



Leishmanization revisited: Immunization with a naturally attenuated cutaneous *Leishmania donovani* isolate from Sri Lanka protects against visceral leishmaniasis

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ABSTRACT

Leishmaniasis is a neglected tropical disease caused by *Leishmania* protozoa and associated with three main clinical presentations: cutaneous, mucocutaneous and visceral leishmaniasis. Visceral leishmaniasis is the second most lethal parasitic disease after malaria and there is so far no human vaccine. *Leishmania donovani* is a causative agent of visceral leishmaniasis in South East Asia and Eastern Africa. However, in Sri Lanka, *L. donovani* causes mainly cutaneous leishmaniasis, while visceral leishmaniasis is rare. We investigate here the possibility that the cutaneous form of *L. donovani* can provide immunological protection against the visceral form of the disease, as a potential explanation for why visceral leishmaniasis is rare in Sri Lanka. Subcutaneous immunization with a cutaneous clinical isolate from Sri Lanka was significantly protective against visceral leishmaniasis in BALB/c mice. Protection was associated with a mixed Th1/Th2 response. These results provide a possible rationale for the scarcity of visceral leishmaniasis in Sri Lanka and could guide leishmaniasis vaccine development efforts.

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1. Introduction

Leishmaniasis is a tropical disease caused by *Leishmania* protozoa. Parasites are transmitted to the mammalian host by a sandfly vector and replicate intracellularly, mainly in macrophage phagolysosomes. Infection is associated with three main forms of disease: cutaneous leishmaniasis, where parasites remain contained at the site of the sandfly bite, mucocutaneous leishmaniasis in which there is metastasis to the mucosal tissues of the nose, mouth and throat, and visceral leishmaniasis where parasites disseminate to visceral organs, including the bone marrow, liver and spleen [1]. Visceral leishmaniasis is one of the most lethal tropical parasitic diseases, with a mortality second only to malaria [2]. Leishmaniasis is also the cause of over two million DALYs lost (disability-adjusted life years) [3]. Although visceral leishmaniasis is such a deadly disease, there are as yet no human vaccines licensed for use, and most vaccine studies have focused on the less severe cutaneous form of the disease [4].

Studying self-resolving infections or naturally immune patients can provide valuable insight into vaccine development. The practice of leishmanization arose from the observation that healing of a cutaneous leishmaniasis lesion was associated with protection against re-infection. Leishmanization involves inoculation with *Leishmania* parasites in a hidden area to protect against lesion

development in visible areas, and was performed on a large scale in Israel and Iran in the 1970s and 1980s, but was abandoned due to concerns about decreased immunity to other vaccines and non-healing lesions. Uzbekistan is currently the only country where it is still performed [5].

Evidence that leishmanization could also protect against visceral leishmaniasis is more limited. A study in Sudan observed that only individuals unresponsive to the leishmanin skin test (not previously exposed to *Leishmania*) developed visceral disease. The authors suggested that prior exposure to a cutaneous parasite could lead to a positive leishmanin skin reaction and protection against visceral disease [6]. The positive skin test could however have been due to asymptomatic *Leishmania donovani* infections.

Immunization with *L. donovani* antigens is protective against cutaneous *Leishmania* species [7–10]. In contrast, although immunization with *Leishmania major* antigen fractions such as soluble promastigote exogenous antigens was protective against visceral leishmaniasis [11], immunization with live *L. major* promastigotes did not protect against *Leishmania chagasi* or *Leishmania infantum* [12,13]. Indeed, prior infection with *L. major* was even associated with increased spleen parasite burden following *L. infantum* challenge [13]. Live immunization against visceral leishmaniasis may therefore require the use of parasites closely related to the challenge strain.

In Sri Lanka, there have been over 2000 cases of cutaneous leishmaniasis caused by an atypical *L. donovani* strain, while visceral leishmaniasis is rare [14]. No host genetic polymorphisms associated with altered susceptibility to leishmaniasis have been

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observed in the population [15,16]. Therefore, either parasite factors or acquired host differences may play a role. One possibility is that prior exposure to a more prevalent cutaneous parasite protects against visceral leishmaniasis. We have recently observed virtually no visceral leishmaniasis in districts reporting high levels of cutaneous leishmaniasis in Sri Lanka [17].

We investigate this here and show that immunization of BALB/c mice with a cutaneous clinical isolate from Sri Lanka protects against visceral disease caused by a visceral clinical isolate from Sri Lanka. Protection is associated with a mixed Th1/Th2 response prior to challenge. The protection resulting from this immunization may be due to the fact that both cutaneous and visceral *L. donovani* isolates are closely related, although the virulence of the cutaneous isolate is significantly attenuated since Sri Lanka *L. donovani* is almost exclusively associated with cutaneous rather than visceral leishmaniasis [14]. Overall, this work provides valuable insight into leishmaniasis disease dynamics in Sri Lanka but also has a broader impact on vaccination perspectives for visceral leishmaniasis.

