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Assessment of Protection Induced by DNA and Live Vaccine Encoding Leishmania MHC Class I Restricted Epitopes against *L. major* Challenge in Balb/c Mice Model

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Abstract

Leishmaniasis is a neglected tropical disease endemic in more than 88 countries and is spreading over and over mainly due to transmigration and drug resistance. Unfortunately, despite increasing incidence, effective vaccine is still lagging behind. Unraveling $CD8^+$ T-cells' contribution in infection control has recently led a new concept into Leishmania vaccine research field as T-cell vaccine. Therefore we studied the efficacy of a CD8 stimulating T-cell vaccine (a string of beads) in two distinctive approaches as homologous DNA-DNA or heterologous DNA-Live prime-boost strategies. Here we hypothesized that $CD8^+$ T-cell stimulation by polytope constructs diverts primary Th2 responses into Th1 resulting in disease control. Four recently reported H-2Kd restricted epitopes from proteins out of Leishmania vaccine candidate repertoire were included in the polytope construct (besides 13 HLA-A2 restricted peptides from Leishmania well known vaccine candidates). Protective effect of the polytope construct was evaluated by clinical (footpad swelling and parasite burden) and immunological (IFN-γ/IL-5 ELISA, IFN-γ ICCS and CFSE) assays after *L. major*^{EGFP} infectious challenge of Balb/c mice. In this study, DNA-DNA prime-boost regimen specifically stimulated $CD8^+$ T-cells resulting in partial protection in test group compared to controls. Protective effect was clearly compromised by $CD8^+$ T-cell depletion at the time of infectious challenge resulting in predominant Th2 response. This directly confirmed $CD8^+$ T-cells' role in early stage Th1 response polarization. Heterologous prime-boost regimen (DNA priming and live *L. tar*$^{PT\text{-}EGFP}$ boosting), however was less effective inducing $CD8^+$ T-cells and partial protection induced did not last long. These preliminary results for polytope constructs seem as sparkles of hope in Leishmania vaccination.

Keywords: *Leishmania major*; $CD8^+$ T-cell; Polytope vaccine

Introduction

Neglected tropical diseases (NTDs) are a complex of viral, bacterial, parasitic and helminthic diseases most common in Middle East, North Africa and South America among low-income populations. The term "neglected" is used not exactly because poor people are more affected but because the mortality rate is less considerable than morbidity rate. However debilitating effects reflected as DALY (disability adjusted life year) remark NTDs as serious global health problem more than ever. Unfortunately, effective vaccine is still lagging behind due to some challenges as antigen discovery, pre-clinical development, clinical trials in resource-poor countries and intricacies of host-parasite interaction [1,2].

Leishmaniasis, both as cutaneous (CL) and visceral (VL), falls within NTDs which highly affect Middle East and in particular Iran [3]. Considerable effort without satisfying outcomes was made to control the disease by leishmanization and killed Leishmania vaccine [4]. However, due to ample evidence showing vaccine feasibility, subunit and live attenuated vaccines are still under massive investigations, but both demand more efforts. Recent advancements in computational immunology (immunoinformatics) and also full genome sequence availability from different species of Leishmania has introduced a new concept of "genome-based-vaccines" in "reverse vaccinology" era versus "conventional vaccinology" [5-7]. This concept will hopefully revolutionize both subunit and live attenuated Leishmania vaccine through genome mining for new antigens (new subunit candidates) and targeted gene manipulations respectively.

Leishmaniasis is a Th1-immune-response demanding infection since Leishmania is an obligatory intracellular parasite residing within host macrophages [8]. $CD4^+$ Th1 type cytokines and in particular IFN-γ play a dual role in intracellular infection control. They potentiate pathogen killing within infected macrophages by up regulating toxic nitrogen-oxygen metabolites and activating $CD8^+$ T-cell mediated apoptosis of infected cells [9]. $CD4^+$ and $CD8^+$ T-cells are activated by sensing short peptides presented within MHC class II and class I context respectively. Researchers now harness Immunoinformatics tools to predict potential $CD4^+$/$CD8^+$ T-cell epitopes *in silico* and then select *in vitro/in vivo* evaluated immunogenic ones to arrange them together in a polytope construct [10,11]. Polytopes or T-cell vaccines as novel subunit vaccines are highly preferred since they carry minimal immunogenic part of the protein where whole protein might have

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adverse effects like suppressors of cytokine signaling proteins [12], contain immune regulatory epitopes or Tregitopes [13] and even potentially stimulate Th2 responses such as *Leishmania* meta-1 protein [14].

$CD8^+$ T-cells' contribution is undoubtedly necessary for VL control but remains controversial regarding CL control [15,16]. Our previous study clearly evaluated $CD8^+$ T-cells stimulation by a polytope construct containing H-2Kd restricted epitopes from non-vaccine protein candidates of *Leishmania major* [17]. Included epitopes originated from a list obtained by Leishmania genome mining for H-2Kd restricted epitopes using immunoinformatics tools [18]. Here we evaluated the protective effect of this construct in Balb/c model of CL by DNA-DNA and DNA-live immunization. Both regimens induced partial protection in Balb/c model further potentiating the $CD8^+$ T-cells' role in primary CL infection control. These results are in concordance with a premise postulated by Uzonna et al. that stimulation of $CD8^+$ T-cells early after infection is so critical for immune deviation toward Th1 response [19,20]. In our study protection was clearly compromised by $CD8^+$ T-cell depletion at the time of infectious challenge resulting in long lasting Th2 responses. These preliminary results for polytope constructs seem as sparkles of hope in Leishmania vaccination and even therapeutic research field while post genomics is flourishing more than ever to influence vaccine design [21].

Materials and Method

Ethics statement

All mouse experiments including maintenance, handling program, blood sampling and euthanasia were approved by Institutional Animal Care and Research Advisory Committee of Pasteur Institute of Iran, based on the Specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education of Iran (issued in 2005). All mice were housed in plastic cages with free access to tap water and standard rodent pellets in an air-conditioned room under a constant 12:12 h light-dark cycle at room temperature and 50–60% relative humidity.

Polytope construct

Here we studied the protective effect of a previously designed and evaluated polytope construct composed of few peptides in tandem [10,17]. Briefly the polytope included HLA-A2 restricted epitopes from 4 known Leishmania vaccine candidates (CPB and CPC, 5 peptides, *Lm*STI-1, 4 peptides and LPG-3, 4 peptides) and 4 recently reported H-2Kd restricted epitopes from non-candidates. Peptides were separated by short spacers. Mouse ubiquitin sequence was added to N-terminus and Tetanus Toxoid Th epitope (TT_{830}) was added to the C-terminus. This package (received as pUC-PT from BIOMATIK-Canada) was further cloned in pcDNA3.1$^+$ (Invitrogen) making pcDNA-PT and was purified to be endotoxin free by Endofree Plasmid Giga kit (QIAGEN). We also evaluated a live vaccination strategy by using recombinant *Leishmania tarentolae* parasite stably transfected with polytope sequence in 18srDNA locus and in non-secretory form (*L. tar*$^{PT\text{-}EGFP}$). Polytope structure and cloning pathways are summarized in Figure S1A and S1B respectively.

Anti-CD8 purification from H35-17.2 hybridoma cell

Rat H35-17.2 hybridoma cells produce monoclonal anti-mouse CD8 beta IgG2b antibodies and secrete it into culture medium. Protein-G affinity chromatography columns are then used to specifically purify IgG antibodies from supernatants. H35-17.2 hybridoma cells (a gift by Prof. Genevieve Milon, Pasteur Institute of Paris) were cultured for weeks to concentrate secreted antibody in about 1500 ml medium. Filtered medium was loaded on protein G coated column (HR 10/10 Pharmacia biotech) and IgG was extracted by acidic buffer (pH=2.7). Acidic buffer was immediately neutralized and further exchanged by PBS through dialysis in 4°C. Poly-ethylene glycol concentrated antibody was aliquoted (500 µg for each injection) and stored in -80°C until use. Intra-vascular (i.v.) injection of 500 µg antibody followed by flow cytometry 48 h later with 488-argon ion-laser equipped BD FACScalibur instrument confirmed 98% reduction in CD8 population after anti-CD8 treatment compared to untreated mice. (As observed in Figure S3, even 7 weeks after anti-CD8 treatment, the CD8 population was only 5-6% of total $CD3^+$ T cells instead of 25% in untreated mice.

Leishmania promastigote propagation

Leishmania major (MRHO/IR/75/ER) expressing EGFP (*L. major*^{EGFP}) and recombinant *L. tar*$^{PT\text{-}EGFP}$ were cultured in M199 medium (Sigma) supplemented with 5% heat inactivated Fetal Calf Serum (FCS-Gibco), 0.5 µg/ml Hemin (Sigma), 2.5 µg/ml Adenosine (Sigma), 0.03% L. glutamine (Sigma) and 70 µg/ml Gentamicin (Biosera) were properly propagated in logarithmic growth phase. Five days before challenge fresh medium feeding was stopped to force promastigotes into stationary phase for either vaccination (*L. tar*$^{PT\text{-}EGFP}$) or infectious challenge (*L. major*^{EGFP}). Inoculums were prepared in 1x PBS buffer and were injected in less than 5 hours after preparation.

Mouse immunization protocol and challenge

Female Balb/c mice (6-8 weeks old) were divided in groups (20 mice per group) to be immunized by DNA-DNA or DNA-Live regimens in right hind footpad (summarized in Table 1). Mice were boosted three weeks after priming. Then three weeks after booster injection, all mice were challenged sub-cutaneously (s.c.) in the left footpad (2 x 10^5 stationary phase promastigotes per mouse). DNA immunizations were preceded by electroporation (BTX®-Harvard Apparatus (ECM 830)) right before DNA inoculation with pre-set pulsing voltages (66 volts, 21 milliseconds). For this, mice were humanly anesthetized by Ketamine (10%) - Xylazine (3%) mixture in normal saline. Mice in G2 were depleted of $CD8^+$ T-cells by i.v. inoculation of rat anti-mouse CD8 antibody (purified from H35 hybridoma), 8 hours before and 48 hours after infectious challenge with *L. major*^{EGFP}.

