Setting the proportion of CD4+ and CD8+ T-cells co-cultured with canine macrophages infected with *Leishmania chagasi*

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ARTICLE INFO

ABSTRACT

New methods for evaluating the canine immune system are necessary, not only to monitor immunological disorders, but also to provide insights for vaccine evaluations and therapeutic interventions, reducing the costs of assays using dog models, and provide a more rational way for analyzing the canine immune response. The present study intended to establish an *in vitro* toll to assess the parasitological/immunological status of dogs, applicable in pre-clinical trials of vaccinology, prognosis follow-up and therapeutics analysis of canine visceral leishmaniasis. We have evaluated the performance of co-culture systems of canine *Leishmania chagasi*–infected macrophages with different cell ratios of total lymphocytes or purified CD4+ and CD8+ T-cells. Peripheral blood mononuclear cells from uninfected dogs were used for the system set up. Employing the co-culture systems of *L. chagasi*–infected macrophages and purified CD4+ or CD8+ T-cell subsets we observed a microenvironment compatible with the expected status of the analyzed dogs. In this context, it was clearly demonstrated that, at this selected T-cell:target ratio, the adaptive immune response of uninfected dogs, composed by *L. chagasi*-unprimed T-cells was not able to perform the *in vitro* killing of *L. chagasi*–infected macrophages. Our data demonstrated that the co-culture system with T-cells from uninfected dogs at 1:5 and 1:2 ratio did not control the infection, yielding to patent *in vitro* parasitism ($\geq$80%), low NO production ($\leq$5 μM) and IL-10 modulated (IFN-γ/IL-10$\leq$2) immunological profile *in vitro*. CD4+ or CD8+ T-cells at 1:5 or 1:2 ratio to *L. chagasi*–infected macrophages seems to be ideal for *in vitro* assays. This co-culture system may have great potential as a canine immunological analysis method, as well as in vaccine evaluations, prognosis follow-up and therapeutic interventions.

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1. Introduction

Leishmaniasis is a protozoonosis that is endemic in several countries across the world, with more than 350 million cases being visceral leishmaniasis (Desjeux, 2004). Infected dogs have a high

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density of cutaneous parasitism, and they are the main domestic reservoir of *Leishmania chagasi* (syn. *Leishmania infantum*) contributing to the propagation of the parasite (Deane and Deane, 1962). The reported seroprevalence of canine leishmaniasis in the metropolitan area of Belo Horizonte, Minas Gerais, Brazil can reach values as high as 16% (Coura-Vital et al., 2011) and the impact of controlling the population of infected dog in endemic areas has been debated by several authors (Dietze et al., 1997; Costa, 2011; Coura-Vital et al., 2011).

The development of a canine visceral leishmaniasis (CVL) vaccine is highly desirable and would represent the most practical and efficient control tool (Dye, 1996). A key goal in the control of 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, extending into years of study (Giunchetti et al., 2007; Giunchetti et al., 2008; Roatt et al., 2012). Thus, researchers need to develop methodological strategies that enable a more rapid and accurate evaluation of the canine immune response triggered by distinct vaccine candidates.

In the immune response to *Leishmania* infection, macrophages (MØ) have been shown to play an important role in the control of the parasite in distinct experimental models (Rodrigues et al., 2007). Although it is well established that macrophages participate in killing parasites through mechanisms that depend on reactive oxygen and nitrogen intermediates, the mechanisms by which macrophages kill *Leishmania* in dogs have not been investigated as thoroughly (Rodrigues et al., 2011). 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, macrophages are fully capable of 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-cells 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-cell subsets (Pinelli et al., 1999; Holzmuller et al., 2005; Rodrigues et al., 2009). Thus, it is necessary to develop additional methods for evaluating the immune system in veterinary medicine, especially in experimental dog models. Such tests could be included in pre-clinical trials of vaccines 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 and provide a more rational way of selecting candidate vaccines against CVL as well as to evaluate therapeutic protocols. These methods would contribute to the identification of biomarkers related to interactions between the innate and adaptive immune responses of dogs. In this context, our main goal was to analyze the canine immune response using a co-culture system of canine *L. chagasi*-infected macrophages with different ratios of purified CD4^{+} and/or CD8^{+} T-cells. This approach, based on the canine immune response, would be useful in dealing with a number of canine diseases, in addition to evaluating various treatments and vaccines.

2. Materials and methods

2.1. Animals

Healthy mongrel dogs of both sexes with a mean age of 7 months, born and maintained in a kennel at the Center of Animal Science, Federal University of Ouro Preto, were used in the experiments ($n = 12$). The animals received all the appropriate health management before entering the experiment, having received anti-helmintic treatment (plus Chemital, Chemitec Agro-Veterinary LTDA., Brazil) and vaccination against rabies (Tecpar, Brazil), distemper, adenovirus type 2, coronavirus, parainfluenza, parvovirus, and *Leptospira* (HTLP 5/CV-L Vanguard, Pfizer, Brazil). The study protocol was approved by the Ethical Committee for the Use of Experimental Animals at the Federal University of Ouro Preto, Minas Gerais, Brazil (protocol number 2010/71).

2.2. Parasites

This study used a single lot of criopreserved wild-type strain of *L. chagasi* (MCAN/BR/2008/OP46) recent isolated from hamster hosts, grown in culture medium NNN/LIT (Sigma Chemical Co., USA) supplemented with inactivated 20% fetal bovine serum (FBS) (Cultilab, Brazil), plus penicillin (200 U/mL) and streptomycin (100 μg/mL), at pH 7.4 and incubation temperature of 23 °C. *L. chagasi* promastigotes used for *in vitro* tests were obtained from culture at the late stationary phase (seven days of culture), presenting approximately 80% of quickly moving and slender forms with long flagella, alike metacyclic promastigotes forms). The cultures were used up to the seventh passage *in vitro*. The promastigote forms were washed twice ($1000 \times g$ for 10 min at 4 °C) with 40 mL of phosphate buffered saline (PBS), counted in a Neubauer hemocytometer, adjusted to 5×10^7 promastigotes/mL, presenting more than 90% of viability.

