JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Immunogenicity and potential protection of DNA vaccine of *Leishmania martiniquensis* against *Leishmania* infection in mice

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Abstract

Introduction: In Thailand, *Leishmania martiniquensis* is the predominant species causing cutaneous and visceral leishmaniasis. Its incidence has been increasing among immunocompetent and immunocompromised hosts. We developed a prototype DNA vaccine using a partial consensus sequence of the cysteine protease B (*cpb*) gene derived from *L. martiniquensis* from Thai patients.

Methodology: The laboratory inbred strain of albino BALB/c mice were immunized intramuscularly three times at 2-week intervals (weeks 0, 2, and 4) with *cpb* plasmid DNA (pcDNA_*cpb*) with or without the adjuvant, monoolein (pcDNA_*cpb*-MO). Mice were challenged at week 6 with *L. martiniquensis* promastigotes. Sera were analysed for IgG1, IgG2a, interferon gamma and interleukin 10 (IFN-γ and IL-10, respectively) levels at weeks 0, 4, and 9. Additionally, livers and spleens were also analysed for parasite burden using immunohistochemistry and real-time polymerase chain (qPCR) assays.

Results: Three weeks after promastigote challenge, vaccinated mice showed significantly increased levels of IgG_{2a} and IFN-γ while IL-10 level was significantly reduced when compared with those in the control group ($p < 0.01$). Parasite burden in the livers and spleens of vaccinated mice significantly decreased. In addition, a significant increase in mature granuloma formation in the livers when compared with those of the control group ($p < 0.05$) was found, indicating increased T-helper cells (Th1)-induced inflammation and destruction of amastigotes. Monoolein produced a booster effect to enhance the mouse Th1 protective immunity.

Conclusions: The prototype DNA vaccine could induce a Th1 immune response that conferred potential protection to the *L. martiniquensis* promastigote challenge in BALB/c mice.

Key words: *Leishmaniasis*; *Leishmania*; *leishmania vaccine*; DNA vaccine; Cysteine protease; Immunization.

J Infect Dev Ctries 2021; 15(9):1328-1338. doi:10.3855/jidc.14472

(Received 09 December 2020 – Accepted 09 March 2021)

Introduction

Leishmania is an intracellular protozoan parasite in host macrophages that causes different forms of disease depending on the *Leishmania* species and host immune status [1]. More than 20 species of *Leishmania* have been reported as human pathogens that cause cutaneous, mucocutaneous, and visceral (CL, MCL, and VL, respectively) leishmaniasis. Clinical outcomes of *Leishmania* infection depend on the balance between levels of T-helper cell (Th1 and Th2) cytokines, which can suppress or enhance each other. Increasing levels of Th1 cytokines and IFN- γ are associated with the cure of some leishmaniasis, while IL-10, a Th2 cytokine, could increase disseminated disease in addition to high rates of mortality [2,3]. Drug therapy in leishmaniasis is limited because of its high cost, adverse side effects, and occurrence of drug-resistant parasites [4]. Vaccination is one of the most promising methods for preventing and safely treating leishmaniasis [5]. Developing antileishmanial vaccines consists of three generations: (1) the first generation used whole-killed promastigotes, fractionated *Leishmania* antigen, and live-attenuated promastigotes that induced an appropriate immune response in canines but failed to protect against leishmaniasis in humans [6–8]; (2) The second generation vaccines, recombinant proteins, were further developed, for example, LEISH-Tech and LEISH-F1entered clinical trials which induced robust immune response and protection against *L. infantum* infection in both healthy volunteers and canines [9,10]; and (3) subsequently, the third generation vaccine, DNA vaccine, is being used in ongoing studies, for

example, ChAd63-KH is a promising candidate vaccine encoding two genes of *L. donovani*, and has revealed high efficiency of $CD8^+$ response in patients with post-kala-azar dermal leishmaniasis (PKDL) [11]. Compared with the first- and second-generation vaccines, the DNA vaccine has several advantages in terms of stability, simplicity, safety, and lower production costs.

Virulence factors are considered potential drug targets and vaccine candidates for controlling leishmaniasis [12]. Cysteine proteinases (CPs) are immunogenic proteins and include cysteine protease types I–III (CPB, CPA, and CPC, respectively). CPs constitute virulence factors of *Leishmania* that have important roles in parasitic endurance, autophagy, and metacyclogenesis [8]. *Leishmania* cannot develop within macrophages in the presence of cysteine proteinase inhibitors [15]. CPB plays a role in reducing the function of Th1 cytokines, which have been associated with leishmaniasis pathogenesis [16]. In addition, *Leishmania* CPB has shown evidence as a suitable VL vaccine candidate antigen in mouse and canine infection models [17,18].

Leishmania infection is usually asymptomatic in immunocompetent individuals; however, leishmaniasis co-infection with acquired human immunodeficiency syndrome (HIV) has frequently been reported [19,20]. HIV causes immunosuppression due to CD4(+) T cell depletion that can lead to reactivation of latent infections among immunocompromised patients [21]. *L. martiniquensis*, a zoonotic species causing CL and VL, was reported in the Caribbean, Europe, the United States (US), and Thailand [22]. Since 2012, the incidence of *L. martiniquensis* infection, a predominant autochthonous species reported in Thailand, has been increasing among immunocompetent and immunocompromised hosts, especially in the northern and southern areas of the country of which 47.5 % of leishmaniasis cases occurred as co-infections with HIV [23]. Because most vaccine studies have focused on *L. infantum, L. donovani*, and *L. major* [24], we were interested in developing a DNA vaccine against autochthonous *L. martiniquensis,* which would be the first report of a vaccine developed against this species. In this study, a prototype DNA vaccine using partial consensus sequences of *cpb* from *L. martiniquensis* isolated from Thai VL patients that could induce high Th1-associated cytokine immune responses in Bagg Albino/c (BALB/c) mice and confer potential protection against *L. martiniquensis* promastigote infection is described.

