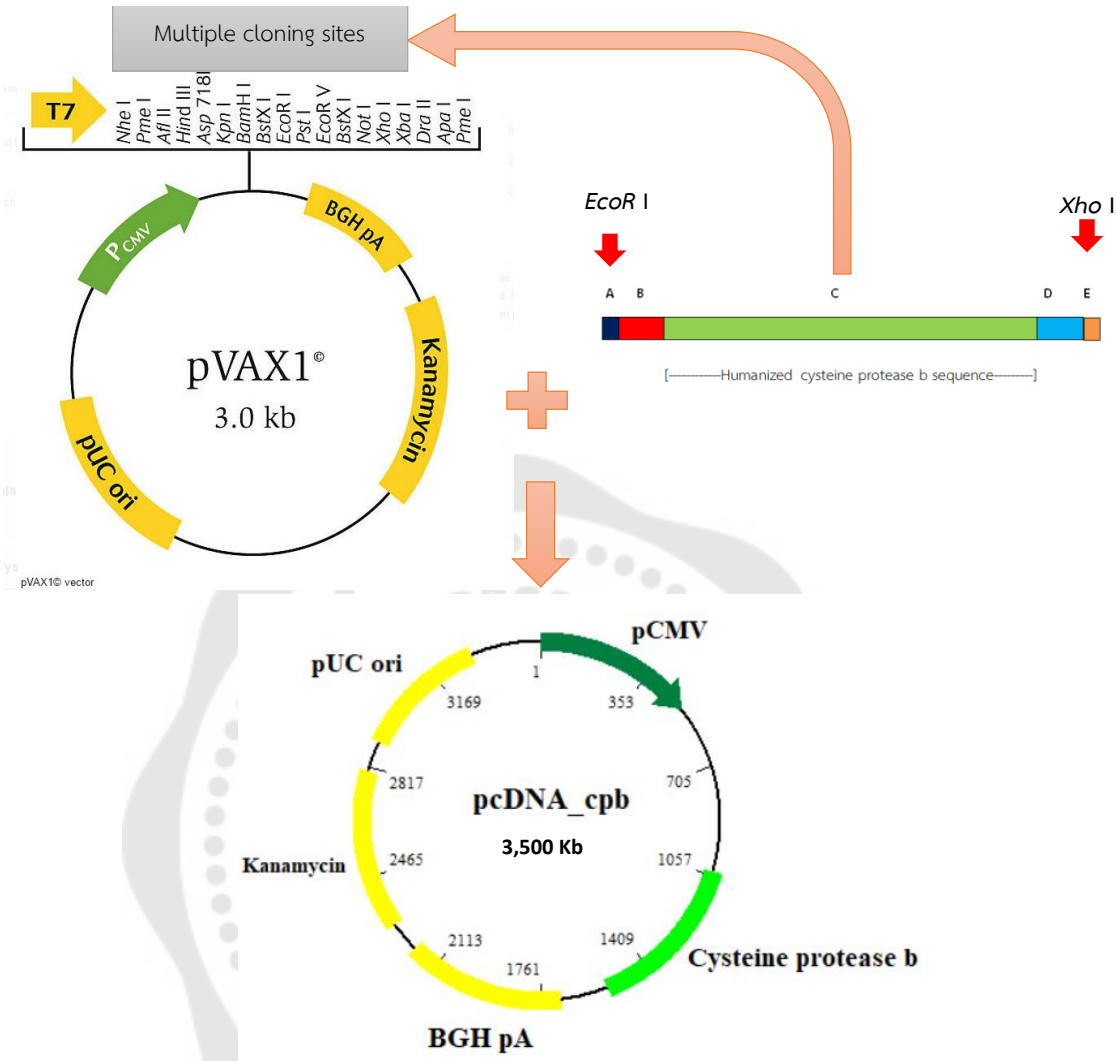

Figure 12 pcDNA_*cpb* DNA vaccine construction

3.2 Sub-cloning procedure

3.2.1 Competent cell preparation

The *E. coli* strains DH5-α was used as a competent cell in this study. In order to prepare the competent cells, the glycerol *E. coli* (DH5-α) stock was slowly thawed on ice and added into 5 mL of LB medium and then incubated overnight at 37 °C in a shaking incubator (250 rpm). Next day, starter *E. coli* was inoculated into 50 mL LB medium *(*1:500 v/v*)* and incubated at 37 °C in a shaking incubator (250 rpm) around 2-3 h or until reach the OD_{600nm} 0.4-0.5. Consequently, the bacterial cells were incubated on ice, 30 minutes and then centrifuged at 4,000 rpm, 10 minutes at 4 °C conditions. The

supernatant was discarded, and then the cell pellet was re-suspended with 10 mL cold sterile 50 nM calcium chloride, left on ice for 30 minutes and subsequently centrifuged at 4,000 rpm. The supernatant was discarded, and the cell pellet was re-suspended in 1 mL of cold sterile 50 mM calcium chloride with 15% glycerol. Aliquots of 100 µL *E. coli* competent cells has been stored at -80 °C until use.

3.2.2 Restriction endonuclease reaction

To prepare the humanized cysteine protease b DNA fragment and Pvax1™ plasmid vector used for ligation, the restriction enzymes, *EcoR* I *and Xho* I, were used. In The microcentrifuge tube were added 5 µl of 5X buffer, 1 µl of restriction enzyme *(EcoR* I, *Xho* I*)* and 1 µg DNA of interest, respectively. Finally, adjust the volume to 20 µl by added ddH_2O. Then, the mixture was incubated at 37 °C for 3 h. After digestion, the digested vector was dephosphorylated to prevent the self-ligation. The restriction products were analyzed by 1% agarose gel electrophoresis.

3.2.3 DNA ligation

The digested *cpb* nucleotide fragment and digested pcDNA mammalian expression vector with compatible restriction sites *(EcoR* I- *Xho* I*)* was ligated by using T_4 DNA ligase *(*Roche, Germany*)*. The ligation mixture ratio of humanized *cpb* fragment and digested pVAX1™ were 8:1. The mixture of ligation were added 10X ligation buffer, dephosphorylated Vector *(100*ng), DNA fragment *(*ng*)*, T_4 DNA ligase, respectively. The final step the ddH_2O were added until the volume was 20 µl. To improve the yield of ligation, the ratio of the *cpb* fragment and digested pVAX1™ should be calculated as the formula below:

$$\text{DNA insert quantity } (ng) = \frac{insert\ (ratio)}{vector(ratio)} \times \frac{vector\ (100\ ng) \times\ insert\ length\ (Kb)}{vector\ lenght\ (kb)}$$

3.2.4 Plasmid transformation

The *E. coli* competent cells were gently thawed on ice. After the competent cells was totally thawed, DNA plasmid was added and chill on ice for 30 min. The

mixture of competent cells and plasmid was quickly put into a water bath at 42 °C (heat-shock) for 90 seconds and immediately put on ice for 10 min. Subsequently, 900 µL of Super Optimal broth with catabolic repression (38) medium was added and shake at 180-200 rpm for 1 h at 37 °C. The suspension of reaction was centrifuged for 2 min at 1500 rpm. All pellets were plated on LB-antibiotic plates. Colonies was observed after incubated for 16-24 h at 37 °C and selected for the transformants.

3.4 Animal experiments

3.4.1 Animal models

Female BALB/c mice, age 3–6 weeks, were purchased from the National Laboratory Animal Center, Bangkok, Thailand. All animal experimental research was approved by the Animal Ethics Committee of Srinakharinwirot University and conducted according to the guidelines of Laboratory Animal Center, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand. The mice were rested for 1 week of adaption and housing under constant room temperature at 25±2 °C, 12 h light-dark cycle at Animal Center, Faculty of Medicine, Srinakharinwirot University, Thailand. During the experiment including immunization and bleeding method, mice were anesthetized by using isoflurane *(*Baxter, IL, USA*).*

3.4.2 Preparation of *Leishmania* parasites.

Leishmania martiniquensis was kindly provided by Professor Dr. Saovanee Leelayoova, by which originally obtained from the skin of infected patient in Thailand and was cultured in Schneider's supplemented with 20% fetal bovine serum and 100 µg/ml of gentamycin at 25°C until early stationary phase. The stationary phase promastigotes were harvested by centrifugation at 2500 rpm (Beckman, Germany) for 15 min at 4 °C. The pellets were washed three times in sterile phosphate buffered saline (PBS) by centrifugation. These parasites were used for animal challenge.

3.4.3 Study design and immunization.

Four groups comprising 5 female BALB/c mice in each group were injected intramuscularly in the tibialis anterior three times in two-week intervals with 50 µL containing plasmid DNA or monoolein (MO, Sigma, USA) in TE buffer. Group I was injected with 50 µL TE buffer containing 100 µg of pcDNA; group II with 50 µg of MO; group III with 100 µg pcDNA_*cpb* and group IV with 100 µg pcDNA_*cpb* plus 50 µg of MO *(*Table 5*)*. For pcDNA_*cpb*-MO preparation, the pcDNA_*cpb* and MO in TE buffer mixture was vigorously vortexed for 5 min, incubated at 60°C for 5 min and this step was repeated three times. Finally, the pcDNA_*cpb*-MO suspension was incubated at 60 °C for 30 min and then placed at room temperature for 30 min before immunization. Mice were mandibular bled at 0, 4 and 9 weeks after the first immunization. Two weeks after the last immunization (at week 6, after the first immunization), all mice were challenged with 50 µl of the early stationary stage of $5x10^6$ L*. martiniquensis* promastigotes via the lateral tail vein. At 21-day post infection, all mice were mandibular bled and sacrificed using isoflurane anesthesia. The liver and spleen of all mice were harvested and kept frozen at -80 ºC until use to determine the parasite burden. The mice immunization plan was shown in Figure 15.

