Application of rapid in vitro co-culture system of macrophages and T-cell subsets to assess the immunogenicity of dogs vaccinated with live attenuated *Leishmania donovani* centrin deleted parasites (LdCen −/−)

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**Abstract**

**Background:** Live attenuated *Leishmania donovani* parasites as LdCen−/− were shown to confer protective immunity against *Leishmania* infection in mice, hamsters, and dogs. Strong immunogenicity in dogs vaccinated with LdCen−/− has been previously reported, including increased antibody response favoring Th1 response lymphoproliferative responses, CD4⁺ and CD8⁺ T-cells activation, increased levels of Th1 and reduction of Th2 cytokines, in addition to a significant reduction in parasite burden after 18 and 24 months post virulent parasite challenge.

**Methods:** Aimed at validating a new method using in vitro co-culture systems with macrophages and purified CD4⁺ or CD8⁺ or CD4⁺:CD8⁺ T-cells of immunized dogs with both LdCen−/− and Leishmune* to assess microbicide capacity of macrophages and the immune response profile as the production of IFN-γ, TNF-α, IL-12, IL-4 and IL-10 cytokines.

**Results and discussion:** Our data showed co-cultures of macrophages and purified T-cells from dogs immunized with LdCen−/− and challenged with *L. infantum* were able to identify high microbicidal activity, especially in the co-culture using CD4⁺ T-cells, as compared to the Leishmune* group. Similarly, co-cultures with CD8⁺ T-cells or CD4⁺:CD8⁺ T-cells in both experimental groups were able to detect a reduction in the parasite burden in *L. infantum* infected macrophages. Moreover, co-cultures using CD4⁺ or CD8⁺ or CD4⁺:CD8⁺ T-cells from immunized dogs with both LdCen−/− and Leishmune* were able to identify higher levels of IFN-γ and IL-12 cytokines, reduced levels of IL-4 and IL-10, and a higher IFN-γ/IL-10 ratio. While the highest IFN-γ levels and IFN-γ/IL-10 ratio were the hallmarks of LdCen−/− group in the co-culture using CD4⁺ T-cells, resulting in strong reduction of parasitism, the Leishmune* immunization presented a differential production of TNF-α in the co-culture using CD4⁺:CD8⁺ T-cells.

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Conclusion: The distinct conditions of co-culture systems were validated and able to detect the induction of immune protection. The method described in this study applied a new, more accurate approach and was able to yield laboratory parameters useful to test and monitor the immunogenicity and efficacy of Leishmania vaccines in dogs.

Keywords: Vaccine, In vitro co-culture, CD4⁺ and CD8⁺ T-cells, Canine visceral leishmaniasis LdCen−/−, Leishmune*
Isolation of peripheral blood mononuclear cells
Blood samples (10 ml each) from the 17 dogs (control group: n = 6; Leishmune group: n = 5; LdCen group: n = 6) were collected in heparinized tubes intended for obtaining peripheral blood mononuclear cells (PBMC), as previously described [13]. Briefly, the whole blood volume collected was placed in a mixture of Ficoll-Hypaque (Sigma Chemical Co.; density: 1.119 g/ml) and Ficoll-Hypaque (Sigma Chemical Co.; density: 1.077 g/ml) at a 1:3 ratio (Ficoll/blood) in sterile polystyrene conical bottom tubes (Falcon, Corning, USA). All samples were centrifuged at 700 × g for 80 min at 22 °C. The PBMC ring was collected at the Ficoll-Hypaque interface and transferred to another tube with 40 ml of Falcon sterile 1× PBS containing 10 % FBS. This tube was centrifuged twice at 400 × g for 10 min at 4 °C. After the supernatant was discarded, the cells were resuspended in 1 ml of cell culture medium RPMI 1640. Cells were counted in a Neubauer hemocytometer chamber to determine the numbers of monocytes or lymphocytes per milliliter.

