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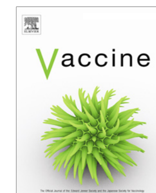

Intranasal but not subcutaneous vaccination with LaAg allows rapid expansion of protective immunity against cutaneous leishmaniasis

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ABSTRACT

Mucosal but not parenteral vaccination with whole *Leishmania amazonensis* promastigotes antigens (LaAg) is known to increase host resistance to infection by an as yet unknown immune mechanism. Since early immune responses are critical for infection establishment, in the present study the differential responses elicited by subcutaneous (s.c.) and intranasal (i.n.) vaccination with LaAg were investigated during the initial stages of infection. For that, BALB/c mice were given two LaAg doses by i.n. or s.c. route prior to *L. amazonensis* infection in the footpad. It was found that mucosal vaccination prevented both T helper (Th) 2-associated cutaneous hypersensitivity and local interleukin (IL)-4 production in the first days after parasite challenge in the footpad. That was accompanied by increased Th1 (T-bet and IL-12) and T_{reg} (Foxp3 and IL-10) transcription factor and cytokine expression in the lesion draining lymph nodes. In contrast, s.c. LaAg predominantly led to higher Th2 (GATA3) and transforming growth factor (TGF)-β expression. Prior i.n. vaccination was able to prevent the disease-exacerbating effect of s.c. vaccination. Although both $CD4^+$ and $CD8^+$ T cells were transiently increased in the cervical lymph nodes (cLN), the numbers of $CD4^+Foxp3^+$ regulatory T (T_{reg}) cells decreased within 48 h of i.n. vaccination as compared to non-vaccinated mice. Adoptive transfer of such cLN cells conferred increased resistance to infected mice, mimicking the effect of i.n. vaccination. Altogether, these data indicate that i.n. vaccination with LaAg may prevent early peripheral expansion of detrimental cells normally elicited by active infection or s.c. vaccination, thus allowing full expansion of protective responses.

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1. Introduction

Leishmaniases are immune-mediated protozoal diseases caused by parasites of *Leishmania* genus transmitted by the bite of infected sandflies. Depending on the parasite specie and host immune status, clinical manifestations may range from cutaneous leishmaniasis (CL) to visceral leishmaniasis (VL). CL is the most frequent form of the disease, reaching 1.2 million cases per year and nearly 4 million cases worldwide [1,2]. Given that many cases are not reported, the actual number could be between 12 and 20 million people living with CL [3]. Current drug treatment has many side effects, and reservoir and vector control is difficult to tackle. Prophylactic vaccination appears as the most effective control strategy, but despite the substantial effort in research, there is yet no licensed vaccine against human leishmaniasis [4]. At present, all that is known about human immune responses to *Leishmania* induced by immunization with killed parasite antigens came from studies with first generation candidate vaccines (killed promastigote extracts) [5]. Although active CL can lead to protection against re-infection [6], subcutaneous (s.c.) or intramuscular (i.m.) vaccination with killed parasites has not succeeded in increasing resistance in monkeys and humans [7,8]. Rather, s.c. vaccination of rhesus monkeys and mice with whole *L. amazonensis* antigens (LaAg) alone has shown to increase susceptibility to infection [9–11]. In BALB/c mice, the disease-promoting effect of s.c. vaccination with whole *L. major* antigens is accompanied by an early-appearing (15–18 h) Jones Mote-type cutaneous hypersensitivity reaction, different from the classical tuberculinic delayed-type (48 h) reaction observed in resistant mice [12].

Local skin events influence the ensuing adaptive immune response to leishmanial infections in man and in mice [13]. In the susceptible BALB/c mouse, the early interleukin (IL)-4 producing T helper (Th) 2 type immune response that follows *L. major* infection is known to be important for parasite establishment.

Abbreviations: CL, cutaneous leishmaniasis; cLN, cervical lymph nodes; LaAg, whole *Leishmania amazonensis* promastigotes antigens; LDA, limiting dilution analysis; pLN, popliteal lymph nodes; VL, visceral leishmaniasis.

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The rapid expansion of IL-4 producing cells has been related to the presence of memory CD4⁺ Vα8Vβ4 TCR cells specific to LACK (*Leishmania* homolog of receptors for activated C-kinase), a leishmanial antigen whose homologue is also expressed by bacteria of normal microbiota of the intestine [14,15]. Julia et al. [16] showed that thymic tolerance achieved by transgenic expression of LACK in the thymus rendered BALB/c mice more resistant to infection. This, together with the finding that the prophylactic intravenous (i.v.) effect as opposed to deleterious s.c. immunization with whole *L. major* antigens was due to early tolerance induction [17], prompted us to use more applicable tolerogenic strategies such as mucosal vaccination. Indeed, oral vaccination with LaAg induced protection against *L. amazonensis* infection in both BALB/c and partially susceptible C57BL/6 mice [10].

The less degradative and more economic intranasal (i.n.) vaccination with LaAg, as compared to the oral route, also induces long-term protection in BALB/c mice against *L. amazonensis* infection, in a manner associated with increased peripheral production of interferon (IFN)-γ [18]. The prophylactic i.n. LaAg effect is not restricted to susceptible BALB/c mice. It is extensive to CL in C57BL/6 mice caused by *L. amazonensis* [19] and golden hamsters against *L. braziliensis* [20], and also VL in BALB/c mice caused by *L. infantum* [21]. In all cases, animals had reduced ability to mount an early-responding cutaneous hypersensitivity, and became more capable to fight infection by a still poorly understood mechanism.