Table 5 Mice immunization plan

Group	DNA vaccines	Immunized dose	Number of mice
1	pcDNA_*cpb*	100 µg	5
2	pcDNA_*cpb* + Monoolein	100 µg	5
3	pcDNA	100 µg	5
4	Monoolein	50 µl	5
5	TE buffer	50 µl	2

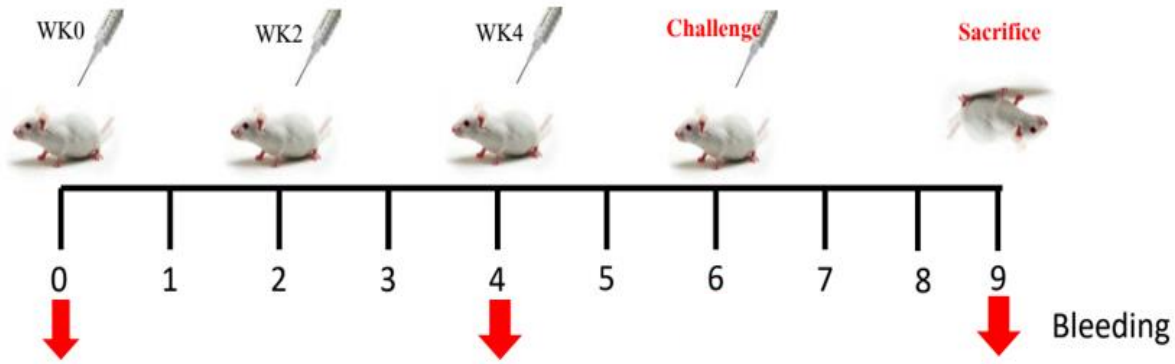

Figure 13 Immunization schedule

3.5 Evaluation of vaccine immunogenicity

3.5.1 The total IgG_1 and IgG_{2a} subclass

Serum samples, collected at 0, 4 and 9 weeks after first immunization, were analyzed for the total IgG_1 and IgG_{2a} antibodies using IgG_1-, IgG_{2a}- Mouse Uncoated ELISA Kit (Invitrogen, ThermoFisher Scientific, USA). Briefly, a 96-well microplate was coated with 100 µl/well anti-IgG_1 or anti-IgG_{2a} at 4ºC overnight and washed twice with washing buffer. Then, 250 µl of blocking buffer was added, incubated at room temperature for 2 h and washed twice with washing buffer. For sample wells, 50 µl of assay buffer B was added, followed by adding 50 µl diluted serum *(1*:10*)* sample and 50 µl diluted HRP-conjugated anti-mouse IgG polyclonal antibody. Then, the plate was sealed and incubated at room temperature with continuous shaking for *2* h (for IgG_1) or 3 h (for IgG_{2a}) followed by further washing. The specific binding was visualized with tetramethylbenzidine (TMB) as a substrate. The absorbance was measured at 450 nm using a microplate reader (Tecan, Switzerland). IgG_1 and IgG_{2a} concentrations were determined using the standard curves.

3.5.2 Quantification of cytokine

Mice sera were determined for IFN-γ, and IL-10 using Mouse IL-10 or IFN-γ DUO-set ELISA Kits (R&D system, USA), In brief, a 96-well plate was coated with 100 µL of either anti-IFN-γ or anti-IL-10, sealed and incubated overnight at room temperature. Each well was washed thrice with 400 µL of washing buffer, incubated with 300 µL of diluent reagent *(1*% BSA in PBS, pH 7.2-7.4*)* for 1 h and washed twice. Then 100 µL of mice serum or standard (recombinant mouse IFN-γ or recombinant mouse IL-10) was added and incubated for 2 h at room temperature, followed by washing twice. After that, 100 µL of anti-IFN-γ or anti-IL-10 detection antibody labeling with biotin was added for 2 h followed by further washing. Then, 100 µL of streptavidin-HRP were added to each well and incubated for 20 min. Finally, 100 µL of tetramethylbenzidine substrate *(*R&D system, USA*)* were added, incubated for 20 min, followed by adding 50 µL of stop solution *(*2 M of H_2SO_4, R&D system, USA*)* and was measured at the 450 nm absorbance. The assay was performed in duplicate wells and cytokines concentrations were determined based on the standard curves.

3.6 Quantification of parasite load

3.6.1 Immunohistochemistry (IHC)

3.6.1.1 Tissue processing

All frozen livers samples used in this research were thawed and embedded in paraffin using an automated tissue processor (Leica TP1050, Leica Instruments GmbH, Nussloch, Germany) and cut in 4 sections that were arranged Super-frost Plus Positively Charged Microscope Slides (Thermo Scientific, USA*)* , incubated at 60°C for 30 minutes and cooled. The experiment was performed at the Forensic Pathology Section, Central Institute Forensic Science, Thailand. To remove liver section paraffin from embedded, tissue sections were three time incubated in xylene (5 min per each), rehydrated in a serial grade of absolute, 95%, and 70% ethanol (5 min per each) and finally twice washed in distill water (5 min per each).

3.6.1.2 Antigen Retrieval and Immunostaining

Antigenic epitopes masked by formalin-fixation were retrieved by incubation in 0.01 M of Citrate-buffer (pH 6.0) (Sigma Aldrich, USA) for 2 min using microwave *(*800 Watts*)* and continuously for 8 min *(*250 Watts*)* , followed by cooling to room temperature in the same buffer. The slide was blocked with 3% hydrogen peroxide for 10 min and rinsed twice with 1X TBS (Tris-buffered saline) washing buffer. The slide was incubated in 5% BSA/0.1% Tween 20 in TBS at 37°C for 2 h and rinsed twice with TBS. Protein casein blocking was performed for 10 min and washed twice with washing buffer, then incubated with 5% of BSA for 2 h and rinsed with washing buffer twice. The mouse anti-*Leishmania* lipophosphoglycan *(* LPG*)* antibody (My BioSource, USA) at concentration 1:10000 in 5% BSA was applied overnight at 4°C in a cold moisture box. After washing, the slide was incubated with the goat anti-mouse secondary antibody for 30 min at room temperature, washed twice, exposed to the Novalink Polymer for 30 min at room temperature, then washed twice using washing buffer and once with deionized water. Diaminobenzidine chromogen was added for 5 min and washed twice with deionized water. Hematoxylin staining is the most commonly used tissue staining for animal histology and routine pathology. The basic dye, of hematoxylin, was stain to basophilic structures, for example nuclei. All slides were counterstained with hematoxylin for 5 min and dehydrated in graded alcohol, xylene substitute and xylene, respectively. All slides were mounted and dried for 2 days.

3.6.1.3 Microscopy

The images resulted from immunohistochemistry reaction of liver tissue section slides were captured under a light microscope using the CellSens Program (Olympus, Japan). The number of *Leishmania* amastigotes and nucleated liver cells were counted from 25 consecutive images captured at 400X magnification using the CellSens Dimension Program (Olympus, Japan). The parasite load was calculated from the number of amastigotes per nucleated liver cell and expressed as a percentage of the amastigote burden. Additionally, non-*Leishmania* infected BALB/c mice (n= 2) was used as negative-LPG binding, the normal control in this experiment.

3.6.2 Quantitative PCR (qPCR)

3.6.2.1 RNA Extraction

To evaluate the quantity of *L. martiniquensis* burden in mice, each of the frozen harvested livers and spleens in all groups were homogenized and extracted for RNA using the PureLink™ RNA Mini Kit (ThermoFisher Scientific, USA). Briefly, fifty mg of tissues were resuspended in 1 mL of TRIzol reagent. Next, 200 µl of chloroform were added and vortexed vigorously for 3 min, and then incubated for 5 min at room temperature. Mixture was then centrifuged at 12,000g for 15 min at 4°C. After that, a clear layer upper phase was gently taken, transferred into a new Eppendorf tube and precipitated by adding 0.7 µl of isopropanol and centrifugation at 12000g for 15 sec at 4°C, discard the flow through and twice time washing the column using 700 µl of washing buffer. after centrifugation at 12,000g for 1 min at 4°C. Then centrifugation at 12000g for 5 min at 4°C. RNA pellet air-dries for 10 min to remove any excess of isopropanol, and then The RNA free water was resuspended in 30 µ into column. RNA concentration was then quantified using the nanodrop machine (ND-1000 Spectrophotometer).

3.6.2.2 cDNA synthesis

To perform quantitative real time PCR. RNA was reverse transcribed into cDNA by using High Capacity cDNA reverse transcription kit ThermoFisher, USA). cDNA is synthesized from RNA extraction using the Reverse Transcriptase (RT) enzyme by random primers, bound to RNA templates, with dNTPs. The cDNA reaction was prepared as described in Table 6. all reactions had the same RNA input (200 ng). The microcentrifuge tube was sealed and centrifuged before process in the thermal cycler, which was pre-set to a program optimized for the High Capacity cDNA reverse transcription kit (Table 7).

Table 6 Reagents and volumes used for cDNA reaction per reaction

Reagent (μL)	Volume (μL)
10X RT Buffer	5
25X dNTP Mix	2
10X RT Random Primers	5
Reverse Transcriptase	2
Nuclease-Free water 10,5	10
RNA	25
Total	50

Table 7 The thermal cycler program optimized for the High Capacity cDNA reverse transcription kit

	Step 1	Step 2	Step 3	Step 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10	120	5	∞

3.6.2.3 qPCR assay

The qPCR protocol was performed as previously described (Jungudomjaroen *et al.* 2015). The specific primers were designed using the rRNA gene of the ITS1 region of *L. martiniquensis* (accession no. KM677931). The forward and reverse primers sequences were 5'-CGATATGCCTTTCCCACACAC-3' and 5'-CTGTATACGCG CGGCATTTG-3' respectively. The qPCR experiment was performed using CFX96™ Optical Reaction Module (Bio-Rad, USA). All samples were performed in duplicate in a total volume of 20 μL/reaction as shown in Table 8. The PCR amplifications started with duplicate steps of denaturation at 50°C for 2 min and 95°C for 10 min, respectively. The subsequent reaction was conducted for 40 cycles of 30 sec at

95°C, 30 sec at 61°C, 30 sec at 72°C followed by the last step consisting of 5 sec at 65°C and 50 sec at 95°C. The PCR expected size was 128 bp. The fluorescence intensity was recorded as Cq. A standard curve was set up using a serial 10-fold dilution of cDNA, reverse transcribed from RNA extracted from in vitro L. martiniquensis promastigotes with a range from 5X107 to 5X104 parasites. All Cq values of samples were used to calculate the parasite number with the standard curve.

