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Original Article

***Leishmania mexicana* Gp63 cDNA Using Gene Gun Induced Higher Immunity to *L. mexicana* Infection Compared to Soluble *Leishmania* Antigen in BALB/C**

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ABSTRACT

Background: Leishmaniasis is a worldwide disease prevalent in tropical and sub tropical countries. Many attempts have been made and different strategies have been approached to develop a potent vaccine against *Leishmania*. DNA immunisation is a method, which is shown to be effective in *Leishmania* vaccination. *Leishmania* Soluble Antigen (SLA) has also recently been used *Leishmania* vaccination.

Methods: The immunity generated by SLA and *L. mexicana* gp63 cDNA was compared in groups of 6 mice, which were statistically analysed by student *t*- test with the *P*-value of 0.05. SLA was administered by two different methods; intramuscular injection and injection of dendritic cells (DCs) loaded with SLA. *L. mexicana* gp63 cDNA was administered by the gene gun.

Results: Immunisation of BALB/c mice with *L. mexicana* gp63 resulted in high levels of Th1-type immune response and cytotoxic T lymphocytes (CTL) activity, which were accompanied with protection induced by the immunisation against *L. mexicana* infection. In contrast, administration of SLA, produced a mixed Th1/Th2-type immune responses as well as a high level of CTL activity but did not protect mice from the infection.

Conclusion: The results indicate higher protection by DNA immunisation using *L. mexicana* gp63 cDNA compared to SLA, which is accompanied by a high level of Th1 immune response. However, the CTL activity does not necessarily correlate with the protection induced by the vaccine. Also, gene gun immunisation is a potential approach in *Leishmania* vaccination. These findings would be helpful in opening new windows in *Leishmania* vaccine research.

Keywords: *Leishmania mexicana*, Gene gun, gp63, *Leishmania* Soluble Antigen, BALB/c

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Introduction

Leishmania is an obligate intracellular parasite of the macrophage-dendritic cell lineage. Although the first species of this parasite was known more than 100 years ago (1), construction of an effective vaccine against it has not yet been achieved (2). As *Leishmania* lives intracellularly in macrophages, the humoral immune system cannot be of great help in immunity and therefore the vaccine-developing strategies must involve the cellular immunity, which has a bias towards the Th1 immune pathway. Due to the complexity of the mechanisms involved in immunity to *Leishmania*, different vaccine strategies have been proposed (3).

DNA vaccination is the latest method of immunisation implicated in *Leishmania* vaccination, shown to have potential to induce immunity to *Leishmania* in mice (4). In this method, DNA sequences that encode a *Leishmania* antigen are spliced into an expression vector, which is administered to the host cells to promote the production of *Leishmania* protein (5, 6). *Leishmania* zinc-metalloproteinase called gp63 is a characterized protein of *Leishmania* species. The immunogenicity of *Leishmania* gp63 has been shown in different studies by several research groups (7-9).

The immunity induced by soluble *Leishmania* antigen (SLA) has also generated interest among *Leishmania* researchers. *L. donovani* promastigote soluble antigens were encapsulated in non-phosphatidylcholine liposomes derived from *E. coli* lipids elicited a protective immune response against experimental visceral leishmaniasis (10). Immunization with soluble *Leishmania* antigen in IFA plus Ad5IL-12 vector induced protection in BALB/c mice against *L. major* infection (11).

Dendritic cells, as professional antigen presenting cells, play a crucial role in immunity

to *Leishmania*. There is a possible role for subsets of DCs in directing the immune response towards either Th1 or Th2 following the encounter of an infectious agent, which may determine whether the host will resist or succumb to that infection (12).

Loading DCs with anti-tumor antigens protected mice from tumor growth (13). In *Leishmania* vaccination the potency and effectiveness of DC-based vaccines has been shown in both immunotherapy and chemotherapy (12, 14, 15). The cytokine profile of mice after DC-based vaccination has demonstrated a shift toward a Th1-type response in which IL-12 has a critical role (15) and because DCs exposed to *L. major* readily produce IL-12, it may further increase the feasibility of using the DC-based vaccines (16).

In the present study, protection induced by *L. mexicana* gp63 cDNA, SLA containing the gp63, and DCs loaded with *L. mexicana* gp63 in *Leishmania* sensitive BALB/c mice against *L. mexicana* was investigated. In addition, the CTL activity and antibody responses rendered by *L. mexicana* gp63 cDNA and SLA were studied.

Materials and Methods

Animals

BALB/c mice were purchased from the Harlan Olac (Oxon, UK) and bred at the Nottingham Trent University animal house. All animals were housed in accordance with the Home Office Codes of Practice for the housing and care of animals.

Leishmania parasites, cells and infection

L. mexicana promastigotes strain M379 were kindly gifted by Dr. Varley, the London School of Hygiene and Tropical Medicine (LSHTM), and cultured in Schneider media

(Sigma, US) supplemented with 10% FCS at 25 °C as described by (17).

Three groups of 6 mice were routinely infected, unless otherwise indicated, by intradermal inoculation of 1×10^6 promastigotes into a shaved area of the back region about 1 cm from the tail base and were monitored at 3- to 4-day intervals. Mice were killed when lesion size exceeded 1 cm². For all experiments, student *t*-test was used to statistically analyze the data. The *P*-value estimated by *t*-test between groups test and control was 0.05.

The CT26 cell line (N-methylurethane-induced BALB/ c murine colon carcinoma) was a kind gift from Prof. Ian Hart (St Thomas Hospital). Cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM)+10% foetal calf serum (FCS) (Bio Whittaker, Europe).

