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Soluble *Leishmania* Antigens Plus Pristane Adjuvant Induce Partial Cross-protection Against Leishmaniasis in BALB/c mice

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Abstract Leishmaniasis is a vector-borne parasitic disease of global concern. The disease is currently controlled by vector management and treatment of infected individuals as there is no approved vaccine. This study evaluated the safety, immunopotency and cross-immunity in BALB/c mice vaccinated with soluble *Leishmania major* or *L. donovani* antigens co-administered with 2,6,10,14-tetramethylpentadecane (pristane) and challenged with either *L major* or *L.donovani* virulent parasites. Safety was assessed by establishing dose-dependent blood-cell and platelet counts at 28 days post-injection, immunopotency was determined by measurement of interferon-gamma (IFN-ɣ) and interleukin 10 (IL-10) production, and disease progression by measurement either footpad lesion and parasite load in case of *L.major* challenge or spleen parasite loads for *L.donovani* challenge at 45 days post-infection. There was no significant effect by pristane on blood cell and platelet counts, indicating that at 20ug/mL, pristane was safe to use as an adjuvant in mice. Mice vaccinated with soluble *L. donovani* antigens plus pristane and challenged with *L. major* had smaller infected footpad lesions and lower parasite loads compared to BCG-vaccinated and unvaccinated mice. Similarly, mice vaccinated with soluble *L. major* antigens plus pristane and challenged with *L. donovani* had lower splenic parasite loads than unvaccinated mice. These corresponded with increased production of IFN-ɣ and suppressed production of IL-10 in each of the vaccinated groups, suggesting an up-regulated protective T helper 1 (Th1) response. The results indicated that vaccination of BALB/c mice with pristane adjuvant co-administered with soluble *Leishmania* antigens promotes Th1 response that confers partial cross-immunity against infection with heterologous *Leishmania* parasites. Further investigations on the safety of pristane in the longer term as well as other factors other than Th1 cytokines production up-regulation that may influence cross-protection in *Leishmania* infections need to be investigated

Keywords: *pristane, adjuvant, leishmaniasis, cross-protection, BALB/c mice*

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1. Introduction

Leishmaniasis is caused by obligate intracellular protozoan parasites of genus *Leishmania*. The parasites are transmitted by infected female phlebotomine sandflies of genus *Phlebotomus* and *Lutzomyia* [1]. Depending on the parasite species and patient's cellular immune system, clinical manifestations of the disease range from the chronic and potentially fatal visceral leishmaniasis (VL) that affects internal organs such as the liver, spleen and bone; to cutaneous leishmaniasis, also called oriental sore, that is characterized by ulcerative skin lesions and is occasionally nodular [2]. The third form of disease, mucocutaneous leishmaniasis causes partial or total destruction of the mucous membrane of the nose, mouth and throat [3]. Leishmaniases impose health and socio-economic burdens on more than a billion people globally, predominantly in economically deprived populations [4].

As an intracellular pathogen, successful immune response to *Leishmania* depends on a coordinated involvement of the innate and adaptive arms of the host's immune system [5]. The key players in innate immunity are neutrophils, macrophages, dendritic cells and natural killer cells that interact to regulate the resultant adaptive immunity, which determines infection outcome [6]. Following phagocytosis, interferon gamma (IFN-ɣ), an effector cytokine secreted by CD4+ T cells, CD8+ T cells and natural killer (NK) cells causes macrophages to secrete nitric oxide synthase (iNOS) that converts L-arginine to nitric oxide (NO), which kills intracellular

amastigotes [7].

Many potential human leishmaniasis vaccines have been tried, but so far none has been approved. This is due to challenges such as lack of adequate funding for research in this field and translational breakdown where most of the research and trial vaccines end at murine and other host animals and are not advanced to human leishmaniasis and lack of an appropriate adjuvant that induces a protective Th 1 immune response [8]. Generally, leishmanial antigens that stimulate Th 1 cell response in *Leishmania* infected hosts are accepted as promising vaccine candidates [9].