2.3. Isolation of peripheral blood mononuclear cells, non-adherent cells and monocytes

Blood samples (20 mL) were collected in heparinized tubes from the 12 healthy dogs. Peripheral blood mononuclear cells (PBMC) were isolated as previously described (Viana et al., 2013). Briefly, the whole blood sample was placed at a 3:1 ratio over dual gradient composed of equal volume of Ficoll-Hypaque (Sigma Chemical Co.; density: 1.119 g/mL) and Ficoll-Hypaque (Sigma Chemical Co.; density: 1.077 g/mL) in sterile polystyrene conical bottom tubes (Falcon, Corning, USA). The samples were centrifuged at $700 \times g$ for 80 min at 22 °C. The PBMC were collected at the dual Ficoll-Hypaque interface and transferred to another tube and washed twice ($400 \times g$ for 10 min at 4 °C) with 40 mL of PBS supplemented with 10% FBS. Afterwards the supernatant was discarded and the PBMC resuspended in 1 mL of cell RPMI 1640 medium. Cells were counted in a Neubauer hemocytometer chamber to determine the numbers of monocytes or lymphocytes per milliliter. Isolated PBMC suspensions adjusted 5×10^5 monocytes/wells were added over circular coverslips (15 mm; Glasscyto, Brazil) on 24-well plates (NUNC, Thermo Fisher Scientific Inc., USA), as previously described (Viana et al., 2013). Briefly, after 24 h, the non-adherent cells (comprising peripheral blood lymphocytes) were removed and transferred to 48-well plates (NUNC, Thermo Fisher Scientific Inc., USA). Non-adherent cells were maintained in culture for 5 days at 37 °C/5% CO$_2$. The adherent cells (comprising peripheral blood monocytes) were cultured with RPMI supplemented with 20% FBS on 20% macrophage colony-stimulating factor medium (obtained from supernatant of cultures of L929 immortalized cells, and incubated at 37 °C/5% CO$_2$). Monocytes differentiating into macrophages were obtained after 5 days of culture at 37 °C/5% CO$_2$.

2.4. Infection of macrophages with L. chagasi *promastigotes* in vitro

Macrophages differentiated from approximately 5×10^5 peripheral blood monocytes as described above (section 2.3) were infected with 5×10^6 *L. chagasi* promastigotes in the stationary phase, using a 10:1 ratio (10 parasites per macrophage) as previously described (Viana et al., 2013). Each well was gently washed 3 h after the infec-

tion and the maintained as a control culture or used for co-culture systems.

2.5. Purification of CD4+ and CD8+ T-cells

CD4+ and CD8+ T-lymphocytes were purified from non-adherent cell suspensions obtained from isolated PBMC preparations using magnetic beads (Miltenyi Biotec Inc., USA) by positive selection using anti-CD4 or anti-CD8-FITC (fluorescein isothiocyanate) antibodies (AbD Serotec, UK) and microbeads coated with anti-FITC, as previously described (Viana et al., 2013). Briefly, the cell suspension was prepared at a concentration of 6×10^7 cells in a 1 mL tube in isolation buffer containing PBS 1×, pH 7.2, 0.5% BSA, and 2 mM EDTA. Monoclonal antibodies (CD4 or CD8-FITC) were added to 2 μL/mL of total lymphocytes and incubated at room temperature (RT) for 15 min. Then, magnetic microbeads were added to 10 μL/mL lymphocytes and incubated for 15 min at RT. The cell suspension was loaded onto a MACS column (Miltenyi Biotec Inc.), which was placed in the magnetic field of a MACS separator. The magnetically labeled CD4+ or CD8+ cells were retained on the column, whereas the unlabeled cells ran through, thus this cell fraction was depleted of CD4+ and CD8+ cells. After removal of the column from the magnetic field, the magnetically retained CD4+ and CD8+ cells were eluted as the positively selected cell fraction by washing the magnetic column with 15 mL of isolation buffer (Viana et al., 2013).

The purity of CD4+ and CD8+ T-cells was evaluated by flow cytometry on a FACSCalibur instrument (Becton Dickinson, USA) interfaced to an Apple G3 workstation. Cell-Quest software (Becton Dickinson) was used for both data acquisition and analysis. A total of 20,000 events were acquired for each preparation. Flow cytometric analysis was performed using canine purified lymphocytes that were first selected on the basis of their characteristic forward (FSC) and side (SSC) light-scatter distributions. Following FSC and SSC gain adjustments, the lymphocytes were selected based on their FSC *versus* SSC properties. Fluorescence was evaluated from FITC spectra (anti-CD4 and anti-CD8 antibodies) on FL1 in dot plot representations. A marker was set as an internal control for nonspecific binding in order to encompass >98% of unlabeled cells, and this marker was then used to analyze data for individual animals. The results were expressed as the percentage of positive cells within the selected lymphocyte gate for CD4+ or CD8+ T-cell. All preparations used in this study presented purity higher or equal 90% (data not shown).