Footpad swelling measurement by metric caliper

Started one week after infectious challenge, mice in different groups were weekly monitored for footpad swelling by metric caliper. Swelling was measured as mean of left footpad thickness and wideness after subtraction of pertinent baseline values of right footpad.

In situ imaging of anesthetized mice for EGFP fluorescence

Anesthetized mice were fixed one by one on imaging stage of KODAK imaging system (*In-Vivo* imaging system F Pro) after position adjustments with door open (exposure time was set on 30 second and filter wavelengths on 470/535 nm). The image was captured in less than a minute. Pixel counting and measurement of the lesions were performed using KODAK molecular image software version 5.3. Results were reported as "net intensity", a quantitative measurement defined as the number of green pixels in a given area (region of interest) multiplied by the average intensity of each pixel [22,23]. This experiment was performed 7 weeks post challenge since earlier imaging barely discriminates fluorescent signal difference among groups.

	Prime (s.c.)	Boost (s.c.)	$CD8^+$ T-cell depletion (i.v.)	Challenge (s.c.)	$CD8^+$ T-cell depletion (i.v.)
G1	pcDNA-PT (50 µg/mouse)	pcDNA-PT (50 µg/mouse)	-	*L. major*^{EGFP}	-
G2	pcDNA-PT (50 µg/mouse)	pcDNA-PT (50 µg/mouse)	8 hours before challenge	*L. major*^{EGFP}	48 hours after challenge
G3	pcDNA (50 µg/mouse)	pcDNA (50 µg/mouse)	-	*L. major*^{EGFP}	-
G4	PBS	PBS	-	*L. major*^{EGFP}	-
G5	pcDNA-PT (50 µg/mouse)	*L. tar*$^{PT\text{-}EGFP}$ $2x10^7$ parasite/ mouse	-	*L. major*^{EGFP}	-
G6	pcDNA (50 µg/mouse)	*L. tar*^{EGFP} $2x10^7$ parasite/mouse	-	*L. major*^{EGFP}	-

Table 1: Immunization schedule and challenge in different groups.

Real-Time PCR measurement of lymph node parasite burden

Real-time PCR was used to quantify parasite burden in draining lymph nodes at both early and late phase of infection as described before [24]. Genomic DNA was extracted from homogenized lymph nodes of 4 individual mice. Two sets of primers targeting a region of kinetoplastid minicircle named RV1 (forward: *5′-CTTTTCTGGTCCCGCGGGTAGG-3′*) and RV2 (reverse: *5′-CCACCTGGCCTATTTTACACCA-3′*) were used. Absolute copy number of the target sequence was extrapolated from a standard curve using Applied Biosystems 7500 real time PCR application. Standards were prepared from genomic DNA extracted from 2×10^7 *L. major* parasite serially diluted 10 folds up to 6 dilutions. PCR reactions were prepared in duplicate including 50 ng genomic DNA, 5 pmol of each forward and reverse primers, 12.5 µl Qiagen QuantiFast SYBR Green Master Mix to a total volume of 25 µl. PCR amplification cyclings included: 1 cycle of 95°C for 2 min; 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 40 s. Data was extrapolated on standard curve using 7500 system SDS software.

Antigenic stimulators

The polytope construct contained 4 H-2Kd restricted 9-mer peptides which were used in *in vitro* assays (Table 2). A 10-mer peptide from Hepatitis C virus' NS-3 protein (KLSGLGLNAV- kindly provided by Dr. Arash Memarnejadian, Pasteur Institute of Iran) was used as irrelevant control. All peptides were synthesized by BIOMATIK-Canada.

Lymphocyte culture and stimulation

Spleens dissected from humanly euthanized mice (6 mice per group) were individually homogenized and treated with freshly prepared ACK buffer (NH_4Cl, 8 gr/L, $KHCO_3$, 1 gr/L, Na_2EDTA 372 mg/L) to prepare erythrocyte free single cell suspension of splenocytes. 3 x 10^6 cells were plated in 48 well culture plates (Orange Scientific) and stimulated by individual P1-P4 peptides (10 µg/ml), irrelevant control peptide (10 µg/ml), F/T (20 µg/ml) and ConA (5 µg/ml) while incubated at 37°C-5% CO_2 incubator for 5 days. Supernatants were then collected for IFN-γ and IL-5 cytokine assay by ELISA. For intra-cellular cytokine staining (ICCS) and cell proliferation using 5-(and-6)-carboxy fluorescein diacetate succinimidyl ester (CFSE), 10^7 splenocytes were plated in 6 well plates and stimulated with 10 µg/ml of each relevant peptide (P1-P4). Three days later, cell cultures were supplemented with recombinant human-IL-2 (CEDARLANE) at 100 U/ml final concentrations and incubated for further 3 days.

Cytokine and antibody ELISA

Secreted IFN-γ and IL-5 were quantified by DouSet ELISA-Mouse IFN-γ and DouSet ELISA-Mouse IL-5 kits (R&D Systems) respectively as indicated by instructions. Briefly, maxisorb ELISA plates (Greiner-Bio one) were coated by anti-cytokine antibody, incubated overnight at ambient temperature (RT), washed and coated with PBS-1% BSA (1 h at RT). After two hours of incubation at 37°C of standard dilutions (100 µl) and supernatants (200 µl), plates were washed and biotin conjugated anti-cytokine antibody was added (2 h at 37°C). Final reaction was revealed by streptavidin conjugated horse-radish-peroxidase (20 min at RT) and enzyme substrate (ABTS® Peroxidase Substrate System (KPL)). Reaction was stopped by 1% SDS solution and absorbance was measured at 405 nm with reference filter.

Antibody ELISA assay was followed by previously described instructions [24]. Briefly maxisorb ELISA plate was coated with either P1+P4 or P2+P3 mixture (5 µg/mL each peptide) then loaded with 100 µl of 1:10 diluted pooled sera of each group. Reaction was revealed by 1:10,000 diluted HRP conjugated goat anti-mouse IgG1 or IgG2a (2 h at 37°C) and KPL enzyme substrate (20 min, 37°C). Reaction was stopped by 1% SDS solution. Absorbance was measured at 405 nm with reference filter.

$CD8^+$/IFN-γ^+ T cells detection by intra-cellular cytokine assay using flow cytometry

Herein standard plate based protocol with some modifications was used [10,25]. Briefly, pre-stimulated splenocytes were harvested from 6 well plates. Cells were washed and restimulated *in vitro* again by relevant peptides (10 µg/ml), irrelevant control (10 µg/ml) and PMA/Ion (Sigma) along with Golgiplug (BD-Biosciences) to prevent excretion of elevated cytokines while incubated at 37°C-5% CO_2 incubator overnight. After overnight culture, plate was centrifuged at 2000 rpm for 5 min to pellet the cells. Cells were washed in staining buffer (PBS 1x, 0.5% FCS and 0.1% NaN_3) and re-suspended in the same buffer containing PE-Hamster anti-mouse CD3e and PerCP-Rat anti-mouse CD8 antibodies. After 30 min at 4°C, cells were pelleted and washed in staining buffer then fixed and permeabilized with 200 µl of Cytofix-Cytoperm buffer (BD Cytofix-Cytoperm kit, BD biosciences) while incubated for further 20 minutes at 4°C. After adequate washing with saponin containing wash buffer (BD Cytofix-Cytoperm kit, BD

Peptide	Protein name	Accession No.	Peptide sequence	HLA	SYFPEITHI(1)	BIMAS(2)
P1	Guanin deaminase	LmjF29.0867	SYSSLVSAL	H-2Kd	28	2880
P2	Fatty acid elongase	LmjF14.0650	SYETGSSTL		25	2400
P3	Protein prenyltransferase	LmjF25.0150	AYSVSASSL		28	2880
P4	Hypothetical protein	LmjF29.2650	FYQEAAELL		27	2400

(1) http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm
(2) http://www-bimas.cit.nih.gov/molbio/hla_bind/

Table 2: Characteristics of relevant peptides in polytope construct.

biosciences), cells were re-suspended with FITC-Rat anti-mouse IFN-γ antibody for 30 min at 4°C then washed and prepared for data acquisition in staining buffer with BD FACScalibur flow cytometer. 100,000 events were acquired and analyzed by FlowJo 7.5.3 (TreeStar, USA). Live lymphocytes were gated on $CD3^+$ T cells then $CD8^+$/IFN-γ^+ T cell population was reported in percent in $CD3^+$ gate of lymphocyte region. PMA/Ion stimulated cells were used as control.

$CD8^+$ T-cell proliferation determination by CFSE flow cytometry assay

Pre-stimulated splenocytes were harvested from 6 well plates in 14 ml falcon tubes filled up to 14 ml with RPMI medium. Cells were washed and resuspended in 1 ml PBS-0.1% BSA then very gently mixed with 1 ml freshly prepared 5-(and-6)-carboxy fluorescein diacetate succinimidyl ester (CFSE-20 μM) solution. Cells were incubated 10 min in 37°C-5% CO_2 incubator and immediately neutralized by 10% FCS supplemented cold RPMI (5 min incubation on ice). Cells were properly washed twice to remove un-integrated CFSE and restimulated with relevant peptides (10 μg/ml), irrelevant peptide (10 μg/ml) and ConA (5 μg/ml). Cells without CFSE staining were used as control [26]. 48 h later cells were re-harvested and stained for CD3 (PE-Hamster anti-mouse CD3e, BD biosciences) and CD8 markers (perCP Rat anti-mouse CD8α, BD biosciences) after 30 min incubation at 4°C in staining buffer. Cells were washed and prepared for data acquisition in staining buffer with BD FACScalibur and analyzed by FlowJo 7.5.3. 100,000 events were acquired and live lymphocytes were gated on $CD3^+$ T cells then $CD8^+$ T cells. CFSE dilution was analyzed as dividing and was reported in percent after subtraction of background proliferation of control peptide stimulated cells. ConA stimulated cells were used as control. CFSA proliferation was fulfilled seven weeks after challenge.