Generation of DCs

BM-DC was generated as described by Inaba and coworkers with some modifications (18). Briefly, hind limbs of naïve BALB/c mice were collected and all muscle was removed using scalpel and tweezers. After cutting the ends of the bone, bone-marrow cells were flushed out and harvested. Then the bone marrow cells centrifuged and resuspended in 1ml BM-DC media, and plated at 1×10^6 cells per well/ml with 100ng/ml of mGM-CSF. The cells were then incubated overnight at 37° C, 5% CO₂. On day 2 and day 4, non-adherent cells were washed out by gently replacing 700µl/well of media with fresh DC media containing GM-CSF. On day 6, BM-DC were repeated and split into two groups. The first group (test) were pulsed with 10µg/ml SLA and the second group was used as control. Control and test groups were pulsed 4-6hrs later by 1µg/ml LPS to induce maturation. The following day, BM-DCs were washed in se-

rum free RPMI 1640 media, counted and injected intradermally at 2×10^6 per mouse.

FACS Analysis

5×10^5 per tube DCs were harvested for FACS analysis. Cells were washed twice in PBS + 0.1% BSA + 0.02% NaN₃. Rat anti-mouse CD80, Macrophage/Monocyte marker (F4/80), DEC205, I-A (murine class II) and CD45, and hamster anti-mouse CD11c monoclonal antibodies were added. Appropriate isotype controls were used in each experiment. The cells were incubated on ice for 30 minutes with primary antibodies. Cells were then washed twice in PBS + 0.1% BSA + 0.02% NaN₃ and incubated for 30 minutes on ice with FITC coupled goat anti-rat IgG or goat anti-hamster IgG as secondary antibodies as appropriate. Finally the cells were washed in PBS + 0.1% BSA + 0.02% NaN₃ and resuspended in 500µl of sheath fluid, and then the FACS analysis was performed.

Preparation of SLA

The *L. mexicana* SLA was prepared according to what previously described by Dumonteil (9). Briefly, late log phase *L. mexicana* promastigotes were washed 4 times in PBS and then the parasites were resuspended in 100mM Tris buffer, pH 7.3 containing 1mM EDTA, 0.5mM PMSF (Sigma, US) and 2.5g/ml Leupeptin (Sigma, US). The parasites were lysed by sonication and the lysate was centrifuged at 13000rpm for 20 min. The supernatant was centrifuged again for 4 h at 39,000rpm, and then was dialyzed against 5 liters of cold PBS overnight with continuous agitation and several changes of the PBS. The lysate was sterilized by passing through 22µm filters (Sartorius, US).

Coating of gold particles by DNA

DNA was coated onto 1.0 Micron gold particles (Biorad, Hemel Hempstead, Hertfordshire, UK) using manufacturers' instruction

and administered by Helios Gene Gun (Biorad, Hemel Hempstead, Hertfordshire, UK). Briefly, 200 µl of spermidine was added to 16.6 µg of gold followed by sonication. 200 µl of 1M calcium chloride was added to the DNA-Spermidine solution followed by incubation at room temperature for 10 minutes. Tubes were centrifuged at 13,000 rpm for 1 min and gold particles resuspended in dry ethanol (Sigma, US). After repeating the above step 2 more times, particles were resuspended in 0.025mg/ml of PVP in dry ethanol. During these steps, the plastic tubing was dried using nitrogen for 15-20 minutes using nitrogen gas. The resuspended gold particles were loaded into the dried tubing using a syringe and the tubing was placed on the roller/dryer (Biorad, Hemel Hempstead, Hertfordshire, UK) followed by incubation for 15 minutes. The PVP-dry ethanol was gently removed using the syringe and the tube was rotated on the roller along with nitrogen gas being passed through it for 5 minutes. Bullets were then cut using guillotine and stored at 4°C until used for immunization.

DNA construct

To assess the immunogenicity of *L. mexicana* gp63, the *L. mexicana* gp63 gene cloned into VR1012 plasmid vector, a gift from Dr Dumonteil Laboratorio de Parasitología Yucatan Mexico. oliver@tunku.uady.mx (9), and used in the investigation. The DNA was bulked up by transformation of *E. coli* followed by purification using Quia-gen EndoFree plasmid purification Maxi Prep Kits and all the products were evaluated by UV spectrophotometer at 260 and 280nm. The ratio OD260/OD280 was always more than 1.7. The construct was also sequenced by MWG-Biotech using 5'-ACATCCTCACCGACGAGAAG-3', 5'-CTTGAAGTCGCCACAGATCA-3', 5'-GCTGCAACAGCTTGGAGTATC-3' and

5'-GATACTACACCGCCCTGTGC-3' primers and checked for mismatches against the sequence obtained from the gene bank.

CTL Assay

LPS Blast

Between 2-3 days prior to the removal of spleens from immunized mice, naïve splenocytes were cultured at 1.5×10^6 cells/ml in 40ml T cell media containing 25µg/ml LPS and 7µg/ml dextran sulphate in a T75 culture flask and incubated at 37°C, 5%CO₂. On the day of isolation of immunized mice splenocytes, LPS treated naïve splenocytes were irradiated at 3000rads for 4 minutes. Cells were washed and pulsed with 100µg/ml of relevant or irrelevant peptides separately for at least 1 hour. Cells were then washed, counted and added to culture plates containing splenocytes from immunized mice at 5×10^5 /well.