2.6. Co-culture systems

In this study, we have used four different co-culture systems: (1) Total lymphocyte LYM:MØ; (2) CD4+:CD8+:MØ; (3) CD4+:MØ and (4) CD8+:MØ. On each co-culture system, lymphocytes suspensions were incubated with *L. chagasi*-infected macrophage (section 2.4). Distinct cell ratios were used including 4:1 (2×10^6 LYM:5×10^5 MON) in the LYM:MØ system; 1:1:1 (5×10^5 CD4+:5×10^5 CD8+:5×10^5 MON) and 2:2:1 (1×10^6 CD4+:1×10^6 CD8+:5×10^5 MON) for the CD4+:CD8+:MØ system; 1:5 (1×10^5 CD4+ or CD8+:5×10^5 MON), 1:2 (2.5×10^5 CD4+ or CD8+:5×10^5 MON), 1:1 (5×10^5 CD4+ or CD8+:5×10^5 MON), and 2:1 (1×10^6 CD4+ or CD8+:5×10^5 MON), and for purified CD4+:MØ or CD8+:MØ systems. Control cultures were run in parallel to each experimental batch where *L. chagasi*-infected macrophages were incubated in the absence of lymphocytes. All culture systems were incubated for 72 h, as described previously (Viana et al., 2013). After this period, the analyses of parasitological biomarkers were performed (frequency of *L. chagasi*-infected MØ and the parasite load)

and the supernatants collected and stored at –80 °C for nitric oxide (NO) and cytokines measurements.

2.7. Parasitological biomarkers—frequency of L. chagasi-infected MØ and the parasite load

The frequency of *L. chagasi*-infected MØ was calculated as the number of infected macrophages in 200 macrophages. The parasite load was determined by counting the numbers of amastigotes in 200 macrophages and then dividing the number of amastigotes by the number of infected macrophages, in order to obtain the average number of amastigotes per macrophage, as previously described (Viana et al., 2013).

2.8. Nitric oxide measurement

NO levels in the supernatant of cell co-culture were quantified indirectly by measuring nitrite in culture supernatants of macrophages alone and in the co-culture system via the Griess reaction (Ding et al., 1988). Aliquots of 100 μL per well were deposited, in duplicate, in 96-well flat-bottom plates (NUNC, Thermo Fisher Scientific Inc.), 100 μL of Griess reagent (0.1% *N*-(1-naphthyl) ethylenediamine, 1% sulfanilamide, 5% phosphoric acid; Sigma Aldrich, St. Louis, MO, USA) was added to each well, and plates were incubated at RT for 10 min in the dark. Then the absorbance was determined in an automatic microplate reader (Multiskan MCC 340, Labsystems, Finland) with a 570-nm filter. Results were determined after interpolating values obtained from a standard curve made with sodium nitrite ($NaNO_2$) at concentrations of 0.78–100 μM for each card. They were expressed considering the amount of nitrite concentration (μM) obtained in each culture.

2.9. ELISA cytokine assay

Culture supernatants were collected at 72 h, and the levels of IFN-γ, TNF-α, IL-12, IL-4, and IL-10 were titrated in culture supernatants using the ELISA method (R&D Systems, USA). Briefly, the plates were coated with anti-IFN-γ, anti-TNF-α, anti-IL-12, anti-IL-4, and anti-IL-10 mAb in PBS, pH 7.4, and incubated at 4 °C overnight. After blocking the wells using buffer containing PBS plus 0.05% (v/v) Tween 20 and 0.1% (w/v) BSA, supernatants were added to each well. Biotin-labeled mAb in incubation buffer was added to each well, and streptavidin-HRP was used as enzyme. The reaction was developed using 3,3',5,5'-tetramethylbenzidine substrate and stopped with 2.5 M H_2SO_4 solution. The plates were washed after each step of incubation using PBS plus 0.05% (v/v) Tween 20. Minimum sensitivity levels were 63 pg/mL for IFN-γ, 63 pg/mL for TNF-α, 62 pg/mL for IL-12, 78 pg/mL for IL-4, and 78 pg/mL for IL-10. All experiments were performed using 96-well plates (COSTAR, Corning Inc., USA) and according to the instructions of R&D Systems. The reading was performed using a microplate automatic reader (EL800; Biotek, Winooski, VT, USA) at a wavelength of 450 nm.

2.10. Statistics and data analysis

The statistical analysis was performed with the software Graph-Pad Prism 5.0 (Prism Software, CA, USA). Data normality was assessed using the Kolmogorov-Smirnov test. The analyses of macrophage cultures, co-cultures, NO, NAG, MPO, and cytokines were performed using repeated measures ANOVA (paired test). Differences were considered significant at $p < 0.05$ and are highlighted in the figures by connecting lines. The selection of parasitological and immunological biomarkers with relevant performance from each co-culture system was carried out using cut-off edge (doted lines provided in the figures) for the frequency of *L. chagasi*-infected MØ ($\geq$80%) and parasite load ($\geq$80%), NO production ($\leq$5 μM) and

IFN-γ/IL-10 balance (≤2). The selection was based on the acceptable values for uninfected dogs. Those biomarkers with relevant performance were highlighted by gray rectangles in the Figures.

3. Results

3.1. Frequency of L. chagasi-infected MØ and the parasite load

The analysis of parasitological biomarkers was carried out in all four co-culture systems (Figs. 1–3A). The analysis demonstrated that in all co-culture systems the frequency of *L. chagasi*-infected MØ was lower as compared to the control condition. However, only in the co-culture systems performed with CD4⁺ or CD8⁺ T-cells at 1:5 and 1:2 ratios yielded to an *in vitro* parasitism profile ≥80% of controls compatible with the expected immunological status of the analyzed dogs. Moreover, similar data was observed for MØ parasite load. Despite an overall decreased parasite load observed in all co-culture systems, only in the LYM:MØ and CD4⁺ or CD8⁺ at 1:5; 1:2 and 1:1 ratios was observed a parasitism profile ≥80% of controls, consistent with the expected immunological status for the uninfected dogs evaluated in this study. A notable reduction of *L. chagasi*–infected macrophages was observed when the co-culture systems were performed using CD4⁺:CD8⁺ T-cells simultaneously or high ratio of purified CD4⁺ or CD8⁺ T-cells (1:1 and 2:1). Moreover, these conditions also let to high percentage of macrophage lysis making the systems not compatible with expected immunological status of the uninfected dogs evaluated in this study (data not shown).