Methodology

Parasites and mice

L. martiniquensis promastigotes (MHOM/TH/2011/PG), a reference strain previously isolated from bone marrow aspiration of a Thai patient, was used in this study. Promastigotes were grown in Schneider's medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% heat-inactivated foetal bovine serum, 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, pH 7.4. Twenty inbred female BALB/c mice, four to six weeks old, obtained from the National Laboratory Animal Center, Mahidol University, Thailand, were used for immunization. All animal experiments were approved by the Animal Care & Use Committee of the Faculty of Medicine, Srinakharinwirot University (ethics approval reference Number 8/2558 and 1/2561). All procedures used in this work complied with the ethical standards of the relevant national and institutional guides for the care and use of laboratory animals. Briefly, mice were rested for one week of adaption and housed under constant room temperature at 25 ± 2 °C, 12 h light/dark cycle at the Animal Center, Faculty of Medicine, Srinakharinwirot University, Thailand. During the experiment including the immunization and bleeding methods, mice were anesthetized using isoflurane (Baxter, IL, USA). All efforts were made to minimize pain in the animals.

Design and construction of cpb DNA vaccine

The *cpb* consensus sequence was generated using the 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, of which three were from GenBank (accession numbers MH752382.1 to MH752384.1) and nine nucleotide sequences were from other Thai VL isolates. The *cpb* consensus sequence was transformed into an amino acid sequence (http://www.kazusa.or.jp/codon/) to predict antigenic amino acid epitopes. The designed humanized chimeric DNA sequences (565 bp) were synthesized by GenScript Company, US in addition to the Kozak consensus sequence, initiation translation region, and the 6x-His tag at the end of *cpb*. The *EcoR* I and *Not* I recognition sites were added at the 5' and 3' ends, respectively, as the cloning sites. The synthesized *cpb* DNA sequence was inserted in the pVax1™ vector (Invitrogen, Thermo Fisher, USA) downstream of the Kozak consensus sequence under the control of the CMV promoter. The DNA construct was designated pcDNA_*cpb* and transformed into competent

Escherichia coli cells (DH5-α strain). The pcDNA_*cpb* clones were extracted for DNA using the Endo-Free Plasmid Giga Kit (Qiagen, USA). The size and sequence of the *cpb* DNA insert sequence was confirmed by gel electrophoresis and nucleotide sequence analyser, respectively (Pacific Science, Thailand) using a pCMVF upstream primer (5'-TAGGCGTGTACGGTGGGAGGAGGTC-3') and downstream primer (3'-CTACTCAGACAATGCGATGC-5').

Analysis of pcDNA_cpb intracellular protein expression

Vero cells (ATCC, US) were grown on glass cover slips in a six-well plate and separately transfected with 5 µg of recombinant plasmids: (1) pcDNA_*cpb* or pcDNA empty vectors or (2) pCMV_kanD2pRME plasmid, which express the pre-membrane and envelop proteins of dengue serotype 2 after lipofectamine 3,000 transfection (Invitrogen, Thermo Fisher Scientific, US) as instructed by the manufacturer. After 24 hours of transfection, cells were fixed and permeabilized with cold acetone for 15 minutes. The cells were incubated with the mouse anti-6x-His-monoclonal antibody (Invitrogen, Thermo Fisher Scientific, USA) or anti-flavivirus (Clone 4G2) monoclonal antibody at 37 °C for 1 hour. The cells were then washed with phosphate-buffered saline (PBS) buffer and incubated with secondary antibody, anti-mouse IgG-FITC diluted at 1:500, for 1 hour. After the cells were washed, the nuclei were counterstained with 4,6-diamino -2-phenylindole hydrochloride (DAPI) from Sigma–Aldrich (MO, USA). The result of protein expression in cells was visualized at 200× magnification under a fluorescence microscope.

Mouse immunization and parasite challenge

Four groups with five female BALB/c mice in each group were injected intramuscularly in the tibialis anterior three times at two-week intervals with 50 µL containing plasmid DNA or monoolein (MO, Sigma-Aldrich) in PBS buffer. Group I was injected with 50 µL PBS buffer containing 100 µg pcDNA (vehicle control); group II was injected with 50 µg MO (vehicle control); group III was injected with 100 µg pcDNA_*cpb;* and group IV was injected with 100 µg pcDNA_*cpb* plus 50 µg MO. For animal ethics concerning saving animal life, we used a vehicle control group as the normal control group [25]. For pcDNA_*cpb*-MO preparation, the pcDNA_*cpb* and MO in the PBS buffer mixture were vigorously vortexed for 5 minutes and then incubated at 60 °C for 5 minutes. This step was repeated three times. Finally, the pcDNA_*cpb*-MO suspension was incubated at 60 °C for 30 minutes and then kept at room temperature for 30 minutes before immunization. Blood samples were obtained from the facial vein at the mandibular region at weeks 0, 4, and 9 after the first immunization. Two weeks after the last immunization (at week 6, after the first immunization), all mice were challenged via the tail vein with 50 µL containing 5×10^6 *L. martiniquensis* promastigotes in the early stationary stage. At 21 days after infection, blood samples were obtained, and mice were then sacrificed using isoflurane anaesthesia. The livers and spleens of all mice were harvested and kept frozen at –80 ºC until used to determine the parasite burden.

Determination of the total IgG_1 and IgG_{2a} antibody responses

Serum samples, collected at weeks 0, 4, and 9 after the first immunization were separately analysed for IgG_1 and IgG_{2a} antibodies using IgG_1 and IgG_{2a} Mouse Uncoated ELISA Kits (Invitrogen, Thermo Fisher Scientific, USA). The assay was performed in triplicate. Briefly, a 96-well microplate was coated with 100 µL/well anti-IgG_1 or anti-IgG_{2a} at 4 ºC overnight and washed twice with washing buffer. Blocking buffer (250 µL) was added, incubated at room temperature for 2 h and washed twice with washing buffer. For sample wells, 50 µL assay buffer B was added followed by adding 50 µl diluted serum sample and 50 µl diluted horseradish peroxidase (HRP)-conjugated anti-mouse IgG polyclonal antibody. Finally, the plate was sealed and incubated at room temperature with continuous shaking for 2 hours (for IgG_1) or 3 hours (for IgG_{2a}) followed by additional washing. The specific binding was visualized using tetramethylbenzidine (TMB) as a substrate. The absorbance was measured at 450 nm using a microplate reader (Tecan, Switzerland). The IgG_1 and IgG_{2a} concentrations were determined using standard curves.

