Veterinary Parasitology xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

# Analysis using canine peripheral blood for establishing *in vitro* conditions for monocyte differentiation into macrophages for *Leishmania chagasi* infection and T-cell subset purification

Kelvinson Fernandes Viana[a,b,c], Rodrigo Dian Oliveira Aguiar-Soares[c], Bruno Mendes Roatt[c], Lucilene Aparecida Resende[a,c], Denise da Silveira-Lemos[d], Rodrigo Corrêa-Oliveira[c], Olindo Assis Martins-Filho[f], Sandra Lima Moura[g], Marcos Santos Zanini[h], Márcio Sobreira Silva Araújo[f], Alexandre Barbosa Reis[c,i], Rodolfo Cordeiro Giunchetti[a,c,e,f,*]

[a] *Laboratório de Biologia das Interações Celulares, Departamento de Morfologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*
[b] *Laboratório de Biotecnologia, Departamento de Ciências Agrárias e Tecnológicas, Universidade Federal do Tocantins, Gurupi, Tocantins, Brazil*
[c] *Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas/NUPEB, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil*
[d] *Laboratório de Imunologia e Genômica de Parasitos – Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*
[e] *Laboratório de Imunologia Celular e Molecular, Centro de Pesquisa René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brazil*
[f] *Laboratório de Biomarcadores de Diagnóstico e Monitoração, Centro de Pesquisa René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brazil*
[g] *Laboratório de Imunoparasitologia – Núcleo de Pesquisas em Ciências Biológicas/NUPEB, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil*
[h] *Laboratório de Microbiologia e Zoonoses – Departamento de Medicina Veterinária, Universidade Federal do Espírito Santo, Alegre, Espírito Santo, Brazil*
[i] *Departamento de Análises Clínicas, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil*

ARTICLE INFO

*Article history:*
Received 11 July 2013
Received in revised form 10 August 2013
Accepted 14 August 2013

*Keywords:*
Canine visceral leishmaniasis
*Leishmania chagasi*
*In vitro* microbicidal analysis
Macrophage
CD4 and CD8 T cells

ABSTRACT

Canine visceral leishmaniasis (CVL) is a parasitic disease endemic in many countries, and dogs present as the major natural reservoir of the parasite, *Leishmania chagasi* (syn. *L. infantum*). Biomarkers in the canine immune system is an important technique in the course of developing vaccines and treatment strategies against CVL. New methodologies for studying the immune response of dogs during *Leishmania* infection and after receiving vaccines and treatments against CVL would be useful. In this context, we used peripheral blood mononuclear cells (PBMCs) from healthy dogs to evaluate procedures related to (i) establishment of *in vitro* conditions of monocytes differentiated into macrophages infected with *L. chagasi* and (ii) purification procedures of T-cell subsets ($CD4^+$ and $CD8^+$) using microbeads. Our data demonstrated that after 5 days of differentiation, macrophages were able to induce significant phagocytic and microbicidal activity after *L. chagasi* infection and also showed increased frequency of parasitism and a higher parasite load. Although

* Corresponding author at: Laboratório de Biologia das Interações Celulares, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brazil. Tel.: +55 31 3409 3003.
*E-mail addresses:* giunchetti@icb.ufmg.br, giunchetti@gmail.com (R.C. Giunchetti).

0304-4017/$ – see front matter. 
http://dx.doi.org/10.1016/j.vetpar.2013.08.014

N-acetyl-β-D-glucosaminidase (NAG) levels presented similar levels of macrophage culture and *L. chagasi* infection, a progressive decrease in myeloperoxidase (MPO) levels was a hallmark over 5 days of culture. High purity levels (>90%) of CD4 and CD8 T cells were obtained on a magnetic separation column. We concluded that monocytes differentiated into macrophages at 5 days and displayed an intermediate frequency of parasitism and parasite load 72 h after *L. chagasi* infection. Furthermore, the purification system using canine T-lymphocyte subsets obtained after 5 days of monocyte differentiation proved efficient for CD4 or CD8 T-cell purification (≥90%). The *in vitro* analysis using *L. chagasi*-infected macrophages and purified T cells presented a prospective methodology that could be incorporated in CVL vaccine and treatment studies that aim to analyze the microbicidal potential induced by specific $CD4^+$ and/or $CD8^+$ T cells.