3.2. NO levels

The measurements of NO levels were performed in the supernatant from all four co-culture systems (Figs. 1–3B). Higher NO levels were for all co-culture systems as compared to the control condition. However, the establishment of a cut-off edge for NO levels (≤5 μM) compatible with the parasitological biomarkers profile (≥80%) expected for uninfected dogs, revealed that only in the co-culture systems performed with CD4⁺ or CD8⁺ T-cells at 1:5 and 1:2 ratios the NO levels were bellow the cutoff levels. An prominent increase in the NO production, incompatible with the immunological status of the dogs included in the study, was observed when the co-culture systems were performed using LYM:MØ, CD4⁺:CD8⁺ T-cells simultaneously or high ratio of purified CD4⁺ or CD8⁺ T-cells (1:1 and 2:1).

3.3. Pro- and anti-inflammatory cytokines

The analysis of cytokines in the supernatant of all four co-culture systems is shown in the Figs. 1–3C. Data analysis demonstrated that regardless the co-culture systems the levels of TNF-α, IL-12 and IFN-γ was higher as compared to the control condition. However, only in the co-culture systems performed with CD4⁺ T-cells at 1:5 and 1:2 ratios and CD8⁺ T-cells at 1:5, 1:2 and 1:1 ratios yielded to low IFN-γ/IL-10 balance (≤2) matching with the expected immunological status of the dogs analyzed. A notable increase of IL-4 and decrease of IL-10 was observed when the co-culture systems were performed using high ratio of purified CD4⁺ T-cells (2:1).

3.4. Overall performance analysis of the co-cultures systems

Aiming to evaluate the applicability of the proposed co-culture systems for pre-clinical trials of canine visceral leishmaniasis, cut-off edges for parasitism (≥80%), NO production (≤5 μM) and IFN-γ/IL-10 balance (≤2), compatible with the expected status of uninfected dogs were established. Using these criteria, the results demonstrated that only the co-culture systems performed with CD4⁺ or CD8⁺ T-cells at 1:5 and 1:2 ratios showed to be applicable conditions for parasitological/immunological assessment in pre-clinical trials of canine visceral leishmaniasis. The other tested conditions led to results not compatible with the overall status of dogs evaluated, probably representing methodological artifact related to over proportion between T-cells and macrophages.

4. Discussion

In this study, we developed co-culture systems using total lymphocytes, CD4⁺ and/or CD8⁺ T-cells over *L. chagasi*-infected macrophages as an alternative strategy for simultaneous evaluation of parasitological/immunological status in dogs. This methodology seems to be useful for analyzing vaccine approaches and treatment against CVL, using peripheral blood samples, instead requiring invasive methods for parasitological/immunological measurements. In general, invasive methods, including bone marrow, spleen and lymph nodes aspirates, despite providing compartmentalized status, relevant to understand the anti-*Leishmania* host response, usually represent risks, require controlled procedures, qualified professional assistance and may interfere in the host response during follow-up studies. Therefore, the use of well standardized peripheral blood-based methods may allow the assessment of compatible parasitological/immunological parameters and overcome the major restrictions of those invasive procedures and also provide additional information than the already available PBMC-based methods. To establish such assay, in the present investigation we have evaluated the performance of co-culture systems to provide a range of biomarkers, including parasitological approaches to measure the *L. chagasi* infection status of PBMC-derived macrophages, the NO levels along with the type I and type II cytokines. These analyses were essential for identifying the ideal T-cell:MØ ratio that yield appropriated parasitological (parasitism frequency and parasite load) and immunological (Nitrite, IFN-γ, TNF-α, IL-12, IL-4 and IL-10) pattern, compatible with the animals analyzed and avoid methodological artifact, that might lead to parasitological/immunological data inconsistent with those expected for a given dog status. Although, the proposed method is an *in vitro* approach that may not include all elements/events that take place *in vivo*. Our results showed that the proposed *in vitro* approach was able to yield laboratorial parameters useful to evaluate the ability of canine immune cells to control the *L. chagasi* infection *in vitro* as a biomarker for monitor their immunological status.