Cytokine assay

Mouse sera were separately assessed for IFN-γ and IL-10 using Mouse IL-10 or IFN-γ Duo Set ELISA Kits (R&D Systems, USA). The assay was performed in duplicate. Briefly, 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 three times with 400 µL washing buffer, incubated with 300 µL diluent reagent (1% BSA in PBS, pH 7.2–7.4) for 1 hour and washed twice. One-hundred microliters of mouse serum or standard

(recombinant mouse IFN-γ or recombinant mouse IL-10) were added and incubated for 2 hours at room temperature followed by two washes. Subsequently, 100 µL of detection antibodies were added, incubated for 2 hours, and washed twice. One-hundred microliters of streptavidin-HRP enzyme (R&D Systems, USA) was added to each well and incubated for 20 minutes. Finally, 100 µL tetramethylbenzidine substrate (R&D Systems, USA) was added and incubated for 20 minutes followed by addition of 50 µL 2 M sulfuric acid (H_2SO_4) to stop the reactions, and the samples were then assessed at 450 nm absorbance. Cytokine concentrations were determined based on standard curves.

Parasite burden assay

Immunohistochemistry

All mice were sacrificed, livers were removed, weighed, and kept at –20 °C. All frozen livers were thawed, cut, and fixed in 10% buffered formalin for 24 hours and embedded in paraffin. The experiment was performed at the Forensic Pathology Section, Central Institute of Forensic Science, Thailand. The liver tissues in paraffin blocks were cut into thin sections (5 µm) using a microtome (Leica Biosystem, USA) and placed on Superfrost Plus Positively Charged Microscope Slides (Thermo Scientific, USA). An immunohistochemical analysis was performed using a Novolink Polymer Detection System (Leica Biosystem). The liver sections were deparaffinized in xylene and xylene substitute and then rehydrated in graded alcohol. Antigen retrieval was performed using 0.01 M sodium citrate (Sigma Aldrich) followed by washing with de-ionized water. The slide was blocked with 3% hydrogen peroxide for 30 minutes and rinsed twice with 1X Tris-buffered saline (TBS) washing buffer. The slide was incubated in 5% BSA/0.1% Tween 20 in TBS at 37 °C for 2 hours after which it was rinsed twice with TBS. Protein casein blocking was performed for 10 minutes, and the samples were then washed twice with washing buffer. The mouse anti-*Leishmania* lipophosphoglycan (LPG) antibody (My BioSource, USA) at a concentration of 1:10,000 in 5% BSA was applied overnight at 4 °C in a cold moisture box. After washing, the slide was incubated with the secondary antibody for 30 minutes at room temperature, washed twice, exposed to the Novolink Polymer for 30 minutes at room temperature, and then washed twice using washing buffer and once with de-ionized water. Diaminobenzidine chromogen was added for 5 minutes and washed twice with de-ionized water. All slides were counterstained with haematoxylin for 5 minutes and dehydrated in graded alcohol, xylene substitute, and xylene. The slides were mounted, and the images were captured under a light microscope using the CellSens Program (Olympus). The *Leishmania* amastigotes and nucleated liver cells were quantified from 25 consecutive images captured at 400× magnification using the CellSens Dimension Program (Olympus) [26]. The parasite load was calculated from the number of amastigotes per nucleated liver cell and expressed in terms of *L. donovani* Units (LDUs). LDU represents the number of amastigotes per host nucleus multiplied by the total organ weight (mg) [27]. Additionally, non-*Leishmania*-infected BALB/c mice (n = 2) were used for negative anti-LPG binding, which was the normal control in this experiment. Granuloma formation in the livers of all mouse groups was quantified from the full image capture at 200X magnification of the whole liver section. The granulomas were classified as immature and mature granulomas. Immature granulomas demonstrated a high number of amastigotes with a small population of Kupffer cells with or without mononuclear cell infiltration. Mature granulomas were classified by condensed amastigote-infected Kupffer cells encircled by cellular infiltrate. Involuting granulomas had the characteristics of cellular infiltrate but with few or no amastigotes; however, they were counted as mature granulomas in this study [26].

Quantitative PCR (qPCR)