In vitro generation of CTLs

One week after the last immunization, spleens were harvested from the immunized mice and single cell suspensions were prepared in sterile conditions. Cells were flushed out from the spleens by serum-free RPMI1640. All cells were centrifuged at 1500rpm for 3 minutes and resuspended in CTL media (RPMI 1640 supplemented with 1% L-glutamine, 10%FCS, 20mM HEPES buffer, 50µM 2-Mercapto Ethanol, 50U/ml penicillin, 50µg streptomycin and 0.25µg/ml fungizone). The cells were counted using white cell counting fluid (0.6% acetic acid in distilled water) and 0.1% Trypan Blue, and plated in a 24 well plate at 2.5×10^6 cells/500µl/well. 5×10^5 /500µl irradiated and peptide pulsed LPS blasts were added to the splenocytes to make a final volume of 1ml in each well of 24 well plate. Supernatants were collected usually on day 3 and 5 for cytokine testing. In certain experiments SLA

was used instead of peptide to stimulate CTL activity in vitro.

Chromium Release Cytotoxicity Assay

On day 5 of in vitro stimulation, splenocytes were harvested, washed twice in serum free medium, counted and resuspended in CTL media and used as the effector cells. CT-26 or CD8+ cells were also harvested, washed and labeled with chromium-51 (Amersham, UK)

followed by 1h incubation at 37°C. The CT26 labeled cells were then pulsed with relevant and irrelevant peptides separately and incubated for 1 hour at 37°C. For DCs, the cells were split into two groups. One group was loaded with SLA and the other was used as control. A standard 4 hour Cr release assay was performed and the specific cytotoxicity was determined using the following formulae.

$$\text{percentage cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

Detection of anti-Leishmania IgG2a and IgG1 isotype antibodies

After immunization, mice were regularly bled for 7 times at a week interval starting a week after the last immunization. The blood samples were harvested and spun at 200rpm for 10 min. The serum was collected and stored at -20 until tested for specific immunoglobulin IgG1 and IgG2a using ELISA. Serum samples from 9 naïve mice were used for control. *L. mexicana* Soluble Antigen (SLA) 1µg/well was coated on the flat bottom 96-well plates (Biorad, Hemel Hempstead, Hertfordshire, UK) and incubated overnight at room temp. After 4 times wash with PBS, 1:100 dilution of the serum samples in dilution reagent (1% BSA, 0.05% Tween 20 in 20mM Trizma base, 150mM NaCl, pH 7.2-7.4) was added in duplicate followed by 2h incubation at room temp and 4 times washes with PBS. The plates were blocked with block buffer (1% BSA, 5% sucrose in PBS with 0.05 NaN₃) for 1hr. And then washed 4 times washes with PBS. Rabbit anti-mouse IgG1 or IgG2a (Serotech, UK) was added at 1:1000 followed by 1hour incubation at room temp and 4 times washes. HRP conjugated goat anti-rabbit antibody at 1:1000 dilution was added and the plates were stored at room temp for 1h followed by 4 times washes. 50µl of HRP substrate

(DAKO, US) was added and kept at room temp for 20minutes for reaction development. 2.5M H₂SO₄ was added to stop the reaction and the OD was measured at 570 nm by spectrophotometer.

Results

Protection induced by *L. mexicana* gp63 cDNA

The gene gun was used in this study to immunize BALB/c mice with *L. mexicana* gp63 cDNA. 1µg of *L. mexicana* gp63 cDNA (VR1012) coated on gold particles (see Materials & Methods) was administered by gene gun into a shaved area of the abdomen. A control group of 3 mice was administered with gold particles coated with empty plasmid by the gene gun. The mice were immunized twice two weeks apart and the immunized mice were monitored regularly followed by challenge with the parasite. Results indicated a significant protection induced by immunization with 1µg *L. mexicana* gp63 cDNA using the gene gun; 66% (4 out of 6) of the immunized mice remained free of lesion (Fig. 1).

Protection induced by SLA + IFA

To investigate the ability of SLA to protect the animals from *Leishmania*, a series of experiments were performed using test and control groups of *Leishmania* sensitive BALB/c mice. The test group was injected with 100µg of *L. mexicana* SLA admixed with the same volume of IFA as adjuvant and the control group was injected with PBS. The results showed that two S.C. injections of SLA+IFA did not significantly decrease the size of the *Leishmania* lesion compared to controls (Fig. 2).

Protection induced by Dendritic Cells (DC) loaded with SLA

Immunity rendered by id inoculation of dendritic cells loaded with SLA against *Leishmania* was investigated. Bone-marrow cells were obtained from BALB/c mouse Femur and Tibia, and cultured with GM-CSF for 6 days with gentle washes every two days (see materials and methods). On day 6, DCs were replanted at 1×10^6 /ml and split into two groups. One group (test) was treated with the SLA at a concentration of 10-15µg/ml and after 4-6 hours pulsed with 1µg/ml LPS to mature. The second group of DCs (control) was only pulsed with LPS. On day 7, DCs were injected at 2×10^6 per mouse into groups of BALB/c mice. A third group of BALB/c mice were also injected with PBS and used as an additional control. DCs phenotype was determined with a number of Abs and FACS analyses (Fig. 3). Mice were immunized twice at two weeks interval and then were challenged with 2×10^6 *L. mexicana* promastigotes. No significant protection was observed (Fig.4).

CTL activity induced by immunization with gp63 cDNA construct

To evaluate the role of cytotoxic T cells in immunity to *Leishmania*, a standard 4 h cytotoxicity assay was to assess the ability of

L. mexicana gp63 cDNA to generate specific cytotoxic T lymphocytes. BALB/c mice were immunized twice at two weeks interval with *L. mexicana* gp63 cDNA by gene gun. Mice were sacrificed two weeks following the 2nd immunization and spleens were collected. Splenocytes were harvested and cultured *in vitro* for 5 days together with blasts cells pulsed with LPS and SLA. On day 5, the splenocyte cells were used as effectors in cytotoxicity assays against CT26 tumor cells transfected with *L. mexicana* gp63 (Materials and Methods section).

