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PD-1 and PD-L1 regulate cellular immunity in canine visceral leishmaniasis

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ABSTRACT

PD-1 is a negative costimulator of chronic infectious diseases In this study, we investigated the expression of PD-1 and its ligands in the spleen of dogs with visceral leishmaniasis and lymphoproliferative response to soluble antigen, in lymph node cells in the presence or absence of antibodies blocking PD-1 and its ligands. Our results showed expression of PD-1 and its ligands is higher after *L. infantum* infection and in the spleen of infected dogs, PD-1 blockage was able to restore the antigen-dependent lymphoproliferative response and regulated production of the cytokines IL-4 and IL-10 and NO production. We concluded that *L. infantum* infection modulates PD-1 and its ligands expression in canine VL and that blockage of PD-1 restores the immune response. Thus, blockage of PD-1 is a target for therapeutic drug development.

1. Introduction

Leishmaniasis occurs in 88 countries, 350 million people at risk, 500,000 new cases of VL per year [1]. Brazil is one of the six countries around the world with high number of cases of visceral leishmaniasis (VL) [2], which is a fatal disease involving uncontrolled parasitism in vital organs [3]. Humans and dogs are the two main host reservoirs for the L. *donovani* complex [4], but dogs have higher numbers of parasites in their skin than humans, which favors infection of the vectors [5,6]

Suppression of cell immunity is the most important aspect of the pathogenesis and progression of canine disease. Absence of T cell response to *Leishmania* (L.) spp. antigens is observed *in vivo*, with a negative Montenegro test [7]. In dogs infected with L. *infantum*, the number of T lymphocytes becomes reduced [8], possibly related to apoptosis of T lymphocytes in dogs with VL [9]. Reduction in the antigen-specific lymphoproliferative response has also been observed in dogs with VL [10,11]. Restoration of proliferation has been observed in *in vitro* infection with L. *infantum* in canine macrophages and was attributed to B7 expression [12]. For pathogens to be eliminated by T cells, two signals are required, the first signal is antigen-specific and is generated by T cell receptors (TCRs) through recognition of the peptide presented by the major histocompatibility complex (MHC) in antigen-presenting cells (APCs), while the second signal is non antigen-dependent and is supplied by binding members of the B7 family [13]. B7 family pathways regulate the balance between the stimulatory and inhibitory signals that are necessary for defense against invading microorganisms [14,15]. Over recent years, many inhibitory pathways within the B7-CD28 family that diminishes T cells response [16] have been identified, including programmed death protein [17].

PD-1, also known as CD279, is expressed in several cell types, such as T cells, natural killer (NK) cells, B cells, monocytes, dendritic cells [17], macrophages [18] and regulatory T cells [17]. It binds to PD ligands (PD-L1 and PD-L2), thereby eliciting their biological functions [17] and decreasing the stimulatory signals through TCRs [19]. It also induces apoptosis of CD3 + T cells in the spleen and peripheral blood of dogs with VL [20]. Anergy has been observed in CD8 + T cells from patients with VL, along with elevated expression of PD-1 mRNA and IL-10 [21]. PD-L1, also known as B7-H1 or CD274, is expressed in dendritic cells, macrophages, T cells and B cells [16]. Its expression is associated with Th1 response and can be induced by lipopolysaccharide

Abbreviations: AP, alkaline phosphatase; ALT, alanine aminotransferase; APC, antigen-presenting cell; AST, aspartate aminotransferase; BSA, bovine serum albumin; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; CK, creatine kinase; ConA, concanavalin-A; VL, visceral leishmaniasis; DAB, diaminobenzidine; FITC, fluorescein isothiocyanate; Fol, foliculus; GTG, glutamyl transferase gamma; L, *Leishmania*; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NK, natural killer; NO, nitric oxide; OD, optical density; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PD, programmed death; PE, phycoerythrin; PHA, phytohemagglutinin; SAg, soluble antigen; TCR, T cell receptor; Th, T helper; VL, visceral leishmaniasis

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(LPS) and IFN-γ [22]. After PD-L1 blocking of B cells in dogs with VL, the Th1 response was found to recover [23], with proliferation of CD4+ and CD8 + T cells in peripheral blood [11]. PD-L2, also known as B7-DC or CD273 [16], is expressed in dendritic cells, macrophages and bone marrow cells [19]. PD-L2 expression increases during the Th2 response [24]. Binding of PD-L2 to PD-1 inhibits proliferation *via* TCRs and reduces production of cytokines by CD4 + T cells [25].

In dogs with VL, depletion of CD4+ and CD8 + T cells in peripheral blood mononuclear cells (PBMCs) has been correlated with increased PD-1 expression on the surface of these cells. After PD-1/PD-L1 in adherent PBMCs was blocked, proliferation was restored in the presence of the antigen [11]. Increased expression of PD-1 and its ligands has also been observed in T lymphocytes, B lymphocytes and macrophages of the peripheral blood and spleen in dogs with VL. This increased expression regulates other functions such as apoptosis of CD3+ cells and TNF-α and nitric oxide (NO) production [20].

In this study, we investigated expression of PD-1 and its ligands in the spleen and in PBMCs, from dogs that were naturally infected with L. *infantum*. We assessed the lymphoproliferative response in lymph nodes and the production of NO and the cytokines IFN-γ, TNF-α, IL-10 and IL-4, after blocking of these receptors *in vitro* in the presence or absence of soluble antigen (SAg) of L. *infantum*.

2. Material and methods

2.1. Ethics Committee approval

This study was approved by the Brazilian College of Animal Experimentation (COBEA) and by the Ethics Committee for Animal Use (CEUA), of the School of Veterinary Medicine of Araçatuba (FMVA), Universidade Estadual Paulista "Julio de Mesquita Filho" (UNESP), under procedural no. 00530/2013.

2.2. Selection of infected dogs and control group

We selected a total of 54 adult dogs aged two to five, of various breeds and weights. Among these, 31 dogs with VL (infected group: 17 females and 14 males), that were obtained from the Araçatuba Zoonosis Control Center (AZCC). These dogs were symptomatic and showed clinical signs of canine VL and they showed cytologically positive in popliteal lymph nodes and seropositive for L. *infantum* by means of indirect ELISA [26].

The control group was formed by 23 clinically healthy dogs (14 females and 9 males), aged 3 to 4 years of various breeds and weights. These dogs had individual owners who signed an informed consent statement to permit collection of samples of PBMCs or spleen fragments at the time when their dogs males and females were castrated. The dogs selected had normal hematological and biochemical plasma parameters (alanine aminotransferase, aspartate aminotransferase, bilirubin, calcium, total cholesterol, creatine kinase, creatinine, alkaline phosphatase, phosphorus, gamma glutamyl transferase, glucose, globulin, lactate dehydrogenase, total protein, triglycerides and urea), and urinalysis. Serum samples from all the dogs were subjected to an indirect ELISA test [26] for total antigens of *Leishmania* spp. were found to be seronegative and were clinically monitored with serological tests for three months before the experiment. They were also found to be negative for DNA of the parasite *Leishmania* spp. in spleen and bone marrow samples, in an analysis by means of qPCR [27].

2.3. Sample collection

The dogs with VL were sacrificed after anesthetization with barbiturate (thiopental sodium, Cristália®, Itapira, SP, Brazil), followed by intravenous injection of 19.1% potassium chloride, in compliance with Decree no. 51,838 of the Brazilian Ministry of Health, of March 14, 1963, which states that domestic animals presenting VL should be euthanized. The method used followed the recommendations of Resolution no. 714 of the Federal Council of Veterinary Medicine, of June 20, 2002. Afterwards, one spleen fragment 2 cm^3 was collected. Half of this sample was fixed in 10% buffered formaldehyde solution for inclusion in paraffin in order to obtain histological sections for immunohistochemical evaluation. The other half was kept at 4 °C in the cell culture medium RPMI-1640, supplemented with 10% heat inactivated bovine fetal serum (Gibco®, USA), 0.03% L-glutamine (Sigma-Aldrich®, USA), 100 IU/mL of penicillin (Sigma-Aldrich®, USA) and 100 mg/mL of streptomycin (Sigma-Aldrich®, USA), until processing.