2. Materials and methods

2.1. Parasite strains and culture

Both *L. donovani* isolates were obtained in Sri Lanka. The cutaneous SL-CL isolate was obtained from a cutaneous lesion on the nose from a 28-year-old male. The SL-VL isolate was derived from the third autochthonous visceral leishmaniasis patient in Sri Lanka, a 57-year-old visceral leishmaniasis patient with chronic fever, hepatosplenomegaly, lymphadenopathy, low hemoglobin and seropositivity for rK39. SL-VL was identified as *L. donovani* MON-37 based on multilocus isoenzyme electrophoresis and sequencing of the 6-phosphogluconate dehydrogenase (6PGDH) gene [17]. It has previously been established that the causative agent of cutaneous leishmaniasis in Sri Lanka is likewise *L. donovani* MON-37 [18,19] and we have confirmed by sequencing the 6PGDH gene that the SL-CL isolate used in this study is the same. Sequencing of the 6PGDH gene in the SL-CL and SL-VL isolates used in this study was performed at the McGill University Genome Quebec Innovation Center.

Leishmania promastigotes were cultured in M199 medium (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum, 40 mM HEPES (pH 7.4), 0.1 mM adenine, 5 mg/L hemin, 1 mg/L biotin, 1 mg/L biopterin, 100 U/mL penicillin and 100 µg/mL streptomycin. Parasites were maintained in this medium at 26 °C.

2.2. Immunizations

Female BALB/c mice (17–20 g) were purchased from Charles River Laboratories and maintained in the animal care facility under pathogen-free conditions. All experiments were performed in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

Mice were immunized subcutaneously in the left hind footpad with 1×10^3 to 1×10^6 SL-CL stationary phase promastigotes in 50 µL PBS. Control mice were immunized with 50 µL PBS alone. Footpad parasite burden prior to challenge was determined by limiting dilution of footpad homogenates.

2.3. Challenge infections

Seven weeks post-immunization, mice were challenged intravenously with 5×10^7 SL-VL stationary phase promastigotes. Mice were sacrificed 28 days following challenge. Liver parasite burden was determined by direct counting of amastigotes from Diff-Quick stained liver impressions and expressed as Leishman-Donovan

Units (LDU): number of amastigotes per 1000 cell nuclei $\times$ liver weight (g) [20].

2.4. In vitro splenocyte restimulation

Splenocytes were separated into a single cell suspension by passing through a fine wire mesh and red blood cells were lysed with 0.17 M NH₄Cl pH 7.2. Splenocytes were then resuspended at 2×10^6 cells/mL in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mM HEPES, 10% heat-inactivated FBS, 20 U/mL penicillin, 20 µg/mL streptomycin and 50 µM β-mercaptoethanol (Sigma). Cells were restimulated at 37 °C in 5% CO₂ with 50 µg/mL of soluble *Leishmania* antigen (SLA) prepared from SL-VL promastigotes or left unstimulated (null) for 72 h. IFNγ, IL4, and IL10 cytokine concentration in the culture supernatant were determined by sandwich ELISA according to the manufacturer's instructions (eBioscience).

Soluble *Leishmania* antigen (SLA) was obtained by sequential freeze-thaw cycles of stationary phase SL-VL promastigotes. Solutions were cleared by centrifugation and the protein concentration in the supernatant was determined by Bradford reaction (BioRad).

2.5. Serum antibody analysis

To determine total IgG serum antibody levels, ELISA plates were coated with 2.5 µg/mL SLA overnight. Wells were blocked in 2.5% FBS in PBS. Two-fold serial dilutions of serum samples in 2.5% FBS in PBS were added to the wells for 2 h, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:20,000; Rockland). Color development was performed with 3,3',5,5'-tetramethylbenzidine substrate (eBioscience) and the reaction was stopped with 2 N H₂SO₄. Absorbance measurements were performed at 450 and 570 nm. The antibody titer was identified as the highest dilution where absorbance values still exceeded $3 \times$ (mean blank OD + S.D. of blanks) as described in [21].

To determine antibody isotypes, ELISAs were performed as above, using 1 in 50 dilutions of serum samples. Incubation with serum samples was followed by 1 h incubation with anti-mouse IgG1 or IgG2a antibodies (1:1000) and 1 h incubation with horseradish peroxidase conjugated anti-goat IgG (1:5000).

2.6. Statistical analysis

The significance of the data was evaluated by two-tailed Student's *t* test.

3. Results

3.1. Subcutaneous immunization with low doses of SL-CL is protective against visceral leishmaniasis

In Sri Lanka, cutaneous leishmaniasis caused by *L. donovani* is common, while visceral leishmaniasis is rare [14]. We were therefore interested to investigate whether cutaneous infection with *L. donovani* derived from a human cutaneous leishmaniasis case can provide immunological protection against visceral leishmaniasis and this was tested in BALB/c mice. Clinical isolates were obtained from a cutaneous leishmaniasis patient (SL-CL) and a visceral leishmaniasis patient (SL-VL) from Sri Lanka. Both isolates are *L. donovani* since this species is reported to be the causative agent of cutaneous leishmaniasis [18,19,22]) and visceral leishmaniasis [17] in Sri Lanka, as detailed in methods. We also confirmed that both parasites used in this study were *L. donovani* MON-37 by sequencing of the 6-phosphogluconate dehydrogenase (6PGDH) gene.

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