Table 8 Reagents and volume used for qPCR assay per reaction

Reagent (µL)	Volume (µL)
LeishF	0.2
LeishR	0.2
SsoAdvanced™ Universal SYBR®Green Supermix	10
DNA Template	2
Distilled H_2O	7.6
Total	20

3.7 Statistical analysis

The comparisons of all parameter values between groups of vaccinated and control mice were analyzed by GraphPad Prism Software, Version 7.0 *(*La Jolla, USA*)*. The statistical analysis was performed using the 2-tailed nonparametric Mann-Whitney U test, by which a *p*-value <0.05 was considered significant difference.

CHAPTER 4
RESULTS

4.1 The construction of pcDNA_*cpb* vaccine

The *cpb* nucleotide sequences of *L. martiniquensis* obtained from 12 Thai and Myanmar patients in Thailand and from GenBank were converted to amino acid and analyzed. The consensus sequence was constructed, and sub cloned into pcDNA mammalian expression vector. The result showed that the amino acid sequences of 12 autochthonous isolates was 90% homology (Figure 16).

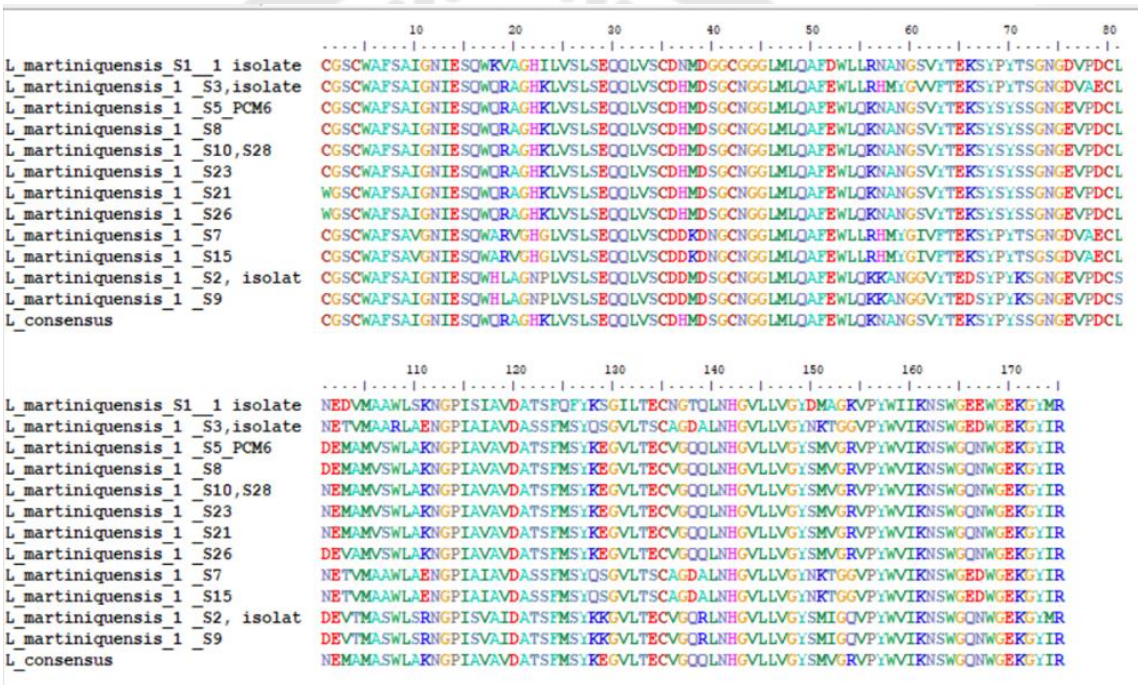

Figure 14 The alignment of CPB amino acid sequences of *L. martiniquensis* isolated from 12 Thai and Myanmar patients. The lowest-line sequence showed the consensus sequence of CPB which was used for humanized codon transformation to DNA sequence.

The synthesized humanized codon of partial consensus *cpb* coding region consisting of 525 bp nucleotide sequences with additional Kozak and 6-His tag sequences, in a total of 565 bp, was inserted in the pVaxI™ vector and cloned in the *E.*

coli host *(*Figure 14*)*. The modification sequence of *cpb* sequence was shown in figure 17

GAATTCACCATGGTGCGGTTCATGCTGGGCTTTTTCTGCGATTGGAAACATTGAGAGTCAGTGGCAGCGGGCAGG
CCATAAATTGGTAAGTCTTTCAGAACAGCAACTGGTATCCTGTGACCACATGGACAGTGGATGCAACGGCGGACT
CATGCTCCAGGCTTTTGAATGGCTGTTGCGCAACGCCAACGGATCTGTGTATACTGAGAAGTCCTACCCGTATAC
GTCTGGTAATGGAGATGTTCCAGACTGCCTTGAGTCTGGAGATCTTGTGGTAGGGGCGCAAATAGACGGGTACGT
GACAATACAAAGTAACGAAATGGTTATGGCGGCGTGGTTGGCAAAGAATGGTCCAATAGCCATAGCAGTTGACGC
CACTTCATTCATGAGTTACAAGTCCGGCGTTCTTACTGAGTGCGTAGGCCAACAGCTTAATCATGGCGTATTGCT
CGTGGGGTACAACATGGTAGGCCGCGTACCTTACTGGGTGATTAAAAATAGCTGGGGCGAGGACTGGGGAGAAAA
GGGTTACATACGCCATCATCACCACCACCATTAGCTCGAG

Restriction site

ACCATGG = **Kozak sequence**

GAATTC = **EcoRI I [Buffer 2]**

CATCATCACCACCACCATTAG = **Histidine-tag [for protein purification in the future]**

CTCGAG = **Xho I [Buffer 2]**

Figure 15 The schematic of modified *cpb* sequence for sub-clone to pVAX™ vector

The *cpb* recombinant vector was confirmed in size by gel electrophoresis (Figure 18) and DNA sequencing (data not shown).

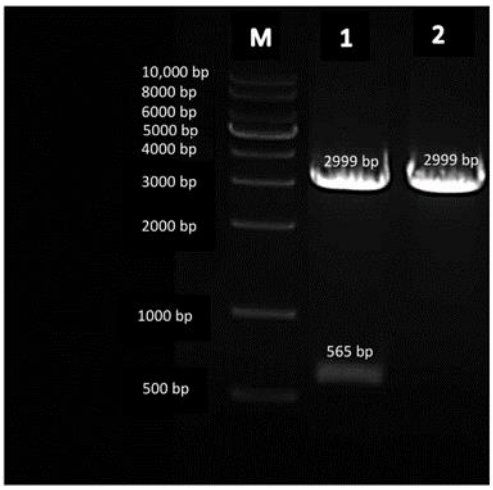

Figure 16 Agarose gel electrophoresis of pcDNA_*cpb* and pVax1™ plasmid DNA after *Xho* I and *Eco*R I digestion; *cpb* insert size was 565 bp. M*:* Hyper ladder 10 kb marker, Lane 1*:* pcDNA_*cpb*, Lane 2*:* Empty pVax1™ vector.

4.2 Total IgG_1 and IgG_{2a} antibodies in BALB/c mice after immunization

Mice sera collected at different time points after immunization with pcDNA, monoolein pcDNA_*cpb,* and pcDNA_*cpb*-MO *(*Figure 15*)* were investigated for total level sera of IgG_1 and IgG_{2a} antibodies as shown in Figure 19A and Figure 19B, respectively and data was summarized as shown in Table 9. In mice immunized with pcDNA, on week 0 before immunization, the total mean sera specific to IgG_1 and IgG_{2a} levels were 71.2 ng/ml and 21.40 ng/ml, respectively, two weeks (week 4) after second immunization were 64.2 ng/ml and 121.42 ng/ml, respectively, and five weeks after third immunization and three weeks after challenge (week 9) were 95.2 and 116.53 ng/ml, respectively.

The sera IgG_1 of monoolein vaccinated mice on week 0, 4, and 9 showed similar levels, whereas sera IgG_{2a} on week 0, 4, and 9 were slightly increased. However, this was not statistically significant *(*Table 9*)*. In mice immunized with pcDNA_*cpb*, the total mean sera specific to IgG_1 levels were increased gradually and statistically significant difference from week 0 to week 4 and week 4 to week 9 as the following: IgG_1: week 0, 99.8 ng/ml and week 4, 187.2 ng/ml (P<0.01); week 4, 187.2 ng/ml and week, 9 282.4 ng/ml (P<0.01). Similarly, in mice immunized with pcDNA_*cpb*-MO, the total mean sera specific to IgG_1 levels were increased gradually and statistically significant difference from week 0 to week 4 and week 4 to week 9 as the following: IgG_1: week 0, 64.6 ng/ml and week 4, 276.6 ng/ml (P<0.01); week 4, 276.6 ng/ml and week 9, 292.4 ng/ml (P<0.01). This indicated that pcDNA_*cpb* and pcDNA_*cpb*-MO could stimulate immune system and had booster effect. In addition, the total mean sera between pcDNA_*cpb* and pcDNA_*cpb*-MO vaccinated mice of IgG_{2a} was higher than IgG_1 *(*week 4 and week 9*)*. Moreover, mice immunized with pcDNA_*cpb*-MO showed higher sera IgG_1 and IgG_{2a} antibodies levels than mice immunized with pcDNA_*cpb* (IgG_1 P<0.01, IgG_{2a} P<0.01). Conversely, on week 9 mice immunized with pcDNA_*cpb*-MO showed slightly higher sera IgG_{2a} and showed statistically significant than mice immunized with pcDNA_*cpb* *(P*<0.01*)* , however IgG_1 antibodies levels was not statistically significant difference (IgG_1 P = 0.64).