A number of studies have also been conducted to assess cross-protection. Using soluble *L. major* promastigote exogenous antigens (LmSEAgs), it was reported that the antigens conferred protection in BALB/c mice infected with *L. donovani*, but did not confer immunity in mice infected with *L. braziliensis*. There was a significant increase in Th1 cytokine, interferon-gamma (IFN-ɣ) mean production and a reduction in Th2 cytokine, interleukin-10 production [10]. Other studies in rhesus monkeys (*Macaca mulatta*) that had self-healed following experimental infection with cutaneous leishmaniasis causing *L. major* were significantly protected from infection with *L. amazonensis* and *L. guyanensis* [11]. In another study involving the vervet monkey (*Cercopithecus aethiops*) version of leishmaniasis, a high cross-reactivity between *L. donovani* and *L. major* was reported, with *L. donovani* conferring protection against *L. major* infection [12].

Oil-in-water emulsions are commonly used as adjuvants in animal vaccines, because they are stable, non-costly, and fairly easy to prepare into vaccine formulations [13]. These vaccines have the antigen as an aqueous phase that is added to the mineral oil phase in the presence of a surfactant, forming micelles consisting of oil droplets that contain antigens. The oil part stimulates mobilization of immune cells to the site of inoculation and also serves to protect the antigen from host immune cells thus elongating its availability and extending the period of time for the immune cells to process the antigen. This results in enhanced immune response as compared to inoculation of antigens alone [14].

Pristane is an isoprenoid hydrocarbon occurring naturally in phytol metabolism in plants, marine algae and copepods [13]. Studies on pristane demonstrated that pristane significantly increased the phagocytic index of macrophages in mice and significantly increased Th1-biased immune response [14]. The objective of this study was to evaluate cross-protection in mice vaccinated with soluble *Leishmania major* antigens (SLmAgs) + pristane and challenged with *L. donovani*; and mice vaccinated with soluble *Leishmania donovani* antigens (SLdAgs) + pristane and challenged with *L. major* by assessing cytokine production and disease progression.

2. Materials and Methods

2.1. Experimental Animals

Eight week-old BALB/c mice weighing 21 ± 2.6 g were used. BALB/c mice were selected because they are the lowest known animals with the highest susceptibility to *Leishmania* infections and are known to produce immune responses similar to human responses to leishmaniases. All experiments were carried out in accordance with the KEMRI Animal Care and Use Committee (ACUC) guidelines. To assess safety, animals were randomly selected and divided into 4 groups of 3 animals each and injected intravenously with 100µl containing 10µg/mL, 20µg/mL and 40µg/mL of pristane using a 22-gauge needle through the tail vein (indicate what volume was injected). The negative control group was injected with phosphate buffered saline (PBS). Whole blood samples were taken via cardiac puncture using a 22-gauge needle and the blood stored in EDTA tubes. Blood count analysis was done using an automated hematology analyzer to give leukocyte, erythrocyte and platelet counts after 28 days.

After safety assessment, animals were divided into 4 groups of 9 animals as follows; group I, injected with PBS (unvaccinated - negative control); group II, injected with BCG + SLmAg/SLdAgs (positive control); group III, injected with soluble *L. major* antigens (SLmAgs) + pristine and infected with *L. donovani* and, group IV, injected with soluble *L. donovani* antigens (SLdAgs) + pristine and infected with *L. major*. The animals were vaccinated with 100 µl total volume of the vaccine through intraperitoneal injection using 21G needles. The trial vaccine contained 20µg/mL of pristane and 45µg/mL of either SLmAgs or SLdAgs, at an adjuvant/antigen ratio of 4:6. The emulsion was mixed with SLAgs and vortexed for 20 seconds and administered through intraperitoneal injection in a volume of 100 µl in each mouse. After 21 days, animals received a second dose of the vaccine. After 10 days, three animals from each group were sacrificed for immunologic assessment and the remaining animals were infected with either 1 x 10^6 *L. major* or 1 x 10^7 *L. donovani* metacyclic promastigotes. At 45 days post-infection, blood was collected from all the animals and assessed for IFN-ɣ and IL-10 production and disease progression.

2.2. Leishmania Parasites

Metacyclic promastigotes of *L. major* strain IDU/KE/83 = NLB-144 and *L. donovani* strain NLB-065 were used. The parasites were cultured in Schneider's Insect medium (Sigma-Aldrich) supplemented with 20% of heat inactivated fetal bovine serum (FBS, Sigma-Aldrich) and 100 U/mL of penicillin/streptomycin (Sigma-Aldrich) at 26°C. Stationery-phase promastigotes were obtained on the 7th day of growth, washed three times with PBS at 1000x *g* for 10 minutes at 10°C, re-suspended in PBS and quantified by using hemocytometer after fixation with PBS/0.4% formaldehyde.

