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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

modifications, and expressed as Leishman Donovan Units (LDU index), which correspond the number of *Leishmania* amastigotes per 1000 nucleated cells. For control purposes, seven dogs that were not infected with CVL according to the IFAT assay were also submitted to parasitological examination.

2.5. Immunophenotyping by flow cytometry

Immunophenotyping analysis of splenocytes by flow cytometry was undertaken as described by Reis et al. (2005). Briefly, 1×10^7 cells/mL of splenocytes were subjected to pre-fixation and erythrocyte lysis by the gentle addition of 13 mL lysis solution (FACS lysing solution, Becton Dickinson, Mountain View, CA, USA) followed by incubation for 10 min at RT. After centrifugation (450 ×*g* for 10 min at RT), the pellet was resuspended in 500 μL phosphate buffered saline supplemented with 10% of fetal bovine serum.

In a 96-well U-bottom plate (Limbro Biomedicals, Inc., Aurora, OH, USA), 30 μL of pre-fixed splenocyte suspension was incubated at RT for 30 min, in the dark, in the presence of 30 μL of anti-canine cell surface marker antibodies. Monoclonal antibodies (mAbs), which define canine cell phenotypes, including purified rat anti-dog CD5 (Rat-IgG2a, clone YKIX322.3), anti-dog CD4 (Rat-IgG2a, clone YKIX302.9), anti-dog CD8 (Rat-IgG1, clone YCATE55.9), anti-MHC-II (Rat-IgG2b, clone YKIX334.2), anti-CD45RA (Rat-IgG2b, clone YKIX753.22.2), and anti-CD45RB (Rat-IgG2b, clone YKIX716.13) were used in an indirect immunofluorescence procedure. FITC-labeled mouse anti-human-CD21 (Mouse-IgG1, clone IOBla) was used in direct immunofluorescence procedures. Unlabeled mAbs used in this study were purchased from Serotec (Oxford, UK) and anti-CD21 from Immunotech Co. (Marselle, France).

During the immunophenotyping the cells were also incubated under the same conditions in the presence of 60 μL of previously diluted FITC-conjugated sheep anti-rat IgG antibody. Before flow cytometric data collection and analysis, labeled cells were fixed for 30 min with 200 μL of FACS fix solution (10.0 g/L paraformaldehyde; 10.2 g/L sodium cacodylate, and 6.65 g/L sodium chloride, pH 7.2).

2.6. Flow cytometry data storage and analysis

Flow cytometric measurements were performed on a FACScan instrument (Becton Dickinson). The Cell-Quest software package was used in both data acquisition and analysis. A total of 10,000 events were acquired for each preparation. Canine whole-blood leucocytes were identified on the basis of their specific forward (FSC) and side (SSC) light-scatter properties as described by Reis et al. (2005). The results were expressed as the percentage of positive cells within the selected lymphocyte gate for $CD5^+$, $CD4^+$, $CD8^+$, and $CD21^+$ cells. Semi-quantitative analyses were performed to evaluate differential expression of cell surface markers presenting a unimodal distribution ($MHC\text{-}II^+$, $CD45RA^+$, and $CD45RB^+$), and the results were expressed as Mean Fluorescence Channel (MFC).

2.7. Splenocytes proliferation assay

The functional activity of splenocytes was determined using the lymphocyte proliferation assay. The cell suspension was adjusted to 10^6 cells/mL of RPMI-1640 medium (Sigma) supplemented with 10% FCS, 100,000 U/L penicillin and 0.2 g/L streptomycin. Two hundred microliters of the suspension was transferred into each well of a 96-well flat-bottomed plate (Nunc, Naperville, IL, USA) containing 25 μl of LSA (25 μg/ml) obtained according Reis et al. (2006c), for the antigenic stimulus assays. The mitogenic lectin Concanavalin A (ConA, Sigma, USA) at 10 μg/mL, and RPMI 1640 medium were used as controls. All samples were run in triplicate. The microplates were incubated at 37 °C under a 5% CO_2 atmosphere for 3 days. Twenty hours before harvesting, 50 μL of medium with ^{3}H-thymidine (5 μCi/mL) was added. The incorporation of ^{3}H-thymidine was measured with a liquid scintillation counter (Tricarb CA 1600, Packard BioScience Company, Meriden, CT, USA). The stimulation index was calculated as the ratio of counts per minute (CPM) in stimulated samples *versus* CPM in non-stimulated controls.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. The normality of the data was assessed using the Kolmogorov–Smirnoff test. Parametric data were analysis by variance (ANOVA) followed by Tukey's test. Non-parametric data were analyzed by the Kruskal–Wallis test followed by Dunns' test. Student's *t*-test and the Mann–Whitney test were used to identify significant differences between the ratio of stimulated samples *versus* non-stimulated control cultures for parametric and non-parametric data, respectively. Correlation analysis was performed by Pearson's rank test in the case of parametric data and Spearman's rank test in the case of non-parametric data. In all cases, significance was defined at $p < 0.05$.