From the control group, a spleen fragment 2 cm^3 was collected by means of surgical excision, in accordance with the protocol of Lima et al. (2012). The sample was divided into two parts for immunohistochemistry and for obtaining leukocytes, as described above.

Peripheral blood samples were obtained from the infected dogs and control group obtained by femoral or jugular veins puncture. The samples were collected in tubes (10 mL) containing sodium heparin (BD Vacutainer®, USA) and were processed immediately after collection.

Bone marrow obtained from the right iliac crest using a Jamshidineedle was placed with citrate-phosphate-dextrose-adenine-1(CPDA-1) anticoagulant (JP Ind. Pharmaceutical, Ribeirão Preto, SP,Brazil) (four parts of bone marrow to one part of anticoagulant).Isolation of bone marrow mononuclear cells followed the same protocol as for PBMC.

The lymph node, after each dog had been sacrificed, its right popliteal lymph node was removed and macerated to obtain lymph node cells (50×10^6/mL in RPMI 1640 medium without calf bovine serum).

2.4. Isolation of PBMCs, spleen leukocytes and lymph node cells

PBMCs from the infected and control groups were isolated by means of gradient concentration using Histopaque 1077 (Sigma-Aldrich®, USA), following the manufacturer's instructions. The mononuclear cells thus isolated were processed in 15 mL tubes (BD Biosciences Falcon®, USA) with 10 mL of erythrocyte lysis buffer containing 7.46 g/L of NH_4ClO_3 at 4 °C for 10 min. The cells were then washed three times in phosphate-buffered saline (PBS) at pH 7.2 and resuspended in supplemented RPMI-1640 (Sigma-Aldrich®, USA) as described previously. They were then kept in a humidified incubator (water jacket; Thermo Scientific®, USA) at 37 °C under 5% CO_2.

The total leukocyte count from the spleen and lymph node was obtained after maceration of the sample, removal of cell debris (Cell strainer, BD Falcon®, USA) and processing to lyse the erythrocytes, as described above. The same procedure was performed to obtain lymph node cells in the lymphoproliferation assays.

2.5. Antibodies

Flow cytometry was performed with dual cell stained. Monoclonal antibodies conjugated with phycoerythrin (PE), were used: anti-human PD-1, which shows a high degree of homology between humans and dogs [28]; anti-human PD-L1, used in accordance with Esch et al. (2013), with modifications [19]; and anti-human PD-L2, presenting 75–85% homology with *Canis lupus familiaris* (BLAST/ Request Identification P7WRT59M014) [19]. The isotype control for the PD-1, PD-L1 and PD-L2, consisted of mouse IgG1. The monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) were anti-canine CD3 and its mouse IgG1 isotype control. Anti-human CD21 and its mouse IgG1 isotype control showed reactivity with dogs. Anti-rat F4/80, a marker for membrane-differentiated macrophage monocyte antigens that are highly conserved in humans and dogs showing 70–97% homology with *Canis lupus familiaris* (BLAST / NE57FKGV014) and its mouse IgG2aκ isotype control.

In the blocking assays on receptors, purified monoclonal antibodies were used: anti-human PD-1, anti-human PD-L1, anti-human PD-L2 and isotype control mouse IgG1 k.

Table 1
Antibodies selected for immunohistochemistry and flow cytometry.

Antibody / Clonality	Host / Isotype	Dilution / Clone	Assay	Company
PD-1 / Monoclonal	Mouse anti-Human / Mouse $IgG_1\kappa$	15 µL/10^5 / MIH4 5 µL/10^5	Cytometry / PE	BD Biosciences, USA
PD-L1 / Monoclonal	Mouse anti-Human / Mouse BALB/c $IgG_1\kappa$	15 µL/10^5 / MIH1 5 µL/10^5	Cytometry / PE	BD Biosciences, USA
PD-L2 / Monoclonal	Mouse anti-Human / Mouse $IgG_1\kappa$	15 µL/10^5 / MIH18 5 µL/10^5	Cytometry / PE	BD Biosciences, USA
CD3 / Monoclonal	Mouse anti-dog / Mouse IgG1	5 µL/10^5 / MOPC-21 5 µL/10^5	Cytometry / FITC	AbD Serotec®, UK
CD21 / Monoclonal	Mouse anti-human / Mouse IgG1	15 µL/10^5 / LT21 5 µL/10^5 / MOPC-21	Cytometry / FITC	AbD Serotec®, UK
F4/80 / Monoclonal	Mouse anti-Rat / mouse IgG2aκ	5 µL/10^5 / 5 µL/10^5 / BM8	Cytometry / FITC	Abcam®, UK
PD-1 / Monoclonal	Mouse anti-Human / Mouse $IgG_1\kappa$	5 µL/mL / J116 5 µL/mL / P36281	Blocking of receptor	eBioscience®, USA
PD-L1 / Monoclonal		5 µL/mL / MIH5 5 µL/mL / P36281	Blocking of receptor	eBioscience®, USA
PD-L2 / Monoclonal	Mouse anti-Human / Mouse $IgG_1\kappa$	5 µL/mL / MIH18 5 µL/mL / P36281	Blocking of receptor	eBioscience®, USA
PD-1 / Polyclonal	Goat anti-dog / Mouse IgG2bκ	1:200 / 1:200	IHQ*	Biorbyt, UK
PD-L1 / Monoclonal	Mouse anti-Human / Mouse IgG2bκ	1:200 / 29E.2A3 1:200 / MG2b-57	IHQ*	Biolegend®, USA
PD-L2 / Monoclonal	Mouse anti-Human / Mouse IgG2bκ	1:200 / MIH 18 1:200 / MG1-45	IHQ*	Biolegend®, USA

* IHQ - Immunohistochemistry.

The following purified anti-dog anti-PD-1 polyclonal antibodies produced in goat were used in the immunohistochemical evaluations: anti-human PD-L1 monoclonal antibody with its mouse IgG2bκ isotype control; anti-human PD-L2 with its mouse IgG1κ isotype control (Table 1).

2.6. Identification and quantification of expression of PD-1 and its ligands through immunohistochemical evaluation on spleen samples from the control and infected groups

The histological sections were deparaffinized in xylene (10 min each) and hydrated in alcohol 100°, 95°, 80°, 75° and distilled water (5 min each). The antigen retrieval was performed using citrate buffer (Labsynth, SP, Brazil; 2.4 g/mL at pH 6.0) in a steamer for 45 min at 90 °C, followed by washing in PBS three times (5 min each). Endogenous peroxidase activity was blocked using methanol (Labsynth, SP, Brazil), with 3% H_2O_2 (Merck Millipore©, Germany) for 20 min at room temperature (RT), followed by washing in PBS three times (5 min each). To block nonspecific sites, 3% bovine serum albumin (BSA) (Sigma-Aldrich®, USA) in PBS was used for 1 h at RT. The primary antibody was then added to PD-1 and its ligands for 24 h at 4 °C, followed by washing in PBS three times (5 min each). The secondary antibody (LSAB2 system, HRP, Dako®, K0690) was applied (100 µL/slide) for 45 min at RT, followed by washing in PBS three times (5 min each). The chromogen diaminobenzidine (DAB) (Dako®, K3468; 20 µL/mL) was applied with nickel chloride (Sigma-Aldrich®, USA; 0.3 mg/mL) for 1 min. Counterstaining was performed using Harris hematoxylin (1:2) for 3 min and the material was mounted in Canada balsam. The immunostaining was evaluated under an Olympus BX61 microscope (Japan) coupled to an Olympus DP71 camera, using Image Pro MC (Media Cybernetics Inc.), version 6.1.0.34C. Two sections were used to quantify the immunostaining, with four fields counted in each section. The immunolabelled cells on the plasma membrane were counted.