In the present study, we have observed that the use of high cell ratios, including the LYM:MØ system, 1:1:1, and 2:2:1 ratios for CD4⁺:CD8⁺:MØ system and 1:1, 2:1 ratios in the purified CD4⁺:MØ or CD8⁺:MØ systems, showed an enhanced frequency of macrophage lysis (data not shown). Although the macrophage lysis mediated by T-cells would not be expected at high levels for uninfected dogs used in the present study, it is possible that methodological artifacts generating non-natural over cytokine microenvironment was created in the co-culture systems using disproportional cell ratios that possibly let to un-specific lysis of *L. chagasi*–infected macrophages. Therefore, it appears that the choice of an ideal cell ratio for co-culture systems using CD4⁺ T-cells or CD8⁺ T-cells ratio that not exceed 1:2 seems to be fundamental to a confident *in vitro* system that resemble the immunological status of the uninfected dogs evaluated in this study. Few studies have included co-culture using canine purified T-cell subsets, as proposed here (Pinelli et al., 1994; Holzmuller et al., 2005; Bourdoiseau et al., 2009). The cytotoxic activity of canine CD8⁺ T-cells co-cultured with *L. infantum*–infected macrophages was first reported by Pinelli et al. (1994). In that study, the lymphocytes from asymptomatic dogs infected with *L. infantum* were able to produce
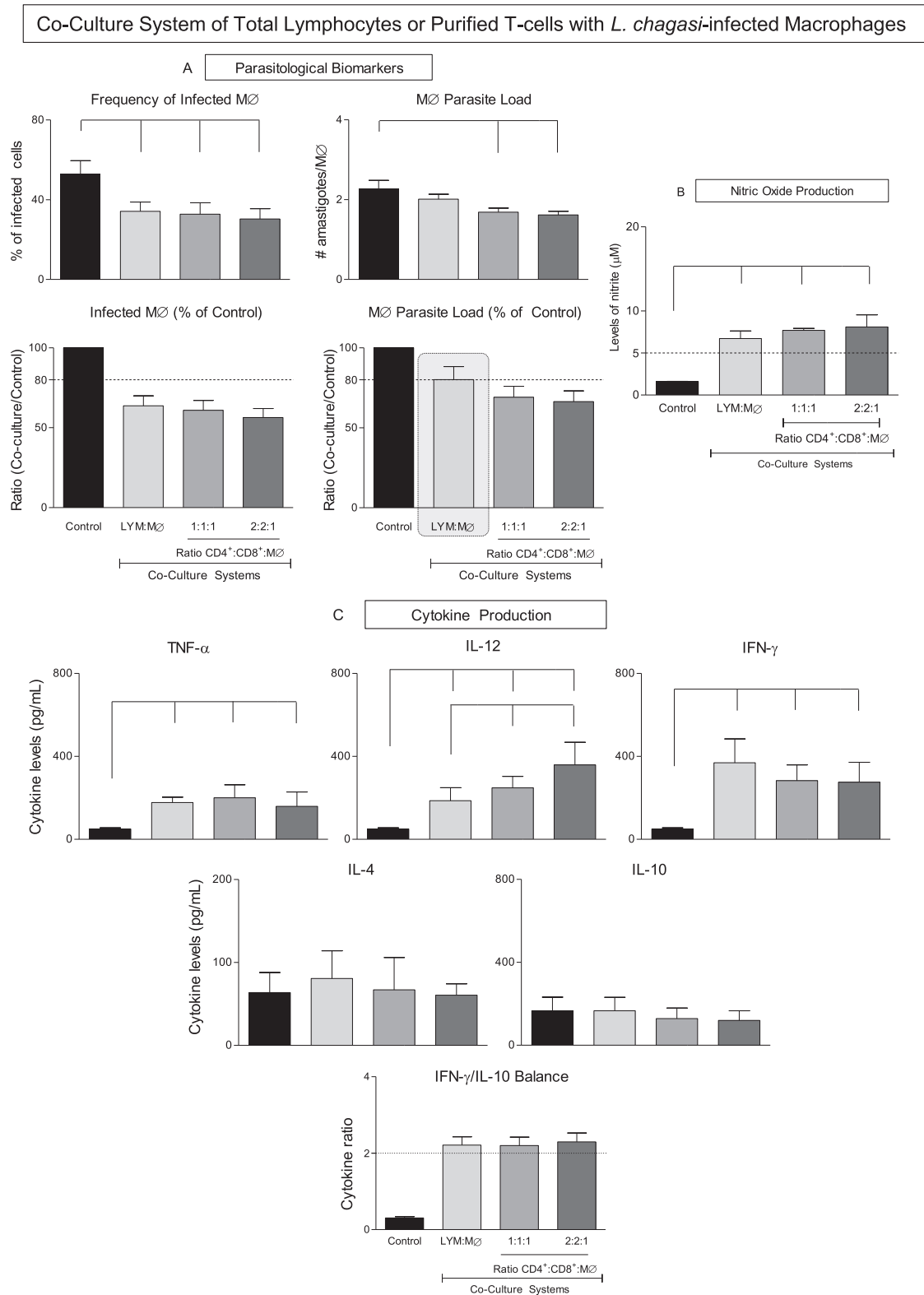
Fig. 1. Parasitological and immunological biomarkers in co-culture systems of total lymphocytes or purified CD4^{+}:CD8^{+} T-cells with *Leishmania* chagasi-infected macrophages. Co-culture systems were set up with total lymphocytes: MØ and also distinct CD4^{+}:CD8^{+} T-cell:MØ ratios (LYM:MØ ▢; 1:1:1 ▨ and 2:2:1 ▨) and compared with the control culture of *L. chagasi*-infected MØ alone (■). (A) Parasitological biomarkers reflecting the overall parasitism, including the frequency of *L. chagasi*-infected MØ (%) and parasite load (# amastigotes/MØ) were evaluated in the co-culture systems (upper panels) and for the ratio of co-culture/control cultures (bottom panels). Immunological biomarkers, including (B) nitric oxide (μM) and (C) the cytokines TNF-α, IL-12, IFN-γ, IL-4 and IL-10 (pg/mL) and IFN-γ/IL-10 ratio, were measured in the supernatant from the co-culture systems. The connecting lines indicate in all cases significant differences at $p < 0.05$. The parasitism ($\geq$80%), NO production ($\leq$5 μM) and IFN-γ/IL-10 balance ($\leq$2) were used as a cut-off edge (doted lines), acceptable for uninfected dogs, to evaluate the co-culture system performance. Biomarkers with relevant performance are highlighted by gray rectangles.