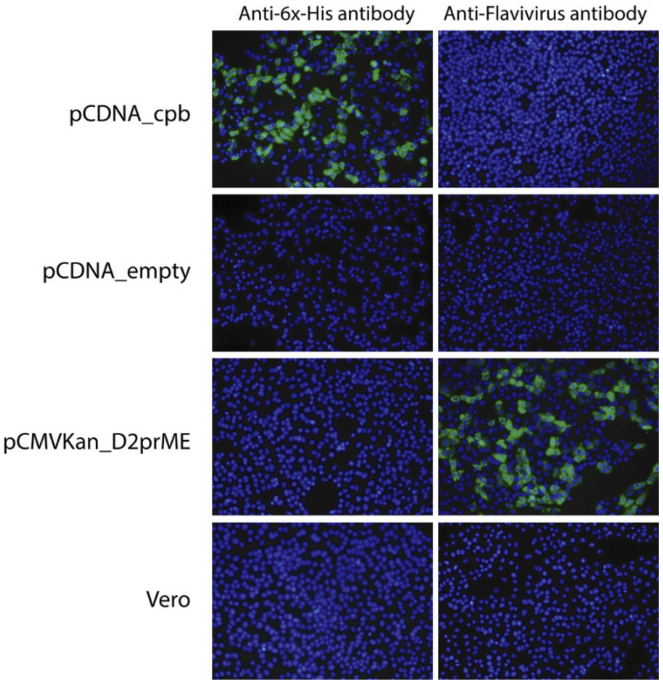
Evaluation of parasite burden in mice was performed using 100 mg each of frozen liver and spleen samples in all groups that were individually homogenized after RNA was extracted using a PureLink™ RNA Mini Kit (Thermo Fisher Scientific, US). The concentration was quantified using a Nanodrop™ spectrophotometer (Thermo Fisher Scientific, USA). Two hundred nanograms of RNA extract were used to reverse transcribe to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). The real-time polymerase chain reaction (qPCR) protocol was performed as previously described [28]. The specific primers were designed using the rRNA gene of the ITS1 region of *L. martiniquensis* (accession no. KM677931). The forward and reverse primer sequences were LeishF, 5'-CGATATGCCTTTCCCACACAC-3'; and LeishR, 5'-CTGTATACGCG CGGCATTTG-3', respectively [28]. The qPCR experiment was performed using the CFX96™ Optical Reaction Module (Bio-Rad, USA). All samples were assayed in duplicate in a total volume of 20 µL/reaction containing 10 µL Sso Advanced™

Universal SYBR® Green Supermix (Bio-Rad), 0.2 µL LeishF and LeishR primers, 7.6 µL ddH_2O and 2 µL DNA template. The PCR amplifications started with duplicate denaturation steps at 50 °C for 2 minutes and 95 °C for 10 minutes. The subsequent reaction was conducted for 40 cycles of 30 seconds each at 95 °C, 30 seconds at 61 °C, and 30 seconds at 72 °C followed by a final step consisting of 5 seconds at 65 °C and 50 s at 95 °C. The expected PCR product size was 128 bp. The fluorescence intensity was recorded as C_q. A standard curve was established using a serial 10-fold dilution of cDNA, which was reverse-transcribed from RNA that was extracted from *L. martiniquensis* promastigotes ranging from 5×10^7 to 5×10^4 parasites. All C_q values of the samples were used to calculate the parasite number using a standard curve.

Statistical analyses

The comparisons of all parameter values between the groups of vaccinated and control mice were performed using GraphPad Prism Software, Version 7.0 (La Jolla, CA, USA). The statistical analysis was performed using the two-tailed nonparametric Mann–Whitney U test. A nonparametric Kruskal–Wallis test was used for analysis of variance (ANOVA) test. A *p*-value < 0.05 of both analyses was considered significant.

Figure 1. Expression of pcDNA_cpb plasmid in Vero cells.



Vero cells were transfected with indicated recombinant plasmids and reacted with monoclonal antibodies specific for Anti-6x-His (IgG) and flavivirus (IgG). The expression of protein was visualized with FITC-conjugated anti-mouse IgG antibody and is shown in green while the nuclei counterstained with DAPI are shown in blue. Vero cells transfection with or without pcDNA empty plasmid served as the negative controls. The pCMV_kanD2pRME plasmid expressing the flavivirus protein was used as control to show the specificity of anti-6x-His which reacted only with pcDNA_*cpb*-transfected cells.

Results

Construction of the pcDNA_cpb vaccine

The *cpb* nucleotide sequences of *L. martiniquensis* obtained from 12 Thai and Myanmar patients in Thailand and from GenBank were aligned and showed 90% nucleotide sequence identity (data not shown). The synthesized partial consensus *cpb* coding region with humanized codons consisting of 525 bp with additional Kozak and 6-His tag sequences in a total of 565 bp was inserted in the pVaxI™ vector and cloned into the *E. coli* host. The size and sequence of the *cpb* recombinant vector was confirmed by gel electrophoresis and DNA sequencing, respectively, (data not shown).

CPB protein expression in Vero cells

Immunofluorescence with mouse anti-6x-His antibody was performed to confirm whether pcDNA_*cpb*-tagged with 6x-His plasmid could express the CPB protein. Green fluorescence was detected in the cells transfected with pcDNA_*cpb* plasmid and incubated with anti-6x-His while green fluorescence was not observed when an anti-flavivirus antibody was used (Figure 1). The green fluorescence was also observed in Vero cells transfected with pCMV_kanD2pRME plasmid and reacted with anti-flavivirus antibody, but no green fluorescence could be observed when anti-6x-His was used. The green fluorescence could not be observed in the negative control groups, the empty pcDNA vector- transfected cells group, and non-transfected Vero that had been treated with both anti-6x-His and anti-flavivirus antibodies. This result suggests that pcDNA_*cpb* plasmid was expressed in eukaryotic cells.

The total IgG_1 and IgG_{2a} antibodies in the BALB/c mice after immunization

Mouse sera collected at different time points after immunization with pcDNA, MO, pcDNA_*cpb,* and pcDNA_*cpb*-MO were investigated for total IgG_1 and IgG_{2a} antibodies. The mean IgG_1 and IgG_{2a} concentrations after immunization are shown in Figures 2 and 3. The IgG_1 concentrations of the vaccinated groups were significantly higher than those of the vehicle control groups (Figure 2). The IgG_1 level at week 9 (three weeks after challenge) of pcDNA_*cpb* with/without MO increased compared with that at week 4, but only the IgG_1 of the pcDNA_*cpb* group

significantly increased compared with the values at week 4 ($p < 0.01$). Interestingly, the IgG_1 levels of the pcDNA_*cpb*-MO group were significantly higher than those of the pcDNA_*cpb* without MO group ($p < 0.01$) at week 4 but did not significantly differ at week 9. Mice immunized with pCDNA_*cpb* with/without MO exhibited significantly higher IgG_{2a} levels than those of the vehicle control mice ($p < 0.01$) as shown in Figure 3. The presence of MO adjuvant (pcDNA_*cpb*-MO) caused a significant increase in IgG_{2a} more than seen in the group receiving only the pcDNA_*cpb* vaccine at weeks 4 and 9. The groups with pcDNA_*cpb* with/without MO was significantly different between weeks 4 and 9 ($p < 0.01$). These results indicate that pcDNA_*cpb* and pcDNA_*cpb*-MO could stimulate immune responses, while MO produced a booster effect.