The results clearly revealed that immunization of mice with *L. mexicana* gp63 cDNA induces specific CTL activity against CT26 tumor cells expressing *L. mexicana* gp63 and DCs loaded with SLA as targets (Fig. 5). The *in vitro* restimulation of CTLs by SLA loaded blast cells was crucial. It was shown that removing the *in vitro* restimulation of the splenocytes highly prevented the generation of CTL activity in immunized mice and levels was comparable with that of naïve mouse splenocytes restimulated *in vitro* by blast cells loaded with SLA. *In vitro* depletion of CD8+ T cells by anti CD8 Ab and complement on day 5 significantly removed the CTL activity suggesting an effector role of CD8+ T cells in the CTL activity.

CTL activity induced by SLA (SLA + IFA & DCs pulsed with SLA)

The potency of SLA in inducing CTL activity was evaluated in BALB/c mice injected with SLA + IFA or DCs loaded with SLA. Mice were either subcutaneously injected with 100µg per mouse of SLA mixed with the same volume of IFA or intradermally immunized with 2×10^6 per mouse matured DCs loaded with SLA at the left flank. One injection of SLA + IFA or SLA loaded matured DC induced high level of CTL activity, when tested against DCs loaded with SLA in cytotoxicity assay (Fig. 6).

Antibody responses to *Leishmania* vaccines

To study the direction of immune response raised by the vaccines, in a set of experiments, five groups of six female BALB/c mice were used. The first group was immunized with 1µg *L. mexicana* gp63 construct (VR1012) using gene gun. The second group was immunized with empty plasmid vector (VR1012) by gene gun. The third group was immunized S.C. with 100µg SLA admixed with the same volume of IFA. The fourth group was injected S.C. with PBS. The immunization was carried out on day 0 and 14, and one week after the second immunization, the mice were bled once a week regularly. Serum was separated and stored at -20 for antibody typing by ELISA to determine the level of anti-*Leishmania* IgG2a and IgG1 isotype antibodies.

The results clearly demonstrated a sharp increase of IgG2a in mice immunized with *L. mexicana* gp63 construct by gene gun as early as 7 days after the immunization, which slightly decreased afterward and remained at that level during the course of experiment. The results also showed that the gene gun immunization induced an increase in the level of IgG1 after day 14 similar to

that obtained by immunization with the empty vector. Immunization of mice with SLA+IFA resulted in high levels of IgG1. The level of IgG2a induced by immunization with SLA+IFA was lower than that of IgG1 during the course of study (Fig. 7).

Antibody responses in mice immunized with DCs pulsed with SLA

To determine the “direction” of the immune system following immunization with of DCs pulsed with SLA, groups of 6 BALB/c mice were either immunized I.D. with 2×10^6 DCs loaded with SLA, or control DCs or PBS. DCs were prepared from bone marrow cells and loaded with 10µg/ml SLA. One µg/ml LPS was also added to compete DC maturation (see materials and methods). Two weeks after the second immunization, all mice were bled to determine the level of mouse IgG, and IgG1 and IgG2a isotypes. The results clearly showed significant increase in levels of total IgG, IgG1 and IgG2a in test groups compared with controls (Fig. 8) indicating a rise of both Th1 and Th2-type antibody response in the immunized mice.

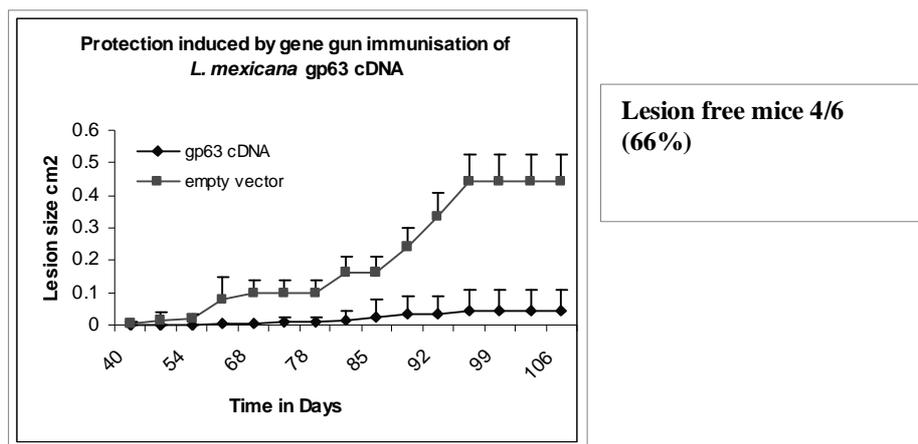


Fig. 1: Gene gun immunisation with *L. mexicana* gp63 cDNA. 1µg per mouse of *L. mexicana* gp63 plasmid DNA (VR1012) coated on gold particles was introduced to abdomen skin of BALB/c mice by gene gun on day 0 and 14. The control group was given 1µg empty vector coated on gold particles. The mice were challenged with 2×10^6 log-phase *L. mexicana* promastigote on day 28, and were monitored regularly. The graph represents 3 independent experiments. Bars represent the standard deviation n=6