Microbicidal activity
The microbicidal activity was assessed using monocytes, which were adjusted and plated at 5 × 10⁵ monocytes/well using 24-well plates (NUNC, Thermo Fisher Scientific Inc., USA), on circular coverslips (15 mm; Glasscyto, Brazil), as previously described [13]. Briefly, cultures were established using RPMI supplemented with 20 % fetal calf serum on 20 % macrophage colony-stimulating factor medium obtained from supernatant of cultured L. amazonensis promastigotes in the stationary phase, using a 10:1 ratio (ten parasites per macrophage). Each well was gently washed 3 h after infection, and the cultures were maintained in co-culture with CD4⁺ or CD8⁺ T-cells or CD4⁺:CD8⁺ T-cells simultaneously to assess microbicidal activity at 72 h after infection. To calculate the rate of parasitic infection, the numbers of amastigotes in 200 macrophages were counted. Afterwards, the total number of amastigotes was divided by the total number of infected macrophages to obtain the average number of amastigotes per macrophage [12, 13].

Purification of CD4⁺ and CD8⁺ T-cells
After obtaining the PBMC by Ficoll-Hypaque gradient, lymphocytes were submitted to further purification, as previously described [12, 13]. CD4⁺ and CD8⁺ T-lymphocytes were isolated 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. Cell suspension was prepared at a concentration of 6 × 10⁷ cells in a 1-ml tube in an 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. Next, magnetic microbeads were added to 10 μl/ml lymphocytes and incubated for 15 min at RT. Cell suspension was loaded into 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 in the column, whereas the unlabeled cells run through, resulting in this cell fraction being depleted of CD4⁺ and CD8⁺ cells. After removing 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.

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 whole blood leukocytes that were 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 by gating 1 on the FSC versus SSC graph. 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 to encompass > 98 % of the 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 gate for T-cell surface markers presenting CD4⁺ or CD8⁺.

Co-culture system
After purification, the CD4⁺ and/or CD8⁺ T-cells were incubated with macrophage (Mac) previously infected with L. infantum promastigotes (10 parasites per macrophage) in the following proportions: to CD4⁺ T-cells: Mac - 1 lymphocyte for each 2 macrophages (1:2) and to CD8⁺ T-cells: ac - 1 lymphocyte for each 2 macrophages (1:2). The culture of CD4⁺:CD8⁺ T-cells simultaneously incubated under the same condition used ratios of 1:1:1 (CD4⁺:CD8⁺:Mac). Cells were grown in this co-culture system for 72 h, as previously described [12, 13]. Afterwards, the well supernatants were collected and stored at -80 °C for later cytokine measurements.

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). 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 an incubation buffer was added to each well, and streptavidin-HRP was used as an enzyme. The reaction was developed using 3,3′,5,5′-tetramethylbenzidine substrate and stopped by adding a 2.5 M H₂SO₄ solution. The plates were washed after each step using PBS plus 0.05 % (v/v) Tween 20. Minimum sensitivity levels were 119 pg/ml for IFN-γ, 109 pg/ml for TNF-α, 102 pg/ml for IL-12, 118,08 pg/ml for IL-4, and 89 pg/ml for IL-10. All experiments were performed using 96-well plates (COSTAR, Corning Inc., USA) and according to R&D Systems instructions. Reading was performed using a microplate automatic reader (EL800; Biotek, Winooski, VT, USA) at a wavelength of 450 nm.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.0 software (Prism Software, CA, USA). Data normality was assessed using the Kolmogorov-Smirnov test. The analyses of co-cultures and cytokines were performed using repeated ANOVA measurements. Differences were considered significant at P < 0.05.