Statistical Analysis

Statistical analysis was performed using Graph-Pad Prism 5.0 for Windows (San Diego, California). The data were analyzed with Student's t-test unless the F value was statistically significant. In this case data were analyzed with Mann-Whitney-U test. The results were considered statistically significant with a precision of $p<0.05$ (One asterisk, $0.05>p>0.01$; two asterisks, $0.01>p>0.001$; three asterisks, $p<0.001$). All over the paper, the results have been considered significant only if the difference of the test groups (G1 or G5) was significant versus all relevant control groups (G2 and G3 and G4 for DNA-DNA experiment, G4 and G6 for DNA-Live experiment). In each case, date presented is the numerical difference of actual stimulation by relevant peptides and background stimulation by irrelevant peptide. All indicated data are representative of two rounds of experiment.

Results

In vitro footpad swelling measurements coupled with *in vivo* qualitative footpad imaging

Started one week after infectious challenge, weekly measurements of footpad swelling (as a clinical sign of infection) by metric caliper showed a significant control ($p<0.05$, t-test analysis) in lesion size in DNA-DNA vaccinated group (G1) lasting during infection (Figure 1A). The difference between G1 and $CD8^+$ T-cell depleted G2 group ($0.01>p>0.001$, unpaired t-test analysis) is very important (G2 has comparable size during infection with G3 and G4). DNA-Live vaccination induced a non-significant difference in lesion size in G5 group early after infection (compared to corresponding controls) which did not last long (Figure 1B). Seven weeks after challenge, 6 randomly selected mice from each group were subject to *in vivo* imaging using fluorescence property of *L. major*^{EGFP} as challenge parasite. As shown in Figure 1C, net intensity comparison of selected ROI of left footpad in G1 was significantly lower at late infection after DNA-DNA immunization ($0.01>p>0.001$, unpaired t-test analysis). This difference with G2 group (which has comparable parasite load with controls) is remarkably important. However no significant difference was detected in DNA-Live vaccinated groups (Figure 1D). All footpad images from G1-G6 groups captured by *in vivo* imaging are illustrated in Figure 1E.

Lymph node parasite burden evaluated by Real-Time PCR

Parasite burden is routinely measured by limiting dilution but where applicable more sensitive approaches as RT-PCR are substituted. Dissected, homogenized and weighed draining lymph nodes at 3 and 7 weeks post challenge were assayed for absolute quantity of parasite in each individual tissue by means of quantitative real-time PCR (based on standard curve). As shown in Figure 2B, significant lower parasite load was detected at 7 weeks post-infection in G1 group ($p<0.05$, unpaired t-test) in comparison to controls (the difference was not significant at 3 weeks post challenge as shown in Figure 2A). This observation was quite consistent with footpad *in vivo* imaging results at 7 weeks point, which is a direct measurement of local fluorescent signals (Figure 1E). Higher level of parasite number in G2 group seven weeks after challenge remarks a significant role for $CD8^+$ T-cells ($p<0.001$, unpaired t-test). Parasite level was even higher in G2 at 3 weeks post challenge but not significant (Figure 2A). DNA-Live immunization confirmed a non-significant difference between G5 and control groups 3 weeks after challenge (Figure 2C) which was negligible at 7 weeks (Figure 2D). As a marker of parasite proliferation, lymph nodes' weight was lower in G1 group (Figure 2E) 7 weeks post-challenge but not in G5 group (Figure 2F).

IFN-γ production by peptide stimulation

Before challenge, 3 weeks and finally 7 weeks after, 6 mice per group were randomly selected and humanly euthanized. Spleens were dissected to prepare single cell suspensions of splenocytes. 3×10^6 cells were distinctly stimulated *in vitro* with 10 μg/ml of each relevant P1-P4 peptides and irrelevant control peptide (10 μg/ml). IFN-γ level was calculated after subtracting background secretion by cells stimulated with control peptide. Before challenge (Figure 3A), IFN-γ was detected in response to P2, P3 and P4 stimulation at a low but significantly different level ($0.05>p>0.01$, Mann-Witney U test) in G1+G2 group (G1+G2 refer to the results from these 2 groups before anti-CD8 treatment, both immunized with pcDNA-PT). Three weeks after challenge, IFN-γ was detectable only in response to P4 stimulation but not statistically significant (Figure 3B). At the end, all 4 peptides induced remarkable IFN-γ compared to controls especially G2 with very low cytokine level ($0.05>p>0.01$, Mann-Witney U test) (Figure 3C). The same analysis was performed for DNA-Live vaccination and as observed, no significant IFN-γ was detected neither before challenge (Figure 4A) nor weeks after at the late phase (Figure 4C). However 3 weeks analysis revealed detectable IFN-γ after P3 and P4 stimulation ($0.05>p>0.01$, Mann-Witney U test) (Figure 4B).

Th1/Th2 ratio determined by cytokine ELISA after F/T stimulation

$3x10^6$ cells were distinctly stimulated *in vitro* with *Leishmania major* F/T derived from multiple consecutive freezing and thawing action as described in previous section. Total Th1/Th2 ratio was verified after F/T stimulation by ELISA determined IFN-γ and IL-5 level. As shown

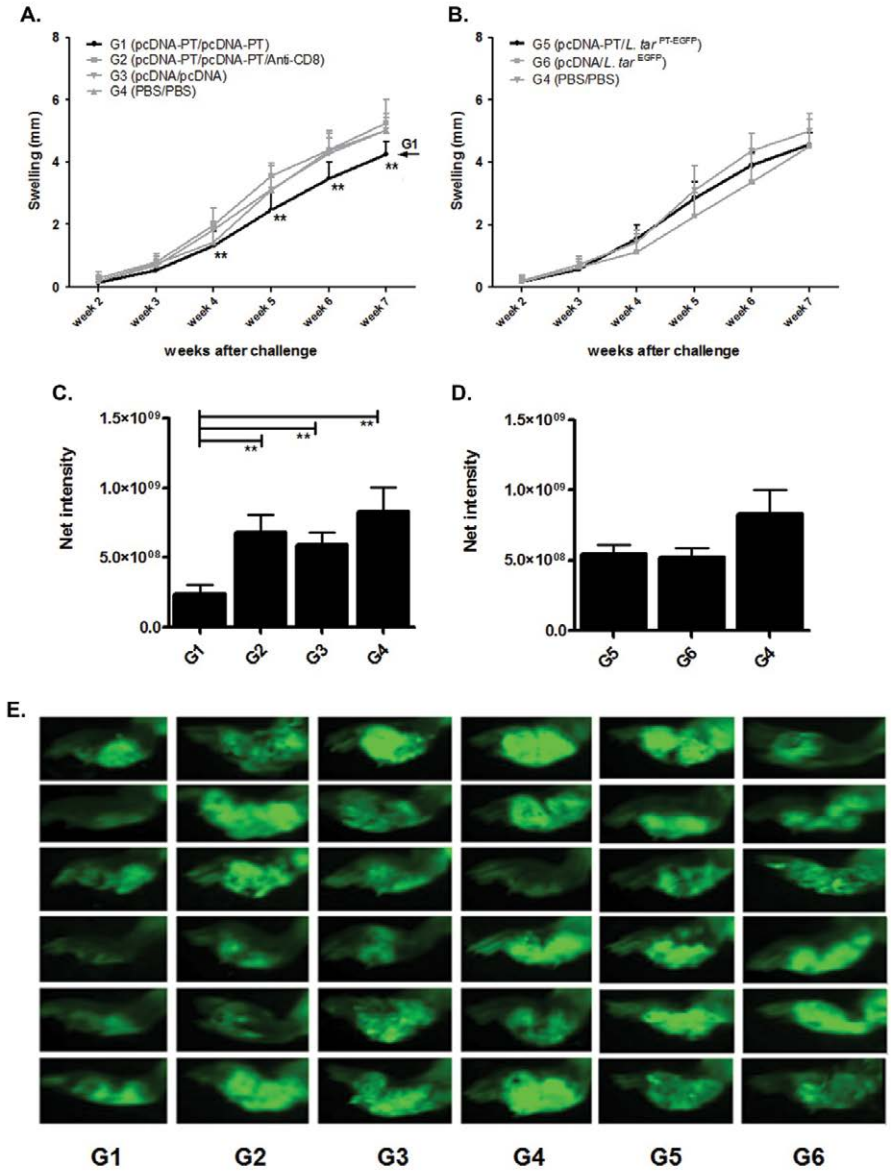

Figure 1: *In vitro* footpad swelling measurements coupled with *in vivo* qualitative footpad imaging. A. Weekly footpad swelling measurement by metric caliper in DNA-DNA vaccinated groups and corresponding controls. B. Weekly footpad swelling measurement by metric caliper in DNA-Live vaccinated groups and corresponding controls. Each dot represents mean+SD of mice in each group. Stars indicate significant lower lesion size in G1 compared to G2 ($0.01>p>0.001$, unpaired t-test analysis). C (DNA-DNA immunization) and D (DNA-Live immunization) represent results of qualitative footpad imaging by illuminating fluorescent parasites inside footpads of live but anesthetized mouse using KODAK imaging system (*in vivo* system FX Pro) 7 weeks after challenge. Each column represents mean+SD of Net intensity of selected ROI in each group. Stars indicate significant difference after unpaired t-test analysis ($0.01>p>0.001$). E illustrates footpad images in each group captured by KODAK imaging system. ROI: Region of Interest.

in Figure 5A, three weeks after challenge of DNA-DNA immunized group (G1), the immune response was roughly skewed towards Th1. However Th1 polarization was quite dominant 7 weeks after challenge due to high levels of IFN-γ to IL-5 in this group (Figure S2-A). $CD8^+$ T-cell depletion in G2 clearly compromised this effect where Th2 response dominated even 7 weeks after challenge. This was directly the result of higher levels of IL-5 compared to IFN-γ in this group (Figure S2-A). It was then concluded that Th1 polarization was a direct effect of $CD8^+$ T cells' contribution. DNA-Live immunization (G5) in contrast was not able to induce Th1 polarization (Figure 5B) although higher levels of IFN-γ were detected both 3 and 7 weeks after challenge (Figure S2-B).