3. Results and discussion

The role of the anti-*Leishmania* immune response underlying the susceptibility/resistance during CVL has been recognized in many *ex vivo* and *in vitro* investigations (Cardoso et al., 2007; Lage et al., 2007; Pinelli et al., 1994, 1995; Reis et al., 2006a). Recently, we demonstrated that immunoglobulin levels, as well as the parasite load are relevant hallmarks of distinct clinical status of CVL (Coura-Vital et al., 2011a; Reis et al., 2009, 2006c). To better understand the events underlying the compartmentalized immune response in the spleen, we performed a detailed analysis of clinical status of CVL and *ex vivo* and *in vitro* phenotypic features of the major splenocyte subsets ($CD5^+$, $CD4^+$, $CD8^+$, $CD21^+$), and several activation markers ($MHC\text{-}II^+$, $CD45RA^+$, $CD45RB^+$). The spleen is one of the tissues where the parasite replicates, and is an important organ responsible for adaptive immune response during CVL infection. Thus, the assessment of cellular immunophenotypic profile may improve our understanding of the relationship between the major events of disease progression with uncontrolled parasite replication in the splenic compartment.

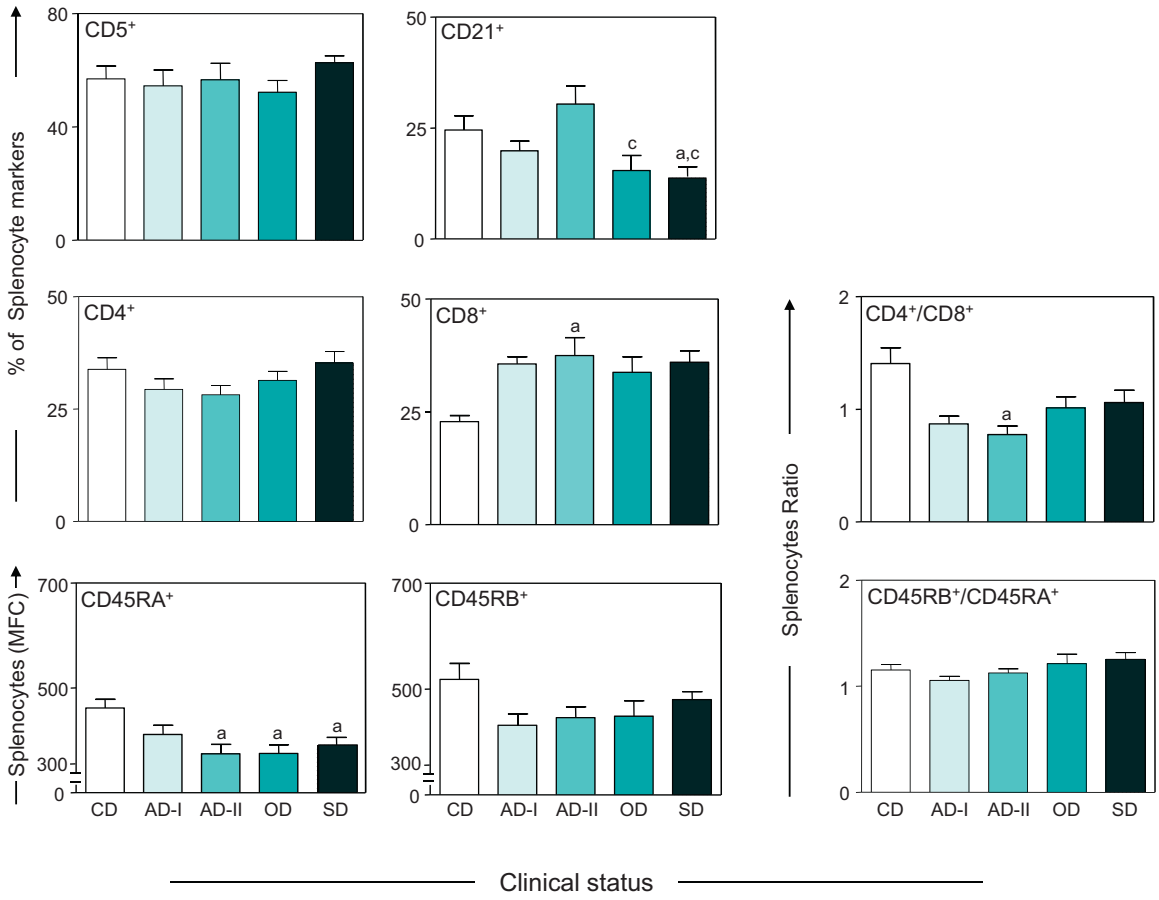

Fig. 1. Immunophenotypic profile of splenocytes in dogs naturally infected with *Leishmania infantum* categorized according to their clinical status as asymptomatic (AD), oligosymptomatic (OD), symptomatic (SD), and control (CD) groups. The results are expressed in bar graphs as mean percentage of $CD5^+$, $CD21^+$, $CD4^+$, $CD8^+$ within gated lymphocytes, and $CD4^+/CD8^+$ ratio plus standard deviation. The results of $CD45RA^+$ and $CD45RB^+$ are expressed as average of mean fluorescence channel (MFC), and $CD45RB^+/CD45RA^+$ ratio plus standard deviation. Significant differences at $p < 0.05$ are indicated by the letters *a* and *c* in comparison with the CD and AD-II groups, respectively.