Results

Immunization with LdCen⁻/⁻ attenuated parasites resulted in significant microbicidal activity of macrophages co-cultured with CD4⁺ T-cells associated with increased levels of Th1 cytokines

The performance of the co-culture system was evaluated, including CD4⁺ T-cells, to analyze the contribution of this cell subpopulation in the immunogenicity establishment. After 3 h of in vitro infection of macrophages with L. infantum, macrophages from the LdCen⁻/⁻ group showed a lower (P = 0.049) frequency of infection (Fig. 1a) as compared to the control group. The parasite burden was reduced in macrophages in both LdCen⁻/⁻ (P = 0.0009) and Leishmune⁺ groups (P = 0.005) compared to the control (Fig. 1b). Interestingly, 72 h after in vitro infection, LdCen⁻/⁻ group displayed an intense reduction (P = 0.012) in parasite burden as compared to the Leishmune⁺ group (Fig. 1c). LdCen⁻/⁻ (P = 0.008) and Leishmune⁺ (P = 0.005) immunizations induced high levels of cytokines IFN-γ (Fig. 1d), TNF-α (Fig. 1e) and IL-12 (Fig. 1f) and low levels of IL-4 (Fig. 1g) and IL-10 (Fig. 1h) in co-cultures as compared to the control group. Remarkably, the LdCen⁻/⁻ presented high levels of IFN-γ when compared to the Leishmune⁺ group (Fig. 1d). Furthermore, both analyzed vaccines presented a high IFN-γ/IL-10 ratio as compared to the control group, with LdCen⁻/⁻ displaying the highest (P = 0.006) cytokine ratio (Fig. 1i).

LdCen⁻/⁻ and Leishmune⁺ immunized dogs induced an intense microbicidal activity of macrophages co-cultured with CD8⁺ T-cells and increased levels of pro-inflammatory cytokines

Since the CD8⁺ T-lymphocytes act as effector cells to control intracellular pathogens, this T-cell subset was included in the co-culture system to analyze the induction of memory. LdCen⁻/⁻ (P = 0.010) and Leishmune⁺ (P = 0.009) groups presented significant reduction in the number of amastigotes in macrophages co-cultured with CD8⁺ T cells as compared to the control group (Fig. 2c). All analyzed pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-12; Fig. 2d-f) presented high levels in co-cultures of both LdCen⁻/⁻ and Leishmune⁺ groups as compared to the control group. In contrast, LdCen⁻/⁻ (P = 0.018) and Leishmune⁺ (P = 0.034) groups displayed lower levels of IL-4 (Fig. 2g) and IL-10 (Fig. 2h) as compared to the control group. Interestingly, LdCen⁻/⁻ (P = 0.006) and Leishmune⁺ (P = 0.012) groups showed increased IFN-γ/IL-10 ratio, in addition to LdCen⁻/⁻ immunization eliciting the highest (P = 0.008) cytokine ratio (Fig. 2i).

The co-culture system using macrophages plus CD4⁺:CD8⁺ T-cells simultaneously revealed high anti-leishmanicidal activity, increased levels of pro-inflammatory cytokines, and a reduction of IL-4 and IL-10 in both LdCen⁻/⁻ and Leishmune⁺ groups

The co-culture system using macrophages associated with CD4⁺: CD8⁺ T-cells simultaneously was employed to analyze the additive effect of adaptive immune response in the context of T-cells subsets. In this sense, LdCen⁻/⁻ (P = 0.001) and Leishmune⁺ (P = 0.007) immunizations induced a reduction in the parasite burden at 72 h post infection with L. infantum as compared with the control group (Fig. 3c). The cytokine analysis revealed high levels of IFN-γ (Fig. 3d), TNF-α (Fig. 3e) and IL-12 (Fig. 3f) in both LdCen⁻/⁻ and Leishmune⁺ groups when compared to control group. Moreover, the Leishmune⁺ immunization was able to induce high amounts of TNF-α as compared to the LdCen⁻/⁻ group (P = 0.042). Furthermore, both analyzed vaccines showed low levels of IL-4 (Fig. 3d) and IL-10 (Fig. 3h). Similar to co-cultures using purified CD4⁺ or CD8⁺ T-cells, the coculture system using CD4⁺:CD8⁺ T-cells simultaneously was able to identify in LdCen⁻/⁻ and Leishmune⁺ groups a high (P = 0.016) IFN-γ/IL-10 ratio, plus the LdCen⁻/⁻ group displayed the highest cytokine ratio (Fig. 2i).
Co-Culture System of Purified CD4+ T-cells with \textit{L. infantum}-infected Macrophages