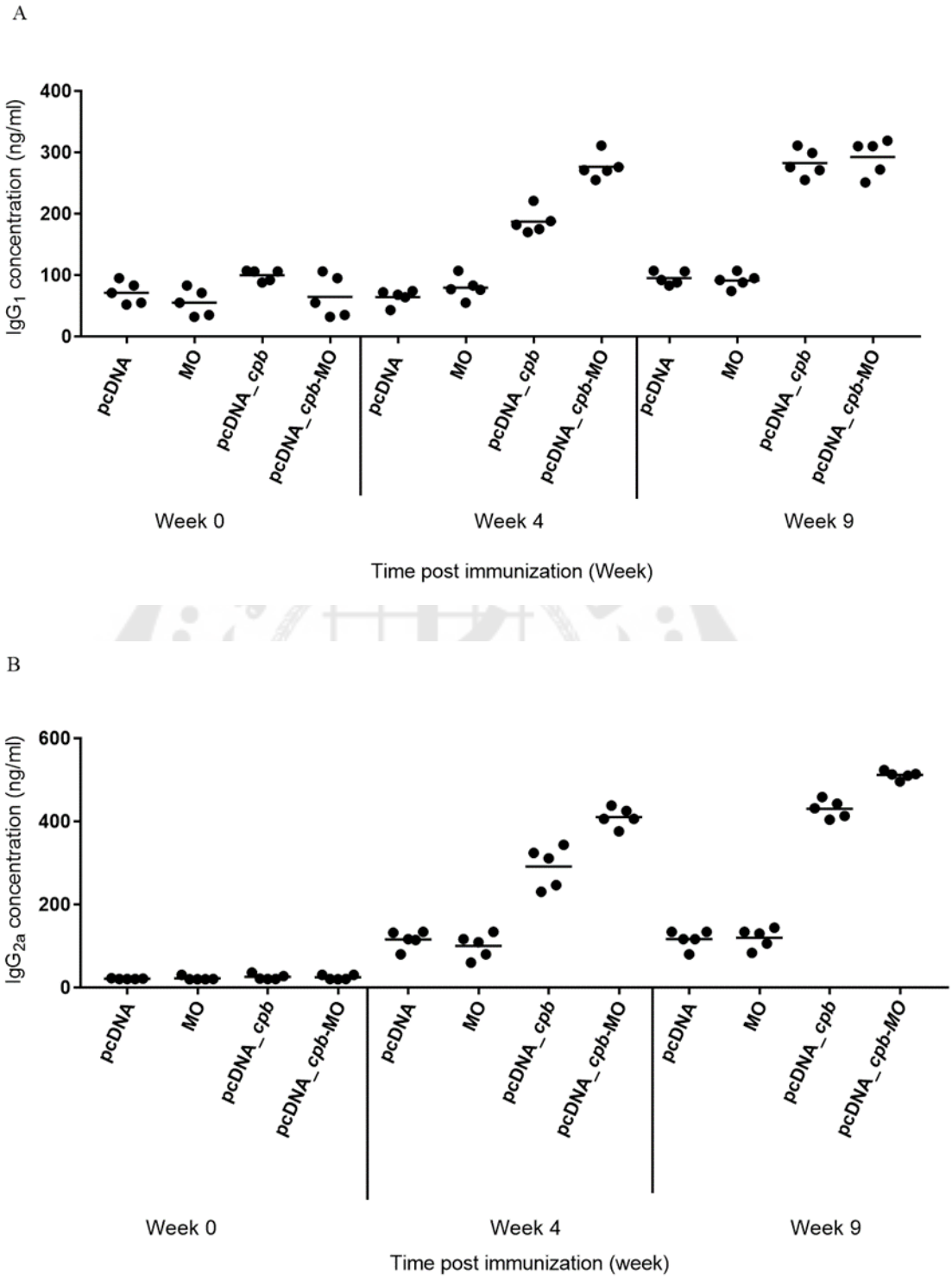

Figure 17 IgG_1 (A) and IgG_{2a} (B) responses in BALB/c mice sera (n=5) after immunization with pcDNA_*cpb*, pcDNA_*cpb*-MO compared with the pcDNA vector and MO control groups.

Table 9 The total IgG_1 and IgG_{2a} concentration quantitated from mice sera collected at different time points.

Group	The mean sera concentration of IgG_1 (ng/ml)			The mean sera concentration IgG_{2a} (ng/ml)		
	Week 0	Week 4	Week 9	Week 0	Week 4	Week 9
pcDNA	71.2	64.2	95.2	21.40	121.42	116.53
Monoolein	55.2	79.6	91.2	22.39	100.16	119.72
pcDNA_*cpb*	99.8	187.2[a, b]	282.4[a, c]	25.5	291.32[a, b]	430.12[a, c]
pcDNA_*cpb*-MO	64.6	276.6[a]	292.4[a]	24.6	410.26[a]	511.54[a, d]

The results are shown as mean + SEM. [a] indicates statistically significant difference compared with the control group (pcDNA or monoolein) in the same week at $p<0.01$. [b] indicates statistically significant difference compared with the pcDNA_*cpb*-MO in the same week at $p<0.01$. [c] and [d] indicate statistically significant difference between week 4 and week 9 of pcDNA_*cpb* and pcDNA_*cpb*-MO at $p<0.01$, respectively.

4.3 pcDNA_*cpb* vaccine induced the production of IFN- γ and IL-10

Sera IFN-γ and IL-10 concentration were determined at week 0, 4 and 9 as shown in Figure 20 and Figure 21, respectively and data was summarized as shown in Table 10. The mean sera IFN-γ in mice immunized with pcDNA on week 4 were slightly higher than sera IFN-γ levels on week 0 (week 0, 23.04 pg/ml, week 4, 52.04 pg/ml) as shown in (Table 10). The mean sera IFN-γ levels on week 9 (214.90 pg/ml) was statistically significantly higher than sera IFN-γ levels on week 4 ($P<0.01$). In mice immunized with pcDNA_*cpb*, the mean sera IFN-γ levels were sequentially increased from week 0 to week 4 and week 9 (week 0, 16.24 pg/ml, week 4, 256.19 pg/ml, week 9, 519.41 pg/ml). The increasing levels were statistically significant difference ($P<0.01$ for week 0 to week 4, $P<0.01$ for week 4 to week 9). Similarly, in mice immunized with pcDNA_*cpb*-MO, the mean sera IFN-γ levels were sequentially increased from week 0

to week 4 and to week 9 (week 0, 24.29 pg/ml, week 4, 270.16 pg/ml, week 9, 573.58 pg/ml). The increasing levels were statistically significant difference (P<0.01 for week 0 to week 4, P<0.01 for week 4 to week 9). When compared the mean IFN-γ levels between mice immunized with pcDNA_*cpb* and pcDNA_*cpb*-MO, the mean IFN-γ levels on week 4 and week 9 between those group of mice did not show significant difference *(*P>0.05 for week 4, P>0.05 for week 9) *(*Figure 20*)*. However, mice immunized with pcDNA_*cpb* or pcDNA_*cpb*-MO showed statistically significant higher mean sera IFN-γ levels than mice immunized with pcDNA or monoolein on both week 4 and week 9 (P<0.01 for week 4, P<0.01 for week 9) *(*Table 10*)*.

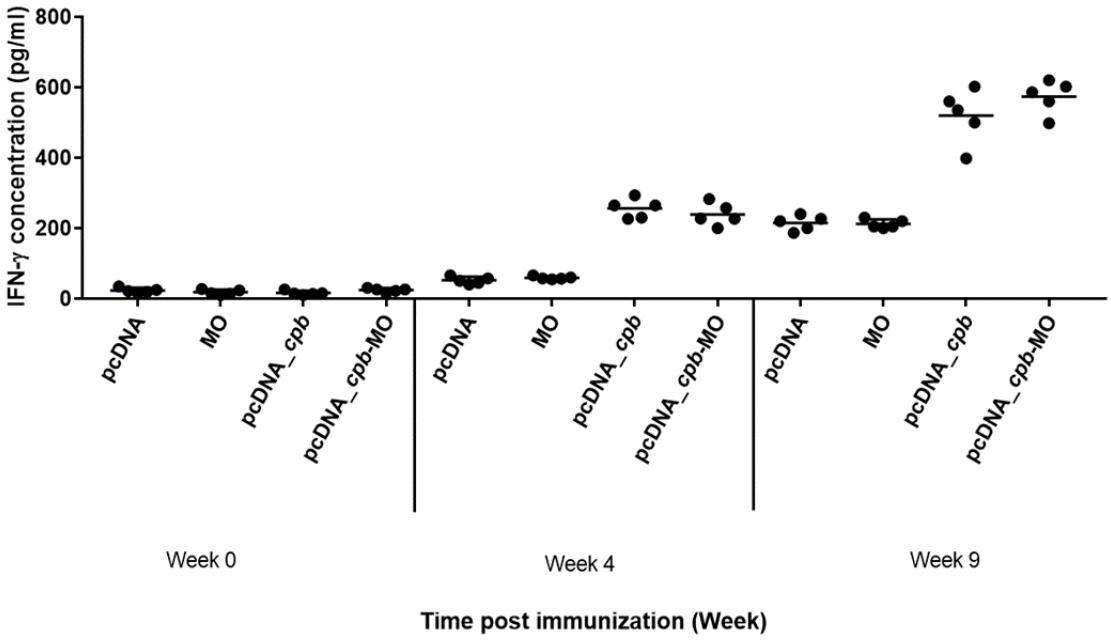

Figure 18 IFN-γ production in BALB/c mice after immunization thrice with pcDNA_*cpb*, pcDNA_*cpb*-MO, pcDNA or MO.