Production of pcDNA_cpb vaccine induced IFN-γ and IL-10

The expression of cytokines was analysed using sera collected at weeks 0, 4, and 9. Mice immunized with pcDNA_*cpb* or pcDNA_*cpb*-MO showed significantly higher IFN-γ levels than those in the vehicle control mice at weeks 4 and 9 ($p < 0.01$) as shown in Figure 4. In addition, the IFN-γ concentrations in mice vaccinated with pcDNA_*cpb* with/without MO correspondingly increased from weeks 4 to 9 with significant differences ($p < 0.01$). At week 4, the pcDNA_*cpb* or pcDNA_*cpb*-MO mice showed significantly higher IL-10 levels than those of the

Figure 2. IgG_1 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; The bars represent significant differences compared among groups using the Mann-Whitney U test at $p < 0.05$.

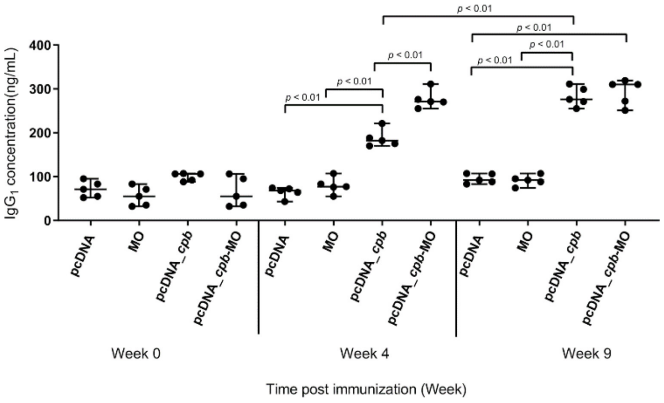


Figure 3. IgG_{2a} responses in BALB/c mice sera (n = 5) after immunization with pcDNA_*cpb*, pcDNA_*cpb*-MO compared with the pcDNA vector and MO control groups, The bars represent significant differences compared among groups using the Mann-Whitney U test at $p < 0.05$.

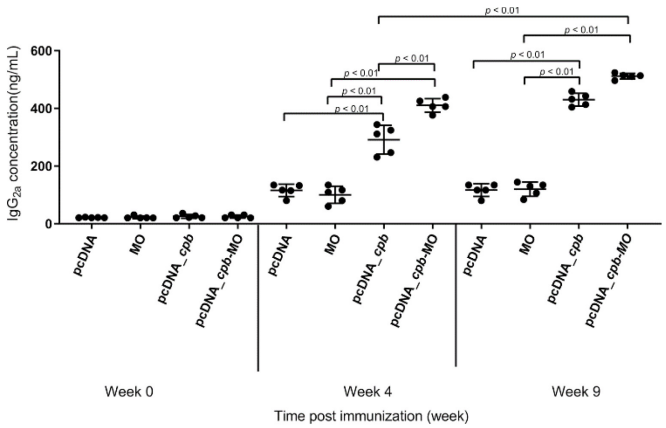


Figure 4. IFN-γ production in BALB/c mice after immunization three times with pcDNA_*cpb*, pcDNA_*cpb*-MO, pcDNA or MO; The bars represent statistical significance compared among groups using the Mann-Whitney U test at $p < 0.05$.

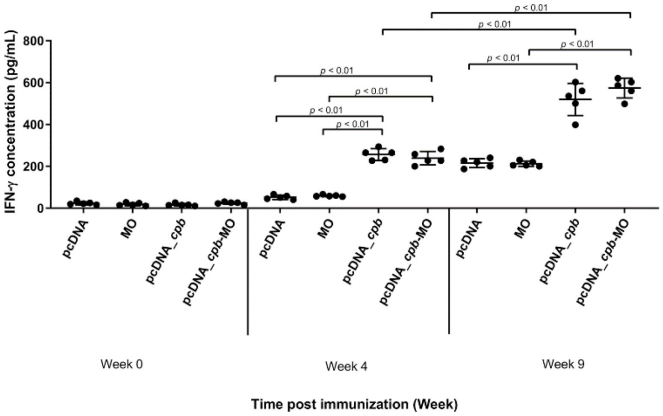
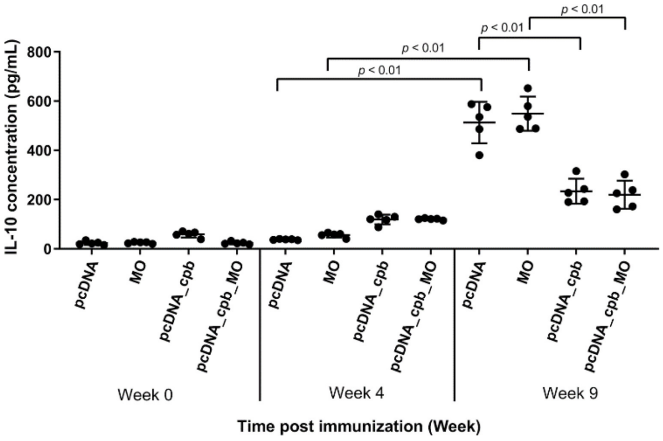


Figure 5. IL-10 levels in BALB/c mice after immunization three times with pcDNA_*cpb*, pcDNA_*cpb*-MO, pcDNA or MO; The bars represent statistically significant comparison among groups using the Mann-Whitney U test at $p < 0.05$.



pcDNA and MO vehicle control groups ($p < 0.01$). Nevertheless, three weeks after the challenge with *L. martiniquensis* promastigotes at week 9, the IL-10 levels in the control groups were induced dramatically, while only a slight increase in IL-10 levels were observed in the pcDNA_*cpb* or pcDNA_*cpb*-MO mouse groups (Figure 5). However, these values were significantly higher than those of the corresponding treatment groups at week 4. Both IFN-γ and IL-10 showed significantly higher and lower levels than those of the vehicle control groups at week 9 ($p < 0.01$), respectively. The finding that mice vaccinated with pcDNA_*cpb or* pcDNA_*cpb*-MO produced higher IFN-γ but lower IL-10 levels than mice vaccinated with only pcDNA or MO indicates the potential of vaccine-induced Th1 responses in a mouse model.