2.3. Preparation of Soluble *Leishmania* Antigens

Soluble *Leishmania* antigens were prepared from promastigote stationary phase *L. donovani* (MHOM/KE/82/LRC-L445=NLB-065) cultures. The parasites were washed 4 times in 1x PBS, centrifuged at 1000x g/10 minutes at 4°C and supernatant removed. The resultant pellets were re-suspended in lysis buffer

containing 100 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8) supplemented with 50 µg/mL leupeptine, 50 µg/mL antipapain, 50 µg/mLaprotinin and 1.6 mM phenylmethylsulfonyl fluoride/1 x 10^9 parasites (Sigma-Aldrich). The suspension was subjected to to three, 10 minutes freeze/thaw cycles, followed by ten sonication pulses of 30 seconds/40W. The sonicate was incubated at 4°C for 18 hours to allow complete antigen extraction, then centrifuged at 4000x g for 30 minutes at 4°C. The supernatant was collected, aliquoted and stored at -70°C until time of use. The protein concentration was determined using the Bradford method [17]

2.4. Adjuvant Preparation

The 2,6,10,14-tetramethylpentadecane adjuvant (Sigma-Aldrich) was prepared using the method described by Stone [18]. Briefly, oil to aqueous (O:A) phase ratio of 4:1 v/v was used. The surfactant TWEEN® 80 (Sigma-Aldrich) was added to the oil phase on a vortexer at maximum speed for 1 minute. The emulsion was used immediately to prepare the vaccine for immunization. BCG (Serum Institute of India Ltd.) was reconstituted in saline water and a dose of 20 µg/mouse in 50 µl was used.

2.5. Measurement of Cytokine Production

Blood was obtained from the experimental animals at 31 days post vaccination and at the 45th day post-infection, by cardiac puncture using a 22-gauge needle into haematocrit tubes. Sera was obtained by centrifuging the blood at 8000x g at 4°C for 10 minutes. IFN-ɣ and IL-10 detection and quantification was done using the BD CBA Soluble Protein Flex Set system as per manufacturer's instructions (BD Biosciences). The data were acquired using FACSCalibur and analyzed using the FCAP Array software v3.0 (BD, Biosciences).

2.6. Measurement of Disease Progression

Disease progression in *L. major* infected mice was assessed by measuring the infected footpad lesion size using a digital caliper and the lesion size expressed in millimeters as the difference between the thickness of the infected footpad and the uninfected footpad. Infected footpad parasite burden was assessed using the limiting dilution assay (LDA) as described by Lima [18]. In *L.donovani* infected mice, disease progression was assessed by determining splenic parasite burden, expressed as Leishman Donovan Unit (LDU) as previously described by Bradley and Kirkely [19].

2.7. Statistical Analysis

All experiments were carried out in triplicate and the results expressed as mean ± standard deviation (mean ± SD). Data was analyzed using ANOVA with *post-hoc* Tukey HSD test. *P* values equal to or less than 0.05 ($p \leq 0.05$) were considered significant.

3. Results

3.1. Effect of Pristane on Blood Cell Count

At 28 days post treatment, blood cell counts showed that the number of white blood cells in mice treated with pristane at concentration of 10µg/mL (9.21±0.23K/µL); 20µg/mL (9.97±0.28K/µL) and 40µg/mL (8.87±0.28K/µL) was not significantly different (p = 0.076) from the white blood cell count (9.29±0.21K/µL) in the untreated control group. Similarly, red blood cells count across the test pristane concentrations 10µg/mL (11.66±0.30M/µL); 20µg/mL (11.86±0.28M/µL) and 40µg/mL (10.89±0.36M/µL) was not significantly different (p = 0.128) from the red blood cell count (12.24±0.46M/µL) in the untreated control group. The platelet count in mice treated with different concentrations of pristane 10µg/mL (1003±50.86K/µL); 20µg/mL (1105.33±77.6K/µL) and 40µg/mL (978.67±57.68K/µL) was not significantly different (p = 0.463) from the platelet count (1070.33±29.83K/µL) in the untreated control group, as shown in Table 1.