Studies have reported a decrease in the percentage of B cells ($CD21^+$) in peripheral blood of dogs naturally infected with *L. infantum* (Bourdoiseau et al., 1997; Reis et al., 2006b). In the present study, *ex vivo* immunophenotypic analysis of splenocytes also showed a reduction in the percentage of B lymphocytes ($CD21^+$) in the OD group as compared with AD-II, as well as in the SD group as compared with both CD and AD-II (Fig. 1). The decrease of B cells in dogs with active CVL is intriguing and unexpected, mainly because these animals exhibit intense polyclonal B cells, as evidenced by increased production of immunoglobulin of various classes and subclasses (Martinez-Moreno et al., 1995; Reis et al., 2006c; Coura-Vital et al., 2011a). Results obtained by Guerra et al. (2009), however, demonstrated no difference in the percentage of this lymphocyte subpopulation in the spleen when the animals were categorized by parasite load. Thus, we suggest that the decreased percentage of B cells ($CD21^+$) in peripheral blood (Reis et al., 2006b), and spleen as well from symptomatic dogs (SD) could reflect an involvement in the production of these cells in the bone marrow and a possible differentiation in plasma cells.

We also observed that $CD8^+$ T cells reflect most accurately both the clinical status and the overall bone marrow parasite density, as increased levels of $CD8^+$ circulating lymphocytes appeared as the major phenotypic feature of asymptomatic disease and dogs bearing a low parasite load (Reis et al., 2006b). In the present study, we did not observe alterations in $CD5^+$ and $CD4^+$ splenocytes in *ex vivo* experiments, whereas the AD-II group presented a high percentage of $CD8^+$ T cells, and reduction in the $CD4^+/CD8^+$ ratio (Fig. 1). Coura-Vital et al. (2011a) observed similar results with higher $CD8^+$ T cells, and lower CD4/CD8 ratio in AD-II and SD groups in the peripheral blood compared with CD. In another study that evaluated the immunophenotyping of leukocytes from liver and spleen from naturally infected dogs showed that the splenic immune responses in both symptomatic and asymptomatic dogs were very similar (Sanchez et al., 2004). Guerra et al. (2009) showed in naturally infected dogs that, the increase frequency of $CD8^+$ T cells in peripheral blood is strongly associated with low splenic parasitism. In our current study, we observed that low parasite load in spleen is found mainly in asymptomatic dogs (AD-I and AD-II) confirming