The IFN-γ/IL-10 concentration ratio of the mouse sera in all groups is shown in Figure 6. As the IFN-γ/IL-10 ratio was previously shown to be an indicator of vaccine success [29], a low ratio has been considered the result of vaccine failure, whereas a high ratio reflects vaccine success. In this study, the mean IFN-γ /IL10 ratio was very high in both the pcDNA_*cpb* with and without MO groups with significant differences compared with the vehicle control groups ($p < 0.01$).

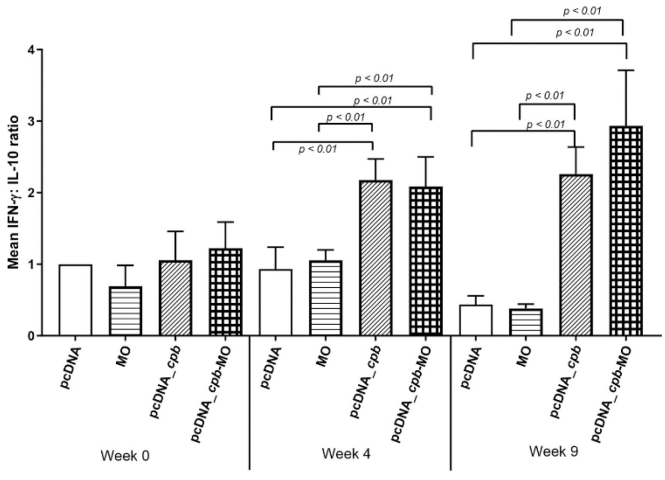
Vaccination with pcDNA_cpb reduced the parasite burden in the BALB/c mice

Immunohistochemistry assay

The parasite burdens in the livers of the animal groups are shown in Figures 7A, B, and D. The 1000× magnification of the liver tissue sections of pcDNA_*cpb*-vaccinated challenged mice (Figure 7D) demonstrated that the anti-LPG-positive *L. martiniquensis* amastigotes were brown, and the nuclei of nucleated cells, such as nucleated liver, Kupffer, and dendritic cells, were counterstained with haematoxylin and are shown in blue in Figure 7D. The liver tissue sections of noninfected normal mice were used to confirm that anti-LPG binding was not observed as all cells were shown in blue (Figure 7C). The parasite burden investigated by immunohistochemical staining of liver tissue sections using anti-LPG revealed the protective potential of the pcDNA_*cpb* group. The livers of the mice immunized with pcDNA_*cpb* (Figure 7B) demonstrated a decreased in brown-stained amastigotes compared with the vehicle control groups, pcDNA (Figure 7A). The average parasite loads, which were counted as the number of amastigotes in 25 consecutive images (five mice in each group), are shown in Figure 8. The Leishman Donovan Units (LDUs) from the pcDNA_*cpb* group was 5,963 LDUs, whereas the pcDNA of the control group was 7,604 LDUs. The total number of immature and mature granulomas in each mouse group are shown in Table 1. More mature granuloma numbers were detected in the pcDNA_*cpb* than in the vehicle control groups with significant differences ($p < 0.01$).

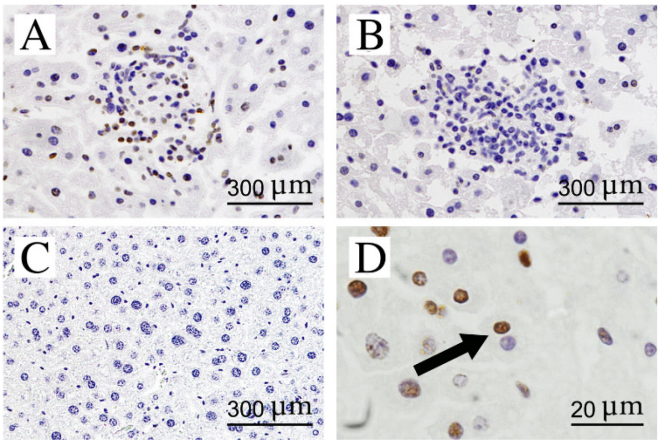
Parasite burden in the liver and spleen determined by qPCR assay

Figure 6. The IFN-γ/IL-10 concentrations ratios.



IFN-γ/IL-10 calculated from the IFN-γ and IL-10 concentrations of each experimental group at different time points; The bars indicate significant differences among the groups using the Mann-Whitney U test at $p < 0.01$.

Figure 7. Immunohistochemical images of the cross-sectioned livers of the vaccinated mice.



Cross-sectioned experimental mice livers after vaccinating with pcDNA (A), pcDNA_*cpb* (B) and challenged 3 weeks with *L. martiniquensis* promastigotes; The liver tissue of noninfected normal mice (negative control) is shown in (C). A 1000X magnification of the pcDNA_*cpb*-challenged mice is shown in (D), while images from 400X magnifications are shown in (A), (B) and (C). The positively stained *L. martiniquensis* amastigotes appear brown (arrow), and the nuclei are shown in blue. The granuloma formation is shown in A and B.

Table 1. Total numbers of hepatic granulomas in mice immunized with pcDNA, and pcDNA_*cpb* and challenged 3 weeks after vaccinating with *L. martiniquensis* promastigotes.