**Analysis of \textit{in vitro} efficacy**

**A** Frequency of infected MØ

**B** MØ Parasite Burden (3h)

**C** MØ Parasite Burden (72h)

**D** IFN-\(\gamma\)

**E** TNF-\(\alpha\)

**F** IL-12

**G** IL-4

**H** IL-10

**I** IFN-\(\gamma\)/IL-10 ratio

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**Experimental groups**

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**Fig. 1** Co-culture system using \textit{L. infantum} infected macrophages (MØ) and CD4+ T-cells, 24 months after experimental challenge with \textit{L. infantum}. Control group (\(n=6\) dogs) and Leishmune\(^*\) (\(n=5\) dogs) received three doses of PBS or vaccine, respectively, at 21 day intervals; LdCen\(\text{−/−}\) group (\(n=6\) dogs) received \(10^7\) LdCen\(\text{−/−}\) stationary phase promastigotes in single-dose. \(a\) Infection rate (\%) of macrophages after 3 h of in vitro infection with \textit{L. infantum} (5 \(\times\) \(10^6\) promastigotes; ratio ten parasites: 1 MØ). \(b\) Number of amastigote forms by macrophages after 3 h of in vitro infection with \textit{L. infantum}. \(c\) Number of amastigote forms by macrophages after 72 h of in vitro infection with \textit{L. infantum}. The cytokine profile (pg/mL) was analyzed in supernatant from \textit{L. infantum} infected macrophages (MØ) co-cultured with CD4+ T-cells for IFN-\(\gamma\) (\(d\)), TNF-\(\alpha\) (\(e\)), IL-12 (\(f\)), IL-4 (\(g\)), IL-10 (\(h\)) and IFN-\(\gamma\)/IL-10 ratio (\(i\)). The x-axis represents the control group (C), Leishmune\(^*\) and LdCen\(\text{−/−}\) vaccines. The connecting lines between the bars indicate significant differences (\(P<0.05\)).
Co-Culture System of Purified CD8⁺ T-cells with *L. infantum*-infected Macrophages

Analysis of *in vitro* efficacy

**A** Frequency of infected MØ

**B** MO Parasite Burden (3h)

**C** MO Parasite Burden (72h)

**D** IFN-γ

**E** TNF-α

**F** IL-12

**G** IL-4

**H** IL-10

**I** IFN-γ/IL-10 ratio

**Experimental groups**

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**Fig. 2** Co-culture system using *L. infantum* infected macrophages (MØ) and CD8⁺ T-cells; 24 months after experimental challenge with *L. infantum*. Control group (*n* = 6 dogs) and Leishmune® (*n* = 5 dogs) received three doses of PBS or vaccine, respectively, at 21 day intervals; LdCen⁺ group (*n* = 6 dogs) received 10⁷ LdCen⁺ stationary phase promastigotes in single-dose. **a** Infection rate (%) of macrophages after 3 h of in vitro infection with *L. infantum* (5 × 10⁶ promastigotes; ratio ten parasites: 1 MØ). **b** Number of amastigote forms by macrophages after 3 h of in vitro infection with *L. infantum*. **c** Number of amastigote forms by macrophages after 72 h of in vitro infection with *L. infantum*. The cytokine profile (pg/mL) was analyzed in supernatant from *L. infantum* infected macrophages co-cultured with CD8⁺ T-cells. **d** IFN-γ, **e** TNF-α, **f** IL-12, **g** IL-4, **h** IL-10 and **i** IFN-γ/IL-10 ratio. The x-axis represents the control group (C), Leishmune® and LdCen⁺/⁻ vaccines. The connecting lines between the bars indicate significant differences (*P* < 0.05).
Co-Culture System of Purified CD4⁺:CD8⁺ T-cells with *L. infantum*-infected Macrophages