Table 1. Blood cells and platelet counts in BALB/c mice at 28 days post treatment with pristane. Results expressed as the mean ± SD

Cells	Control (PBS)	Pristane dose (µg/mL)			P values
		10 µg/mL	20 µg/mL	40 µg/mL	
WBC (K/µL)	9.29±0.2	9.21±0.2	9.97±0.2	8.87±0.2	0.076
RBC (M/µL)	12.24±0.4	11.66±0.3	11.86±0.2	10.89±0.3	0.128
Platelets (K/µL)	1070.33±29	1003±50	1105.33±77	978.67±57	0.463

3.2. Cytokine Production

At 31 days post-vaccination, the amount of IFN-ɣ produced by SLmAgs + pristane-vaccinated mice was 1321.58±50.61 pg/mL, which was significantly higher ($p < 0.01$) than IFN-ɣ produced by SLdAgs + pristane-vaccinated mice (1177.59±9.53 pg/mL). Mice vaccinated with BCG + SLdAgs had a mean IFN-ɣ production of 705.22±27.73 pg/mL, that was significantly lower ($p < 0.01$) compared to SLdAgs + pristane-vaccinated mice. For IL-10 at 31 days post-vaccination, the amount produced in SLmAgs + pristane-vaccinated, 15.63±1.08 pg/mL was not significantly different (p = 0.78) from the level of IL-10 in SLdAgs + pristane-treated mice (14.59±1.14 pg/mL). Compared to BCG + SLdAgs-treated mice that had a mean production of 22.25±0.45 pg/mL, the amount of IL-10 in SLdAgs + pristane-treated mice was significantly lower ($p < 0.01$). At 45 days post-infection, the amount of IFN-ɣ produced in SLmAgs + pristane-treated mice (1547.05±100.6 pg/mL) was significantly higher ($p < 0.01$) compared to that in SLdAgs + pristane-treated mice (1258.62±63.94 pg/mL). However, the level of IFN-ɣ in the SLdAgs + pristane-

treated mice was significantly higher ($p < 0.01$) than that in BCG + SLdAgs-treated group (705.22±27.73 pg/mL). For IL-10, the amount produced in the SLmAgs + pristane-treated mice (17.4±0.83 pg/mL) not significantly different ($p > 0.01$) from the amount of IL-10 in SLdAgs + pristane-treated mice (15.54±1.79 pg/mL). However, BCG + SLdAgs-treated mice produced 23.27±0.39 pg/mL of IL-10 which was significantly higher ($p < 0.01$) compared to the SLdAgs + pristane-treated mice. Similarly, against the unvaccinated group that produced 82.07±19.15 pg/mL, the level of IL-10 was significantly lower ($p < 0.01$) in SLdAgs + pristane-treated mice as shown in Figure 1.

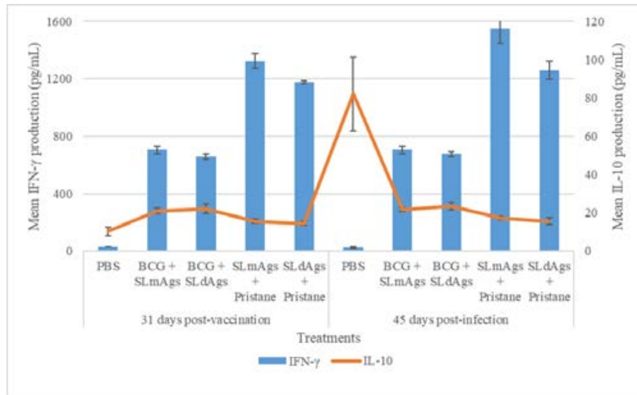

Figure 1. IFN-ɣ and IL-10 production in BALB/c mice at 31 days post-vaccination with SLAgs + pristane and 45 days post-infection with virulent *Leishmania*. Results presented as mean ± SD

3.3. Footpad Parasite Load

The footpad parasite load in SLdAgs + pristane-vaccinated and *L. major* infected mice (16.82±0.72 x 10^6) was significantly higher ($p = 0.02$) compared to the SLmAgs + pristane-vaccinated group that had a parasite load of 12.56±1.33 x 10^6. The BCG + SLdAgs-vaccinated group of mice had a mean parasite load of 21.33±0.35 x 10^6 that was significantly higher ($p < 0.01$) than the SLdAgs + pristane-vaccinated mice. Similarly, at a parasite load of 22.57±0.78 x 10^6, the unvaccinated group had a significantly higher ($p < 0.01$) mean parasite load than the SLdAgs + pristane vaccinated-mice as shown in Figure 2.