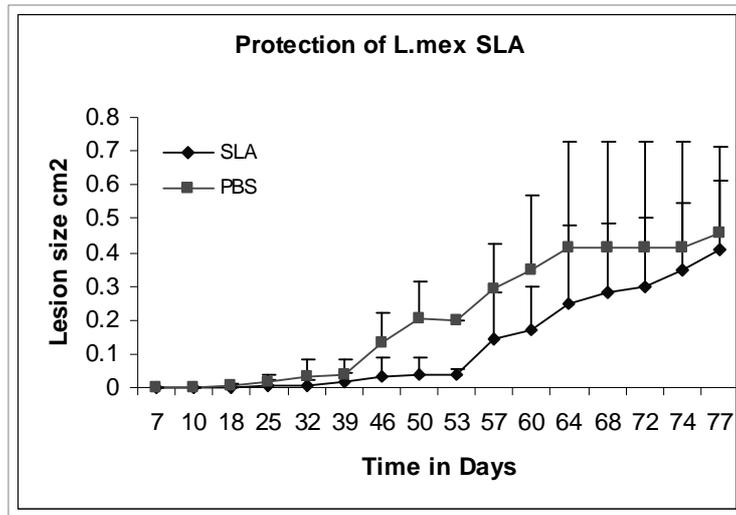


Fig. 2: Protection induced by SLA admixed with IFA in BALB/c mice. Two groups of 6 BALB/c mice were immunized S.C. with 100 μ g+IFA or PBS twice at 2 weeks interval. Meanwhile controls were injected with PBS. Two weeks later mice were challenged with 2×10^6 *L. mexicana* promastigotes. The mice were monitored regularly and average of the surface of the lesions was measured. Student t test was used to statistically analyze the data. The graph represents 3 independent experiments. Bars represent the standard deviation n=6

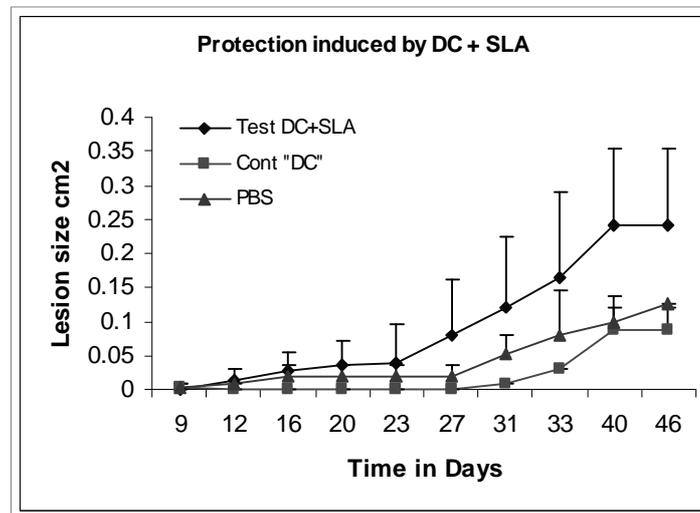


Fig. 3: Protection induced by DCs loaded with SLA in BALB/c mice. Bone-marrow cells derived from BALB/c mice were cultured with GM-CSF for 7 days. One day before immunization, they were loaded with 10-15 μ g/ml SLA and pulsed with LPS 1 μ g/ml (see materials and methods). On day 7 DCs were phenotyped and then, 2×10^6 per mouse of each DC preparation was administered in a group of 6 BALB/c mice intradermally twice at two weeks interval. A control group of 3 mice were injected with PBS. The mice were challenged with 2×10^6 *L. mexicana* log phase promastigotes two weeks after the last immunization and were monitored regularly. Student t-test was used to analyze the data. The graph represents 3 independent experiments. Bars represent the standard deviation n=6