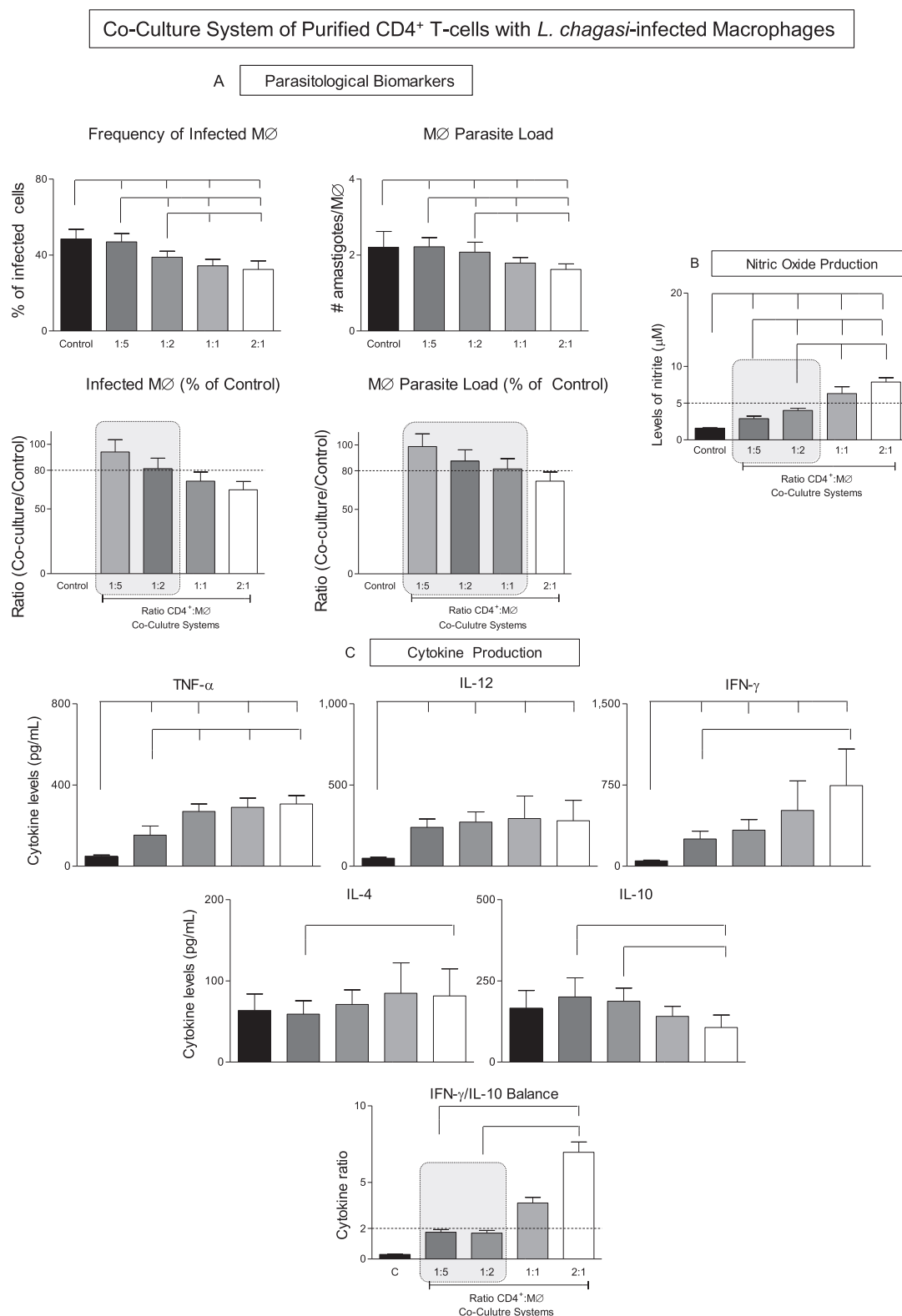
Fig. 2. Parasitological and immunological biomarkers in co-culture systems of purified CD4+ T-cells with *Leishmania chagasi*-infected macrophages. Co-culture systems were set up with distinct CD4+ T-cell:MØ ratios (1:5 ■; 1:2 ■; 1:1 ■ and 2:1 ☐) and compared with the control culture of *L. chagasi*-infected MØ alone (■). (A) Parasitological biomarkers reflecting the overall parasitism, including the frequency of *L. chagasi*-infected MØ (%) and parasite load (# amastigotes/MØ) were evaluated in the co-culture systems (upper panels) and for the ratio of co-culture/control cultures (bottom panels). Immunological biomarkers, including (B) nitric oxide (μM) and (C) the cytokines TNF-α, IL-12, IFN-γ, IL-4 and IL-10 (pg/mL) and IFN-γ/IL-10 ratio, were measured in the supernatant from the co-culture systems. The connecting lines indicate in all cases significant differences at $p < 0.05$. The parasitism ($\geq$80%), NO production ($\leq$5 μM) and IFN-γ/IL-10 balance ($\leq$2) were used as a cut-off edge (doted lines), acceptable for uninfected dogs, to evaluate the co-culture system performance. Biomarkers with relevant performance are highlighted by gray rectangles.
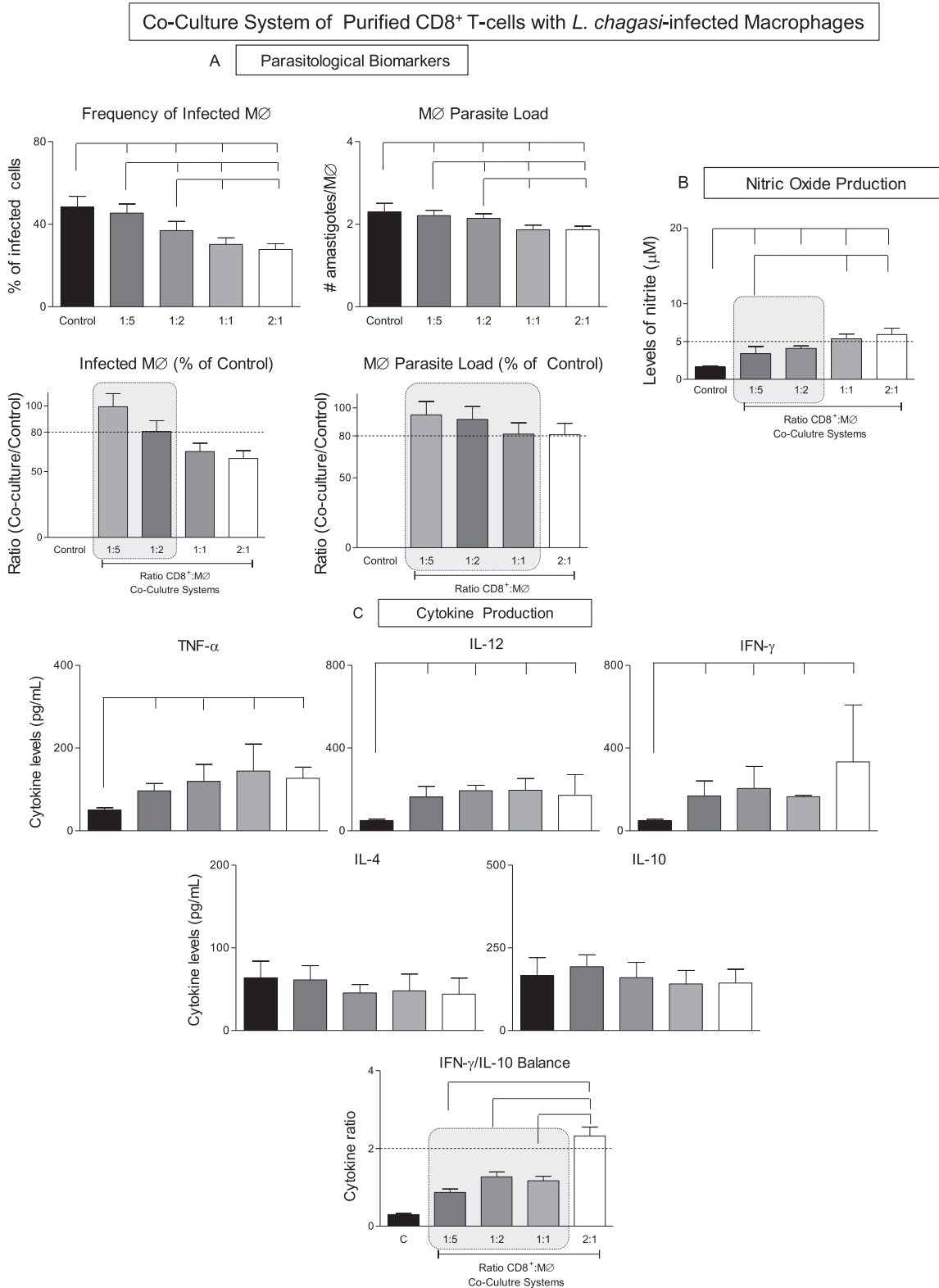
Fig. 3. Parasitological and immunological biomarkers in co-culture systems of purified CD8^{+} T-cells with *Leishmania chagasi*-infected macrophages. Co-culture systems were set up with distinct CD8^{+} T-cell:MØ ratios (1:5 ▨; 1:2 ▨; 1:1 ▨ and 2:1 ▢) and compared with the control culture of *L. chagasi*-infected MØ alone (■). (A) Parasitological biomarkers reflecting the overall parasitism, including the frequency of *L. chagasi*-infected MØ (%) and parasite load (# amastigotes/MØ) were evaluated in the co-culture systems (upper panels) and for the ratio of co-culture/control cultures (bottom panels). Immunological biomarkers, including (B) nitric oxide (μM) and (C) the cytokines TNF-α, IL-12, IFN-γ, IL-4 and IL-10 (pg/mL) and IFN-γ/IL-10 ratio, were measured in the supernatant from the co-culture systems. The connecting lines indicate in all cases significant differences at $p < 0.05$. The parasitism ($\geq$80%), NO production ($\leq$5 μM) and IFN-γ/IL-10 balance ($\leq$2) were used as a cut-off edge (doted lines), acceptable for uninfected dogs, to evaluate the co-culture system performance. Biomarkers with relevant performance are highlighted by gray rectangles.