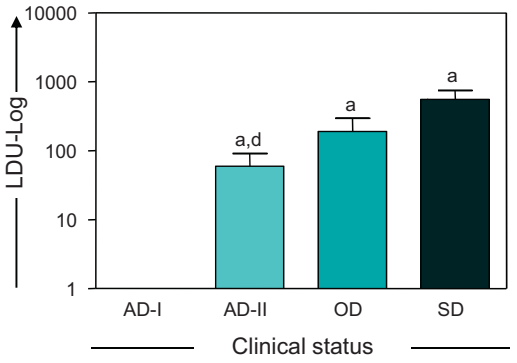

Fig. 2. Splenic parasite load in dogs naturally infected with *Leishmania infantum* categorized according to their clinical status as asymptomatic I (AD-I), asymptomatic II (AD-II), oligosymptomatic (OD) and symptomatic (SD) groups. The results are expressed in bar graphs as average of Leishman Donovan Units (LDU) in log scale plus standard deviation. Significant differences at $p < 0.05$ are indicated by the letters *a* and *d* in comparison with the AD-I and SD groups, respectively.

this hypothesis. In fact, different studies have correlated the level of $CD8^+$ T cells with protection during *L. infantum* infection (Pinelli, 1997; Reis et al., 2006b) or during the immunogenic response after vaccine administration against CVL (Giunchetti et al., 2007, 2008; Roatt et al., 2012). Thus, our data also suggest that $CD8^+$ T cells in several compartments may control the parasite replication.

Furthermore, we evaluated the *ex vivo* $CD45RA^+$ and $CD45RB^+$ expression on splenocytes in different clinical groups. The results showed a decreased expression of $CD45RA^+$ in the animals after seroconversion. However, no differences were observed for $CD45RB^+$ expression or the $CD45RB^+/CD45RA^+$ expression ratio among the groups (Fig. 1). Guerra et al. (2009) also observed decreased $CD45RA^+$ expression on splenocytes from animals infected with a moderate parasite load. These changes may be associated with the high splenic parasitism frequently noticed in dogs with high parasite load or exhibiting severe CVL clinical status.

Spleen parasite load was assessed using Giemsa-stained microscopy to determine the Leishman Donovan Units (LDU) (Fig. 2). Data analysis demonstrated a higher parasite load in SD, OD, and AD-II groups compared with AD-I dogs. Interestingly, we observed lower parasitism in AD-II than SD dogs in the splenic compartment (Fig. 2). Sanchez et al. (2004) suggested that the striking differences in parasite burden between AD and SD animals could be explained by a diminished infection or a more efficient control of the parasite replication in the former group, as we observed in our study.

To evaluate a *Leishmania*-specific cell-mediated immune response, splenocytes from dogs with CVL were stimulated by a mitogen (ConA) (Fig. 3A) or specific antigen (LSA) (Fig. 3B). Our results demonstrated that a lymphoproliferative response decreased in all seropositive dogs in comparison with the CD group, when simulated with ConA or in all infected dogs after simulation with LSA. Furthermore, a correlation analysis between non-specific and LSA-specific lymphoproliferative response and clinical status revealed that severe CVL disease is correlated with a low proliferation index during the infection ($r = -0.3627$, $p = 0.0113$; $r = -0.5263$, $p = 0.0001$, respectively; Fig. 3A and B). Similar results have been documented by other research groups (Cabral et al., 1998; Pinelli et al., 1994; Rodriguez-Cortes et al., 2007).