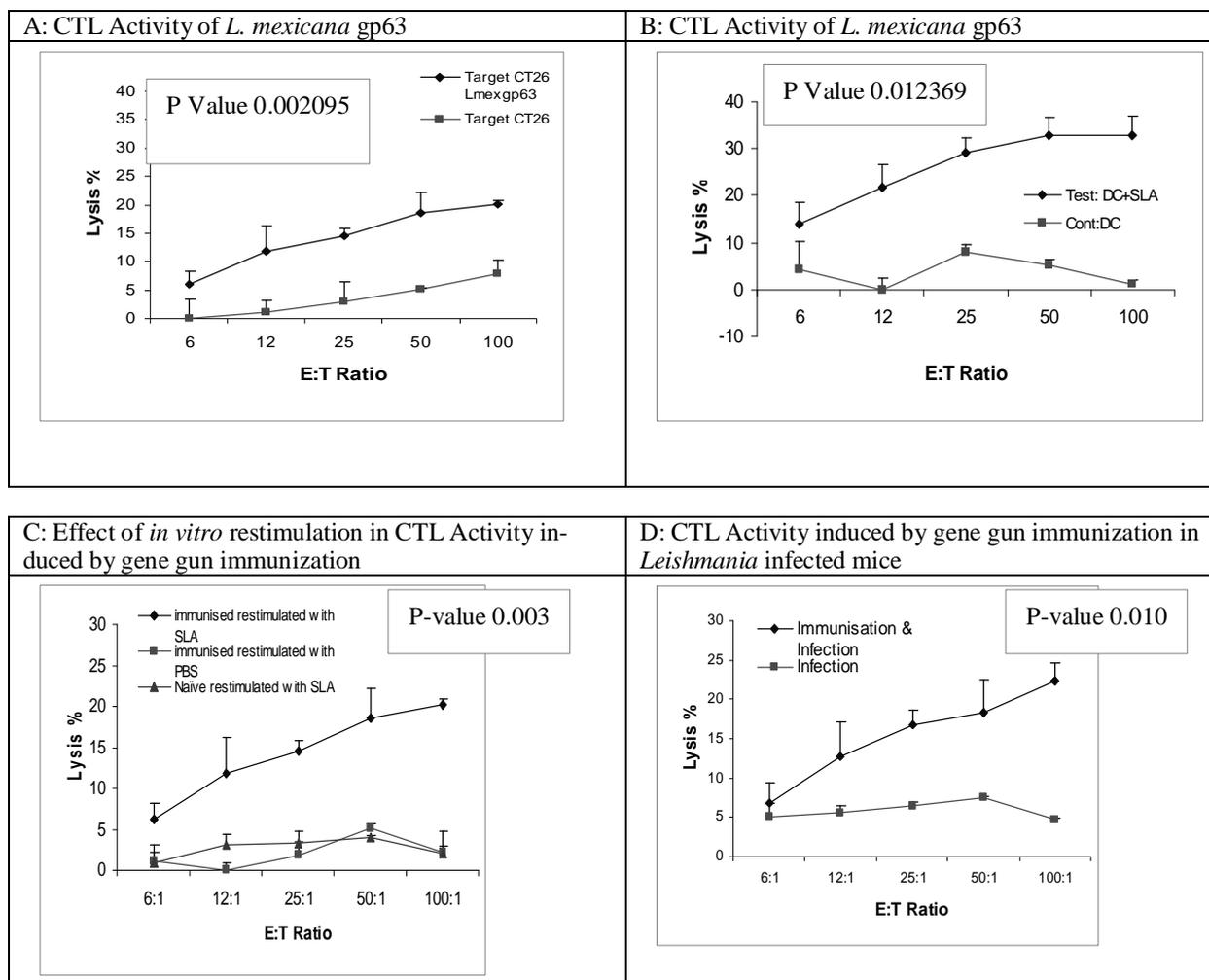


Fig. 4: CTL activity induced by gene gun immunisation using *L. mexicana* gp63 cDNA. BALB/c mice were immunized twice with *L. mexicana* gp63 using the gene gun twice; on day 0 and 14. On day 28 mice were sacrificed and the splenocytes were cultured *in vitro* with blast cells pulsed with LPS and SLA for 5 days. On day 5 the cells were used as effector in standard cytotoxicity assay. A: DNA immunized mice restimulated with blast cells+SLA tested against CT26 *L. mexicana* gp63 cells. B: DNA immunized mice restimulated with blast cells+SLA tested against DCs pulsed with SLA C: naive mice restimulated with blast cells+SLA tested against CT26 *L. mexicana* gp63 cells. D: DNA immunized mice restimulated with blast cells+PBS tested against CT26 *L. mexicana* gp63 cells. The results represent 8 mice in 4 independent experiments

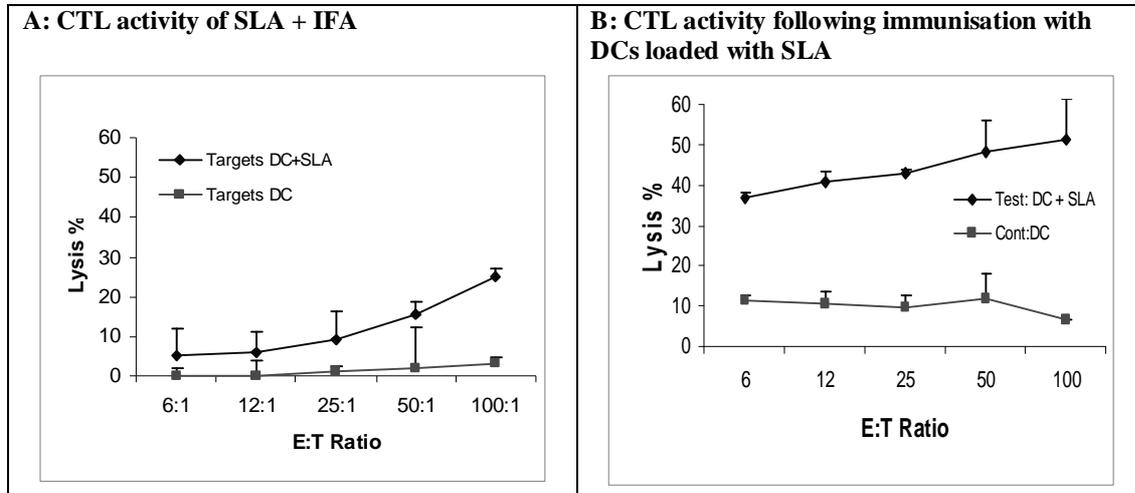


Fig. 5: CTL activity in mice immunised with SLA+IFA or DCs pulsed with SLA. BALB/c mice were injected S.C. with 100µg of SLA admixed with the same volume of IFA or immunized I.D. with 2×10^6 DCs loaded with SLA per mouse. After two weeks the mice were sacrificed and their splenocytes were cultured *in vitro* for 5 days together with blast cells pulsed with LPS and SLA. On day 5 they were used as effector cells in a cytotoxicity assay against DCs pulsed with SLA

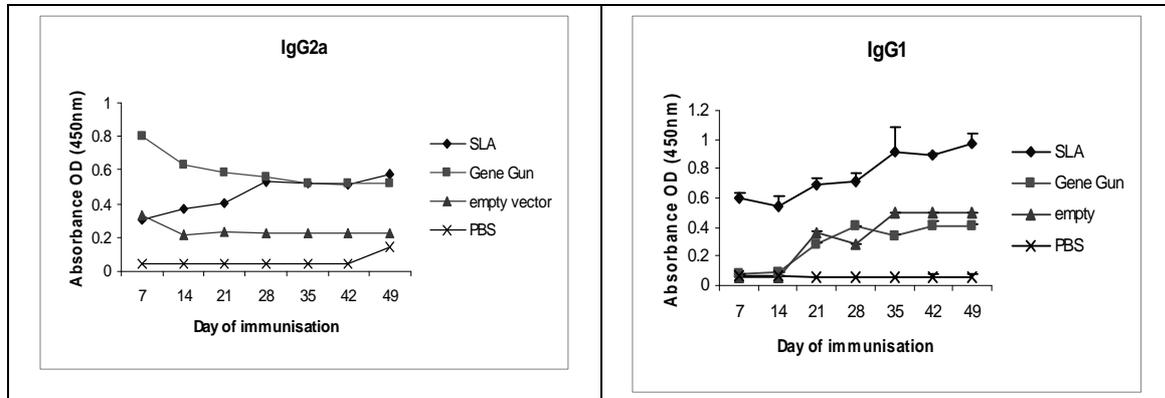


Fig. 6: Th1/Th2 direction of immune response in mice immunized with DNA or SLA. BALB/c mice were immunized with *L. mexicana* gp63 cDNA using the gene gun or injected S.C. with 100µg/mouse SLA mixed with the same volume of IFA. A control group of mice was immunized with the empty vector. An additional control group was also injected with PBS. All groups of mice were immunized twice at two week interval. After two immunizations, blood samples were collected every 7 days and ELISA was implicated to determine IgG2a and IgG1 isotype antibodies against SLA