high levels of IFN-γ, whereas cells of symptomatic dogs did not. However, the authors reported that in some animals, CD4^{+} T-cells were also capable of lysing *L. infantum*–infected macrophages, but without specifying the ways in which lysis triggered by this subpopulation of lymphocytes could occur (Pinelli et al., 1999; Holzmuller et al., 2005). In the present study, we also observed cell lysis in the co-culture system employing CD4^{+} T-cells and *L. chagasi*–infected macrophages, especially at the ratio 1:1 (data not shown). Indeed, these findings suggest that CD4^{+} may contribute to the cytotoxic function in the canine model.

In the present study, the co-culture system that uses total lymphocytes or simultaneously use CD4^{+}:CD8^{+} T-cells in both cell ratios (1:1:1 and 2:2:1) led to enhanced production of NO, all leading to levels above the cut-off. The NO levels were similar between the macrophage co-cultured with CD4^{+} and/or CD8^{+} T-cells, showing NO production over the cut-off edge of 5 μM only when high ratio of purified T-cells were used. The NO levels in co-cultures using CD4^{+} or CD8^{+} T-cells at 1:5 and 1:2 ratios showed values below the cut-off edge, similar to the parasitological profile observed for the tested uninfected dogs. Our hypothesis is that as when a dog present a efficient anti-*Leishmania* response, achieve by effective vaccination, asymptomatic infection or successful treatment, a compatible parasite control mediated by enhanced NO production would be detected in the proposed co-culture method. In the context of biomarkers regarding *Leishmania* control, NO is considered as a crucial mediator for controlling intracellular pathogens, and its analysis was incorporated into this study. However, NO production may vary depending on cell type. For example, murine cell line J774 macrophages produce higher levels of NO than canine macrophages (Pinelli et al., 2000; Gantt et al., 2001; Holzmuller et al., 2002). Furthermore, in a study using a strain of Darby canine macrophages, the authors did not find significant differences between the levels of NO cell line in comparison with PBMC cultures (Panaro et al., 1998). However, the levels of phagocytosis and parasitism control were significantly higher in cultures of PBMC stimulated with concavaline A (Panaro et al., 1998). Various studies have shown the importance of NO in the control of *Leishmania* infection as an effector molecule, indicating immunogenicity induction (Giunchetti et al., 2007; Araújo et al., 2009; Reis et al., 2010) and reduction in the parasitic index in *L. infantum*-infected macrophages, and thus contributing to the establishment of an immunoprotection profile in vaccinated dogs (Holzmuller et al., 2005; Lemesre et al., 2007).