2.7. Flow cytometry

The expression of PD-1 and its ligands on CD3, CD21 and F4/80 cells was quantified as described by Chiku et al. (2016). Ten thousand events were analyzed, and cell debris excluded. The data were acquired using a flow cytometer (BD Accuri® C5), with analysis using the BD Accuri C6 software, version 1.0.264.21 (BD Biosciences, CA, USA).

2.8. Evaluation of expression of PD-1 and its ligands in control group after in vitro infection of macrophages with L. infantum for 72 hours

PBMCs from the control group (n = 13) were diluted in 1 mL of supplemented RPMI 1640 (Sigma-Aldrich®, USA). Cell counting and viability assessment (Countess, Invitrogen®, USA), a concentration of 2×10^6 cells per 200 µL of supplemented RPMI 1640 (Sigma-Aldrich®, USA), at pH 7.2, was obtained. Culturing was performed in sterile 24-well plates (Corning Costar, Sigma-Aldrich®, USA), with sterile LPS-free circular glass coverslips of 15 mm in diameter (Perfecta coverslips, Sao Paulo, Brazil) so that the volume would not overflow. After 4 h at 37 °C under 5% CO_2, with the aim of achieving complete cell adhesion, the wells were washed with supplemented RPMI 1640 at 37 °C to remove any non-adherent cells. The culture medium was replaced with 1 mL of supplemented RPMI 1640 at 37 °C and the cells were cultured in a humidified incubator (water jacket; Thermo Scientific®, USA) at 37 °C under 5% CO_2 for 7 days, in order to allow differentiation into macrophages.

After 7 days of culturing, the macrophages were infected with promastigotes of *L. infantum* (MHOM/BR/00/MERO2) in RPMI 1640 without antibiotic (Sigma-Aldrich®, USA), at a ratio of 10 parasites for each macrophage. After 4 h of infection at 37 °C under 5% CO_2, the wells were washed with PBS at pH 7.2 and at 37 °C, in order to remove any promastigotes that had not become phagocytized. The culture medium was replaced with a total volume of 1 mL at 37 °C. After 72 h of incubation, the coverslips were scraped and the cells were transferred to sterile tubes and incubated with phycoerythrin-conjugated monoclonal antibodies to PD-1 and its ligands, along with the respective isotype controls. Following this, the cells were recovered from the plate, placed in wells, centrifuged at 2000 rpm for 3 min to remove the supernatant and stored at 4 °C protected from light until the time of making readings through flow cytometry (BD Accuri® C5).

2.9. Evaluation of PD-1 and its ligands on T lymphocytes, B lymphocytes and macrophages in PBMCs from the control group after in vitro infection with L. infantum for 6 days

To ascertain whether there was a need for cell contact between macrophages and other cell types for regulation of the expression of PD-1 and its ligands, total PBMCs ($2 \times .10^6$) from healthy dogs were isolated, resuspended in 1 mL of supplemented RPMI 1640 (Sigma-Aldrich®, USA) and inserted into sterile 24-well culture plates (Costar, Sigma-Aldrich®, USA). The cell cultures were infected with promastigotes of *L. infantum* (MHOM/BR/00/MERO2) in the proportions of 10 parasites for each cell and were incubated in a humidified incubator (water jacket; Thermo Scientific®, USA) at 37 °C under 5% CO_2 for 6 days. After this period, the cells were recovered, put into 1.5 mL microtubes and centrifuged at 2000 rpm for 3 min to remove the supernatant. The immunostaining was performed using monoclonal antibodies conjugated with PE for PD-1, its ligands and the isotype controls. The material was then maintained at 4 °C, protected from light, until the time of data acquisition by means of flow cytometry (BD Accuri® C5).

2.10. Blocking of PD-1 and its ligands in cultures of PBMCs and spleen leukocytes in the infected group

To evaluate the expression of PD-1 and its ligands and the altered production of IFN-γ and IL-10 after blocking, PBMCs and spleen leukocytes from the dogs with VL were isolated and resuspended at a concentration of 5×10^6 cells in 1 mL of supplemented RPMI 1640 (Sigma-Aldrich®, USA), as previously described. These suspensions were then placed in 24-well sterile culture plates (Costar, Sigma-Aldrich®, USA) in the presence or absence of blocking antibodies for PD-1 and its ligands. The cell cultures were placed in a humidified incubator (water jacket; Thermo Scientific®, USA) at 37 °C under 5% CO_2 for 72 h. Following this, the material was placed in microtubes and centrifuged at 2500 rpm for 5 min. The supernatant thus obtained was removed and stored in a freezer at −80 °C for quantification of the cytokines IFN-γ and IL-10.

2.11. Lymphoproliferation assays

For the lymphoproliferation test, the popliteal lymph nodes in the infected group were removed from the dogs after euthanasia. These were kept in supplemented RPMI 1640 (Sigma-Aldrich®, USA), at pH 7.2, until the processing as described above. A quantity of 10×10^6 lymph node cells was stained with 2.5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-(CellTrace®, Invitrogen, USA), in accordance with the manufacturer's instructions. Cell concentrations of 2×10^6/well were cultured in duplicates in a final volume of 300 ul of supplemented RPMI 1640 (Sigma-Aldrich®, USA) in sterile 96-well culture plates (Sigma-Aldrich®, USA). The negative control consisted of absence of stimulation. The positive controls consisted of presence of mitogens: 5 μg/mL of concanavalin-A (ConA) (Sigma-Aldrich®, USA) and 5 μL/mL of phytohemagglutinin-M (PHA) (Gibco®, NY); presence of 20 μg/mL of SAg of *L. infantum* (MHOM/BR/00/MERO2), produced as described by Scott et al. (1987); and wells containing blocking antibodies for PD-1 and its ligands in the presence or absence of SAg. The cells were maintained in a humidified incubator (water jacket; Thermo Scientific®, USA) at 37 °C under 5% CO_2 for 6 days. Following this, the cells were recovered from the plate, placed in wells, centrifuged at 2000 rpm for 3 min to remove the supernatant and stored at 4 °C with protection from light until the time of making readings through flow cytometry.

The analysis was performed using the BD Accuri C6 software, v. 1.0.264.21 (BD Accuri®, USA). The cell population was selected so as to have size and complexity compatible with the lymphocyte population.

2.12. Culture supernatant for assaying the cytokines TNF-α, IFN-γ, IL-4 and IL-10, and NO

The lymphoproliferation assay was performed in duplicates without labeling the cells with CFSE, in a 96-well plate (Sigma-Aldrich®, USA), under the same conditions and at the same concentrations as used for the blocking antibodies, in order to obtain the cell culture supernatant. After 6 days of incubation in a humidified incubator (water jacket; Thermo Scientific®, USA) at 37 °C under 5% CO_2, the cells were recovered from the plate and centrifuged at 2000 rpm for 3 min. The supernatant was divided into aliquots of 100 μL and was stored at −80 °C for subsequent measurement of the cytokines TNF-α, IFN-γ, IL-4 and IL-10, and NO.