$CD8^+$/IFN-γ^+ T cell quantification by intra-cellular cytokine staining

Single cell suspensions of splenocytes were *in vitro* stimulated with individual relevant and irrelevant peptides plus rh.IL-2 to moderately augment frequencies of responding clones. As observed in Figure 6, ICCS successfully detected $CD8^+$/IFN-γ^+ T cells in G1 group against P1 and P4 stimulations ($0.05>p>0.01$, Mann-Witney U test) both early after infection (6A) and later (6B). So IFN-γ detected in ELISA in response to P1 and P4 was directly attributable to $CD8^+$ T-cells. However $CD8^+$ T-cells' frequency in response to P2 and P3 might have been less than enough to be adequately sensed by ICCS even at late phase of infection

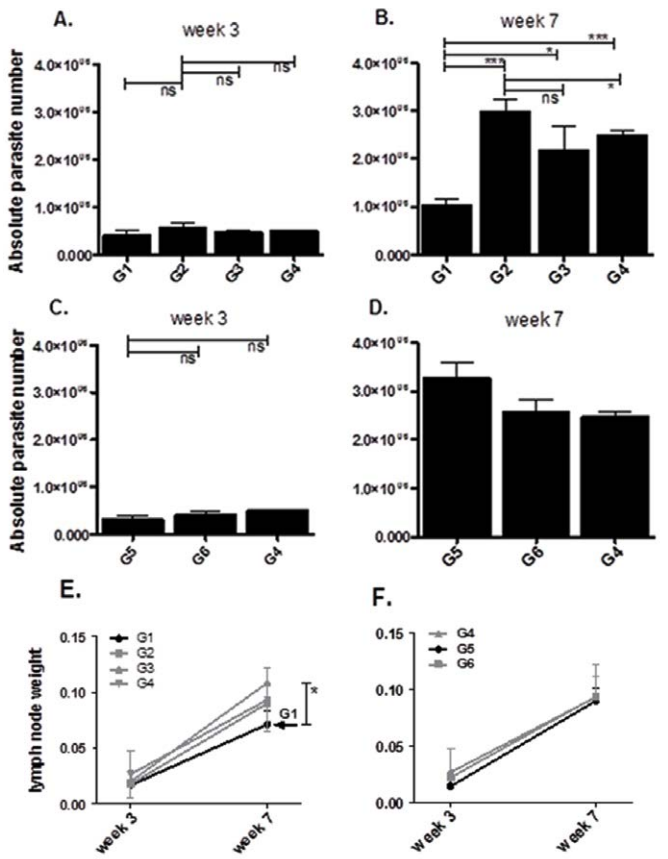

Figure 2: Lymph node weight and parasite burden. *Three and seven weeks after challenge,* dissected draining lymph nodes (next to challenge site) from humanly euthanized mice in different groups (4 mice per group) were individually weighed and homogenized in *Schneider's insect medium.* Homogenates were subject to genomic DNA extraction and RT-PCR with Leishmania kinetoplast specific primers. *The A and B plots represent results from DNA-DNA immunization at 3 and 7 weeks post-challenge respectively. Stars represent significant difference (p<0.05, unpaired t-test). The C and D plots represent results from DNA-Live immunization at 3 and 7 weeks post-challenge respectively.* E and F represent lymph nodes' weight during infection (Each dot represents mean+SD of lymph node weight in each group). Columns represent mean+SD of absolute parasite load in each group. ns: not significant.

in contrast to ELISA results. The lower avidity of P2 and P3 could have been compensated by complementary rounds of *in vitro* stimulation and IL-2 supplementation. $CD8^+$ T-cell depletion was clearly reflected in flow cytometric results both early and late infection. Furthermore ICCS analysis after DNA-Live immunization revealed higher but not statistically significant $CD8^+/IFN\text{-}\gamma^+$ T cell frequencies in G5 group compared to relevant controls (Figure 7A and 7B). Representative plots of ICCS strategy are illustrated in Figure S3.

$CD8^+$ proliferation in response to peptide stimulation evaluated by CFSE

CFSE stained splenocytes were restimulated *in vitro* for further 48 hours and evaluated for $CD8^+$ T-cell proliferation in response to individual P1-P4 epitopes and control peptide. As shown in Figure 8A, 7 weeks post- challenge DNA-DNA immunization stimulated $CD8^+$ T-cell proliferation against P1 and P4 epitopes ($0.05>p>0.01$, Mann-Witney U test) but not P2 and P3. These results further confirmed ICCS and higher avidity of P1 and P4 compared to P2 and P3. In contrast no considerable proliferation was observed against P1-P4 peptides after DNA-Live immunization (Figure 8B). This was in good concordance with ICCS results in DNA-Live immunized mice. Representative plots of CFSE dilution are illustrated in Figure S4. So based on common results of ELISA, ICCS and CFSE at 7 weeks post infection, we concluded that P1 and P4 have effectively raised $CD8^+$ T cell responses and have contributed in partial protection but P2 and P3 might have less effectively contributed due to lower avidity.

Humoral immune response evaluation by ELISA as Th1/Th2 deviation marker

Serum IgG2a and IgG1 antibodies were evaluated before and 5 weeks after challenge as a reliable marker of Th1 and Th2 directed response respectively. P1 and P4 were pooled together since were supposedly stronger peptides. P2 and P3 were then pooled together. Before challenge no significant difference was detected within groups (data not shown). As shown in Figure 9A, IgG2a to IgG1 ratio stands at least 2 folds higher in DNA-DNA immunized group (G1) compared

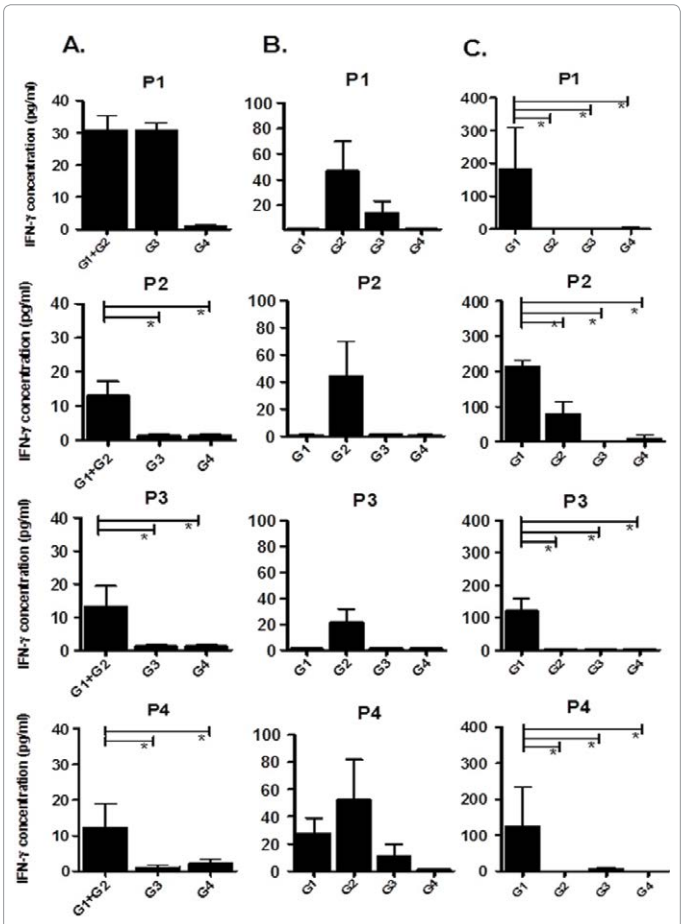

Figure 3: IFN-γ production after DNA-DNA immunization. Before infectious challenge (column A), 3 weeks (column B) and 7 weeks (column C) after challenge, 6 mice per group were randomly selected and humanly euthanized. Spleens were dissected to prepare single cell suspensions of splenocytes. 3 x 10^6 cells were distinctly stimulated *in vitro* with relevant P1-P4 peptides (10 µg/ml each) and irrelevant control peptide (10 µg/ml). IFN-γ level was calculated after subtracting background secretion by cells stimulated with control peptide. G1+G2 refer to the results from these 2 groups before anti-CD8 treatment. In each plot, columns represent mean+SD in each group. Stars represent significant difference between groups analyzed by Mann-Whitney-U test ($0.05>p>0.01$).

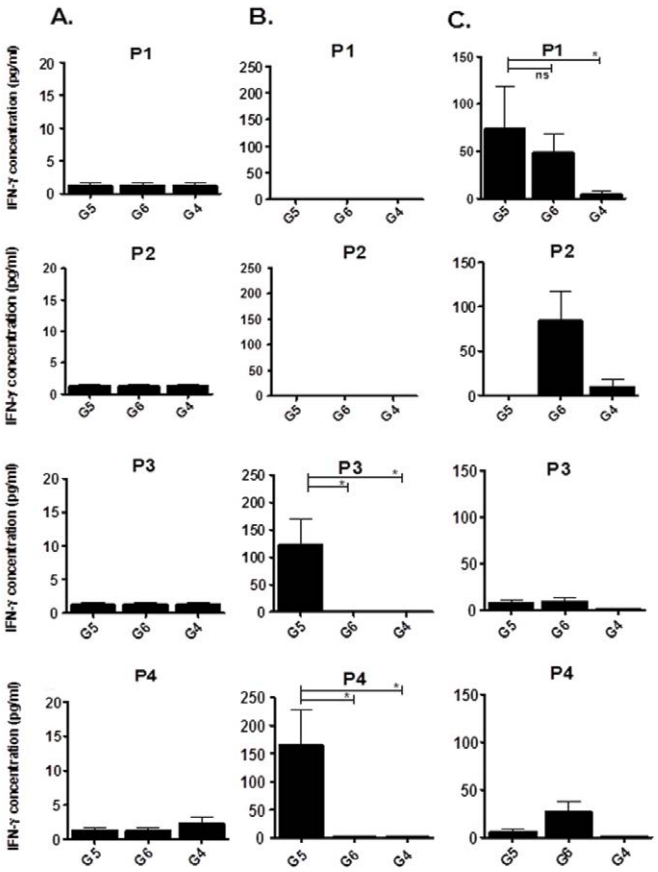

Figure 4: IFN-γ production after DNA-Live immunization. Before infectious challenge (column A), 3 weeks (column B) and 7 weeks (column C) after challenge, 6 mice per group were randomly selected and humanly euthanized. Spleens were dissected to prepare single cell suspensions of splenocytes. 3 x 10^6 cells were distinctly stimulated *in vitro* with relevant P1-P4 peptides (10 µg/ml each) and irrelevant control peptide (10 µg/ml). IFN-γ level was calculated after subtracting background secretion by cells stimulated with control peptide. In each plot, columns represent mean+SD in each group. Stars represent significant difference between groups analyzed by Mann-Whitney-U test ($0.05>p>0.01$).

to controls. Higher IgG1 level in CD8$^+$ T-cell depleted group (G2) compared to G1 is noteworthy (Figure S5). These results notify a clear Th1 dominant response in DNA-DNA vaccinated group versus Th2 dominant response in CD8$^+$ T-cell depleted G2 group. As observed, the ratio was not too big. This could reflect the function of ubiquitin molecule which directs the polytope into proteasome right after synthesis reducing the chance for B-cell stimulation. This promisingly validates the ubiquitination concept in polytope vaccine design. Furthermore humoral response is detected against both peptide pools including P2 and P3. This further confirms that P2 and P3 peptides could raise the recall response in ICCS and CFSE by complementary rounds of stimulations and IL-2 supplementation *in vitro*. Analysis after DNA-Live immunization did not discriminate any remarkable Th1 polarized response (Figure 9B). IgG1 and IgG2 levels are separately illustrated in Figure S5.