Moreover, it was conducted an analysis of the phenotypic features in the spleen compartment in the presence or absence of LSA stimulation (Fig. 4). We observed an increased percentage of $CD8^+$ splenocytes in OD and SD as compared with CD in non-stimulated cultures. Moreover, the stimulated cultures with LSA antigens showed increased percentage of $CD8^+$ T cells in AD-II as compared with the CD group or non-stimulated culture in the same group (Fig. 4A). Similarly, Pinelli et al. (1995) also observed that T lymphocytes from symptomatic dogs failed to proliferate after *Leishmania* antigen stimulation *in vitro*. On the other hand, these authors reported that asymptomatic dogs showed both $CD8^+$ T and $CD8^+$ T lymphocyte proliferation, and were capable of lysing infected macrophages.

We also investigated whether dogs with distinct clinical forms of CVL present alterations in the expression of activation markers ($CD45RA^+$, $CD45RB^+$, and $MHC\text{-}II^+$) on splenocytes (Fig. 4B). The development of a T-cell mediated specific immune response requires *Leishmania* antigen presentation to Th cells together with major

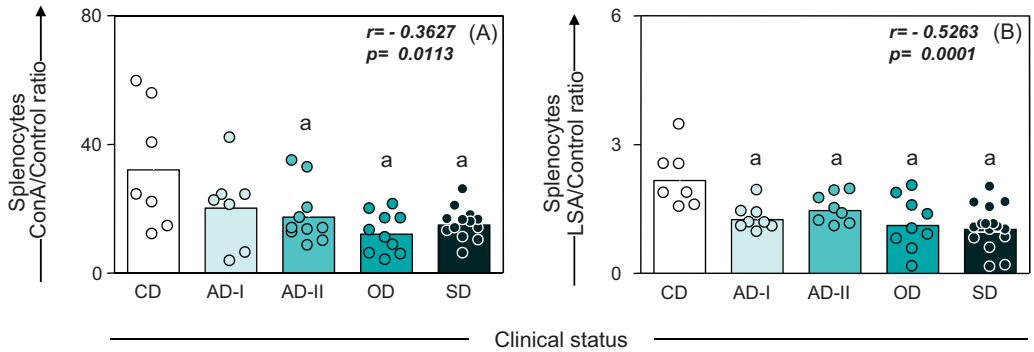

Fig. 3. Proliferative index of splenocytes in dogs naturally infected with *Leishmania infantum* categorized according to their clinical status as asymptomatic (AD), oligosymptomatic (OD), symptomatic (SD), and control (CD), after *in vitro* Concanavalin A (ConA) or *L. infantum* soluble antigen (LSA) stimulation. The results are expressed in bar graphs as scattering of individual values and mean ConA/Control ratio (A) or LSA/Control ratio (B). Correlations between proliferation indices and clinical status are also inserted, and Pearson's correlation coefficient (r) and p-values are shown within graphs.