2.13. Assay for detecting the cytokines TNF-α, IFN-γ, IL-4 and IL-10

The assay for detecting the cytokines TNF-α, IFN-γ, IL-4 and IL-10 was performed using a capture ELISA kit (DuoSet canine R&D Systems, Minneapolis, USA), in accordance with the manufacturer's instructions. The detection limits of the IFN-γ curve were from 7.81 to 2000 pg/mL; TNF-α curve, 1.95 to 1000 pg/mL; IL-10 curve, 7.81 to 2000 pg/mL; and IL-4, 5.85 to 6000 pg/mL. The plates were read using a spectrophotometer (Spectra Count, Packard Bioscience Company, USA) with a 450 nm filter.

2.14. Nitrite assay

The NO assay was performed using the Griess method [29] on the supernatants of the PBMC cultures of leukocytes and spleen tissue. The supernatants were collected and centrifuged for 10 min at 2000 rpm, in order to remove suspended cells. For quantitative analysis, a standard curve was produced at the time of the experiment (1.5–200 μmoles of sodium nitrite (Labsynth, SP, Brazil).

2.15. DNA extraction and real-time qPCR

DNA extraction and quantification of the parasite burden of the spleen in the infected and control groups were performed as described by Perosso et al. (2014). The cycle temperatures used for the real-time PCR (CFX96®, C1000 Thermal Cycler, Bio Rad) were in accordance with the manufacturer's instructions.

For each reaction, a standard curve was produced with DNA from promastigotes of *L. infantum* (MHOM/BR00/MER02) at dilutions of 10^8 to 1 parasites.

2.16. Statistical analysis

The immunohistochemical assays were analyzed using the Mann-Whitney test. The *in vitro* infection with *L. infantum* and measurements of the IFN-γ and IL-10 concentrations in culture supernatants from PBMCs and leukocytes from spleen tissue with blocking of PD-1 and its ligands were analyzed using the Wilcoxon test. The lymphoproliferation assays with detection of the concentrations of NO and the cytokines IFN-γ, TNF-α, IL-10 and IL-4 in the supernatant of lymph node culture were analyzed using the Wilcoxon test. Correlations between the parasite load of the spleen in the infected group and the expression of PD-1 and its ligands were made using the nonparametric Spearman test. The statistical analysis was performed using the GraphPad Prism 6.0 software and values were considered significant when $p < 0.05$. All data were expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Selection of dogs for study: control and infected groups

The most common clinical signs in the dogs of the infected group

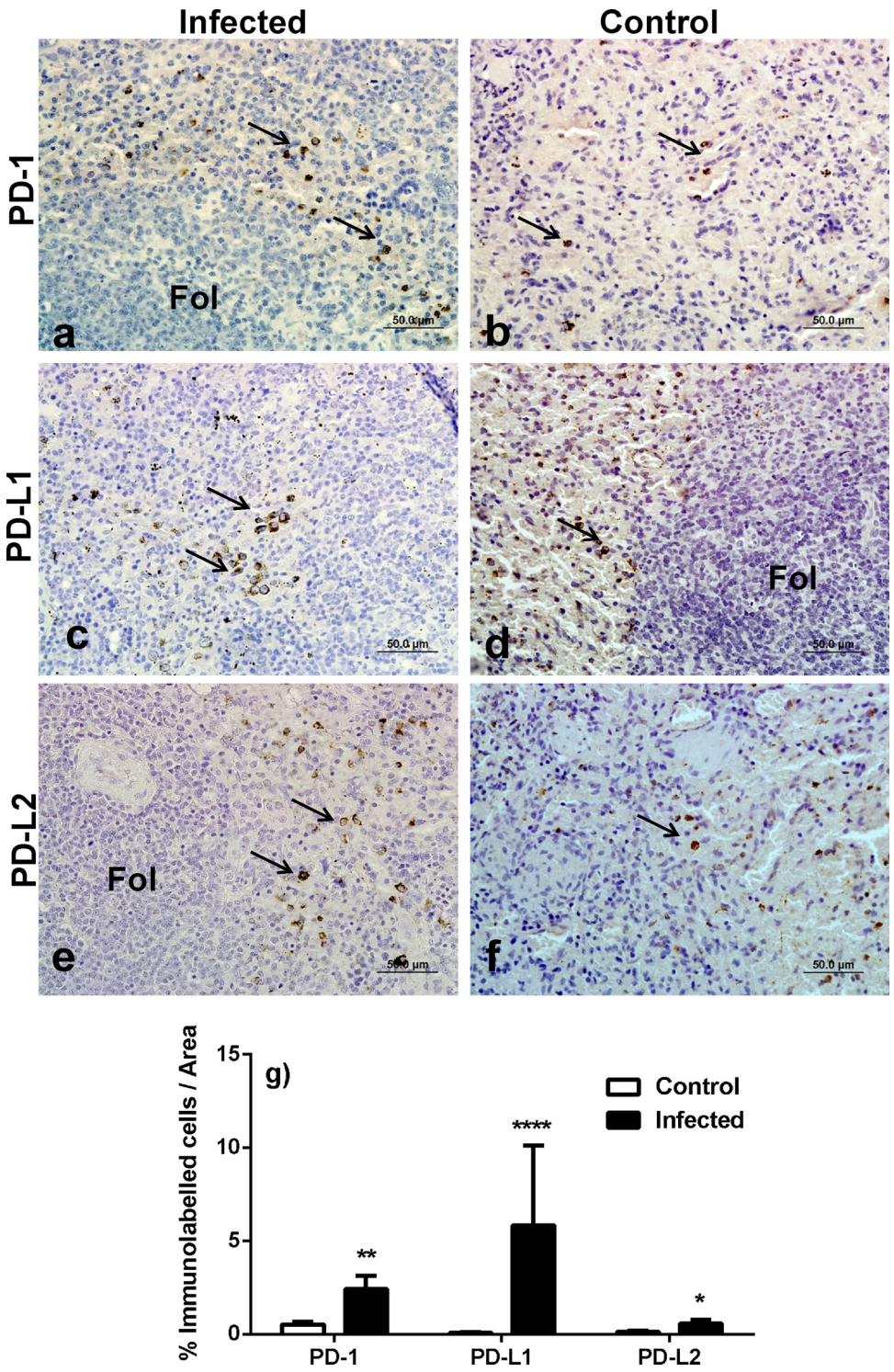

Fig. 1. Immunohistochemical of PD-1 and its ligands and frequency immmunolabelled cells in spleen tissue of dogs. Representative images showing immunohistochemical staining of PD-1 and its ligands in spleen tissue of dogs in the infected group (n = 20) and control group (n = 10). Two sections were made from spleen tissue and four fields were counted in each section. The immunolabelled cells on their plasma membrane were also counted. Fig. 2. a – f: positive cells are brown stained (arrows) and are located in marginal zone and red pulp (bar = 50 µm); Fig. 2. g: Percentage of cells in immunohistochemical assay with labelled monoclonal antibodies for PD-1 and its ligands. The results are presented as mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.001. Fol = spleen lymphoid follicles.

were lymphadenopathy, skin lesions and onychogryphosis. All the infected dogs were considered positive cytology and also positive serology for indirect ELISA for the total antigens of *Leishmania* spp. The optical densities (ODs) found in the ELISA serological tests in the dogs of the control group (n = 23) and the infected group (n = 31). The ODs of the antibody concentrations in the samples from the control and infected groups were obtained using a spectrophotometer in an ELISA reader, and these showed that the control group was below the cutoff point (0.270) and that the infected group was above the cutoff point, as had been determined in a previous study [26].