Discussion

It has been a consensus for years that CD4$^+$ T cells are the pivotal immune correlates of leishmaniasis control which determine the fate of infection. Such a premise was based on data from laboratory mice challenged with high doses (10^4-10^7) of *L. major* parasite. Most of the mice species as CBA and C57BL/6 are resistant to infection due to Th1 immune deviation except than Balb/c mice which succumbs to high dose challenge and is known as susceptible in this context [27]. Although leishmaniasis is an intracellular infection, the contribution of CD8$^+$ T-cells as immune correlates of disease at primary infection remained to be addressed [28,29] until the data from low dose experimental challenge in both Balb/c and C57BL/6 mice was extrapolated. The data from Balb/c mice infected by low dose challenge was controversially CD8$^+$ T-cell dependent, but these mice were able to elevate Th1 type immune response and control the primary and secondary infection [30-32]. However data from C57BL/6 mice clearly shed light on CD8$^+$ T-cells as contributors to CL control. CD8$^+$ T-cells depletion at primary infection abolished resistance in C57BL/6 mice infected intra-dermally by 100-1000 metacyclic promastigotes (approximation of low dose natural infection) [33]. Uzonna et al. further elucidated that IFN-γ secreted by CD8$^+$ T-cells is important to direct early Th2 type responses towards Th1 and establish protection which will end in a long term memory protecting against subsequent infections [19,20].

Unraveling CD8$^+$ T-cells' contribution in leishmaniasis control could profoundly impress vaccine design procedure. CD8$^+$ T-cell epitopes are 8-10 small fragments easily predictable by online immunoinformatics software. These epitopes if evaluated immunogenic *in vitro* and *in vivo*, which is the precise mark of previous activation following natural infection, could be further benefited in polytopes or T cell vaccines. Although both unassembled epitopes used individually in cocktail or assembled peptides in tandem are effective in stimulating relevant T cell clones, nucleic acid based constructs or DNA polytopes expressing epitopes in tandem are potentially preferred due to self-adjuvanting characteristics of plasmid DNAs and their intrinsic potential to induce both CD4$^+$ and CD8$^+$ T-cells [34,35].

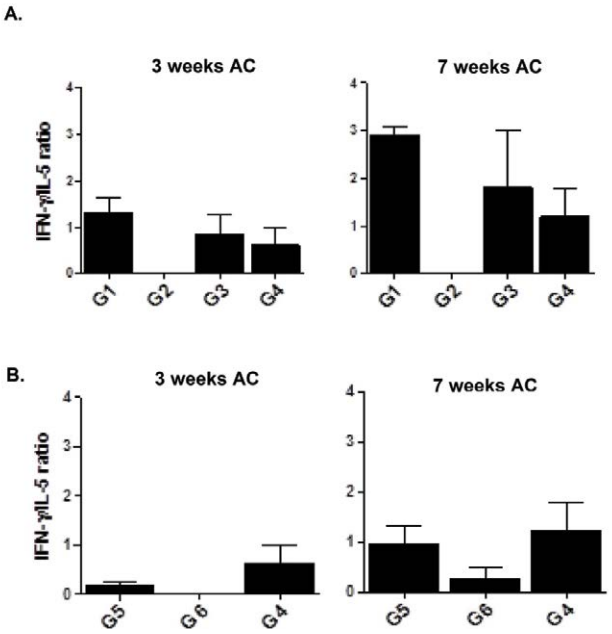

Figure 5: Th1/Th2 ratio determined by cytokine ELISA after F/T stimulation. 3 x 10^6 cells were distinctly stimulated *in vitro* with *L. major* F/T (20 µg/ml). Total Th1/Th2 ratio was verified after F/T stimulation by ELISA determined IFN-γ and IL-5 level. A. Results for DNA-DNA immunization. B. Results for DNA-Live immunization. Each column represents mean of IFN-γ to mean of IL-5 in each group.

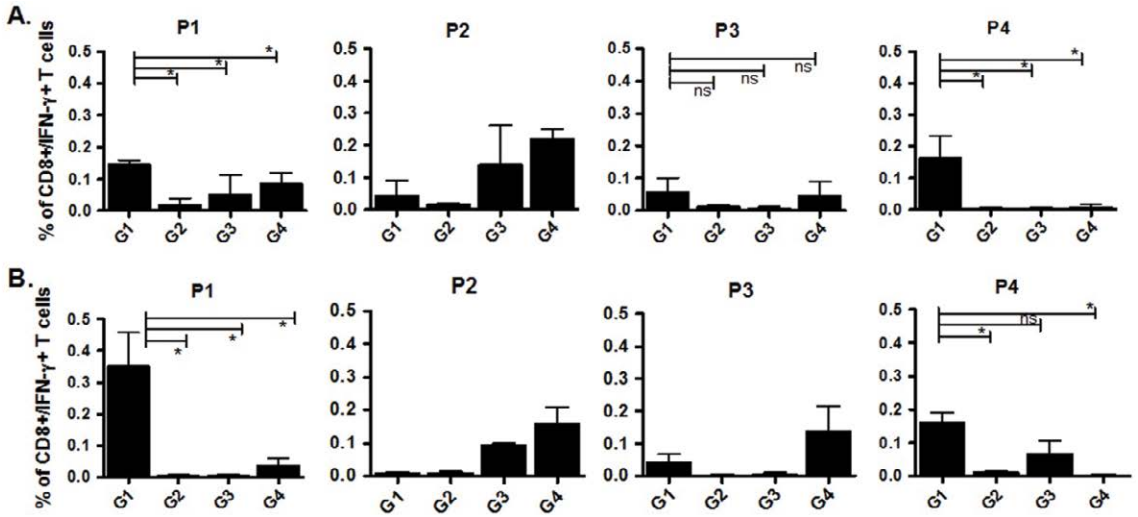

Figure 6: Intra-cellular Cytokine Staining for CD8⁺/IFN-γ⁺ T cells after DNA-DNA immunization. 10^7 splenocytes were plated in 6 well plates and stimulated with relevant peptides (P1-P4). 3 days later, cell cultures were supplemented with rh.IL-2 at 100U/ml final concentration and incubated for further 3 days. Cells were harvested and restimulated *in vitro* again by relevant peptides, irrelevant control and PMA/Ion along with Golgiplug while incubated at 37°C and 5% CO_2 incubator overnight. Cells were further stained for CD3/CD8/IFN-γ and data was acquired by BD FACScalibur on 100,000 events and analyzed by FlowJo software. A. 3 weeks after challenge. B. 7 weeks after challenge. Each column represents mean+SD of CD8⁺/IFN-γ⁺ T cells in each group. Significant differences are presented by stars after analysis by Mann-Whitney assay (0.05>*p*>0.01). ns: non-significant difference.

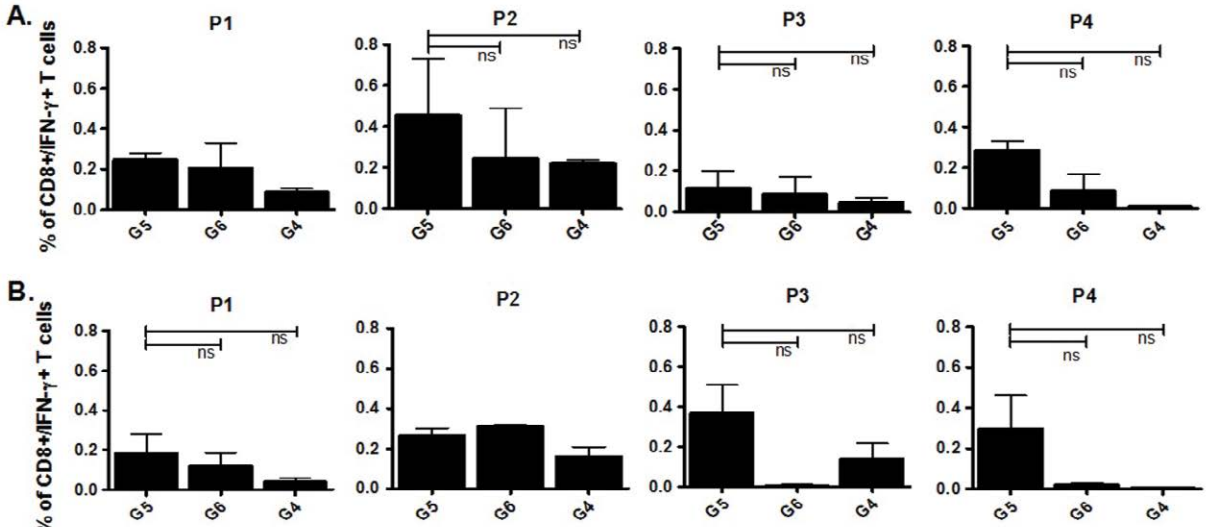

Figure 7: Intra-cellular Cytokine Staining for CD8⁺/IFN-γ⁺ T-cells after DNA-Live immunization. 10^7 splenocytes were plated in 6 well plates and stimulated with relevant peptides (P1-P4). 3 days later, cell cultures were supplemented with rh.IL-2 at 100 U/ml final concentrations and incubated for further 3 days. Cells were harvested and restimulated *in vitro* again by relevant peptides, irrelevant control and PMA/Ion along with 1 µl per reaction Golgiplug while incubated at 37°C and 5% CO_2 incubator overnight. Cells were further stained for CD3/CD8/IFN-γ and data was acquired by BD FACScalibur on 100,000 events and analyzed by FlowJo software. A. 3 weeks after challenge. B. 7 weeks after challenge. Each column represents mean+SD of CD8⁺/IFN-γ⁺ T-cells in each group. ns: non-significant difference (*p*>0.05) after analysis by Mann-Whitney test.