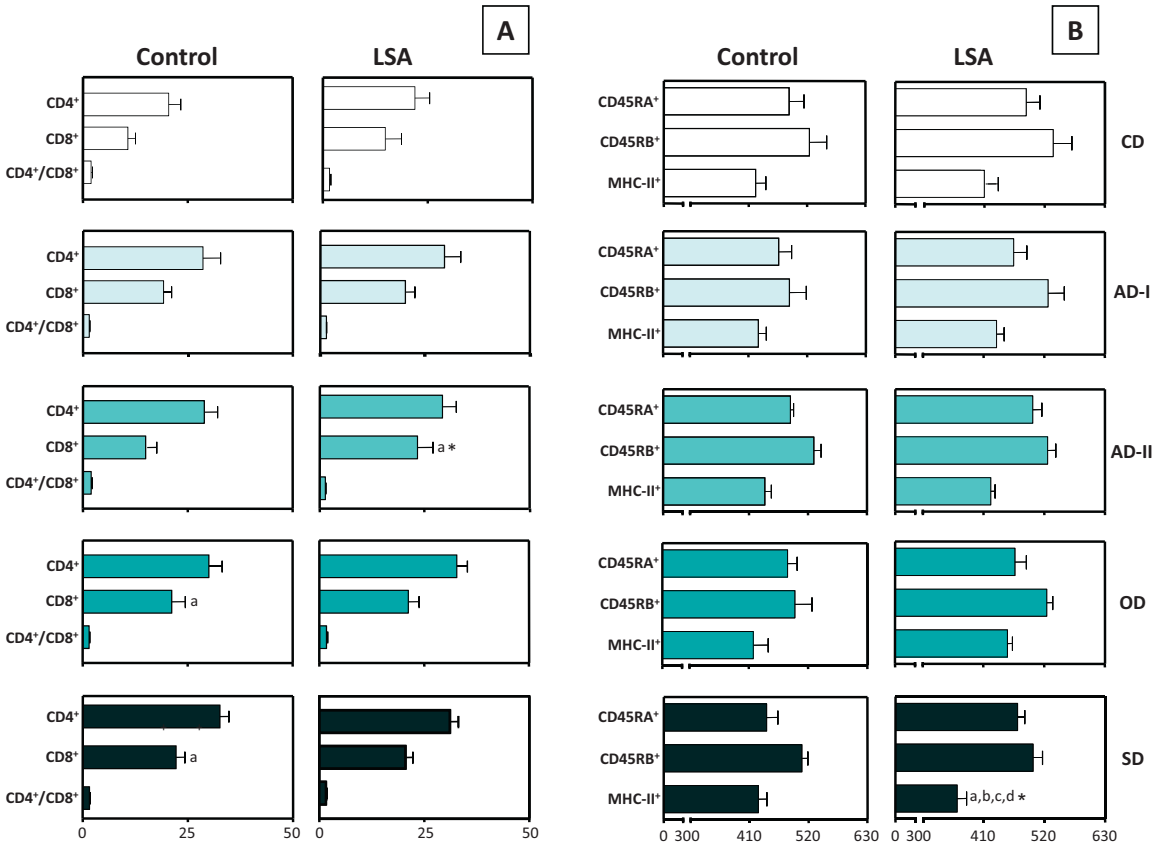

Fig. 4. Immunophenotypic profile and activation status of splenocytes in dogs naturally infected with *Leishmania infantum* categorized according to their clinical status as asymptomatic (AD), oligosymptomatic (OD), symptomatic (SD), and control (CD), after *in vitro* culture in the presence of medium (Control) or *L. infantum* soluble antigen (LSA) for 120 h. The results are expressed in bar graphs as mean percentage of $CD4^+$ and $CD8^+$ within gated lymphocytes and $CD4^+/CD8^+$ ratio plus standard deviation (A), and average of major histocompatibility complex ($MHC\text{-}II^+$), $CD45RA^+$, and $CD45RB^+$ as mean fluorescence channel (MFC) within gated splenocytes plus standard deviation (B). Significant differences at $p < 0.05$ are indicated by the letters *a*, *b*, *c*, and *d* in comparison with the CD, AD-I, AD-II, and OD groups, respectively. Significant differences at $p < 0.05$ between control and *L. infantum* soluble antigen (LSA) cultures are indicated by asterisk.

histocompatibility complex (MHC) molecules. However, *Leishmania* can impair antigen presentation in several ways. Reis et al. (2006b) demonstrated enhanced MHC-II density as well as a higher CD45RB/CD45RA expression index in peripheral blood from asymptomatic dogs, which seems to represent a key element to control CVL morbidity. In the present study, we observed decreased MHC-II expression in splenocytes after LSA stimulation in SD group as compared with all groups or non-stimulated culture in the same group (Fig. 4B). Cobbold and Metcalfe (1994) performed pioneering investigations of MHC-II molecules in dogs. They observed that, in contrast with other species, the MHC-II of dogs is expressed constitutively in all circulating lymphocytes. Increased expression of MHC-II may reflect an antigenic priming-related immunological event. Based on this proposal, our findings suggest that symptomatic dogs displayed lower MHC-II expression, probably promoting more vulnerability to disease.

In conclusion, our findings highlighted the complexity of cellular immunological events related to the natural infection of dogs by *L. infantum*. Our results showed that the role of $CD8^+$ T splenocytes seems to be key for an effective immunological response, a hallmark of asymptomatic CVL, whereas the pronounced loss of MHC-II expression on splenocytes upon specific- antigen stimulation is a biomarker of symptomatic CVL.

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