3.2. Increased expression of PD-1 and its ligands by means of immunohistochemistry on spleen samples from the dogs in the infected group, compared with the control group

Positive staining for PD-1 and its ligands was found at several intensities on plasma membranes, both over the complete circumference and over part of it. Cytoplasm staining that could be attributed to cytoplasmic PD-1 and its ligand protein was also observed (Fig. 1 a–f).

The analysis on the images showed that there were higher percentages of PD-1 +, PD-L1 + ($p < 0.05$) and PD-L2 + ($p < 0.001$) cells in the spleen sections of the infected group than in the control group

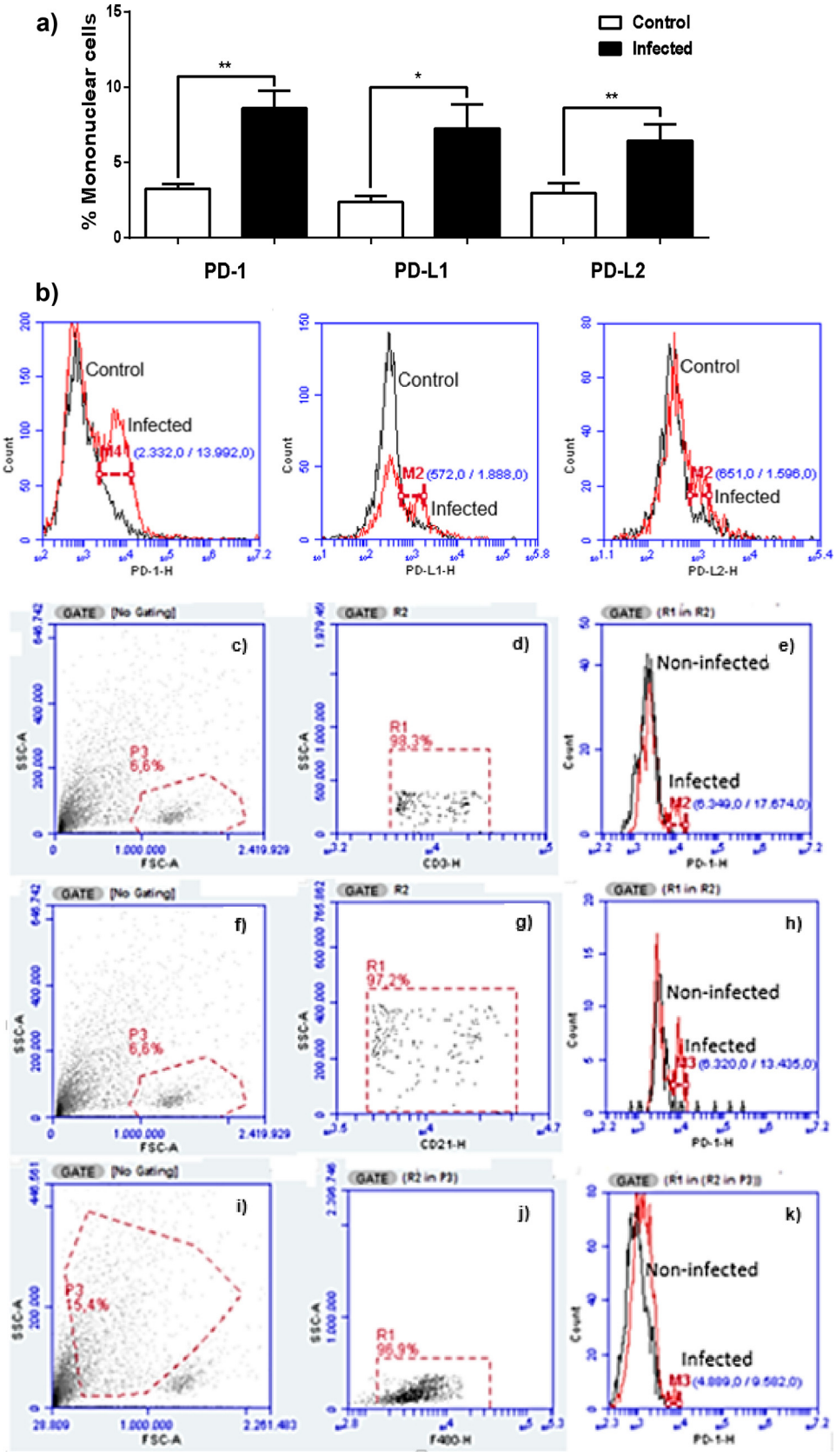

Fig. 2. Expression and representative flow cytometry of PD-1 and its ligands in PBMC, CD3+, CD21+ and F4/80+ cells. Expression of PD-1 and its ligands according to flow cytometry. (a) Cultured total PBMCs (n = 8), *in vitro* with and without infection with *L. infantum* for 6 days. Statistical analyses were performed using the *t*-test and Wilcoxon test, and graphical presentations show the mean ± SEM. *p < 0.05, **p < 0.01. (b) Representative flow plot demonstrating how the evaluation of PD-1 and its ligands was made. Representative images demonstrating how the flow cytometry plots were made for evaluating PD-1 and its ligands in CD3+, CD21+ and F4/80+ cells, in PBMCs from healthy dogs infected *in vitro* by *L. infantum*, compared with non-infected cells. The gate was made using SSC/FSC (size and granularity) for the lymphocytes (c, f), and the gate was made using SSC/FSC (size and granularity) for the macrophages (i). In FL1, CD3 + T lymphocytes (d), CD21 + B lymphocytes (g) and F4/80+ macrophages (*j*) were gated, using the isotype control as a reference. The gated CD3+, CD21+ or F4/80+ cells in FL1 were overlain in FL2 with non-infected (Black) and infected (Red) PD-1+, PD-L1+ and PD-L2+ cells (e, h, k).

Table 2
Expression of PD-1 + and its ligands in CD3 +, CD21 + and F4/80 + cells from PBMCs.

PBMC	PD-1 ($\bar{x}$ % ± SD)		PD-L1 ($\bar{x}$ % ± SD)		PD-L2 ($\bar{x}$ % ± SD)	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
T lymphocyte	11.09 ± 7.54	16.52 ± 8.64**			9.12 ± 7.47	
B lymphocyte				6.98 ± 4.67*		
Macrophage			26.88 ± 17.91	23.16 ± 20.47		

Statistical analyses were performed using the *t*-test and Wilcoxon test, and graphical presentations show the mean ± SEM. *p < 0.05, **p < 0.005.

(Fig. 1g).

3.3. Evaluation of the expression of PD-1 and its ligands in T and B lymphocytes and macrophages after in vitro infection by L. Infantum in cultures on total PBMCs of the control group

PD-1 and its ligands not showed difference at 3 days after *L. infantum* infection (data not shown). The expression of PD-1 ($p < 0.05$), PD-L1 and PD-L2 ($p < 0.01$) was higher at 6 days in after *L. infantum* infection (Fig. 2a) than in cultured total PBMCs from non-infected dogs. A representative histogram of flow cytometry analysis of expression of PD-1, PD-L1 and PD-L2 was showed in Fig. 2b.

After observing the increased expression of PD-1 and its ligands in the total culture of PBMCs of healthy dogs after *in vitro* infection with *L. infantum*, we investigated the expression of these receptors in T cells, B cells and macrophages, by means of flow cytometry. A representative histogram of flow cytometry analysis of expression of PD-1 on CD3 +, CD21 +, F4/80 + cells was showed in Fig. 2 c–k The expression of PD-1 and its ligands increased after *L. infantum* infection on the surface of T lymphocytes (CD3 +); PD-L1 expression increased after infection of B lymphocytes (CD21 +); and the expression of PD-1 and PD-L2 increased after infection of macrophages (F4/80 +) (Table 2) (*p < 0.05, **p < 0.01).