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Table 10 The mean sera concentration of IFN-γ and IL-10

Group	The mean sera concentration of IFN-γ (pg/ml)			The mean sera concentration IL-10 (pg/ml)		
	Week 0	Week 4	Week 9	Week 0	Week 4	Week 9
pcDNA	23.04	52.04	214.90[E]	23.30	58.83	513.15[E]
monoolein	18.28	59.19	212.19[F]	27.56	56.79	564.96[F]
pcDNA_*cpb*	16.24	256.19[a]	519.41[a, c]	16.11	119.46[a]	233.96[a, c]
pcDNA_*cpb*-MO	24.29	239.16[a]	573.83[a, d]	20.72	116.37[a]	206.31[a, d]

The results are shown as mean ± SEM. [a] indicates statistically significant difference compared to the control group (pcDNA or monoolein) in the same week at $p<0.01$. [b] indicates statistically significant difference compared to the pcDNA_*cpb*-MO in the same week at $p<0.01$. [c] and [d] indicate statistically significant difference between week 4 and week 9 of pcDNA_*cpb* and pcDNA_*cpb*-MO at $p<0.01$, respectively. [E] and [F] indicate statistically significant difference between week 4 and week 9 of pcDNA and monoolein at $p<0.01$.

Conversely, the mean sera IL-10 levels in mice immunized with pcDNA and monoolein on week 4 were slightly higher than sera IL-10 levels on week 0 (week 0, 23.30 pg/ml, week 4, 58.83 pg/ml) (Table 10). The mean sera IL-10 levels on week 9 (513.15 pg/ml) were statistically significantly higher than sera IL-10 levels on week 4 ($P<0.01$) (Figure 21). Similar to IFN-γ levels, in mice immunized with pcDNA_*cpb*, the mean sera IL-10 levels were sequentially increased from week 0 to week 4 and week 9 (week 0, 16.11 pg/ml, week 4, 119.46 pg/ml, week 9, 233.96 pg/ml). The increasing levels were statistically significant difference ($P<0.01$ for week 0 to week 4, $P<0.01$ for week 4 to week 9) (Figure 21). In mice immunized with pcDNA_*cpb*-MO, the mean sera IL-10 levels were sequentially increased from week 0 to week 4 and week 4 to week 9 (week 0, 20.76 pg/ml, week 4, 116.37 pg/ml, week 9, 206.31 pg/ml). The increasing

levels were statistically significant difference. (P<0.01 for week 0 to week 4, P<0.01 for week 4 to week 9) (Figure 21). When compare the mean IL-10 levels between mice immunized with pcDNA_*cpb* and pcDNA_*cpb*-MO, the mean IL-10 levels on week 4 and week 9 between those group of mice did not show significant difference (P= 0.69 for week 4, P= 0.42 for week 9) *(*Figure 21*)*.

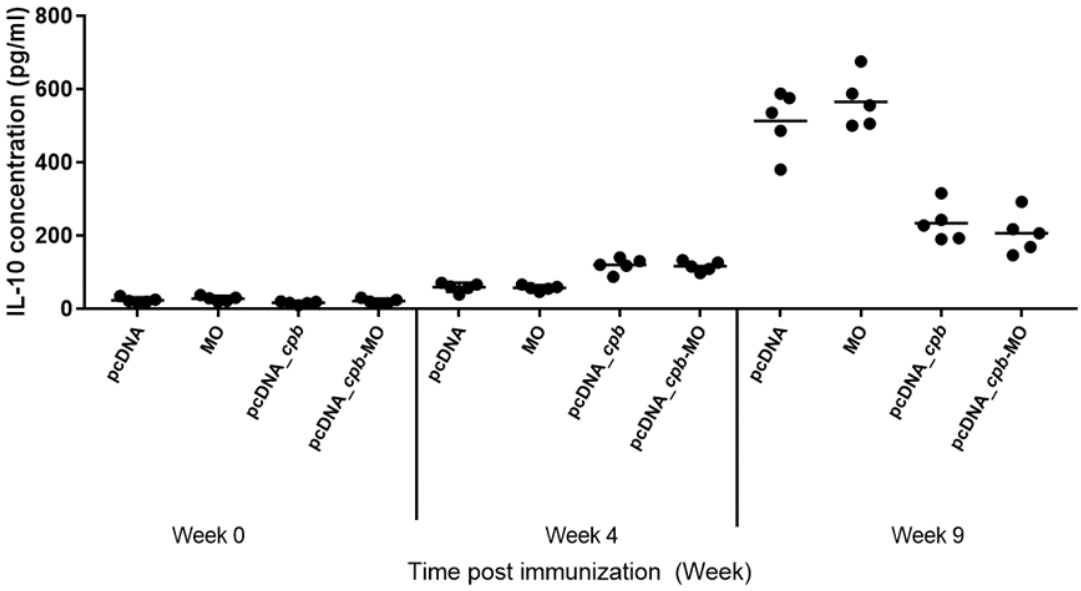


Figure 19 IL-10 levels in BALB/c mice after immunization thrice with pcDNA_*cpb*, pcDNA_*cpb*-MO, pcDNA or MO.

Nowadays, the robust production of IFN-γ is not argued for parasite clearance. The IFN-γ knockdown mice fail to clear parasites[(216)]. The mainly issues of a leishmanial vaccine development is to identify the crucial immunological correlating to the protection. The previous study showed the ratio of IFN-gamma: IL-10 was important associated with the persistence of parasite in mice. thus, the ratio of IFN-gamma: IL-10 is pre-challenge indicator of vaccine success [(217)]. When compared the mean IFN-γ and IL-10 levels between mice immunized with pcDNA or monoolein and mice immunized with pcDNA_*cpb* or pcDNA_*cpb*-MO. On week 4, mice immunized with pcDNA_*cpb* or pcDNA_*cpb*-MO showed statistically significant higher mean sera IFN-γ and IL-10

levels than mice immunized with pcDNA or monoolein (*P*<0.01 IFN-γ, *P*<0.01 IL-10), whereas on week 9, mice immunized with pcDNA_*cpb* or pcDNA_*cpb*-MO showed statistically significant higher IFN-γ but lower IL-10 levels than mice immunized with pcDNA or monoolein on week 9 (*P*<0.01 IFN-γ, *P*<0.01 IL-10) (Figure 21). Figure 22 shows the IFN-γ / IL-10 ratio which indicated that mice vaccinated with pcDNA_*cpb or* pcDNA_*cpb*-MO produce higher IFN-γ but lower IL-10 levels than negative control mice which vaccinated only pcDNA or monoolein.

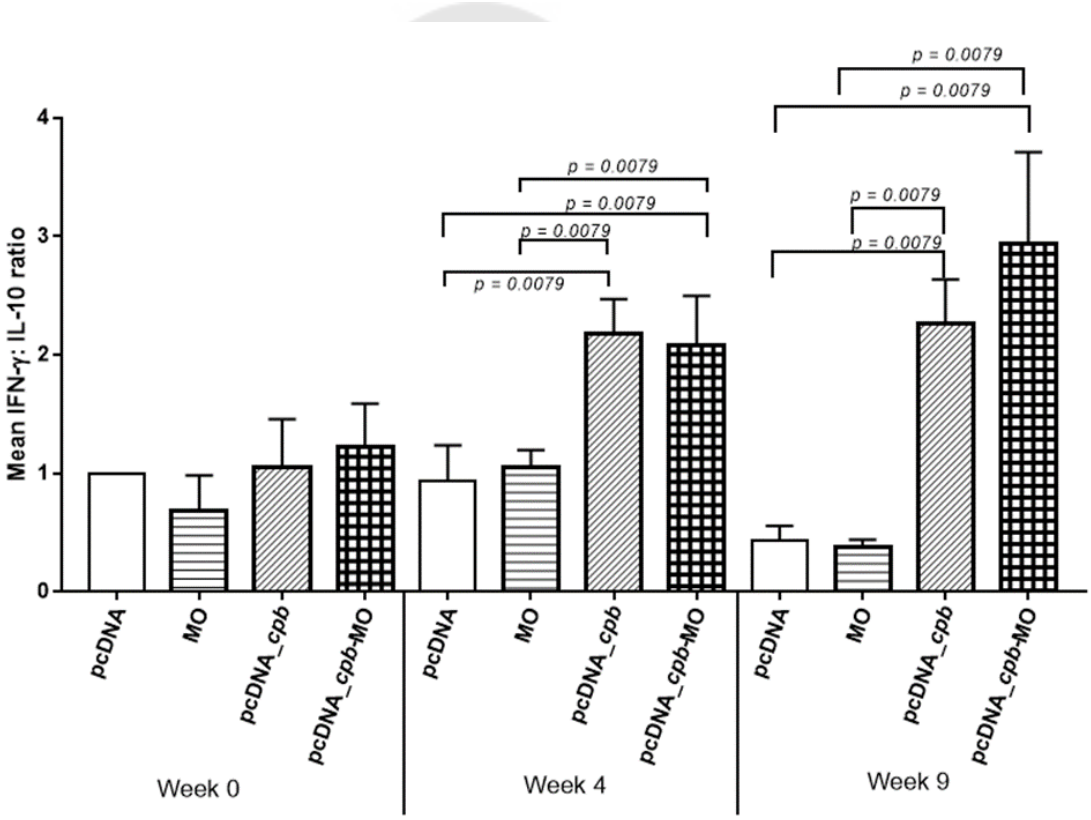


Figure 20 IFN-γ/IL-10 ratios calculated from IFN-γ and IL-10 concentrations of each experimental group at different time points. The bars represent the statistically significant comparison among groups using the Mann-Whitney U test at *p*<0.05.