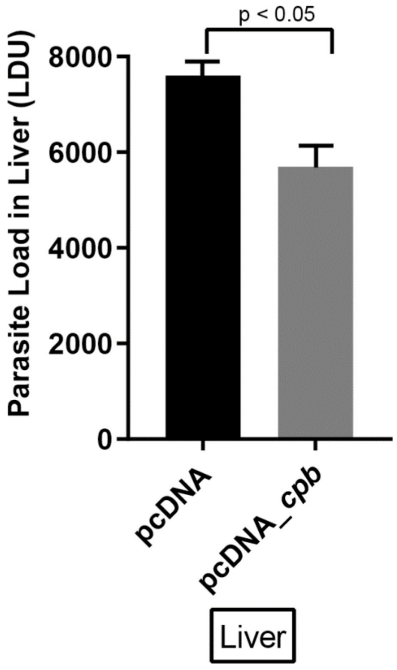
Granuloma type	Mice immunized with			
	pcDNA	MO	pcDNA_*cpb*	pcDNA_*cpb*-MO
Immature	404	392	141[a]	132[a]
Mature	154	173	565[b]	583[b]
Total	558	565	706	715

[a] indicates a statistically significant difference compared with the vehicle control group (pcDNA) at $p < 0.01$; [b] indicates a statistically significant difference between the immature and mature granuloma numbers of pcDNA_*cpb* at $p < 0.01$.

At week 9 (three weeks after the challenge), mice livers and spleens were harvested to quantitate the *L. martiniquensis* amastigote burden. The extracted RNA samples of the liver and spleen samples and *in vitro* cultures of promastigotes were reverse-transcribed to cDNA and used for amplification. Figure 5 shows the quantity of parasite DNA from the liver and spleen samples derived from the qPCR standard curve. The average C_q values of the livers from the mice receiving pcDNA_*cpb* was 30.20 while the average C_q value of the mouse spleens receiving pcDNA_*cpb* was 29.12. The average C_q value of the livers of control mice receiving only the pcDNA empty vector was 27.09. The qPCR standard curve obtained from the plot of the quantification cycle (C_q) against the logarithm of the starting *L. martiniquensis* parasite DNA was linear in the range test ($R^2 = 0.950$) although the data are not shown. Based on a standard curve, the C_q of the mice was converted to parasite numbers and used to evaluate the parasite load in the liver and spleen. The average parasite number of the livers of mice that were immunized with pcDNA_*cpb* was 5.04×10^5 cells, while in the mice immunized with vehicle pcDNA, the parasite number was 4.2×10^6 cells. Likewise, the average parasite load in the spleens of the mice immunized with pcDNA_*cpb* was 1.72×10^6 cells, while in the vehicle pcDNA immunization group, the parasite number in the spleen was 4.5×10^6 cells.

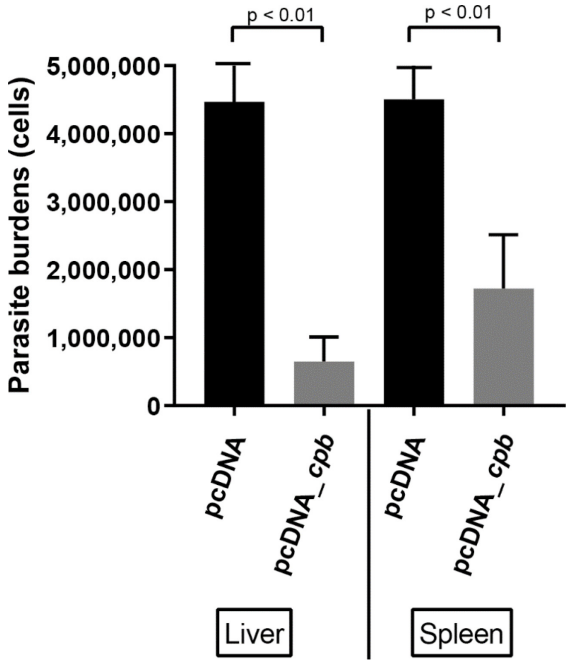
The amastigote burdens obtained from immunohistochemical studies and qPCR (Figures 8 and 9, respectively) showed good correlation with the decreased parasite load in the vaccinated group and showed a significant difference compared with the vehicle control (pcDNA) groups.

Figure 8. Parasite loads in the livers of the vaccinated mice.



Parasite count (LDUs) determined by totaling the anti-LPG-stained amastigotes harboring in liver tissue sections of mice immunized with pcDNA and pcDNA_*cpb* after 3-week challenge with *L. martiniquensis* promastigote; The bars represent significant differences among the groups, analyzed using the Mann-Whitney U test at $p < 0.05$.

Figure 9. Parasite loads in the livers or spleens of the mice.



Parasite burden in the livers and spleens of mice after vaccinating with pcDNA and pcDNA_*cpb* and challenged 3 weeks with *L. martiniquensis* promastigote quantitated by qPCR; The bars represent the significant differences among the groups, analyzed using the Mann-Whitney U test at $p < 0.05$.

Discussion

At present, many candidate vaccines have been studied and verified for immunogenicity and safety for animals and humans. The efficacy of vaccines varied depending on various factors, such as species of studied parasites, vaccine types, administration routes, and adjuvants in addition to adjuvant doses [3]. In this study, we demonstrated a DNA vaccine after which immunizations of genetically susceptible BALB/c mice could induce the Th1 response and showed potential against *L. martiniquensis* promastigote infection. The modified humanized codon *cpb* DNA vaccine was constructed to increase CPB expression in mice because codon optimization is required for increased expression and induces an antigen-specific response in mammals [30]. 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) [31]. Due to the limitations of specific antibodies against *cpb*, we used an anti-6x-His monoclonal antibody to investigate protein expression. Our findings showed that the pCDNA_*cpb*-transfected mammalian cell line could express the CPB protein. This result indicates that *cpb* encoded in the plasmid was fully cloned in the plasmid vector.