Based on the opposing effects achieved with peripheral and mucosal routes of vaccination, in this study we proposed to investigate the critical early responses elicited by s.c. and i.n. vaccination with LaAg.

2. Materials and methods

2.1. Mice

BALB/c female mice of 6–8 weeks old from our own animal house were kept at our facilities receiving filtered water, pelleted food and sterilized bedding. All experiments were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH) and were approved by the Committee on the Ethics of Animal Use of the Federal University of Rio de Janeiro under the number CAUAP180. Prior to any tissue removal, animals were killed with 5% isofluorane inhalation, with death assured by rapid cervical dislocation.

2.2. Parasites

Promastigotes of *L. amazonensis* (strain MHOM/BR/75/Josefa) were kept at 26 °C in Minimum Essential Medium 199 (Cultilab, Brazil) supplemented with 10% of heat-inactivated fetal calf serum (Cultilab, Brazil), antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin, Stemcell Technologies, USA) and hemin (5 μg/mL, Sigma-Aldrich, USA). After isolation from lesion, parasites were never used after the fourth *in vitro* passage, and always used at stationary growth phase for both vaccine preparation and infection.

2.3. LaAg

LaAg was prepared as previously described [10]. Briefly, *L. amazonensis* promastigotes were washed 3 times in phosphate buffered saline (PBS) by centrifugation. Parasites were ressuspended in water to 2×10^8 parasites/mL and submitted to three cycles of freezing and thawing. The resulting lysate (LaAg) was lyophilized, assayed for protein content by Lowry method, and stored at −80 °C. For use, LaAg was reconstituted in phosphate buffered saline (PBS).

2.4. Intranasal and subcutaneous vaccination

For i.n. vaccination, mice held upwards were intranasally instilled with LaAg (10 μg of protein/dose) in 20 μL of PBS [18]. For s.c. vaccination, animals were injected in the lower back with LaAg (25 μg of protein/dose) in 50 μL of PBS with hypodermic needle (0.30 × 13 mm – Becton Dickinson, Brazil) [10]. Animals received a boost vaccination 7 days later. Where indicated, animal received 2 i.n. doses of LaAg or PBS alone followed by 2 s.c. doses with LaAg or PBS with 7-day interval between each dose. Non-immunized mice were used as controls.

2.5. Infection, cutaneous hypersensitivity and lesion growth

Seven days after the boost vaccination, or 24 h after cell transfer as indicated, mice were injected in the hind footpad with 2×10^5 *L. amazonensis* promastigotes in 20 μL of PBS. For cutaneous hypersensitivity, footpad thickness was measured with a digital caliper (Mitutoyo, Brazil) prior to (0h) and 18, 24 and 72 h after parasite challenge. For lesion growth, footpad thickness was measured once a week.

2.6. Parasite loads

Parasite loads were determined by Limiting Dilution Analysis (LDA) as previously described [22]. Briefly, 56 days post infection, each infected foot was homogenized in medium using a tissue grinder. The tissue debris were removed by gravity sedimentation for 10 min, and each supernatant was serially diluted in quadruplicates in flat-bottom culture microplates. After incubation for 15 days at 26 °C, the original numbers of amastigotes/footpad were calculated from the last dilution showing parasite colony.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To evaluate *in situ* cytokine production, infected footpad homogenates were prepared as above, and centrifuged at 2000*g*/10 min [19]. IL-4 was measured in the clarified supernatants by ELISA using paired monoclonal antibodies: rat IgG1 anti mouse IL-4 (clone 11B11), and biotinilated rat IgG1 anti mouse IL-4 (clone BVD6-24G2), (R&D Systems, USA). The IL-4 standard curve was prepared with recombinant IL-4 according to the manufacturer instructions (R&D Systems, USA).

2.8. Quantitative real time PCR (qRT-PCR)

Seven days after infection challenge, lesion-draining popliteal lymph nodes (pLNs) were homogenized in guanidine isothiocyanate solution 4 M. Complementary DNA (cDNA) was synthetized through ImProm-II™ Reverse Transcription System (Promega, USA) according to manufacturer instructions. qRT-PCR reactions were performed in triplicates for each target in StepOne system (Applied Biosystems, USA) using StepOne v2.1 software. Each reaction consisted of yielded cDNA, 2x SYBER® Green Master Mix (Applied Biosystems, USA), forward primer (F) and reverse primer (R) (5 mM each, Promega Corporation, USA). Primers were designed using Primer Express® software version 3.0 (Applied Biosystems, USA): **Foxp3:** F 5′-GGC CCT TCT CCA GGA CAG A-3′, Foxp3R 5′-GGC ATG GGC ATC CAC AGT-3′, **GATA3:** F 5′-GAC CCG AAA CCG GAA GAT GT-3′, GATA3R 5′-GCG CGT CAT GCA CCT TTT-3′, **IL-10:** F 5′-GAT GCC CCA GGC AGA GAA-3′, IL-10R 5′-CAC CCA GGG AAT TCA AAT GC-3′, **IL-12:** F 5′-ACG CAG CAC TTC AGA ATC ACA-3′, IL-12R 5′-CAC CAG CAT GCC CTT GTC TA-3′, **Tbet:** F 5′-GCC AGG GAA CCG CTT ATA TG-3′, TbetR 5′-AAC TTC CTG GCG CAT CCA-3′, **TGF-β:** F 5′-CTA TCA GGT CCT GGC ACT TTA CAA-3′, TGF-βR 5′-CCC CGT GCA TCT CTT CCA-3′. qRT-PCR reaction condi-

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