3.4. IFN-γ and IL-10 concentrations in the culture supernatant from PBMCs and leukocytes from the spleen in the infected group after blocking of PD-1 receptor and its ligands

IL-10 levels were increased in the presence of monoclonal antibody blocking of PD-L2 ($p < 0.05$) in the culture supernatant from PBMCs of infected dogs (Fig. 3a). IL-10 levels were decreased in the presence of monoclonal antibody blocking of PD-1 ($p < 0.05$) in the culture supernatant of spleen leukocytes of infected dogs (Fig. 3b).

After 72 h of blocking of PD-1 and its ligands in culture supernatants from PBMCs and spleen leukocytes from the infected group, there was no significant difference in IFN-γ concentration (data not shown).

3.5. Proliferative response of lymphocytes to the SAg of L. infantum in the cells from lymph nodes of the infected group after blocking of PD-1 receptor and its ligands and evaluation of NO and the cytokines IFN-γ, TNF-α, IL-10 and IL- 4 in the culture supernatant

We observed that blocking PD-L1 increased the antigen-specific lymphoproliferative response ($p < 0.05$), while blocking PD-1, PD-L1 and PD-L2 increased nonspecific proliferation ($p < 0.05$) (Fig. 4a). A representative histogram of flow cytometry analysis of expression of proliferative response was showed in Fig. 4 b–g.

The IFN-γ production in the culture supernatant from the lymph node cells of the infected group that were subjected to the lymphoproliferation assay was lower when PD-1 ($p < 0.01$) and its ligands were blocked in the absence of SAg (Fig. 4h). It was not significantly

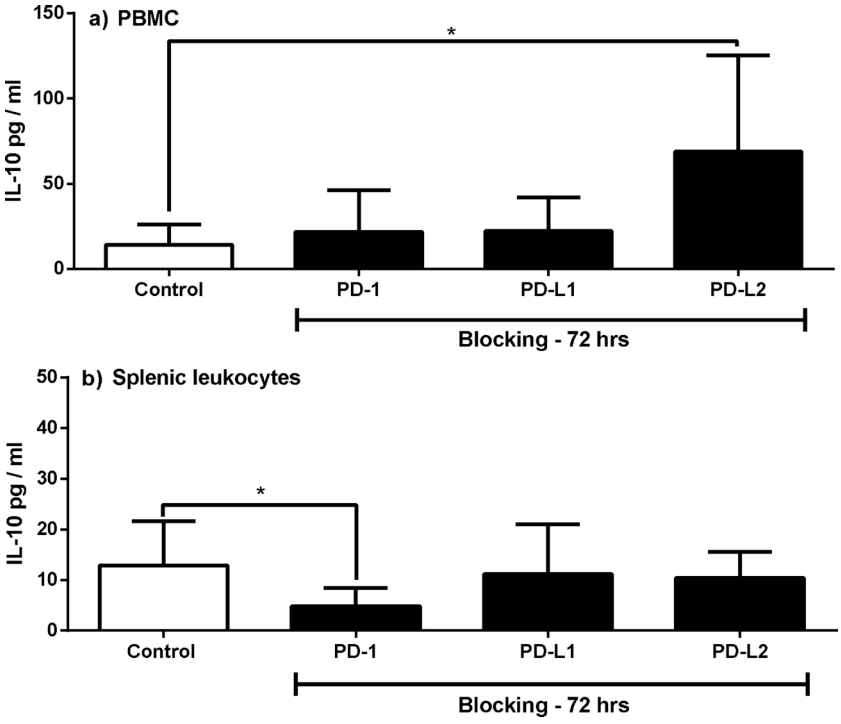

Fig. 3. IL-10 concentration in the culture supernatant of PBMCs and splenic leukocytes. IL-10 concentration in the culture supernatant of (a) PBMCs (n = 14) and (b) spleen leukocytes, from the infected group (n = 13) after blocking of the PD-1 receptor and its ligands for 72 h. Statistical analyses were performed using the *t*-test and Wilcoxon test, and graphical presentations show the mean ± SEM. *p < 0.05.