Based on these relevant data, we proposed a polytopic DNA vaccine encompassing small 9-mer fragments from 4 different non-vaccine candidates previously predicted by immunoinformatics mining of *Leishmania major* genome [18]. We hypothesized that if polytope constructs induce multiple CD8⁺ T-cell clones [17] and if CD8⁺/IFN-γ⁺ T cells effectively direct Th1 responses early after infection [19], then a multi-CD8 inducing polytope could induce protection in animal models of CL. So, here we described the protective efficacy of a homologous (DNA-DNA) and also a heterologous prime-boost regimen (DNA-Live) with a rationally designed polytope encoding four H-2Kd restricted immunogenic peptides after high dose challenge with *L. major*^{EGFP} in Balb/c mice (the immune response is absolutely investigated against H-2Kd restricted peptides and not the others restricted to human HLA-A2 with low affinity for H-2Kd allele).

DNA-DNA immunization (preceded by electroporation) induced partial protection which was immunologically correlated with CD8⁺/IFN-γ⁺ T cell clones. Immune response was totally skewed towards Th1 at late weeks after challenge. This was further confirmed by

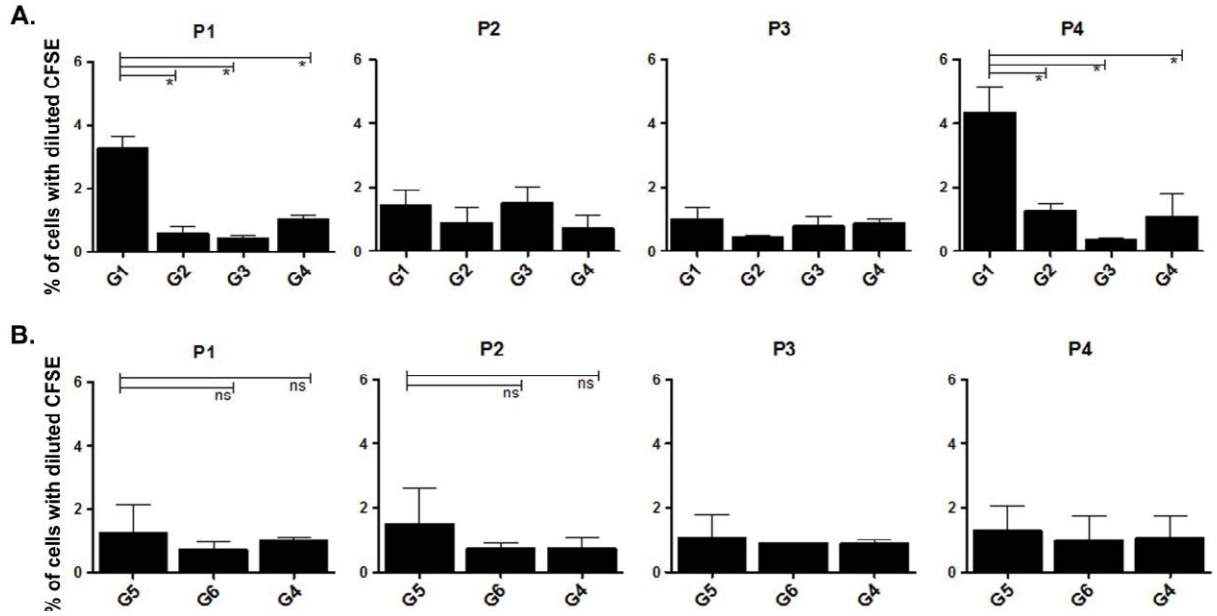

Figure 8: CD8⁺ T-cell proliferation evaluated by CFSE 7 weeks after challenge. Pre-stimulated splenocytes were harvested from 6 well plates, resuspended in PBS-0.1%BSA then gently mixed with freshly prepared CFSE solution. Cells were restimulated in 96 well plates with relevant and irrelevant peptides for further 48 hours. Cells without CFSE staining and ConA stimulated cells were used as control. Cells were then re-harvested and stained for $CD3^+/CD8^+$ markers and analyzed by BD FACScalibur for CFSE dilution. A. DNA-DNA immunization. B. DNA-Live immunization. Significant differences are presented by stars ($0.05>p>0.01$, Mann-Witney U test).

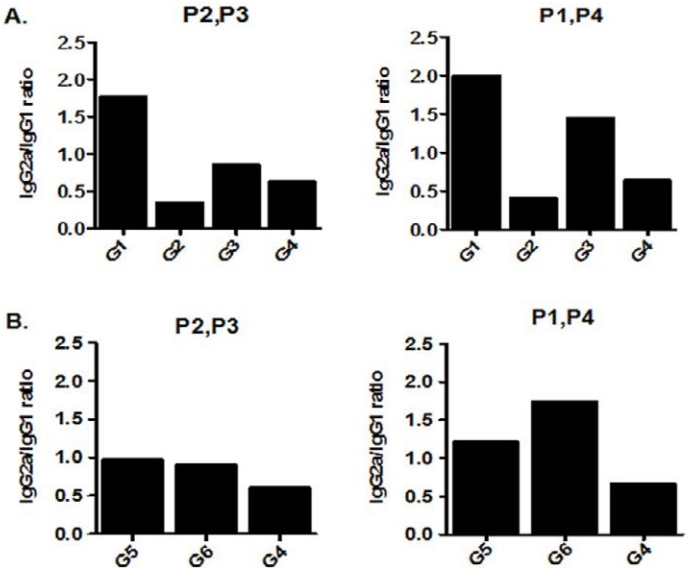

Figure 9: Humoral immune response evaluation by ELISA as Th1/Th2 deviation marker. Serum IgG2a and IgG1 antibodies were evaluated 5 weeks after challenge for Th1/Th2 polarized response by Anti-IgG2a/Anti-IgG1 HRP conjugated antibodies. Stimulations were conducted against 2 different peptide pools including P1 and P4 in one pool and P2 and P3 in the other pool. Each column represents mean IgG2a antibody to mean IgG1 antibody level in each group. A. DNA-DNA immunization. B. DNA-Live immunization.

humoral response evaluation at mid infection (about 5 weeks after challenge). We concluded that peptide affinity is the detrimental factor in protection efficiency. Focusing on ICCS responses we found P1 and P4 as high avidity peptides since both induced $CD8^+/IFN\text{-}\gamma^+$ during early and late phases of infection detected in ELISA, ICCS and CFSE. But response to P2 and P3 was barely detected in ICCS and CFSE showing that these 2 peptides demand further *in vitro* stimulations. P1-P4 was selected among high *in silico* scored peptides (predicted by SYFPEITHI/BIMAS – Table 2) with high *in vivo* IFN-γ production potential after peptide immunization. This was previously reported by Herrera-Najera et al. [18]. In our previous experiment, the DNA construct encoding the same peptides was used to immunize Balb/c mice and we found all 4 peptides immunogenic in ELISpot assay [17]. In this study our results confirmed that after *in silico*

prediction, peptide immunogenicity should be evaluated by infectious challenge to include high avidity epitopes in vaccine construct like P1 (SYSSLVSAL) and P4 (FYQEAAELL) because these 2 were highly promising at pre- and post-challenge conditions quite contrary to P2 and P3. However this is almost a difficult task in Balb/c mice due to disease progression instead of healing after infection. *In vivo* cleavage of peptides included is another important determining factor which was precisely managed by ubiquitin complementation and spacer inclusion for optimal proteasomal degradation. Clinical evaluations by observational methods (footpad swelling and imaging) marked milder disease progression in G1 compared to the rest. Real-Time PCR technique revealed much restricted parasite load at late infection. Totally this is a promising result regarding high dose *L. major* primary infection control in susceptible Balb/c mice by two immunizations. Further boosters (3 or even 4) could potentiate the results.

In line with our results, concerning DNA vaccine efficiency inducing protection against high dose parasite challenge in Balb/c mice by priming $CD8^+$ T-cells, Guranathan et al. demonstrated that immunization with a plasmid encoding Leishmania LACK protein was more efficient than immunization with recombinant LACK protein and recombinant IL-12, as induced protection was more durable against *L. major* challenge. They clearly demonstrated $CD8^+$ T-cell contribution in this effect [36-38]. Furthermore, Campos-Neto et al. proved that vaccination with a plasmid DNA encoding both TSA and *Lm*STI-1 fusion proteins confers protection against *Leishmania major* high dose challenge in Balb/c mice. This effect was attributed to $CD8^+$ T-cells' stimulation by TSA protein [39]. Also a cocktail of 4 plasmids encoding *L. infantum* histone proteins cross protected against high dose *L. major* challenge in Balb/c mice with both $CD4^+$ and $CD8^+$ T-cells as contributors [40]. Heterologous prime-boosts are proof of concept in this regard since this type of immunization is fully accepted where $CD8^+$ T-cells' activation is necessary [41]. Jayakumar *et al.* examined a prime-boost immunization with DNA-MVA encoding tryparedoxin peroxidase (TRYP) with TLR1/2 adjuvant and induced $CD4^+$/$CD8^+$/IFN-γ^+ T cell related protection in Balb/c mice against *L. panamensis* infection [42]. Same results were obtained by LACK DNA-MVA immunization reported by Sanchez-Sampedro [43]. Overally these results suggested that DNA vaccines as equivalents of low dose parasite or antigen administration are able to induce Th1 skewed immune responses against primary high dose parasite challenge. This happens because of $CD8^+$ T-cell activation. Antigen dose effect of DNA vaccine could have been augmented by electroporation which demands further elucidation. G2 group provided the proof of concept in this study. Partial protection was compromised while $CD8^+$ T-cells were efficiently depleted at infectious challenge time after pcDNA-PT prime-boost immunization. Th2 response was evidently dominant and resulted in significant difference in clinical features. Since $CD8^+$/IFN-γ^+ T cells were detected in G1 both at early and late phase; the difference was attributed to the function of these cells. Similar findings were reported in studies in which depletion of $CD8^+$ T-cells abolished induced protection by impeding the frequency of IFN-γ producing $CD4^+$ T cells and reducing the level of IL-12 production and it's receptor. Jayakumar et al. clearly demonstrated that immunity in Balb/c mice against *L. panamensis* high dose challenge induced by DNA-TRYP priming with Pam3CSK4 is compromised by $CD8^+$ but not $CD4^+$ T-cell depletion at the time of infectious challenge [42]. However our study was characterised by peptidic stimulants instead of whole proteins in vaccine construct which is proposed as novel subunit vaccine for Leishmania infection. Polytope ensembles are applicable since peptide epitopes from one potential protein or different proteins of one strain or conserved proteins from different strains of a species are easily assembled together.

