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Original Article

Immunogenicity in dogs and protection against visceral leishmaniasis induced by a 14 kDa *Leishmania infantum* recombinant polypeptide ☆

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ABSTRACT

In areas were human visceral leishmaniasis (VL) is endemic, the domestic dog is the main parasite reservoir in the infectious cycle of *Leishmania infantum*. Development of prophylactic strategies to lower the parasite burden in dogs would reduce sand fly transmission thus lowering the incidence of zoonotic VL. Here we demonstrate that vaccination of dogs with a recombinant 14 kDa polypeptide of *L. infantum* nuclear transport factor 2 (*Li-ntf2*) mixed with adjuvant *Bp*MPLA-SE resulted in the production of specific anti-*Li-ntf2* IgG antibodies as well as IFN-γ release by the animals' peripheral blood mononuclear cells stimulated with the antigen. In addition, immunization with this single and small 14 kDa polypeptide resulted in protracted progression of the infection of the animals after challenging with a high dose of virulent *L. infantum*. Five months after challenge the parasite load was lower in the bone marrow of immunized dogs compared to non-immunized animals. The antibody response to K39, a marker of active VL, at ten months after challenge was strong and significantly higher in the control dogs than in vaccinated animals. At the study termination vaccinated animals showed significantly more liver granulomas and lymphoid hyperplasia than non-vaccinated animals, which are both histological markers of resistance to infection. Together, these results indicate that the 14 kDa polypeptide is an attractive protective molecule that can be easily incorporated in a leishmanial polyprotein vaccine candidate to augment/complement the overall protective efficacy of the final product.
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1. Introduction

More than a dozen identified and characterized species of *Leishmania* cause diseases in humans ranging from simple self-healing lesions in cutaneous leishmaniasis (CL) to debilitating and lethal (if untreated) visceral leishmaniasis (VL) also known as kala-azar. Transmission of all forms of leishmaniasis occurs naturally by the bites of female sandflies either from infected humans or from infected animals. Direct transmission of VL by contaminated needles in drug-abusers has also been documented [1]. Moreover, effective drugs to treat VL are toxic, non-sterilizing, expensive and difficult to administer. There is no vaccine to human VL.

In addition to being a human disease VL, is zoonotic infection as well. Dogs are major reservoirs of the parasite [2,3]. Canine VL (CVL) is widely distributed in Latin America [4,5] and Mediterranean basin [6,7]. In the USA, the potential for CVL to be a significant problem has been recently highlighted [8–10].

Two vaccines to CVL are commercially available in Brazil, Leishmune® [11], and Leish-Tec® [12] and one in Europe, CaniLeish® [13–15] but these products have not been approved for human use due to low protection efficacy observed in vaccinated dogs as well as because they use saponin-based adjuvant, which is not suitable for humans. In addition, Leishmune® and CaniLeish® are glycoprotein fractions purified from whole extracts of *Leishmania donovani or Leishmania infantum* promastigotes [13,16], therefore have possible complications with standard operating procedures (SOP) for their manufacture. Consequently, better vaccines for both CVL and human VL are still in high demand [17].

☆ *Disclosures:* CA is an employee of DetectoGen Inc. and has no ownership or ownership option in the company. ND, SP and GB have no conflicts of interests. AC-N is a consultant for DetectoGen Inc.

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We have recently used an innovative approach for the direct identification of VL vaccine candidate molecules that are abundantly produced *in vivo* during disease and that are present in bodily fluids (e.g., urine) of human patients with VL. This approach led to the identification of several polypeptides of *L. infantum*. When tested as vaccine candidates formulated with the adjuvant monophosphoryl lipid A (MPLA) from *Bordetella pertussis* emulsified with squalene (Butantan Institute, São Paulo, SP, Brazil) one of the polypeptides, *L. infantum* nuclear transport factor-2 (*Li-ntf*2) induced marked parasite burden reduction in the vaccinated animals compared to control mice [18].

In the present study, using a dog model of the VL we evaluated the immunogenicity and protective efficacy induced by *Li-ntf*2 formulated with *Bp*MPLA-SE. The results confirm that this formulation induces a biased Th1 type of immune response in these animals and that the anti-K39 antibody response, a biomarker of disease activity was significantly lower in vaccinated dogs compared to controls.

2. Material and methods

2.1. Animals and veterinary care

Beagle dogs were purchased from Marshall Bio Resources, North Rose, NY, and housed at the animal facility at The Cummings School of Veterinary Medicine at Tufts University, Grafton, MA. The investigation followed all the guidelines of National Institute of Health for animal experimentation and of the Institutional Animal Care and Use Committee at Tufts University.

2.2. Parasites

L. infantum (MHOM/BR/00/1669) was kindly supplied by Dr. Mary E. Wilson (University of Iowa, Iowa City, IA) and was maintained *in vivo* in hamsters as previously described [19]. Parasites were isolated from the spleen of infected hamsters and cultured in Schneider's medium (Invitrogen, Carlsbad, CA) supplemented with 20% FBS (Hyclone, Thermo Scientific, Rockford, IL) and 2 mM L-glutamine (Gibco-Invitogen, Carlsbad, CA) for 7–10 days at 26 °C. Metacyclic promastigotes forms of the parasite were used for challenge infections of dogs. Challenge was performed ten days after the last immunization of vaccinated dogs and control animals of matching sex and age. Dogs were inoculated intravenously with 10^7 live, virulent metacyclic *L. infantum* promastigotes.