**Analysis of *in vitro* efficacy**

A. Frequency of infected MØ

B. MØ Parasite Burden (3h)

C. MØ Parasite Burden (72h)

**Cytokine Profile**

D. IFN-γ

E. TNF-α

F. IL-12

G. IL-4

H. IL-10

I. IFN-γ/IL-10 ratio

Experimental groups

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**Fig. 3** Co-culture system using *L. infantum* infected macrophages (MØ) and CD4⁺:CD8⁺ T-cells, 24 months after experimental challenge with *L. infantum*. Control group (n = 6 dogs) and Leishmune® (n = 5 dogs) received three doses of PBS or vaccine, respectively, at 21 day intervals; LdCen*−/−* group (n = 6 dogs) received 10⁷ LdCen*−/−* stationary phase promastigotes in single-dose. **a** Infection rate (%) of macrophages after 3 h of in vitro infection with *L. infantum* (5 × 10⁶ promastigotes; ratio ten parasites: 1 MØ). **b** Number of amastigote forms by macrophages after 3 h of in vitro infection with *L. infantum*. **c** Number of amastigote forms by macrophages after 72 h of in vitro infection with *L. infantum*. The cytokine profile (pg/mL) was analyzed in supernatant from *L. infantum* infected macrophages (MØ) co-cultured with CD4⁺:CD8⁺ T-cells for IFN-γ (d), TNF-α (e), IL-12 (f), IL-4 (g), IL-10 (h) and IFN-γ/IL-10 ratio (i). The x-axis represents the control group (C), Leishmune® and LdCen*−/−* vaccines. The connecting lines between the bars indicate significant differences (P < 0.05).
Discussion

This study applied previously standardized protocols [12, 13] of co-culture systems using *L. infantum*-infected macrophages and purified CD4⁺ or CD8⁺ T-cell subsets from immunized dogs, 24 months after experimental challenge with a virulent strain of *L. infantum*. This method was previously developed to analyze vaccine candidates against canine visceral leishmaniasis [12, 13]. LdCen⁺/− and Leishmune⁺ vaccines were used as they have demonstrated a strong immunogenicity [10, 11, 14–18].

In this sense, it has been described that immunization with LdCen⁺/− in dogs elicited high levels of serum immunoglobulins, a lymphoproliferative response, and T-cell activation, which increased production of Th1 cytokines and decreased secretion of Th2 cytokines [14]. Furthermore, it was also shown that LdCen⁺/− and Leishmune⁺ immunizations resulted in significant protection, as revealed by the low parasite burden in the bone marrow at 18 months and even 24 months post challenge [11, 14]. The parasite burden level observed in the LdCen⁺/− and Leishmune⁺ immunized group is comparable to that typically observed in asymptomatic dogs, which are PCR positive [19], suggesting a robust degree of protection. These data support the application of LdCen⁺/− and Leishmune⁺ immunizations in the current evaluation as reference vaccines to validate the proposed co-culture system.

Most studies involving co-culture of macrophages and lymphocytes are empirically developed, using total lymphocytes, and do not reflect the cytokine levels of the T-cells. The method described in this study applied a new, more accurate approach that yields useful laboratory parameters to evaluate the ability of canine immune cells to control in vitro *L. infantum* infection as a biomarker to identify the immunogenicity mechanism.

Traditional immunogenicity studies in vaccines against CVL, such as Leishvacin or Leishmune⁺, have described the biomarkers regarding increased percentage of T lymphocytes producing IFN-γ, especially CD4⁺ T-cells [15, 16]. Moreover, LiESAp-MDP vaccine elicited protection that was correlated with the early establishment of a long-lasting, predominantly Th1-type cellular immune response relationship to enhanced in vitro leishmanicidal activity of macrophages in response to higher IFN-γ production by T-cells [20]. Recently, a study evaluating the LiESP/QA-21 (CaniLeish⁺) vaccine demonstrated strong cell-mediated immune responses against the parasite despite a virulent challenge associated with a lower probability of developing active infections [21]. Furthermore, tests for microbicidal activity of macrophages cultured with autologous total lymphocytes showed that the vaccinated group inhibited twice the parasite replication in comparison with the control group [21]. However, the ideal method for identifying the protective mechanisms induced by vaccine against canine visceral leishmaniasis would analyze the effector immune response. In this sense, the leishmanicidal effect could be evaluated as an effector mechanism induced by T-cells subsets co-cultured with *L. infantum* infected macrophages.