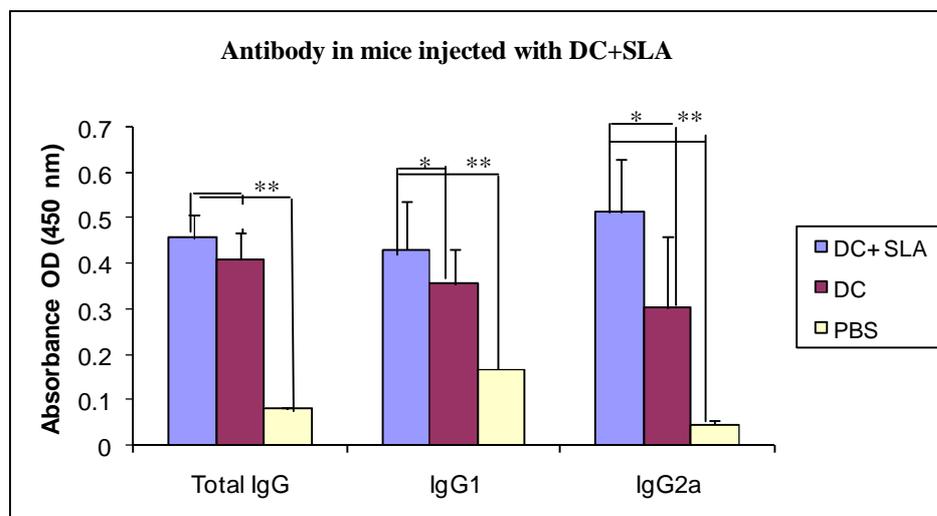


Fig. 7: Ab responses in mice immunised with DCs loaded with SLA. Groups of 6 BALB/c mice were immunised S.C. either with 2×10^6 /mouse DCs alone or pulsed with SLA (see materials and meethods) on day 0 and day 14. On day 28 serum samples were collected and analysed by ELISA to determine the level of IgG, IgG1 and IgG1a. Data were analysed by student *t*-test. * $P > 0.05$, ** $P > 0.01$, *** $P > 0.001$

Discussion

Protection induced by L. mexicana gp63 or SLA

DNA immunization is a method that has recently been used in *Leishmania* vaccination. Different studies using different genes have shown the potency of this method in generating immunity to *Leishmania* (19, 20). Gp63 is an immunogenic protein in *Leishmania* parasites. It has been shown that administration of DNA encoding *Leishmania* gp63 protein can generate immunity and partially protect BALB/c mice from the infection (9, 21). Gene gun immunization is a new approach recently been implicated in *Leishmania* DNA immunization (22).

The immunity induced by Soluble *Leishmania* Antigen has also generated interest among *Leishmania* researchers. In a study by Sharma (10) *L. donovani* promastigote soluble antigens were encapsulated in non-phosphatidylcholine liposomes derived from *E. coli* lipids elicited a protective immune response against experimental visceral

leishmaniasis. In another study, Immunization with soluble *Leishmania* antigen in IFA plus Ad5IL-12 vector induced protection in BALB/c mice against *L. major* infection (11).

Here we compared the protection induced *L. mexicana* gp63 cDNA construct to that induced by *L. mexicana* soluble antigen containing *L. mexicana* gp63 protein in a protection investigation in BALB/c mouse model. For administration of the DNA, 1 μ g of the *L. mexicana* (VR1012) construct coated on gold particles was administered I.D. by gene gun. It was shown that gene gun immunization significantly protected mice from *Leishmania* infection, where 66% of immunized mice were free of lesions. The immunogenicity of SLA was examined in two modes of immunization. In the first approach, two injections of BALB/c mice with 100 μ g/mouse of SLA mixed with 100 μ g/mouse of IFA induced reduction in the size of lesions but did not significantly

prevented *L. mexicana* infection. However, administration of SLA induced levels of Th1 and Th2 immune responses indicating the existence of immunogenic proteins in this preparation, which provokes immunity to *Leishmania*. Therefore, the identification of these immunogenic proteins and using them in potential vaccines as well as developing new methods for vaccine administration to enhance immunogenicity are future areas for *Leishmania* vaccine investigation.

The second approach tested was the application of DCs loaded with SLA. DCs are shown to be a potent adjuvant in *Leishmania* vaccination (16) and their potency in the generation of immunity to intracellular pathogens is dependent on the production of IL-12, which results in shifting the immune response toward Th1-type. It has been shown that the protective potential of DCs pulsed with a given *Leishmania* Ag correlated with the level of their IL-12 expression (15). In a similar study, animals receiving DCs pulsed with *L. donovani* soluble antigen either before or following infection had 1-3 log lower parasite burdens as well as enhancement of the parasite-specific IFN- γ response. When SLA pulsed DCs were transfected with the IL-12 gene, the number of live parasites in the liver of vaccinated mice was further reduced and the parasitological response was associated with a nearly normal liver histology (12).