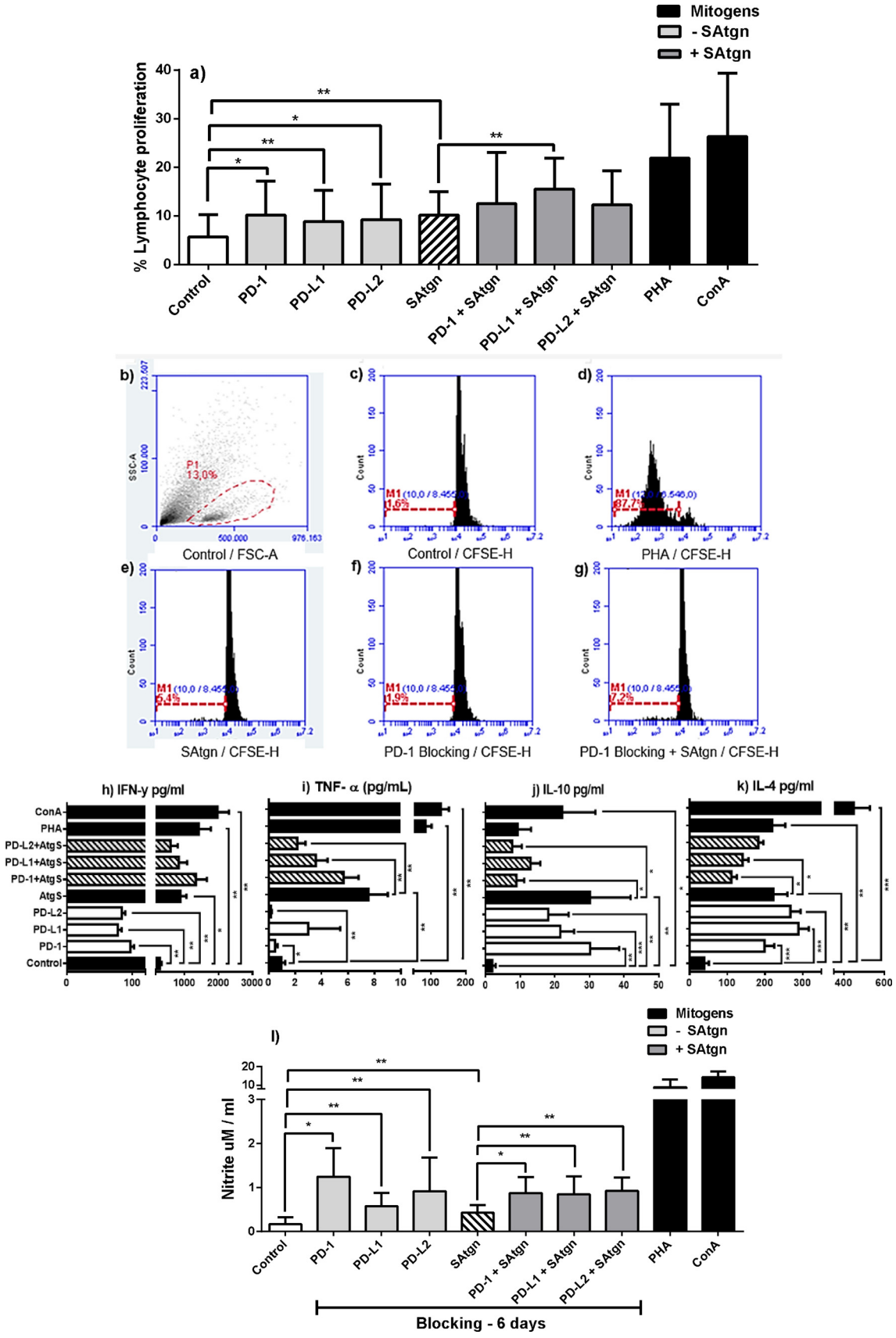

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Fig. 4. Lymphoproliferation, representative flow cytometry, cytokines and nitrite from lymph nodes of the infected group. (a) Lymphoproliferative response of cell cultures from lymph nodes of the infected group (n = 15), after *in vitro* blocking of the PD-1 receptor and its ligands (PD-L1 and PD-L2), in the absence or presence of SAg of *L. infantum*. Statistical analyses were performed using the *t*-test and Wilcoxon test, and graphical presentations show the mean ± SEM. *p < 0.05, **p < 0.01. Fig. 5 b – g. Representative images demonstrating how the lymphoproliferation flow plots were evaluated: (b) gated cell control; (c) histogram of control cells; (d) phytohemagglutinin (PHA); (e) SAg; (f) PD-1 blocking; and (g) PD-1 blocking + SAg. Figs. 5 h – k. Production of cytokines: (h) IFN-γ, (i) TNF-α, (j) IL-10 and (k) IL-4, in the culture supernatant of the lymphoproliferation assay on lymph node cells from infected dogs (n = 11), with culturing for 6 days after blocking of PD-1 and its ligands, with SAg of *L. infantum* (+SAg) and without it (-SAg). Statistical analyses were performed using the *t*-test and Wilcoxon test, and graphical presentations show the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Fig. 5 l. NO concentration in the culture supernatant from the lymph node cells of the infected dogs (n = 11) stained with CFSE and cultured for 6 days without treatment (control) and in the presence of the mitogens PHA and ConA and the SAg of *L. infantum*. Statistical analyses were performed using the *t*-test and Wilcoxon test, and graphical presentations show the mean ± SEM. *p < 0.05, **p < 0.01.

different in the presence of SAg, and high IFN-γ production was observed in the supernatant in the presence of the mitogens used.

The TNF-α production in the culture supernatants from lymph node cells of the infected group that were subjected to the lymphoproliferation assay decreased when PD-1 ($p < 0.05$), and PD-L2 ($p < 0.01$) were blocked in the absence of SAg, and in the presence of SAg when PD-L1 and PD-L2 ($p < 0.01$) was blocked (Fig. 4i).

The IL-10 production in the culture supernatant from lymph node cells of the infected group that were subjected to the lymphoproliferation assay increased with blocking of PD-1 ($p < 0.01$), PD-L1 ($p < 0.001$) and PD- L2 ($p < 0.01$) in the absence of SAg, and it was lower with blocking of PD-1 and PD-L2 ($p < 0.05$) in the presence of SAg (Fig. 4j).

The IL-4 production in the culture supernatant from lymph node cells of the infected group that were subjected to the lymphoproliferation assay increased with blocking of PD-1 and PD-L1 ($p < 0.001$) and PD-L2 ($p < 0.01$) in the absence of SAg; and decreased with blocking of PD-1 and PD-L1 ($p < 0.05$) in the presence of SAg (Fig. 4k).

The NO production in the culture supernatant from lymph node cells of the infected group that were subjected to the lymphoproliferation assay increased with blocking of PD-1 ($p < 0.01$), PD-L1 ($p < 0.05$) and PD-L2 ($p < 0.01$), (Fig. 4l).

3.6. Correlation between expression of PD-1 and its ligands and the parasite load

The PCR parasite load of the spleen from the infected group showed a significant positive correlation ($R^2 = 0.5$, $p < 0.05$) in the analyses on PD-1 and PD-L1 expression in spleen CD21+ cells, through flow cytometry (Fig. 5 a, b). No correlation was observed between parasite load and the expression of PD-1 and its ligand cells in the F4/80+ and CD3+ cells of the spleen from the infected group, data not showed.

4. Discussion

In this study, we showed that there was higher expression of PD-1 and its ligands in the spleen cells of the dogs in the infected group than in the dogs of the control group. *In vitro* infection by *L. infantum* in PBMCs of the healthy dogs also showed higher expression of PD-1 and its ligands. In the spleen leukocytes from dogs in the infected group, blocking of PD-1 decreased IL-10 production. Immune blockade of PD-L1 was able to restore the antigen-dependent lymphoproliferative response in lymph node cell cultures in the infected group.

The expression of PD-1 and its ligands increased in the spleen of the dogs in the infected group (Fig. 1 a–f), similarly PD-1 expression had previously been investigated by means of flow cytometry in PBMCs and leukocyte cells from the spleen of dogs infected with VL, and increased expression was also observed [11], confirming the role of PD1 in canine VL.

PD-1 and its ligands increased in the total PBMCs of the healthy dogs after *in vitro* infection by *L. infantum* (Fig. 2a). However, it is important to emphasize that when the macrophages were differentiated *in vitro* from monocytes of the blood of infected dogs, the expression of PD-1 and its ligands did not show any increase. This suggests that soluble factors derived from T cells and the interaction between the PD-1 receptor and its ligands may contribute towards regulation of this expression. Furthermore, the bidirectional signal [30] may be important in regulating the expression of PD-1 and its ligands.

Increased concentrations of PD-1 and its ligands on the surface of PBMCs were observed after *in vitro* infection with *L. infantum* in CD3+ cells. PD-L1 increased in the B lymphocyte cells and PD-1 and PD-L2 increased in the macrophages, after *in vitro* infection (Table 2). This confirmed that the parasite is capable of regulating the expression of these receptors on the surface of various cell types, as previously observed in naturally infected dogs [20].

Production of IL-10 was observed in culture supernatants from the PBMCs cells and spleen of the infected group (Fig. 3a, b), thus confirming its regulatory role in canine VL. Its production has been observed in various target organ diseases [31,32]. In addition, PD-L2 blockade in PBMCs from infected dogs showed increased IL-10 production (Fig. 3a), similarly to peritoneal cells of mice infected with *Trypanosoma cruzi*, which also showed increased IL-10 [29]. These results show that there is a possible alternative receptor for PD-L2, with stimulatory functions that differ from binding with PD-1, as already suggested [29,33].

Blocking of PD-1 reduced the IL-10 concentration in the supernatant from spleen cell cultures of the infected group (Fig. 3b), thus suggesting

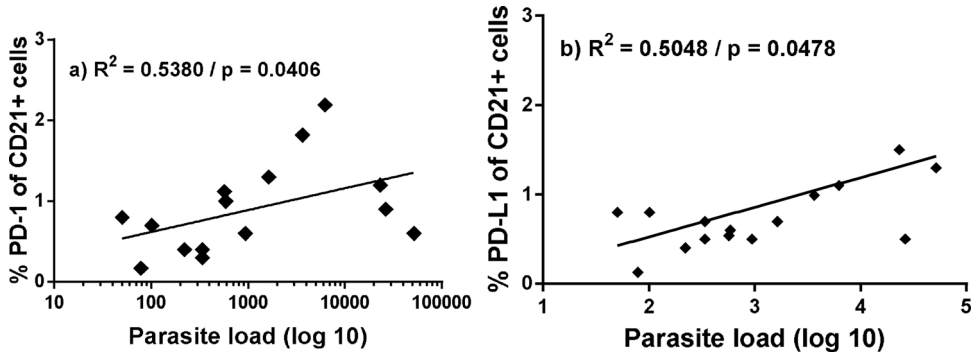

Fig. 5. Association between spleen parasite load and PD-1 and PD-L1 in CD21+ cells from infected dogs. Positive correlation between spleen parasite load in the dogs in the group infected with *Leishmania spp*. (n = 15) and the expression of (a) PD-1 and (b) PD-L1 in CD21+ from the infected group.

that secretion of this cytokine is related to signaling triggered by PD-1 and its ligands. The reduction of IL-10 in the supernatant from spleen cells may be associated with polarization of macrophages to the M1 profile, given that use of blocking antibodies for PD-1 in PBMCs has also been found to have the capacity to increase nitrite production in dogs with VL [20]. It will be important to conduct further studies to clarify the interaction between these receptors.