4.4 Immunohistochemistry

Regarding the immunohistochemical staining of liver tissue sections using an anti-LPG antigen, the major cell surface glycoconjugate of *Leishmania* parasites revealed the potential protection of the pcDNA_*cpb* and pcDNA_*cpb*-MO groups (Figure 23). The anti LPG bound to the amastigote surface was positively stained with Diaminobenzidine chromogen appeared as brown (Figure 23A) while the nucleated cells such as nucleated liver cells, Kupffer cells and dendritic cells were counter-stained with hematoxylin and shown in blue. The 1000X- magnification of the liver tissue section (Figure 23A) demonstrated the anti-LPG positively stained *L. martiniquensis* amastigote (brown stained) in the liver cell nucleus (blue stained). The percentage of amastigote burden in the liver tissue compared with nucleated cells was used to show parasite burdens in the livers of animal groups. The 400X- magnification of pcDNA, MO, pcDNA_*cpb* and pcDNA_*cpb*-MO livers tissue cross section was compared (Figure 23B, C, D and E, respectively). The livers of mice immunized with pcDNA_*cpb* and pcDNA_*cpb*-MO demonstrated decreased brown-stained amastigote burden in liver tissues compared with control groups; pcDNA or MO. The granulomas were observed in all parasite challenge groups, by which most involuted granuloma *(*IVG*)* and mature granuloma *(*MG*)* were observed in pcDNA_*cpb* with MO and without MO groups, respectively *(*Figure 23B and Figure 23D*)* This result indicates the enhancement of IFN-γ and the minute of IL-10 related with the effective killing from the activated macrophage by the production of nitric oxide play critical role to execute amastigote[145]. The percentage of amastigote burden counted from 25 consecutive images of pcDNA_*cpb* and pcDNA_*cpb*-MO groups were 60.1 and 61.3, respectively whereas the non-vaccinated mice, pcDNA and MO showed 83.3 and 90.87, respectively (Figure 24A).

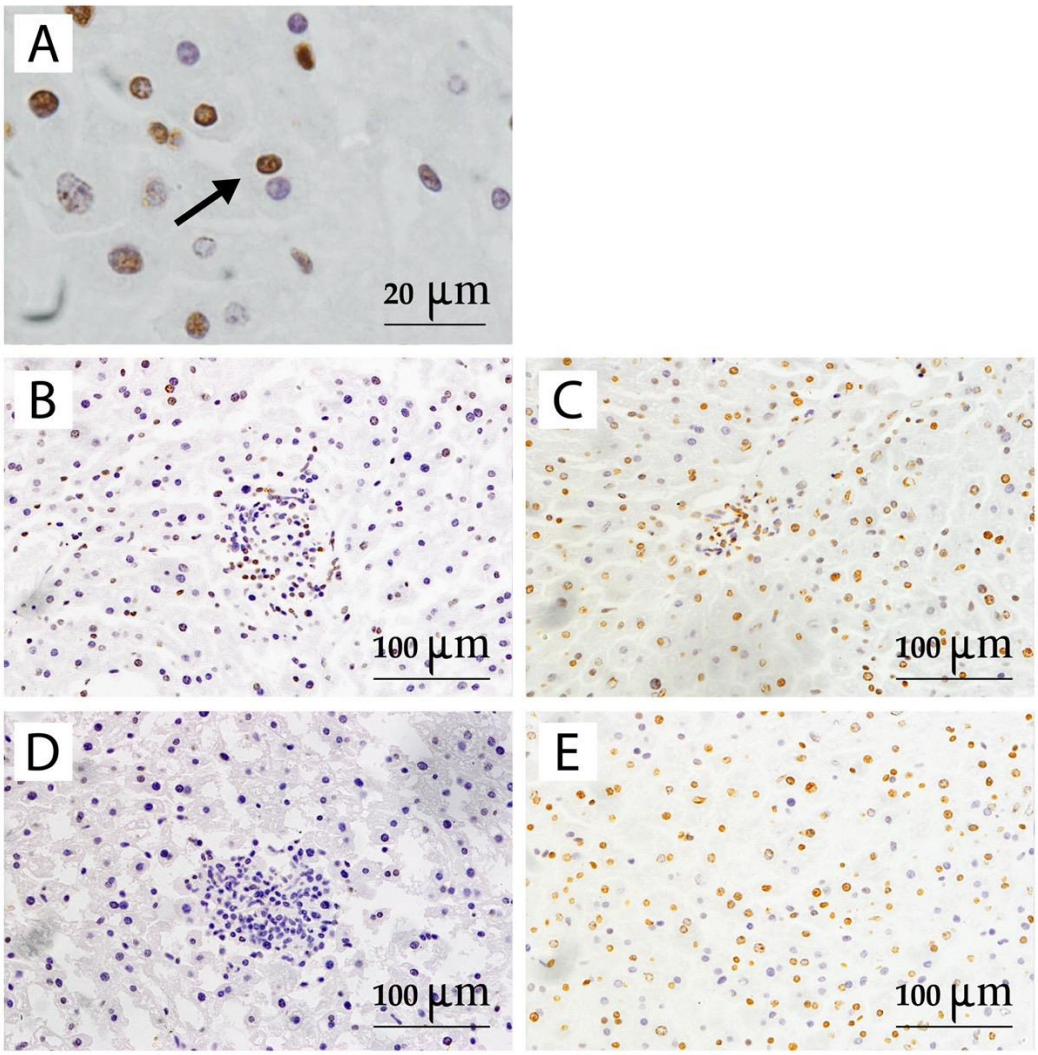


Figure *21* immunohistochemical images of vaccinated and non-vaccinated mice after three weeks challenged with L. martiniquensis promastigotes. The liver sections of mice were stained with anti-LPG monoclonal antibody, developed with DAB and counterstained with hematoxylin. The brown spots indicated positively stained L. martiniquensis amastigotes and the nucleated cells were shown in blue color. The 1000X magnification of the pCDNA_cpb liver section shown in Figure 23A demonstrated the positive anti-LPG binding amastigotes (arrow). The 400X magnifications are shown in Figure 23B, C, D, and E *(*pcDNA, MO, pcDNA_cpb and pcDNA_cpb-MO, respectively). The mature- and involuted-granuloma formations are observed in D and E.

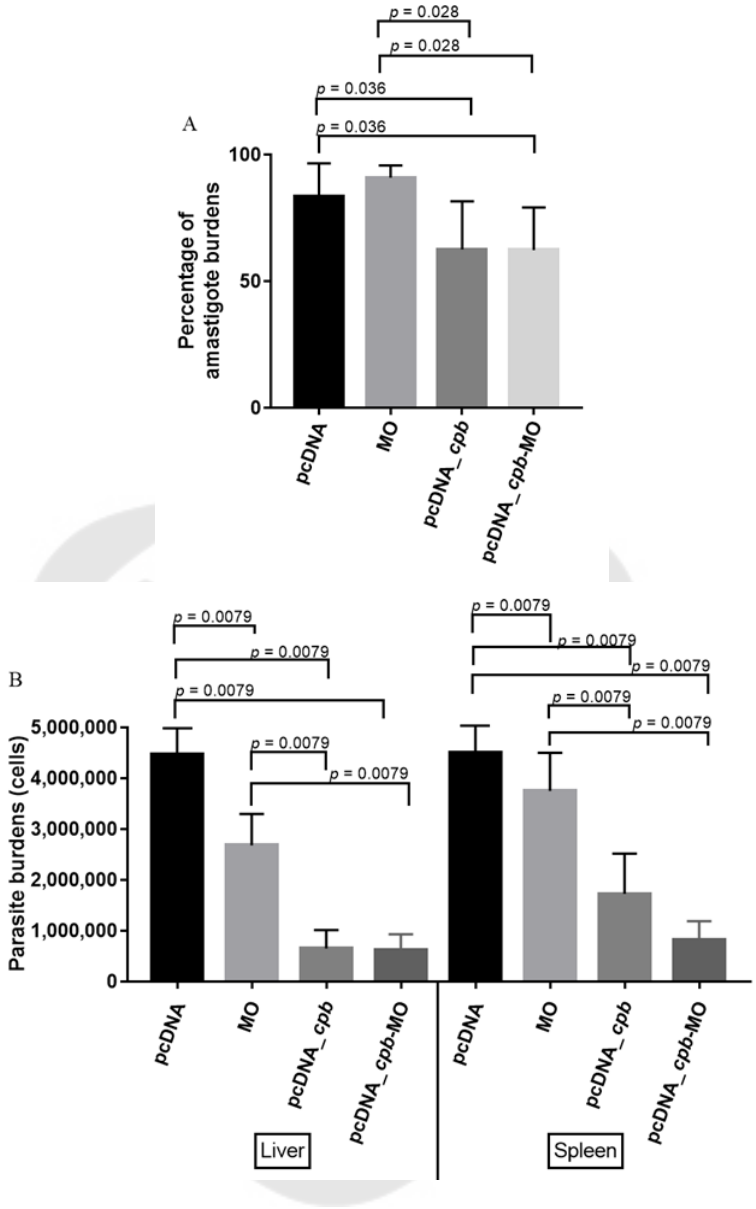


Figure *22* Comparative study of parasite loads in the livers or spleens of vaccinated and non-vaccinated mice groups after 21 days post-infection investigated by immunohistochemistry staining experiments (A*)* and qPCR (B*)*. The bars represent the statistically significant comparisons among groups, analyzed using the Mann-Whitney U shows test at $p<0.05$.