Our results showed that immunization with DCs pulsed with SLA obtained from *L. mexicana* did not protect BALB/c mice from *Leishmania* infection. This was in contrast with the results obtained by Moll in which DCs pulsed by SLA protected BALB/c mice from *L. major* infection (16). The discrepancy could be due to the difference between the species of *Leishmania* used. However, other results showed that DCs pulsed with SLA were potent in generating CTL activity and inducing a mixed Th1/Th2 immune response. Further studies are required to clar-

ify the role of these immune responses in protection against the infection.

CTL activity induced by L. mexicana gp63 and SLA

The role of CD8+ T cells in immunity to *Leishmania* parasites is not yet fully established. There are studies demonstrating that *Leishmania* patients show high proportions of *Leishmania*-reactive CD8+ T cells (23) and it is thought that that CD8+ T cells help raise immunity to *Leishmania* in two different ways. These cells release a large amount of IFN- γ that in turn promotes a Th1 immune response to activate macrophages against the parasite. Also, activated CD8+T cells can kill macrophages, which are invaded by the parasite (see chapter 1) and it has been shown that DNA and DC-based vaccines elicit CD8+ immune response (24, 25).

In the present study, the role of CTL T cells in the immunity induced *L. mexicana* gp63 and SLA was investigated. The results revealed that immunization of mice with *L. mexicana* gp63 presented significant CTL activity in BALB/c mice tested against CT26 tumor cell transfected with the relevant gene. Similar results were also obtained when DCs were transfected with *L. mexicana* gp63 cDNA and used for target cells (data not shown). When the CTL activity was checked against DCs pulsed with SLA containing gp63 protein, the effectors were still able to recognize and kill the targets showing that DCs successfully processed the gp63. *In vitro* re-stimulation with SLA had a crucial role in inducing CTL activity as splenocytes from mice immunized with *L. mexicana* cDNA or DC's transfected with *L. mexicana* gp63 cDNA without *in vitro* re-stimulation did not generate CTL activity, indicating the importance of boosting vaccination in *Leishmania* immunization. In this animal model, T cells derived from non-immunized, infected mice did not have significant CTL

activity confirming that the CTL activity was induced by the DNA immunization not the infection, contrasting with results obtained in human studies (23, 26). Lack of CTL activity in *Leishmania* infected BALB/c mice might be a reason for the susceptibility of these mice to the parasites, which requires further investigation. The CTL activity induced by the DNA immunisation was detectable after four months of immunization and was similar to what has been reported from human patients with mucosal and cutaneous leishmaniasis before and after cure (26).

CTL activity was also detected in mice immunized with SLA+IFA and DCs loaded with SLA. The mice showed a high level of CTL activity against DCs loaded with SLA. The CTL activity induced by SLA was much higher than that of mice immunized with *L. mexicana* gp63 cDNA indicating the presence of other immunogenic proteins in the SLA.

Antibody responses to Leishmania vaccines

It has been shown that the immunity to *Leishmania* is mainly based upon the induction of a Th1-type immune response. Therefore, the type of the immune response induced by the vaccine has a direct association to generating resistance to the parasite (27, 28). It is thought that the different parameters including the nature of antigen, the adjuvant and the method of immunization affect the direction of immune system toward Th1 or Th2 (29, 30).

In the present study we sought to define the type of the immune response induced by a single antigen "*L. mexicana* gp63" or a cocktail of antigens "*L. mexicana* soluble antigens" using different methods of immunization. The type of immune response was determined by establishing the level of IgG2a and IgG1 antibody subtypes in the blood serum that represent the Th1 or Th2-type immune response respectively.

L. mexicana gp63 cDNA was administered by gene gun using gold. There are studies demonstrating that application of gene gun using gold particles bombardment recruits inflammatory cells and leads to Th2 immune response (29, 31). It has also been shown that application of adjuvant such as IL-12 or CpG motif as Th1 immune response enhancers in DNA vaccination (29, 32), shift the gene-gun-mediated DNA immune response from Th2 towards Th1 (33). Some studies indicated that the Th2 induction of gene gun is not due to the decreased amount of DNA used in gene gun immunization (34) but the nature of the antigen strongly influences whether a Th1 or Th2 immune response is induced (35).

In the present studies, the results obtained from the gene gun immunization was in contrast with the previous studies (29), where they suggested a Th2 inducing role for gene gun immunization. Mice administered with 1µg of the DNA by gene gun in BALB/c mice induced a sharp rise of IgG2a, which was detected one week after immunization. The level of IgG1 was quite low for two weeks and slightly increased afterward.

Administration of SLA with IFA induced a high level of IgG1 and less IgG2a, however, both antibodies increased during the course of the experiments. The kinetic responses of the antibody isotypes in the blood serum revealed mixed Th1/Th2 immune responses, which might be due to the presence of several immunogenic antigens in the SLA. The effect of IFA in directing the immune response towards Th1 or Th2 was not determined in the study. DC-based vaccine potency in producing antibodies has already been shown in HIV vaccine studies (24). Application of DCs loaded with SLA resulted in similar profile of IgG2a and IgG1 isotype antibodies to that induced by SLA. Levels of antibodies in control groups injected with DCs alone or PBS could be due to the cross reactivity of natural antibodies,

which detected by the secondary antibody in ELISA; the presence of natural antibodies cross reacting with *Leishmania* parasites was already reported in pigs, rats, mice, hamsters, gerbils and humans (36, 37).

In conclusion, gene gun immunization using *Leishmania mexicana* gp63 cDNA rendered higher immunity to the parasite compared to the SLA either by intramuscular injection or loaded on DCs. However, the role of DCs in induction of immunity against *Leishmania* needs to be more investigated. These results open a new approach for vaccination against *Leishmania* infection and/or other intracellular infectious agents.

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