## 1. Introduction

Leishmaniasis is endemic in 88 countries in tropical and subtropical regions of the Old and New Worlds, with more than 350 million cases being visceral leishmaniasis (VL) (Desjeux, 2004). Infected dogs have a high density of cutaneous parasites, and they are the main domestic reservoir of *Leishmania chagasi* (syn. *Leishmania infantum*) contributing to the propagation of the parasite (Deane and Deane, 1962). Thus, the current strategy for managing VL in humans centers on the detection and elimination of seropositive dogs alongside vector control and therapy for individual cases (Tesh, 1995).

A key goal in the control of canine visceral leishmaniasis (CVL) has been the development of vaccines with high protective capability to interrupt the cycle of parasite transmission (Reis et al., 2010). Assessments of vaccine safety and anti-CVL efficacy generally require a long follow-up, stretching into years of study (Giunchetti et al., 2007, 2008; Roatt et al., 2012). In this context, the development of methodological strategies that enable optimal evaluation of the dog's immune system would be highly relevant. Such tests could be included in clinical trials vaccine against CVL, so that the time needed for the experiments could be reduced. This would likely reduce the costs of experimentation using the dog model as well as provide a more rational way of selecting candidate vaccines against CVL.

Macrophages play an important role in the control of *Leishmania* infection in distinct experimental models. It is well established that macrophages participate in killing parasites through mechanisms that depend on reactive oxygen and nitrogen intermediates. However, the mechanisms by which macrophages kill *Leishmania* in dogs have not been investigated as thoroughly (Rodrigues et al., 2007). The immune response against *Leishmania* sp. is highly dependent on the microbicidal action of macrophages, which are actually the host cell target of this protozoan; however, they have full capacity for antigen presentation and establishment of an effective response against the parasite (Pinelli et al., 1999).

Thus, to develop new approaches for analyzing the immune response of naturally *L. chagasi*-infected dogs or dogs immunized against CVL, *in vitro* co-culture systems with macrophages and purified T-lymphocytes would be useful. However, there is so far no standardized methodology for this purpose, and these tests usually only involve a system with peripheral blood mononuclear cells (PBMCs) without purified T-lymphocyte subsets (Holzmuller et al., 2005; Rodrigues et al., 2007, 2009). The development of additional methodologies for evaluating the immune system in veterinary medicine, especially in experimental dog models, is required. Such an advance would contribute to the identification of biomarkers related to interactions between innate and adaptive immune responses of dogs. In this context, we aimed to further analyze the immune response by using standardized methodologies for a co-culture system of canine *L. chagasi*-infected macrophages and for obtaining purified $CD4^+$ and $CD8^+$ T cells. This approach could contribute to identifying specific immune response biomarkers for developing a resistance or susceptibility profile in CVL, which could be used in both vaccine and treatment strategies against the parasite.

## 2. Materials and methods

### 2.1. Animals

Healthy mongrel dogs, both sexes with a mean age of 7 months, born and raised in a kennel at the Center of Animal Science, Federal University of Ouro Preto, were used in the experiments of (i) establishment of *in vitro* conditions of monocytes differentiated into macrophages infected with *L. chagasi* ($n = 5$) and (ii) purification procedures of T-cell subsets ($CD4^+$ and $CD8^+$) using microbeads ($n = 12$). The animals received all the appropriate health management before entering the experiment, having received anti-helmintic treatment (plus Chemital®, Chemitec Agro-Veterinary LTDA., BRA) and vaccination against rabies (Tecpar, BRA), distemper, adenovirus type 2, coronavirus, parainfluenza, parvovirus, and *Leptospira* (HTLP 5/CV-L Vanguard®, Pfizer, BRA). The study protocol was approved by the Ethical Committee for the Use of Experimental Animals of the Universidade Federal de Ouro Preto, Ouro Preto – MG, Brazil.

### 2.2. Parasites

This study used a wild-type strain of *L. chagasi* (C46) isolated from an infected dog of Governador Valadares, MG, and previously characterized in hamsters (Moreira et al., 2012). This strain was grown in culture medium NNN/LIT (Sigma Chemical Co., USA) supplemented with 20% fetal bovine serum (FBS) inactivated (Cultilab, BRA), plus penicillin (200 U/mL) and streptomycin (100 μg/mL), at pH 7.4 and incubation temperature of 23 °C. Parasites used for

Please cite this article in press as: Viana, K.F., et al., Analysis using canine peripheral blood for establishing *in vitro* conditions for monocyte differentiation into macrophages for *Leishmania chagasi* infection and T-cell subset purification. Vet. Parasitol. (2013), http://dx.doi.org/10.1016/j.vetpar.2013.08.014