Heterologous prime-boost vaccine regimens as mentioned above have proven protective by $CD8^+$ T-cell induction against intracellular pathogens such as Leishmania. Among possible vector combinations, Human Adenovirus [44], Modified Vaccinia Ankara [42,43] and Salmonella [45] have received significant attention. However anti-vector immunity is the main drawback linked with these vectors. Recently introduced live non-pathogenic *Leishmania tarentolae* (*L. tarentolae*) [46], could be a promising surrogate for viral/bacterial vectors in Leishmania vaccine research due to high resemblance to pathogenic *L. major* strain [47]. Herein a recombinant *L. tarentolae* stably expressing 4 H-2Kd restricted peptides in non-secretory form (intra-cytoplasmically) was used in DNA-Live regimen which roughly protected against high dose challenge early after infection. The results indicated dominant Th2 response which was consistent with clinical manifestations. Humoral immunity 5 weeks after challenge also indicated that the balance was not clearly in favor of Th1 response. Totally both clinical and immunological parameters failed to support protection.

Previously *L. tarentolae* has proven promising by long lasting protection while expressing multiple candidate antigens (all among potential vaccine candidates as A2) [24,48] especially along with sandfly salivary immune-stimulatory proteins [49]. This is a confirmatory proof that peptides included must be among high avidity ones previously evaluated in *in vivo* infectious challenge. Besides, the recombinant *L. tarentolae* used in this study expresses an ubiquitinated polytope which guides the newly synthesized polypeptide into proteasome for cleavage. It is recommended to replace the cytoplasmic form of synthesis with secretory to further expose relevant peptides to immune system. For this ubiquitin could be replaced by leader sequences. This way the nascent polytope will be efficiently translocated into endoplasmic reticulum both for degradation and secretion out of the cells. It is also recommended to fulfill a dose escalation for *L. tarentolae* immunization to meet the requirements of $CD8^+$ T-cell activation (in this study while polytope construct is expressed under the control of a strong promoter (18s rRNA), the recombinant parasite is inoculated in 2 x 10^7 parasite per mouse). Furthermore promising adjuvants are now available such as CpG oligonucleotides that are used to potentiate live Leishmania vaccine and help $CD8^+$ T-cell induction *in vivo* [50].

As a whole, DNA-Live regimen roughly protected against high dose challenge due to weak CD8 T cell induction. In contrast, DNA-DNA immunization correlated with a partial protection where immune correlates were induced early after infection and expanded to the end. The polytope construct included only 4 H-2Kd restricted epitopes, 2 of which were lower in avidity. As previously mentioned, these peptides were predicted by immunoinformatics and were evaluated to induce IFN-γ producing clones after peptide immunization in Balb/c mice and not after infectious challenge. As evidenced, Balb/c mice are highly susceptible to Leishmania infection and complete protection by vaccine is somewhat imaginary and not fully achievable. Therefore the limited level of protection observed in this model by this construct is not negligible and is noteworthy to be further potentiated by increasing the number of high-avidity peptides and by advantaging competent adjuvants and delivery systems.

A literature review on T-cell vaccines for *Leishmania* gives very few results. Most of them are at pre-vaccine *in silico* prediction-*in vivo* validation stage to predict potential MHC class I/II restricted peptides from known [51-55] or genome wide vaccine candidates [18,56,57].

Since $CD4^+$ T-cells are known as important contributors of immunity, the few vaccine outputs are confined to MHC II epitopes [58-60]. Very recently Das et al. published the results from an innovative polytope design from conserved regions of 5 known Leishmania candidate antigens. CD4 and CD8 inducing pentadecapeptides evaluated immunogenic in human populations of endemic regions were mapped onto sequence of the original antigens and redesigned to include as many as possible T cell epitopes. This was to meet the requirements of MHC polymorphism in human population with large number of epitope inclusion. A cocktail of 5 different DNA constructs based on each individual peptidic region protected against VL in Balb/c mice. This data is the very first report of a DNA-based T cell vaccine development in Leishmania [61]. Here we reported the results from a prototypic polytope vaccine specifically inducing $CD8^+$ T-cell responses with previously evaluated H-2Kd epitopes. The source proteins of used peptides are among non-vaccine candidates introduced by *L. major* genome mining using immunoinformatics. P4 is also derived from a hypothetical protein so is noteworthy to be further characterized as novel vaccine candidate. Therefore the partial protection conferred by DNA-DNA or DNA-Live (lower efficiency compared to DNA-DNA) regimens in a susceptible CL model could change vaccine concepts. First, new vaccine candidates with both $CD4^+$ and $CD8^+$ T-cell inducing potential could be introduced. Second, polytopic constructs with multiple high avidity epitopes provoking both $CD4^+$ and $CD8^+$ T-cell responses could enter the *Leishmania* vaccine research. In this context non-pathogenic live vectors with high resemblance to pathogenic *L. major* could end in promising results.

References

1. Bethony JM, Cole RN, Guo X, Kamhawi S, Lightowlers MW, et al. (2011) Vaccines to combat the neglected tropical diseases. Immunol Rev 239: 237-270.
2. Rafati S, Modabber F (2014) Cutaneous Leishmaniasis in Middle East and North Africa. Neglected Tropical Diseases-Middle East and North Africa: Springer 117-139.
3. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, et al. (2012) Leishmaniasis worldwide and global estimates of its incidence. PloS one 7: e35671.
4. Khamesipour A, Rafati S, Davoudi N, Maboudi F, Modabber F (2006) Leishmaniasis vaccine candidates for development: a global overview. Indian J Med Res 123: 423-438.
5. Moise L, Cousens L, Fueyo J, De Groot AS (2011) Harnessing the power of genomics and immunoinformatics to produce improved vaccines. Expert Opin Drug Discov 6: 9-15.
6. Terry FE, Moise L, Martin RF, Torres M, Pilotte N, et al. (2014) Time for T? Immunoinformatics addresses vaccine design for neglected tropical and emerging infectious diseases. Expert rev vaccines 14: 21-35.
7. Cantacessi C, Dantas-Torres F, Nolan MJ, Otranto D (2015) The past, present, and future of Leishmania genomics and transcriptomics. Trends parasitol 31: 100-108.
8. Natarajan G, Oghumu S, Varikuti S, Thomas A, Satoskar A (2014) Mechanisms of Immunopathology of Leishmaniasis. Pathogenesis of Leishmaniasis: Springer 1-13.
9. Griffiths KL, Khader SA (2014) Novel vaccine approaches for protection against intracellular pathogens. Curr opin immunol 28: 58-63.
10. Seyed N, Zahedifard F, Safaiyan S, Gholami E, Doustdari F, et al. (2011) In silico analysis of six known *Leishmania major* antigens and *in vitro* evaluation of specific epitopes eliciting HLA-A2 restricted CD8 T cell response. PLoS Negl Trop Dis 5: e1295.
11. Patronov A, Doytchinova I (2013) T-cell epitope vaccine design by immunoinformatics. Open biol 3: 120139.
12. Srivastav S, Ball WB, Gupta P, Giri J, Ukil A, et al. (2014) *Leishmania donovani* prevents oxidative burst-mediated apoptosis of host macrophages through selective induction of suppressors of cytokine signaling (SOCS) proteins. J Biol Chem 289: 1092-1105.
13. De Groot AS, Moise L, Liu R, Gutierrez AH, Tassone R, et al. (2014) Immune camouflage: relevance to vaccines and human immunology. Hum Vaccin Immunother 10: 3570-3575.
14. Serezani CHC, Franco AR, Wajc M, Yokoyama-Yasunaka JKU, Wunderlich G, et al. (2002) Evaluation of the murine immune response to Leishmania meta 1 antigen delivered as recombinant protein or DNA vaccine. Vaccine 31: 3755-3763.
15. Stäger S, Rafati S (2012) CD8+ T cells in Leishmania infections: friends or foes? Front immunol 3: 5.
16. Novais FO, Scott P. CD8+ T cells in cutaneous leishmaniasis: the good, the bad, and the ugly; 2015. Springer 251-259.
17. Seyed N, Taheri T, Vauchy C, Dosset M, Godet Y, et al. (2014) Immunogenicity Evaluation of a Rationally Designed Polytope Construct Encoding HLA-A*0201 Restricted Epitopes Derived from *Leishmania major* Related Proteins in HLA-A2/DR1 Transgenic Mice: Steps toward Polytope Vaccine. PLoS One 9: e108848.
18. Herrera-Najera C, Piña-Aguilar R, Xacur-Garcia F, Ramirez-Sierra MJ, Dumonteil E (2009) Mining the Leishmania genome for novel antigens and vaccine candidates. Proteomics 9: 1293-1301.
19. Uzonna JE, Joyce KL, Scott P (2004) Low dose *leishmania major* promotes a transient T helper cell type 2 response that is down-regulated by interferon γ–producing CD8+ T cells. J exp med 199: 1559-1566.
20. Okwor IB, Jia P, Mou Z, Onyilagha C, Uzonna JE (2014) CD8+ T cells Are Preferentially Activated during Primary Low Dose *Leishmania major* Infection but Are Completely Dispensable during Secondary Anti-Leishmania Immunity. PLoS Negl Trop Dis 8 e3300.
21. Schussek S, Trieu A, Doolan DL (2014) Genome-and proteome-wide screening strategies for antigen discovery and immunogen design. Biotechnol adv 32: 403-414.
22. Bolhassani A, Taheri T, Taslimi Y, Zamanilui S, Zahedifard F, et al. (2011) Fluorescent Leishmania species: development of stable GFP expression and its application for *in vitro* and *in vivo* studies. Exp parasitol 127: 637-645.
23. Taheri T, Saberi Nik H, Seyed N, Doustdari F, Etemadzadeh M-H, et al. (2015) Generation of stable *L. major*+ EGFP-LUC and simultaneous comparison between EGFP and luciferase sensitivity. Exp parasitol 150: 44-55.
24. Saljoughian N, Taheri T, Zahedifard F, Taslimi Y, Doustdari F, et al. (2013) Development of novel prime-boost strategies based on a tri-gene fusion recombinant L. tarentolae vaccine against experimental murine visceral Leishmaniasis. PLoS Negl Trop Dis 7: e2174.
25. Lamoreaux L, Roederer M, Koup R (2006) Intracellular cytokine optimization and standard operating procedure. Nat protoc 1: 1507-1516.
26. Parish CR, Glidden MH, Quah BJ, Warren HS (2009) Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. Curr protoc immunol 4.9.1-4.9.13.
27. Sacks D, Noben-Trauth N (2002) The immunology of susceptibility and resistance to *Leishmania major* in mice. Nat Rev Immunol 2: 845-858.
28. Wang ZE, Reiner SL, Hatam F, Heinzel FP, Bouvier J, et al. (1993) Targeted activation of CD8 cells and infection of beta 2-microglobulin-deficient mice fail to confirm a primary protective role for CD8 cells in experimental leishmaniasis. J Immunol 151: 2077-2086.
29. Huber M, Timms E, Mak TW, Rollinghoff M, Lohoff M (1998) Effective and long-lasting immunity against the parasite *Leishmania major* in CD8-deficient mice. Infect Immun 66: 3968-3970.
30. Menon JN, Bretscher PA (1996) Characterization of the immunological memory state generated in mice susceptible to *Leishmania major* following exposure to low doses of L. major and resulting in resistance to a normally pathogenic challenge. Eur J Immunol 26: 243-249.
31. Doherty TM, Coffman RL (1996) *Leishmania major*: effect of infectious dose on T cell subset development in BALB/c mice. Exp Parasitol 84: 124-135.
32. Courret N, Lang T, Milon G, Antoine JC (2003) Intradermal inoculations of low doses of *Leishmania major* and *Leishmania amazonensis* metacyclic promastigotes induce different immunoparasitic processes and status of protection in BALB/c mice. Int j parasitol 33: 1373-1383.