4.5 Quantitative PCR

After challenging at week 9, the livers of mice were harvested to quantitate the *L. martiniquensis* amastigotes by qPCR. The extracted RNA of the liver samples and *in vitro* culture of promastigotes were reverse transcribed to cDNA and used for amplification. The standard curve obtained from a plot of the quantification cycle *(C_q)* against the logarithm of starting parasite DNA was linear in the range test *(R^2 = 0.950)* (Figure 25). Figure 24B shows the quantity of parasite DNA of the mice liver samples derived from the qPCR standard curve. The average C_q of mice receiving pcDNA_*cpb* or combined with MO was 30.20 and 30.13, respectively whereas control mice receiving only pcDNA or MO were 27.09 and 27.81, respectively. Based on a standard curve, the C_q of mice were converted and used to evaluate parasite numbers. The average parasite burden of pcDNA_*cpb* and pcDNA_*cpb*-MO were $5.04x10^5$ and $7.36x10^5$ cells, respectively. In contrast, pcDNA and MO were $4.2x10^6$ and $2.9x10^6$ cells, respectively

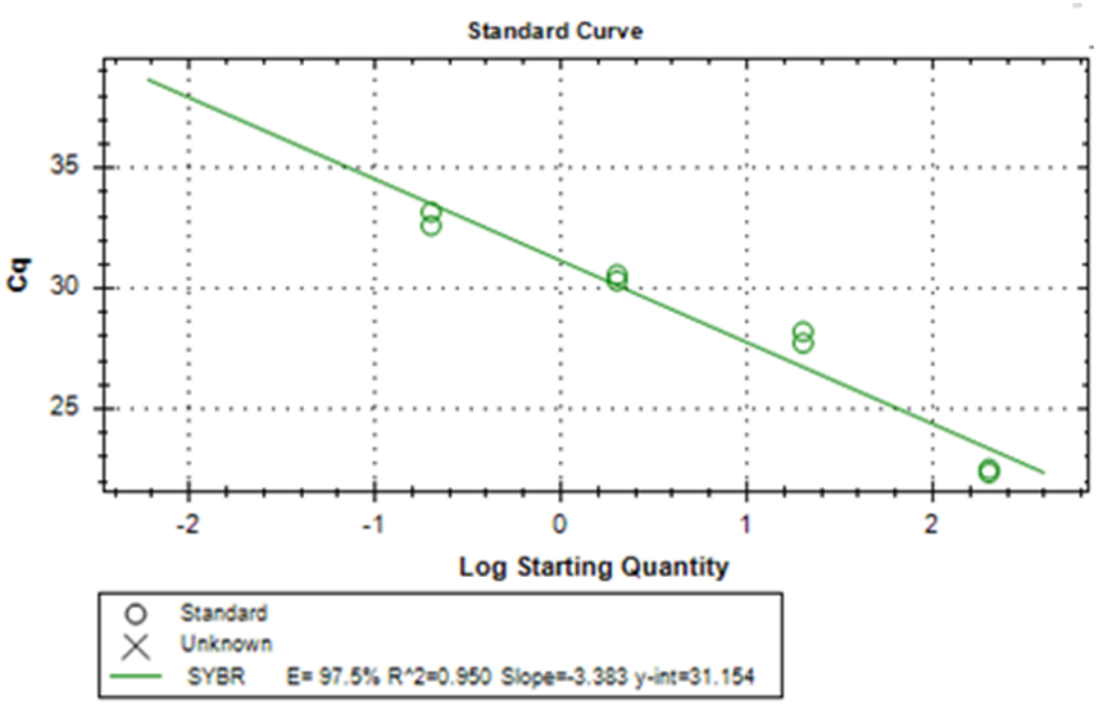


Figure 23 Standard curve of qPCR obtained from a plot of the quantification cycle *(C_q)* against the logarithm of starting L. martiniquensis parasite DNA. The curve was linear in the range test *(R^2 = 0.950)*.

The comparative results of parasite burdens obtained from the amastigote burden counting from immunohistochemical studies (Figure 24A) and qPCR (Figure 24B) showed good correlation of the decreased parasite burden in the vaccinated group with significant difference to controls (pcDNA and MO) groups. However, no significant difference was observed in parasite burden between the pcDNA_*cpb* with and without MO mice groups. From these results, we could conclude that the pcDNA_*cpb* vaccine could induce potential protection against *L. martiniquensis* infection.

CHAPTER 5
DISCUSSION

The previous studies of *Leishmania* vaccine during the last decade showed the prophylactic potential of CPs; CPA, CPB and CPC in mouse and dog models [195, 198, 218-220]. The DNA was used to prime certain specific immune response and then boosted with the recombinant protein(s), either alone or in combination with other CP type, demonstrated the high levels of IgG_{2a}, but not $IgG_{1.}$ Some of studies showed that the protective immunity could be induced only when two genes of *cpa* and *cpb* were co-injected but using a separate gene immunization was not protective [30, 195, 198]. However, the DNA-DNA prime-boost was also shown to have more effective protective responses than DNA/protein, protein/protein immunization strategy in *L. major* signal peptidase vaccination [221]. In this study, we demonstrated three times immunization of genetically susceptible BALB/c mice using DNA vaccine that could induce Th1 response and had potential protection against *L. martiniquensis* infection. The modified humanized codon *cpb* DNA vaccine was constructed in order to increase CPB expression in mice since the codon optimization is required for increased expression and induces antigen specific response in mammals [222]. We have primarily converted this partial 525 bp consensus nucleotide sequence of *L. martiniquensis cpb* to the amino acid sequence and predicted the potential of T cell receptor epitopes (data not shown) [223]. Our results showed that the pcDNA_*cpb* could induce Th1 response as the outcome prediction. The balance of Th1 and Th2 responses plays a critical role in *Leishmania* pathogenesis and protective immunity. The recovery from the disease is associated with the Th1 immune response and production of pro-inflammatory cytokine [224]. The IFN-γ and IL-10 are pro- and anti- inflammatory cytokines, respectively produced by Th1 effector cells and were used as indicators of Th1 immune response in this study. IFN-γ stimulated nitric oxide production in activated macrophage and inhibited intracellular parasite growth [225] while IL-10 associated with the *Leishmania* pathogenesis leads to disease progression and parasite persistence [226]. In addition, IgG_{2a} levels are depended on IFN-

γ, while IgG_1 levels are associated with IL-4 [217]. Therefore, IgG_{2a} and IgG_1 were used as representative markers for Th1 and Th2 responses, respectively. In this study the vaccinated mice with pcDNA_*cpb* showed the increased of IgG_{2a} and IFN-γ before challenged infection with *L. martiniquensis* and sharp increased after infection (Figure 19B and Figure 20). In contrast, 3 weeks after challenge with *L. martiniquensis,* IL-10 levels in mice immunized with pcDNA_*cpb* and pcDNA_*cpb*-MO declined and significantly lower than the control groups. (Figure 21). While IFN-γ in in mice immunized with pcDNA_*cpb* and pcDNA_*cpb*-MO enhanced higher than the control group (Figure 20). This result suggested that mice vaccinated with pcDNA_*cpb* and pcDNA_*cpb*-MO developed Th1 immune response and could have protective immunity. The intramuscular immunization using pcDNA_*cpb* prototype DNA vaccine could induce Th1 immune response that confers potential protection of *L. martiniquensis* challenge in BALB/c mice. We evaluated the reduction of L. *martiniquensis* in liver and spleen, using quantitative PCR *(*Figure 25*)*, the result was correlated with the concentration of IFN-γ indicates the potential of Th1 cytokine response of pcDNA_*cpb* vaccinated mice with or without MO adjuvant. The use of lipid adjuvant such as liposome or cationic lipid or surfactant as vaccine adjuvants was explored since 1974, where mice immunized with diphtheria toxoid (DT) adjuvanted with phospholipid-based liposomes enhanced antibody titers compared with those having the non-adjuvanted DT [227]. MO, a non-ionic surfactant is a kind of monoglycerides which used as a food ingredient and recognized as Generally Recognized As Safe (GRAS) [228] to be used in humans. Monoglycerides have a low solubility in buffer, although certain unsaturated monoglycerides (e.g. 1-mono-olein, 1-monolinolein, 2-monoolein) may interact with buffer to form liquid crystalline states [229]. The neutral lipids/monoglycerides/surfactants could encapsulate DNA obtaining the structures of intercalated liquid-crystal phases of DNA and surfactants without the need to use cationic lipids [230]. MO was used as an adjuvant in the cocktail of pcDNA_*cpb*-MO in this study. The pcDNA_*cpb*-MO formulation remained uniformly opaque without any aggregation at room temperature and was immediately used for immunization. MO could induce higher production of IgG_1 and IgG_{2a} compared

with the controls and without MO only at week 4. Our results showed good potential of MO as a vaccine adjuvant; however, the optimization is needed to be investigated for future vaccine design to achieve higher enhancement of immunity and protection.

We used the liver as a target organ to observe the parasite burden after parasite challenge. The data from this experiment derived from 3-week post infection which we predicted the highest immune response achieved that could confer protective immunity and the outcome result was as we expected. The parasite burdens in the vaccinated mice with pcDNA_*cpb* with or without MO were much lower and showed statistically significant difference than those in the control group (Figure 25). In addition, the results of parasite burden using the two sensitive methods, the qPCR and immunohistochemistry, were correlated by the pcDNA_*cpb* vaccinated mice with or without monoolein showed the numerous of mature granuloma formation compared with non-vaccinated mice which indicated the enhanced Th1 response and protective response in leishmaniasis[231]. Therefore, it can be concluded that pcDNA_*cpb* vaccinated mice were potentially protected from parasite challenge and confirmed the potential protection of pcDNA_*cpb* vaccine as a prophylactic vaccine. The pcDNA vector control, containing the CpG motif backbone as pcDNA_*cpb* vaccine. Nevertheless, these mice produced similar levels of IFN-γ, IL-10, IgG_1 and IgG_{2a} as the MO control *(*Table 9-10*)*. Therefore, this result indicated that the specific antigenic activation of *cpb* gene contained in the vaccine. The IFN-γ/IL-10 ratio has been shown to be an indicator of vaccine success. In this study, IFNγ/IL-10 ratio was high in both pcDNA_*cpb* with and without MO (Figure 22) which implied successfully of pcDNA_*cpb* DNA vaccine in induction of protective cytokines. This also certify the strong antigenic property of CPB against *Leishmania* parasite and also supported that full-length or partial cysteine protease vaccines induced protective immune response against *L. martiniquensis* in a mouse model [232].