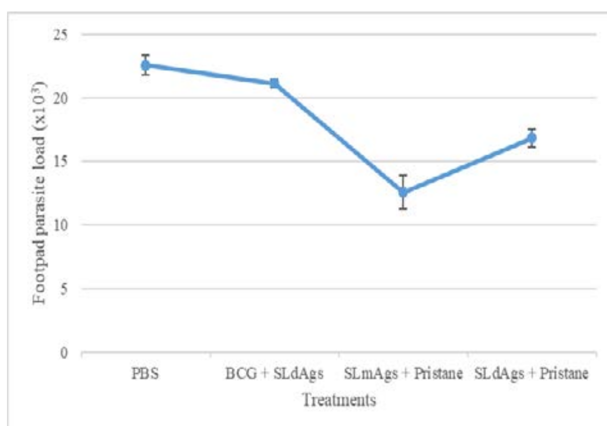

Figure 2. Footpad parasite load in BALB/c mice at 45 days post infection following vaccination with SLAgs + pristane and challenge with *L. major*. Results presented as mean ± SD

3.4. Footpad Lesion Size

The infected footpad mean lesion size of SLdAgs + pristane-vaccinated mice (0.48±0.16 mm) was not significantly different ($p = 0.89$) from that of the SLmAgs + pristane-treated group (0.43±0.08 mm). Compared with the BCG + SLdAgs-vaccinated mice (0.72±0.03 mm), the footpad lesion size in SLdAgs + pristane-vaccinated group was significantly smaller ($p = 0.04$). Similarly, SLdAgs + pristane-treated mice had significantly smaller ($p = 0.03$) lesion size compared to unvaccinated mice (0.77±0.06 mm) as shown in Figure 3.

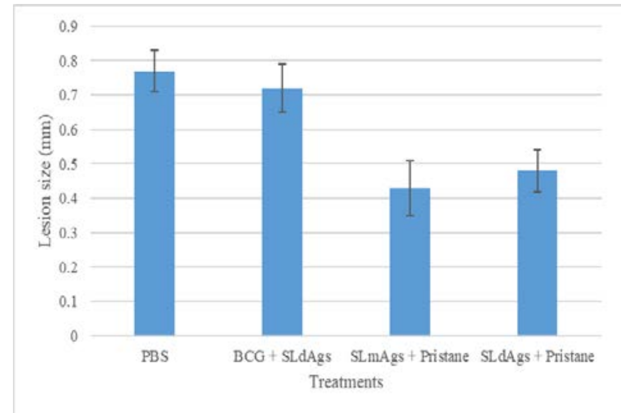

Figure 3. Footpad lesion size in BALB/c at 45 days post-infection following vaccination with SLAgs + pristane and challenge with *L. major*. Results presented as mean ± SD

3.5. Splenic Parasite Load

At 45 days post-infection, the splenic parasite burden in mice vaccinated with SLmAgs + pristane (7.91±1.22 x 10^3) was not significantly different ($p = 0.59$) from the SLdAgs + pristane-vaccinated mice (5.9±1.4 x 10^3). Similarly, there was no significant difference ($p = 0.66$) in the splenic parasite count between SLmAgs + pristane-vaccinated mice and BCG + SLmAgs-vaccinated mice (9.7±1.37 x 10^3). Compared to the unvaccinated group (12.64±3.06 x 10^3), the SLmAgs + pristane-vaccinated group had a significantly lower ($p = 0.03$) splenic parasite load, as shown in Figure 4.