Aiming to incorporate additional biomarkers regarding resistance or susceptibility to *Leishmania* infection, we also analyzed type I and type II cytokines in the supernatant of the co-culture systems. We demonstrated that the levels of TNF-α, IL-12 and IFN-γ and were significantly higher in the all co-culture systems of total lymphocytes and also T-cells with *L. chagasi*–infected macrophages. These results are similar to those obtained in other studies using cell cultures with whole lymphocytes (Holzmuller et al., 2005; Lemesre et al., 2007). Furthermore, it was reported that *L. infantum*–infected macrophages co-cultured with whole lymphocytes from dogs immunized with the *LiESAp* vaccine showed increased levels of IFN-γ in addition to reduction in the *in vitro* parasite load (Holzmuller et al., 2005; Lemesre et al., 2007). Vaccination of dogs also induces an increased percentage of T lymphocytes producing IFN-γ, especially CD4^{+} T-cells (Araújo et al., 2009; Araújo et al., 2011). Likewise, the levels of IFN-γ measured in our study were higher (~1000 pg/mL) in co-cultures using purified CD4^{+} T-cells than in those using CD8^{+} T-cells (~400 pg/mL). Moreover, co-cultures using PBMC and *L. chagasi*–infected macrophages from healthy dogs demonstrated a positive correlation between mRNA levels of IFN-γ/IL-4 and parasite load (Rodrigues et al., 2009). For all the co-culture ratios in this study, IL-4 and IL-10 levels were similar to those of the control group. However, there were significant reductions in IL-10 between the 1:1 and 1:2 co-cultures with CD4^{+} T-cells and the 1:5 and 1:2 co-cultures with CD8^{+} T-cells. Importantly, with the exception of the first studies (Pinelli et al., 1994; Pinelli et al., 1995; Pinelli et al., 1999) in which the co-cultures were performed with purified T-cells, the other studies in the literature (Holzmuller et al., 2005; Lemesre et al., 2007; Rodrigues et al., 2007; Rodrigues et al., 2009) were performed using whole lymphocytes. However, because the proposed co-culture system may be applied in detailed analysis of the canine immune response, it would be useful and highly relevant to employ purified T-cell subsets in this *in vitro* system.

The analysis of the overall performance of all co-cultures systems evaluated in the present investigation revealed that the methodology employing co-culture of *L. chagasi*–infected macrophages and purified CD4^{+} or CD8^{+} T-cell subsets at 1:5 and 1:2 ratios induced a microenvironment compatible with the expected status for the uninfected dogs analyzed in this study and might be useful tool to monitor the status of other dogs in the canine visceral leishmaniasis pre-clinical trials. It is important to mention that as *L. chagasi* and *L. infantum* have been considered synonymies, the proposed methodology should also be applicable to other regions where *L. infantum* is cited as etiological agent and not only in South America where most studies refer to *L. chagasi* as the species causing visceral leishmaniasis.

5. Conclusion

In conclusion, the results presented in this study suggested that the choice of optimal T-cells:MØ ratio is an essential issue when establishing *in vitro* tolls to assess the parasitological/immunological status of dogs, applicable in pre-clinical trials of vaccinology, prognosis follow-up and therapeutics analysis of canine visceral leishmaniasis. The proposed *in vitro* approach is able to yield laboratorial parameters useful to evaluate the ability of canine immune cells to control the *L. chagasi* infection *in vitro* as a biomarker for monitor their immunological status. The methodology employing co-culture of *L. chagasi*–infected macrophages and purified CD4^{+} or CD8^{+} T-cell subsets induced a microenvironment compatible with the expected status of the analyzed dogs. In this context, co-culture system with T-cells from uninfected dogs at 1:5 and 1:2 ratio did not control the infection, yielding to patent *in vitro* parasitism (≥80%), low NO production (≤5 μM) and IL-10 modulated (IFN-γ/IL-10≤2) immunological profile *in vitro*. CD4^{+} or CD8^{+} T-cells at 1:5 or 1:2 ratios to *L. chagasi*–infected macrophages seems to be ideal for *in vitro* assays, as this ratio was able to induce *in vitro* biomarkers profile related to the expected status of the dogs analyzed. This co-culture system seems to have great potential as a method to be incorporated in canine immunological analysis, applied to vaccine evaluations, prognosis follow-up and therapeutic schemes.

Competing interests

The authors declare that they have no competing interests.

Authors contributions

KFV participated in all aspects of the study; RDOAS, ABW and HGK participated in the implementation of co-cultures; LAR performed cytokine quantification; DSL performed the cell purification and analysis of flow cytometry; and RCOS, EMSF, RTF, WOD, MSA, OAMF, ABR, and RCG designed the study.

Acknowledgements

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