The analysis of the immune response showed that pcDNA_*cpb* could induce a Th1 response. IFN-γ and IL-10 are pro- and anti-inflammatory cytokines produced by Th1 effector cells, and IgG2a and IgG1 immunoglobulin isotypes were used as markers for Th1 and Th2 lymphocytes, respectively [32]. The balance of Th1 and Th2 responses plays a critical role in *Leishmania* pathogenesis and protective immunity [33]. In this study, the mice vaccinated with pcDNA_*cpb* showed an increase in levels of IgG_{2a} and IFN-γ before undergoing infection with *L. martiniquensis* and sharply increased levels after infection (Figures 3 and 4, respectively). In contrast, three weeks after challenge with *L. martiniquensis,* the IL-10 levels in the mice immunized with pcDNA_*cpb* and pcDNA_*cpb*-MO declined and became significantly lower than those in the control groups (Figure 5). This result suggests that the mice vaccinated with pcDNA_*cpb* and pcDNA_*cpb*-MO developed a Th1 immune response and showed protective immunity leading to reduced parasite loads in the liver and spleen. The *in vitro* CPB expression using pcDNA_*cpb* in Vero cells (Figure 1) with changes in immune profiles and parasite burdens in the livers and spleens of mice vaccinated with pcDNA_*cpb* group compared with the empty plasmid group indicated that the antigen was expressed in mice. Related studies of *Leishmania* vaccine development during the last decade revealed the prophylactic potential of CPs (CPA–C) in mouse and canine models [34,35]. The DNA was used to prime certain specific immune responses and then boosted with the recombinant protein (s), either alone or combined with other CP types, and then demonstrated high levels of IgG_{2a} but not IgG_1 [17,36]. Moreover, in this study, mice vaccinated with pcDNA_*cpb* and pcDNA_*cpb*-MO could induce a Th1 immune response, which agreed with a related study using the ChAd63-KH vaccine against *L. donovani* infection [11]. However, the efficiencies of the two DNA vaccines are difficult to compare due to differences between vaccine platform, route of immunization, adjuvant, and species of studied parasite. The homology of the *cpb* sequences of *L. martiniquensis* isolates was compared with those of *L. donovani* and revealed 75% sequence homology. Thus, we predict that the *cpb* vaccine is species-specific. However, cross-protection among species should be further investigated.

A related study demonstrated that *L. martiniquensis* could cause VL in BALB/c mice for which intravenous administration was the best inoculation route, enabling the highest parasite load detection in the liver at seven days after infection [37]. Therefore, we used the liver as the main target organ to observe the parasite burden after parasite challenge. The parasite burdens were observed three weeks after infection using two sensitive methods, qPCR and immunohistochemistry. The results of these two techniques also correlated (Figures 8 and 9, respectively). However, the number of parasite burdens in the immunohistochemical technique was less than those found with qPCR. Whole liver extracts were used for qPCR while immunohistochemical staining detected the amastigotes that were positively stained with anti-LPG binding in the cross-sectioned liver tissue.

Leishmania is an obligate intracellular parasite found in macrophages of which parasite clearance requires both innate and adaptive immune systems. The effector cells of both systems are recruited to the infection sites then produced enzymes and cytokines to eliminate the parasite. *L. donovani*-induced granuloma formation represents an example of innate and adaptive immunity combined in a unique microenvironment to eradicate an intracellular pathogen [38]. T-cells are the major cell type present within the granuloma, and mature granuloma T-cells have the capacity to produce IFN-γ [39]. Enhanced levels of IFN-γ were associated with stimulated nitric oxide production in activated macrophages and inhibition of intracellular parasite

growth while IL-10 was associated with *Leishmania* pathogenesis, leading to disease progression and parasite persistence [40]. The IFN γ /IL-10 ratio was high in the pcDNA_*cpb* groups compared with the vehicle control (Figure 6), implying pcDNA_*cpb* DNA vaccine in induction of protective cytokines. The reduced parasite loads and increase in mature granuloma numbers in the vaccinated mice with pcDNA_*cpb* compared with those in the control groups also indicated the effectiveness of the DNA vaccine.

The low immunogenicity of the DNA vaccine from degradation by DNase and other enzymes has been reported [41]. Thus, the strategies to enhance vaccines such as encapsulated DNA vaccines for gene delivery haves been studied [42]. MO, a non-ionic surfactant, is a type of monoglyceride used as a food ingredient and is generally recognized as safe among humans [43]. The neutral lipids/monoglycerides/surfactants could encapsulate DNA to yield the structures of intercalated liquid-crystal phases of DNA and surfactants without the need to use cationic lipids [44]. Mice immunized with DODAB:MO-based liposomes loaded with *Candida albicans* proteins displayed strong humoral and cell-mediated immune responses against specific yeast cell wall proteins and exhibited an immune protective strategy against *Candida* infections [45]. Therefore, we used MO for DNA encapsulation, and the results showed that MO could induce a higher production of IgG_1 and IgG_{2a} compared with that of the controls by week 4. Thus, the use of MO should be carefully optimized and evaluated for future vaccine design to achieve protective immunity.

Conclusions

A prototype DNA vaccine, developed from the partial *cpb* of autochthonous *L. martiniquensis* DNA, was demonstrated in this study. Intramuscular immunization using the pcDNA_*cpb* prototype DNA vaccine could induce a Th1 immune response that conferred potential protection to the *L. martiniquensis* challenge in BALB/c mice. However, the results starting at three weeks after infection, which were expected to stimulate the highest immune response, showed protective potential but incomplete protection from this prototype vaccine concerning *L. martiniquensis* infection. Thus, further research is needed to investigate vaccine efficacy by incorporating a prolonged challenge time and including studying cross-protection against different *Leishmania* species.

Acknowledgements

This research work was supported by the Faculty of Medicine, Srinakharinwirot University (grant nos. 249/2559, 363/2559, 130/2560, 345/2561) and the Graduate Student Research Grant, Year 2017 from the National Research Council of Thailand awarded to Mr. Thuntawat Aunguldee.

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Conflict of interests: No conflict of interests is declared.