33. Belkaid Y, Von Stebut E, Mendez S, Lira R, Caler E, et al. (2002) CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with *Leishmania major*. J Immunol 168: 3992-4000.

34. Mendez S, Gurunathan S, Kamhawi S, Belkaid Y, Moga MA, et al. (2001) The potency and durability of DNA- and protein-based vaccines against *Leishmania major* evaluated using low-dose, intradermal challenge. J Immunol 166: 5122-5128.

35. Karpenko LI, Bazhan SI, Antonets DV, Belyakov IM (2014) Novel approaches in polyepitope T-cell vaccine development against HIV-1. Expert rev vaccines 13: 155-173.

36. Gurunathan S, Sacks DL, Brown DR, Reiner SL, Charest H, et al. (1997) Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. J Exp Med 186: 1137-1147.

37. Gurunathan S, Stobie L, Prussin C, Sacks DL, Glaichenhaus N, et al. (2000) Requirements for the maintenance of Th1 immunity *in vivo* following DNA vaccination: a potential immunoregulatory role for CD8+ T cells. J Immunol 165: 915-924.

38. Gurunathan S, Prussin C, Sacks DL, Seder RA (1998) Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. Nat Med 4: 1409-1415.

39. Campos-Neto A, Webb JR, Greeson K, Coler RN, Skeiky YA, et al. (2002) Vaccination with plasmid DNA encoding TSA/LmSTI1 leishmanial fusion proteins confers protection against *Leishmania major* infection in susceptible BALB/c mice. Infect Immun 70: 2828-2836.

40. Iborra S, Soto M, Carrión J, Alonso C, Requena JM (2004) Vaccination with a plasmid DNA cocktail encoding the nucleosomal histones of Leishmania confers protection against murine cutaneous leishmaniosis. Vaccine 22: 3865-3876.

41. Vasconcelos JR, Dominguez MR, Araujo AF, Ersching J, Tararam CA, et al. (2012) Relevance of long-lived CD8(+) T effector memory cells for protective immunity elicited by heterologous prime-boost vaccination. Front Immunol 3: 358.

42. Jayakumar A, Castilho TM, Park E, Goldsmith-Pestana K, Blackwell JM, et al. (2011) TLR1/2 activation during heterologous prime-boost vaccination (DNA-MVA) enhances CD8+ T Cell responses providing protection against Leishmania (Viannia). PLoS Negl Trop Dis 5: e1204.

43. Sanchez-Sampedro L, Gomez CE, Mejias-Perez E, Sorzano CO, Esteban M (2012) High quality long-term CD4+ and CD8+ effector memory populations stimulated by DNA-LACK/MVA-LACK regimen in *Leishmania major* BALB/c model of infection. PLoS One 7: e38859.

44. Resende DM, Caetano BC, Dutra MS, Penido ML, Abrantes CF, et al. (2008) Epitope mapping and protective immunity elicited by adenovirus expressing the Leishmania amastigote specific A2 antigen: correlation with IFN-γ and cytolytic activity by CD8+ T cells. Vaccine 26: 4585-4593.

45. Schroeder J, Brown N, Kaye P, Aebischer T (2011) Single dose novel Salmonella vaccine enhances resistance against visceralizing *L. major* and *L. donovani* infection in susceptible BALB/c mice. PLoS Negl Trop Dis 5: e1406.

46. Saljoughian N, Taheri T, Rafati S (2014) Live vaccination tactics: possible approaches for controlling visceral leishmaniasis. Front Immunol 5: 134.

47. Raymond F, Boisvert S, Roy G, Ritt JF, Legare D, et al. (2012) Genome sequencing of the lizard parasite Leishmania tarentolae reveals loss of genes associated to the intracellular stage of human pathogenic species. Nucleic acids res 40: 1131-1147.

48. Mizbani A, Taheri T, Zahedifard F, Taslimi Y, Azizi H, et al. (2009) Recombinant Leishmania tarentolae expressing the A2 virulence gene as a novel candidate vaccine against visceral leishmaniasis. Vaccine 28: 53-62.

49. Zahedifard F, Gholami E, Taheri T, Taslimi Y, Doustdari F, et al. (2014) Enhanced protective efficacy of nonpathogenic recombinant leishmania tarentolae expressing cysteine proteinases combined with a sand fly salivary antigen. PLoS Negl Trop Dis 8: e2751.

50. Laabs EM, Wu W, Mendez S (2009) Vaccination with live *Leishmania major* and CpG DNA promotes interleukin-2 production by dermal dendritic cells and NK cell activation. Clin vaccine immunol 16: 1601-1606.

51. Saffari B, Mohabatkar H (2009) Computational analysis of cysteine proteases (Clan CA, Family CI) of *Leishmania major* to find potential epitopic regions. Genomics, proteomics and bioinformatics 7: 87-95.

52. Elfaki ME, Khalil EA, De Groot AS, Musa AM, Gutierrez A, et al. (2012) Immunogenicity and immune modulatory effects of *in silico* predicted L. donovani candidate peptide vaccines. Hum vaccin immunother 8: 1769-1774.

53. Rezvan H (2013) Immunogenicity of HLA-DR1 restricted peptides derived from *Leishmania major* gp63 using FVB/N-DR1 transgenic mouse model. Iran j parasitol 8: 273-279.

54. Agallou M, Athanasiou E, Koutsoni O, Dotsika E, Karagouni E (2014) Experimental validation of multi-epitope peptides including promising MHC Class I- and II-restricted epitopes of four known *Leishmania infantum* proteins. Front Immunol 5: 268.

55. Duarte A, Queiroz AT, Tosta R, Carvalho AM, Barbosa CH, et al. (2015) Prediction of CD8+ Epitopes in *Leishmania braziliensis* Proteins Using EPIBOT: *In Silico* Search and *In Vivo* Validation. PLoS One 10: e0124786.

56. Guerfali F, Ben-Abdallah H, Sghaier R, Ben-Aissa K, Mkannez G, et al. (2009) An in silico immunological approach for prediction of CD8+ T cell epitopes of *Leishmania major* proteins in susceptible BALB/c and resistant C57BL/6 murine models of infection. Infect genet evol 9: 344-350.

57. John L, John GJ, Kholia T (2012) A reverse vaccinology approach for the identification of potential vaccine candidates from *Leishmania* spp. Appl biochem biotechnol 167: 1340-1350.

58. Sachdeva R, Banerjea AC, Malla N, Dubey ML (2009) Immunogenicity and efficacy of single antigen Gp63, polytope and polytopeHSP70 DNA vaccines against visceral Leishmaniasis in experimental mouse model. PLoS One 4: e7880.

59. Kedzierska K, Curtis JM, Valkenburg SA, Hatton LA, Kiu H, et al. (2012) Induction of protective CD4+ T cell-mediated immunity by a Leishmania peptide delivered in recombinant influenza viruses. PLoS One 7: e33161.

60. Agallou M, Margaroni M, Karagouni E (2011) Cellular vaccination with bone marrow-derived dendritic cells pulsed with a peptide of *Leishmania infantum* KMP-11 and CpG oligonucleotides induces protection in a murine model of visceral leishmaniasis. Vaccine 29: 5053-5064.

61. Das S, Freier A, Boussoffara T, Das S, Oswald D, et al. (2014) Modular multiantigen T cell epitope–enriched DNA vaccine against human leishmaniasis. Sci transl med 6: 234ra256-234ra256.