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Short communication

Cellular immunophenotypic profile in the splenic compartment during canine visceral leishmaniasis

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ABSTRACT

To determine the role of the spleen in the pathogenesis of canine visceral leishmaniasis (CVL), we analyzed cellular immunophenotypic profiles of 52 dogs naturally infected with *Leishmania infantum*, clinically classified as follows: asymptomatic dogs-I (AD-I), seronegative/PCR+; asymptomatic dogs-II (AD-II), seropositive/PCR+; oligosymptomatic dogs (OD) and symptomatic dogs (SD). Seven non-infected dogs (CD) were included as a control group. AD-II presented higher levels of CD8+ T splenocytes and lower TCD4+/TCD8+ ratio in comparison with CD. OD and SD showed lower percentages of CD21+ as compared with AD-II. All seropositive dogs presented lower levels of CD45RA+ than CD. Regardless of the stimuli used, the proliferation index from splenocytes *in vitro* was inversely correlated with clinical status. After LSA stimulation, there was a higher percentage of specific CD8+ T in AD-II than CD and non-stimulated culture. In contrast, splenocytes from SD under *in vitro* LSA stimulation induced decreased MHC-II+ expression in comparison with all groups, and non-stimulated culture. In conclusion, the role of CD8+ T splenocytes seems to be important for an effective immunological response, a hallmark of asymptomatic CVL, whereas the pronounced loss of MHC-II expression upon LSA stimulation is a biomarker of symptomatic CVL.

1. Introduction

Visceral leishmaniasis (VL) is a potentially fatal human disease caused by the intracellular protozoan parasite *Leishmania infantum* (Kaye et al., 2004). Canine visceral leishmaniasis (CVL) represents a problem for both veterinary medicine and public health in approximately 50 countries in various endemic areas of the world (Coura-Vital et al., 2011b; Desjeux, 2004). CVL is a systemic,

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chronic, and severe disease that is often fatal because no efficacious drugs exist to cure these animals (Baneth et al., 2008; Reis et al., 2006c). According to Mancianti et al. (1988), CVL can be categorized into three distinct clinical forms, based on major clinical features observed in infected dogs: asymptomatic (AD), with no suggestive signs of the disease; oligosymptomatic (OD), with a maximum of three clinical signs; and symptomatic (SD), with typical clinical signs, showing the most severe clinical signs of CVL.

Recently, Coura-Vital et al. (2011a) proposed a new classification scheme for the canine disease according to serological, molecular, and clinical features. The asymptomatic form was divided in two subgroups: asymptomatic dogs I (AD-I), with negative serological tests for *Leishmania* but positive molecular results, and asymptomatic dogs II (AD-II), showing positive results for both serology and molecular analyses for *L. infantum*.

VL is a disease associated with the inability of lymphocytes to activate MΦ to kill *Leishmania* (Nylen and Sacks, 2007). Parasites can be found in mononuclear phagocytic cells in the spleen and liver, which are the major affected sites (Kaye et al., 2004; Stanley and Engwerda, 2007). In a progressive disease, cellular immune response are impaired, as indicated by studies showing that peripheral blood mononuclear cells (PBMCs) from affected humans and dogs fail to respond to parasite soluble antigens both *in vitro* and *in vivo* (Boggiatto et al., 2010; Goto and Prianti, 2009). On the other hand, a protective immune response (observed in asymptomatic VL) is manifested by a strong proliferative response of PBMCs from affected humans and dogs to *Leishmania* antigens, and the production of cytokines, such as IFN-γ and TNF-α, which are required for MΦ activation and killing of intracellular parasites (Cabral et al., 1992, 1998; Pinelli et al., 1994, 1999; Seixas Duarte et al., 2008; Strauss-Ayali et al., 2005).

Some studies have shown that the proliferation capability of PBMCs from dogs with CVL was decreased upon both antigenic and mitogenic stimulation (Cabral et al., 1998; Cardoso et al., 2007; Pinelli et al., 1995). However, the influence of the populations and subpopulations of the splenocytes derived from dogs with CVL upon antigenic stimulation is not completely understood. Because the spleen is one of the major organs affected during CVL, and the contribution of the compartmentalized immune response in the genesis of splenomegaly during this infection remains unclear, it is relevant to investigate significant alterations in the phenotypic features from infected dogs, performing a detailed *ex vivo* and *in vitro* immunophenotyping of their splenocytes.

2. Materials and methods

2.1. Dogs and experimental design

All procedures in this study were according to the guidelines set by the Brazilian Animal Experimental College (COBEA). This study was approved by the Ethical Committee for the Use of Experimental Animals at the Universidade Federal de Minas Gerais, Brazil (Protocol no. 020/2007).

Fifty-two mixed-breed adult dogs of both genders (age ranging from 2 to 6 years) were selected. They were maintained in the kennel at the Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais or provided by the Centro de Zoonoses–Belo Horizonte/Minas Gerais, Brazil. The dogs used in this study were stray or domiciled mongrel dogs, selected based on their serological results on IFAT (IFI-Leishmaniose-Visceral-Canina-Bio-Manguinhos, Rio de Janeiro, Brazil), which is the gold standard immunological test for diagnosis of CVL. Animals presenting IFAT titers ≥1:40 were considered positive and included into the infected groups. Animals with IFAT negative at 1:40 were considered non-infected and included as a control group. Positive infection was confirmed by PCR in at least one skin sample (Degrave et al., 1994), and the species of *Leishmania* responsible for the infection were determined by restriction fragment length polymorphism–PCR (Volpini et al., 2004).

2.2. Clinical classification

The dogs were clinically classified according to presence/absence of infection signs serological, and molecular tests: asymptomatic dogs I (AD-I, $n = 8$), with no suggestive signs of disease, negative serology, and PCR+ for *Leishmania*; asymptomatic dogs II (AD-II, $n = 10$), with no suggestive signs of disease, positive serology, and PCR+ for *Leishmania*; oligosymptomatic dogs (OD, $n = 11$), with a maximum of three clinical signs including opaque bristles, and/or localized alopecia, and/or moderate loss of weight; and symptomatic dogs (SD, $n = 16$), with characteristic clinical signs of CVL, such as opaque bristles, severe loss of weight, onychogryphosis, cutaneous lesions, apathy, and keratoconjunctivitis, and both groups with positive serology, and PCR+ for *Leishmania*; and control dogs (CD, $n = 7$), classified according to negative serological and PCR− for *Leishmania*.

2.3. Spleen sample collection

The collection of spleen specimens was carried out after euthanasia of the dogs with a barbituric anesthesia (Thiopental at 30 mg/kg body weight). Spleen fragments (5 mm) were stored on ice up to 12 h in a Petri dish in sterile RPMI-1640 (Gibco, Grand Island, NY, USA) prior to use in *ex vivo* and *in vitro* procedures. The tissue was minced in a tissue grinder and transferred to 2 mL of RPMI-1640. The cells suspension was then filtered on stainless steel gauze to obtain a single cell suspension. The mononuclear splenocytes were isolated by differential centrifugation ($800 \times g$ for 40 min at room temperature [RT]) on a Ficoll-Hypaque cushion (Histopaque 1.077, Sigma, St. Louis, MO, USA). The cell suspension was washed twice in RPMI-1640 and resuspended to obtain 10^7 cells/mL.

2.4. Determination of parasite load index – LDU

Following necropsy, fragments of spleen were collected and imprints prepared on two microscope slides. Slides were air-dried, fixed in methanol, stained with Giemsa, and examined under an optical microscopy to detect amastigote forms of *Leishmania*. Parasite densities were determined according to Stauber (1955) with some