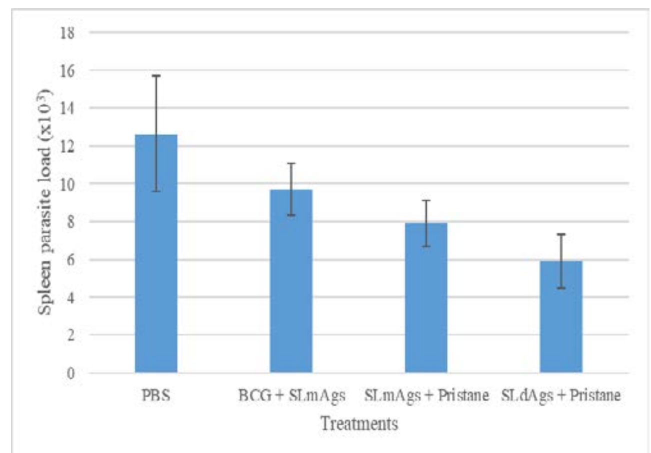

Figure 4. Spleen parasite load in BALB/c mice at 45 days post-infection following vaccination with SLAgs + pristane and challenge with *L. donovani*. Results presented as mean ± SD

4. Discussion

The results of the current study show that injection of BALB/c mice with 20µg/mL of pristane caused an insignificant rise in the total blood cell and platelet counts. These findings are consistent with earlier reports that the number of white blood cells in mice at 4 weeks (28 days) post pristane administration was almost equal in the treated and control groups [21]. Basing on this, a lower concentration of pristane, 20 µg/mL was used in this study, although further investigations need to be done to determine other hematological and biochemical effects of pristane at different concentrations and establish the most suitable concentration in terms safety.

From this study, it was observed that vaccination of BALB/c mice with soluble *Leishmania donovani* antigens formulated with pristane as an adjuvant generates partial protective immunity against infection by *Leishmania major*. Likewise, the results showed that vaccination of BALB/c mice with soluble *L. major* antigens formulated with pristane as an adjuvant generates partial protective immunity against infection by *L. donovani*. These results are consistent with earlier findings in vervet monkeys (*Cercopithecus aethiops*) that demonstrated high immunological cross-reactivity between *L. donovani* and *L. major*; with animals previously exposed to *L. donovani* being protected against *L. major* infection [12]. Findings in this study lay further credence to earlier assertions that *Leishmania*-specific antibodies produced by susceptible experimental animals bind to cross-reacting antigens shared by *Leishmania* species and could be responsible for cross-protection [22].

The observed protective immunity was characterized by high production of interferon gamma (IFN-ɣ) and low production of interleukin-10 (IL-10). As IFN-ɣ is an indicator of induction of the protective Th1 response while IL-10 is an indicator of non-protective Th2 immune response, these results indicate that formulation of soluble *Leishmania* antigens with pristane as an adjuvant promotes Th1 response that significantly represses parasite multiplication thus altering the course of *Leishmania* infection. Compared to BCG, an adjuvant that is associated with strong Th1 immune response when formulated with an antigen, pristane showed a higher induction of protective cytokine production that corresponded with repressed disease development.

As reported earlier, pristane induces the continuous formation of oil granulomatous tissue on peritoneal surfaces as a result of two defense processes which may explain the observed antileishmanial activities. These processes are the engulfing of oil droplets and the attempted encystment of non-metabolizing pristane in small cystic structures lined by macrophages. These lead to the recruitment of large numbers of phagocytic cells, neutrophils and monocytes into the peritoneal cavity, which phagocytose pristane and stick to peritoneal surfaces [16]. Significance of this observation is that a vaccine developed against one *Leishmania* species can be used to protect against other Leishmania species

5. Conclusion

In conclusion, the findings in this study have shown pristane to be a strong candidate for the development of effective anti-leishmanial vaccine due to its ability to induce a protective Th1-skewed immune response when formulated with soluble *Leishmania* antigens. We however recommend for further investigations on the safety of pristane in order to optimize its effective and safe concentration, and an assessment of its suitability as an adjuvant for anti-leishmanial vaccines in non-human primates.

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Competing Interests

Authors have no competing interests.

LIST OF ABBREVIATIONS

BCG Bascillus Calmette-Guerin
CBA Cytometric Bead Array
IFN-ɣ Interferon gamma
IL Interleukin
LDA Limiting Dilution Assay
LDU Leishman Donovan Units
PBS Phosphate Buffered Saline
SLAgs Soluble *Leishmania* Antigens
SLdAgs Soluble *Leishmania donovani* Antigens
SLmAgs Soluble *Leishmania major* Antigens
Th1 T helper 1
Th2 T helper 1

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