The lymphoproliferative specific antigen capacity increased after blocking of PD-L1 in dogs with VL, but there was only a tendency towards increased proliferation through blocking of PD-1 and PD-L2 (Fig. 4a). PD-L1 was previously found to be associated with failure of the proliferative response in canine VL and receptor blocking was found to be able to restore proliferation [11], thus confirming what we observed in this study. The low proliferation observed through blocking PD-1 suggests that perhaps the antibody concentration used was insufficient to restore the lymphoproliferative response, given that the PD-1 expression in dogs with VL was high in both PBMCs and spleen tissue. In agreement with our results, a previous study also found that T cell proliferation in *T. cruzi* infection after blocking PD-L2 was not restored [29]. Nonspecific proliferation became higher through blocking PD-1 and its ligands, similarly to what was observed in other studies [16].

A significant increase in IFN-γ production in the lymph node within the culture supernatant of the infected group in the presence of SAg was observed (Fig. 4h). Expression of this cytokine has been associated with a protective response in canine VL. IFN-γ production was investigated and increased expression in both asymptomatic and symptomatic infected dogs, compared with uninfected dogs, was observed [32]. After using the blocking antibody for PD-1 and its ligands in the absence of SAg, we observed that the production of this cytokine decreased. This result is different from what was previously published by Esch et al. [11], who found that IFN-γ production increased in the *in vitro* culture supernatant from adherent PBMCs with *L. infantum* infection after blocking of PD-L1. However, in our study, we used the supernatant from lymph node cells.

No significant increase in IFN-γ production SAg of the lymph node within the culture supernatant of the infected group was observed after blocking of PD-1 and its ligands (Fig. 4h), but there was a tendency towards an increase. In a previous study, adherent cells in the PBMCs of dogs with VL treated with the blocking antibody for PD-L1 showed increased IFN-γ production by CD4+ cells, but not CD8+ [11]. In our study, we evaluated the total culture supernatant, including CD4+ and CD8+, which may have been why we did not find any significant difference. Another explanation could be that cell depletion may have occurred during production of this cytokine, due to prolonged *in vivo* stimulation, in an attempt to contain the infection by *L. infantum*. Furthermore, different clones and a half concentration of blocked antibody were used in our study.

A significant increase in TNF-α production in the lymph node within the culture supernatant of the infected group in the presence of SAg was observed (Fig. 4i), thus confirming the role of TNF-α in regulation of the immune response in canine VL [20,33].

In the antigen-specific response after the blocking of PD-L1 and PD-L2, the TNF-α concentration in the lymph node within the culture supernatant of the infected group decreased (Fig. 4i), although improvement in the lymphoproliferative response was observed only with PD-L1 blockade. Unlike our results, a previous study showed that blocking of PD-1 and its ligands without SAg increased TNF-α production in spleen cell cultures from dogs with VL after 72 h [20]. It is possible that, in that study, the duration of culturing may have influenced the result obtained and the modulation of the production of this cytokine by PD-1 may be immediate. In our study, the supernatant was evaluated for the presence of TNF-α after 6 days and in the presence of SAg. These results suggest that earlier than 72 h of culturing could be a better time point to look at, for determining the difference in TNF-α production in canine VL.

IL-10 production increased within the supernatant from lymph node cells dogs with VL in response to stimulation by SAg (Fig. 4j). IL-10 has been indicated as suppressing the immune response in VL [34,35]; and, in dogs with VL, increased IL-10 production was observed in the lymph node cells of symptomatic dogs, while there was a reduction in asymptomatic dogs [32]. However, the IL-10 levels were found to decrease after stimulation with specific-antigen blocking of PD-1 and PD-L2, thus confirming that the PD-1 receptor regulates IL-10 production in the immune response in canine VL [11]. Possibly, with PD-1 blockade, antigen-specific T cell proliferation increases the specific T population that produces cytokines, thus generating productive immunity through decreased IL-10, with increases in other cytokines, as was observed in a previous study by Chiku et al. (2016) in spleen leukocytes of canine VL.

IL-4 production increased in the culture supernatant in a lymphoproliferation assay on the lymph nodes of dogs with VL in response to the SAg (Fig. 4k), thus suggesting that it was involved in regulation of the immune response. This differed from what had been observed previously, in which the mRNA for IL-4 from lymph node cells of dogs with VL did not show any difference in response to infection [32]. Furthermore, in the lymphoproliferative response to PBMCs, after stimulation with recombinant L. *(L.) chagasi* in oligosymptomatic and asymptomatic dogs with VL, there were low levels of secretion of the cytokine IL-4 with NO production, unlike to what happened in symptomatic dogs [36]. That finding was similar to what we observed in our study, in the dogs of the infected group.

On the other hand, increased IL-10 and IL-4 production in the culture supernatant from proliferating lymph node cells of dogs with VL in the absence of SAg after blockade of PD-1 and its ligands, may be related to secretion of this cytokine by other cells, since Lamichhane et al. (2017), reported increased IL-10 in dendritic cells after blockade of PD-1. In addition, the presence of soluble molecules such as sPD-1 and sPD-L1 may also influence the regulation of the cytokine response [37], although this has not been investigated yet in canine VL.

The IL-4 concentration decreased in the culture supernatant of the antigen-specific lymphoproliferation from the dogs in the infected group after blocking of PD-1 and PD-L1 (Fig. 4k). This suggests that the Th2 response became reduced, which may facilitate control over parasite multiplication. It is possible that blocking PD-1 and PD-L1 changed the profile of macrophages for M1, since an increase in NO was also observed with use of these antibodies, in the supernatants of these cultures.

In our study, in the supernatant from the lymph node cells, we observed increased production of NO in the dogs with VL, in the presence of blocking antibodies for PD-1 and its ligands in the nonspecific antigen response (Fig. 4l). The specific antigen response was similar to these findings. Increased NO had previously been observed in PBMCs after blocking of these receptors [20].

The positive correlation observed between the splenic parasite load and the expression of PD-1 and PD-L1 on CD21+ cells in the dogs with VL (Figs. 5 a, b) suggests that this cell population may play a role in regulating the immune response in canine VL. B cells are responsible for strong antibody production that is not protective, and hyperglobulinemia is observed. Inefficient cellular response, associated with persistence of the antigen, favors formation of immune complexes that deposit in various tissues and organs. This results in glomerulonephritis, vasculitis, uveitis, myositis and polyarthritis, which are characteristics of the disease [38]. Increased PD-L1 on B cells has been observed in dogs with VL, and after blocking PD-L1, the Th1 response was restored [23]. Expression of PD-1 and its ligands in B cells appears to be associated with selection and survival of plasma cells in the germinal center [39]. In canine VL, high expression of PD-1 and its ligands in B cells may be perpetuating the production of non-protective antibodies in canine VL, which increases the splenic parasitic load.

5. Conclusion

We conclude that *L. infantum* may modulate the expression of PD-1 and its ligands in cells from dogs with VL, thus suppressing lymphoproliferation and altering cytokine production so as to favor persistence and survival of *L. infantum* in macrophages. PD-1 may therefore be a target for drug development that can act as stimulators of the immune response in canine VL.

Conflict of interest

The authors declare that there was no financial or commercial conflict of interest.

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