2.3. Immunization and challenge

To confirm in dogs the immunogenicity of the vaccine formulation that we have previously tested in mice [18], 2 groups of 3 dogs (6 month old, male, Beagle) were immunized subcutaneously three times (three weeks apart) with either *Li ntf2* (50 μg) + *Bp*MPLA-SE (50 μg), group 1, or with *Li ntf2* (50 μg) and no adjuvant, group 2. *Bp*MPLA-SE (Institute Butantan, São Paulo, Brazil) is an oil-in water emulsion containing monophosphoryl lipid A (MPLA) derived from *B. pertussis* [18].

2.4. IgG ELISA

ELISA testing to detect anti-leishmania K39 antibody was performed on dogs' sera obtained before immunization, before challenge, and every two months thereafter for the14 months of the study. Briefly, ELISA MaxiSorp plates (Thermo Fisher Scientific) were coated with 0.1 μg rK39 (InBios International, Inc., Seattle WA) per well in carbonate buffer overnight following by blocking with PBS–Tween 0.5%–BSA 5%. Serial twofold dilutions of serum samples were tested, starting at 1:25. Antibodies to rK39 were revealed with peroxidase-conjugated anti-IgG secondary antibody (Abcam Inc, Cambridge, MA) and the substrate 3,3′,5,5′-tetrame thylbenzidine (TMB). Optical density was determined at 450 nm. IgG ELISA was also performed to detect anti-*Li-ntf2* antibodies in the sera of humans with visceral leishmaniasis as well as in dogs with this disease. Human sera were de-identified samples kindly provided to us by Dr. Ricardo Fujiwara, Federal University of Minas Gerais, Belo Horizonte, Brazil. Dog sera were from animals challenged with *L. infantum* at The Cummings School of Veterinary Medicine animal facility.

2.5. Cytokine assay

Three weeks after the last boost peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Histopaque (Sigma, St. Louis, MO) and suspended in RPMI supplemented with 10% FBS (Hyclone), 100 μg/ml streptomycin, 100U/ml penicillin, 25 mM HEPES, 2 mM L-glutamine, 0.05 mM 2-ME (all Sigma). Cells (2×10^5) were added to the wells of a 96-well flat-bottomed culture microplate (Costar, Lowell, MA) and stimulated at 36 °C for 72 h with 5 μg/ml *Li-ntf2*. Supernatants were collected and assayed for the presence of IFN-gamma using dog specific ELISA kits (DuoSet, canine IFN-γ, R&D Systems, Minneapolis, MN). Cells cultured in presence of 5 μg/ml Phytohaemagglutinin (PHA) or complete medium alone were included as controls.

2.6. Bone marrow sampling

Five and eleven months after challenge dogs were anaesthetized and bone marrow (BM) samples were obtained by puncturing the trochanteric fossa of proximal femur and dispensed into citrated tubes. Microscopic observation of Giemsa stained smears was performed to determine the presence of parasites. If no parasites were observed after at least 30 min on each of the slides, the sample was declared negative. The presence of *Leishmania* parasites was also determined in BM and lymph node (LN) aspirates by parasite growth in culture on biphasic NNN medium. The tubes were incubated at 25–27 °C for one week. A sample was considered as positive when parasites were observed by microscopic examination of the culture media.

2.7. Real-time PCR

From each BM sample, white blood cells were isolated by centrifugation over Histopaque (Sigma, St. Louis, MO). DNA was then extracted by DNeasy Blood and Tissue Kit (Quiagen, USA) according to manufactures' instructions. Two Taq-Man systems were developed: the *Leishmania* Taq-Man system and the dog Taq-Man system. For the *Leishmania* Taq-Man system the target DNA was *L. infantum* DNA polymerase (GeneBank accession number AF009147), which is a single copy number gene [20]. The amplification primers (forward primer, 5′-TGTCGCTTGCAGACCAGATG-3′; reverse primer, 5′-GCATCGCAGGTGTGAGCAC-3′) were designed to amplify a 90-bp fragment and the fluorogenic probe was (5′FA M-CAGCAACAACTTCGAGCCTGGCACC-3′TAMRA). A calibration curve was prepared using purified DNA from 5×10^6 *Leishmania* parasites. Two copies was the lowest target level reliably quantified and linearity was maintained up to 2×10^6 parasites per reaction. For the dog Taq-Man system, the target DNA was intron 12 of the albumin gene (NCBI Reference Sequence: NC_006595.3). An intron was chosen in order to amplify only albumin genomic DNA and thus diagnose the amount of cells present in the sample [21]. The amplification primers (forward primer 5′-ATAGTGT-TATCTTGGGGGTTCT-3′; reverse primer 5′-GACGGAAGCTGG-

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