APPENDIX

APPENDIX A INSTRUMENT

1. Autoclave, HICLAVE HVE -25/50, Japan
2. Automatic Pipettes, Gilson, France
3. Balance (2 digits), Adam, UK
4. Biosafety cabinet class II, NapCo
5. Centrifuge 4°C GS-15R, Beckman, Germany
6. CO_2 humidified incubator, Binder, Germany
7. DNA Thermal Cycler 2400, 9700, Perkin Elmer, USA
8. Filter paper 0.2 µm, Millipore, USA
9. Fixed angle rotor, Eppendorf, Germany
10. Fluorescence microscope, Olympus UIS2, USA
11. Gel Doc, Bio-Rad, USA
12. Gel electrophoresis apparatus, Bio-Rad, USA
13. Heat block, Scientific, USA
14. High speed centrifuge Avanti J-251, Beckman, USA
15. Incubator for 37 °C, Memmert, UK
16. Inverted microscope, Nikon, Japan
17. Nanodrop, Thermo scientific, USA
18. pH meter 220, Corning, UK
19. Refrigerated centrifuge, Eppendorf, USA., Beckman, USA
20. Shaking incubator, Labcon, South Africa
21. Shaking Waterbath, Memmert, UK
22. Spectophotometer, Bio-Rad, USA
23. UV Transilluminator, Funafuti, Japan
24. Vortex Mixer, Stuart scientific, UK
25. Water bath 37 °C, Mammert, USA
26. Histocore PELORIS 3 Premium Tissue processing, Leica System, USA

27. Histocore Bio cut, Leica System, USA
28. Leica CV5030 Fully Automated glass covered slide, Leica System, USA
29. Histocore Acadia, Leica System, USA

Mice experiment

1. Polycarbonate cage dimension 17"x30"x13", National laboratory center, Thailand
2. Water Hyacinth, National laboratory center, Thailand
3. Mouse and Rat diet food No. 082G , National laboratory center, Thailand
4. Corncob , National laboratory center, Thailand
5. Bend tube , National laboratory center, Thailand
6. Rubber bung , National laboratory center, Thailand
7. Bottle 250 mL, National laboratory center, Thailand
8. Isoflurane® R&D system, USA

APPENDIX B REAGENT

Enzymes and reagent for cloning

1. *Not*I, NEB, England
2. *EcoRI*, NEB, England
3. Taq DNA polymerase, Promega, USA
4. Shimp Alkaline Phosphatase, Roche, Germany
5. T4 DNA ligase, Roche, Germany
6. pVax1™ vector Invitrogen, ThermoFisher, USA
7. Certificate low melt agarose gel BioRad, USA
8. 1 kb HyperLadder™, Bioline, USA
9. 100 bp DNA Ladder, BioRad, USA

Reagent Kits

1. IgG_1 Mouse Uncoated ELISA Kit ,Invitrogen, ThermoFisher Scientific, USA

2. IgG_{2a} Mouse Uncoated ELISA Kit ,Invitrogen, ThermoFisher Scientific, USA
3. IFN-γ DUO-set ELISA Kits, R&D system, USA
4. IL-10 DUO-set ELISA Kits, R&D system, USA
5. Hi-speed plasmid Giga prep, Qiagen, Netherland
6. Hi-speed plasmid mini prep, Qiagen, Netherland
7. QIAquick® gel extraction, Qiagen, Netherland
8. Novalink Polymer Detection Systems, Leica Biosystem, UK
9. PureLink™ RNA Mini Kit, ThermoFisher Scientific, USA
10. High-Capacity cDNA Reverse Transcription Kit, ThermoFisher, USA
11. SsoAdvanced™ Universal SYBR® Green Supermix, Bio-Rad, USA

APPENDIX C REAGENTS

Reagents/Buffers

3M Sodium acetate (NaOAe), pH4.8

1. 16.40% (w/v) Sodium acetate
2. Dissolved in distilled water and adjusted pH to 4.8 with glacial acetic acid. Distilled water added to 100 ml. The solution was mixed and autoclaved.

6X DNA loadingdye (10mL)

1. 60 g glycerol
2. 12 mL 0.5 M EDTA, pH 8
3. 10 mg bromophenol blue
4. Distilled water up to 10 mL

50 mM Calcium Chloride

1. 2.77 g $CaCl_2$
2. Distilled water up to 500 mL

50X Tris-acetate buffer (TAE buffer)

1. 2M Tris-acetate
2. 100 mM Na_2EDTA (pH 8.0)

3. Distilled water up to 1L
4. The solution was mixed and diluted with distilled water to 1X
5. before use.

LB agar plate

1. 1.5% Degranulated Agar (Becton Dickinson, USA)
2. LB broth

Wash buffer

1. 0.05% (v/v) Tween -20 in PBS buffer

Phosphate buffer saline (PBS)

2. 1.76 mM KH_2PO_4
3. 8 mM Na_2HPO_4. $2H_2O$
4. 137.9 mM NaCl
5. 2.7 mM KCl

2 mol/dm^3 HCl

Component	Volume
ddH_2O	83.8 ml
12.3 N (HCl)	16.2 ml

2 mol/dm^3 NaOH

Component	Volume
NaOH	8 g
ddH_2O	100 ml

Sodium citrate buffer

Reagents	Volume

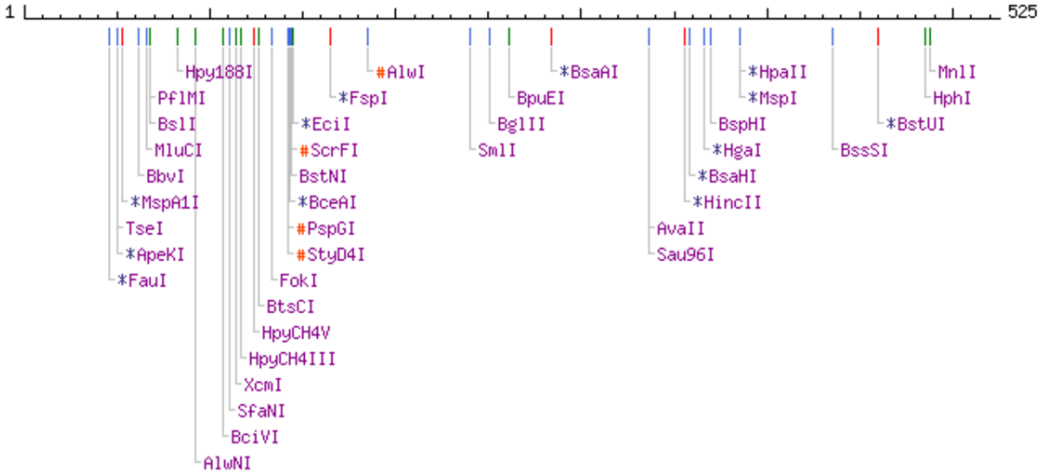
Trisodium citrate	2.94 g
Tween-20	0.5 mL
1 mol/dm^3 of HCl	Adjust to the pH 6
Distilled water	Adjust final volume to 1 dm^3

APPENDIX D SUPPLEMENTAL RESULT

Cpb humanized sequence

TGCGGTTCATGCTGGGCTTTTTCTGCGATTGGAAACATTGAGAGTCAG
TGGCAGCGGGCAGGCCATAAATTGGTAAGTCTTTCAGAACAGCAACTGGTATCCTGT
GACCACATGGACAGTGGATGCAACGGCGGACTCATGCTCCAGGCTTTTGAATGGCTG
TTGCGCAACGCCAACGGATCTGTGTATACTGAGAAGTCCTACCCGTATACGTCTGGTA
ATGGAGATGTTCCAGACTGCCTTGAGTCTGGAGATCTTGTGGTAGGGGCGCAAATAG
ACGGGTACGTGACAATACAAAGTAACGAAATGGTTATGGCGGCGTGGTTGGCAAAGA
ATGGTCCAATAGCCATAGCAGTTGACGCCACTTCATTCATGAGTTACAAGTCCGGCGT
TCTTACTGAGTGCGTAGGCCAACAGCTTAATCATGGCGTATTGCTCGTGGGGTACAAC
ATGGTAGGCCGCGTACCTTACTGGGTGATTAAAAATAGCTGGGGCGAGGACTGGGG
AGAAAAGGGTTACATACGC

cpb nucleotide sequence restriction enzyme mapping



Modify_*cpb*_sequence for sub-clone to pVAX vector

GAATTCACCATGGTGCGGTTCATGCTGGGCTTTTTCTGCGATTGGAAA
CATTGAGAGTCAGTGGCAGCGGGCAGGCCATAAATTGGTAAGTCTTTCAGAACAGCA
ACTGGTATCCTGTGACCACATGGACAGTGGATGCAACGGCGGACTCATGCTCCAGGC
TTTTGAATGGCTGTTGCGCAACGCCAACGGATCTGTGTATACTGAGAAGTCCTACCCG
TATACGTCTGGTAATGGAGATGTTCCAGACTGCCTTGAGTCTGGAGATCTTGTGGTAG
GGGCGCAAATAGACGGGTACGTGACAATACAAAGTAACGAAATGGTTATGGCGGCGT
GGTTGGCAAAGAATGGTCCAATAGCCATAGCAGTTGACGCCACTTCATTCATGAGTTA
CAAGTCCGGCGTTCTTACTGAGTGCGTAGGCCAACAGCTTAATCATGGCGTATTGCTC
GTGGGGTACAACATGGTAGGCCGCGTACCTTACTGGGTGATTAAAAATAGCTGGGGC
GAGGACTGGGGAGAAAAGGGTTACATACGCCATCATCACCACCACCATTAGCTCGAG

Restriction site

ACCATGG = Kozak sequence

GAATTC = *EcoR*I *(*Buffer 2*)*

CATCATCACCACCACCATTAG = Histidine-tag *(*for protein purification in the future*)*

CTCGAG = *Xho* I *(*Buffer 2*)*

Pvax™ vector sequence (CMV promoter, 2999 bp)

GACTCTTCGCGATGTACGGGCCAGATATACGCGTTGACATTGATTATT
GACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTT
CCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCC
GCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCA
TTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTG
TATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGG
CATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATT
AGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAG
CGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGT

TTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGAC
GCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCT
AACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAG
ACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCC
AGTGTGGTGGAATTCTGCAGATATCCAGCACAGTGGCGGCCGCTCGAGTCTAGAGG
GCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTT
GTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTT
CCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGG
GGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATG
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ACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAA
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pcDNA_*cpb* vaccine sequence.

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CGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAAC
GCGGCCTTTTTACGGTTCCTGGGCTTTTGCTGGCCTTTTGCTCACATGTTCTT

Total nucleotide sequence = 3525 bp

ACCATGG = Kozak sequence

GAATTC = *EcoR*I restriction site *(*Buffer 2*)*

CATCATCACCACCACCATTAG = Histidine-tag *(*for protein purification in the future*)*

CTCGAG = *Xho*I restriction site *(*buffer 2, 3*)*

TGCGGC = CPB_ sequence

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