Our data demonstrated that the three co-culture systems (*L. infantum* infected macrophages with purified CD4⁺ or CD8⁺ T-cells or CD4⁺:CD8⁺ T-cells simultaneously) from LdCen⁺/− and Leishmune⁺ were able to identify a significant reduction in the parasitic burden. Noteworthy is the fact that the co-culture system containing a purified CD4⁺ T-cell subset was able to recognize a differential capacity of LdCen⁺/− vaccine to induce a strong in vitro leishmanicidal control when compared to Leishmune⁺ immunization. In fact, the strong leishmanicidal ability could be associated with differential IFN-γ production in addition to a higher IFN-γ/IL-10 ratio, as presented by the co-culture system containing the purified CD4⁺ T-cell subset. In previous studies regarding asymptomatic *L. infantum*-infected dogs, co-cultures with CD8⁺ T-cells and infected-macrophages were able to produce high levels of IFN-γ, whereas cells from symptomatic dogs did not [22].

Type 1 cytokine analysis has been considered a prerequisite for immunogenicity analyses before and after experimental challenge with *L. infantum* in clinical anti-CVL vaccine trials [6, 12]. In fact, the co-culture system proposed herein was able to identify the type 1 cytokine pattern, in agreement with an earlier studies for both LdCen⁺/− and Leishmune⁺ immunizations [11, 14]. Moreover, Leishmune⁺ immunization elicited high TNF-α production in a co-cultured system with CD4⁺:CD8⁺ T-cells simultaneously, but without effect in producing differential parasitism control when compared to LdCen⁺/−. Interestingly, IFN-γ, but not TNF-α levels, was associated with immunogenicity and protection in dogs immunized with LBsap vaccine [23]. Furthermore, it was reported that dogs immunized with purified excreted-secreted antigens of *Leishmania infantum* presented high levels of IFN-γ in co-culture with *L. infantum* infected macrophages and total lymphocytes, inducing a microbicidal effect [24, 25].

Considering the previous studies describing strong immunogenicity elicited by LdCen⁺/− and Leishmune⁺ immunizations, this study applied a new approach based on a co-culture system using *L. infantum* infected macrophages and purified T-cell subsets after vaccination and experimental *L. infantum* challenge. Data from this study indicated that the co-culture system was able to identify the immunogenicity mechanism induced by LdCen⁺/− and Leishmune⁺ vaccination in dogs, in addition to associating an effector mechanism (in vitro leishmanicidal control by *L. infantum* infected macrophages) regarding an induction of T-cell memory.
Conclusions
The low levels of type 2 cytokines (IL-4 and IL-10), high amounts of type 1 cytokines (IFN-γ, TNF-α, and IL-12), and increased IFN-γ/IL-10 ratio were associated with a leishmanicidal effect of L. infantum infected macrophages, using co-culture systems with CD4+ or CD8+ or CD4+CD8+ T-cells simultaneously. This strategy was able to validate the distinct conditions of the analyzed co-culture systems. Thus, taken together, the data point to the in vitro co-culture systems providing a rapid assay to test and monitor the immunogenicity and efficacy of Leishmania vaccines in dogs.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Conceived and designed the experiments: KFV, DSL, and LLB. Analyzed the data: KFV, DSL, LLB, RGG, and RCG. Contributed reagents/materials/analysis tools: SG, RD, AS, DCB, DSL, LLB, WOD, RTF, HLN, and RCG. Wrote the paper: KFV, DSL, LLB, AND RCG. All authors read and approved the final version of the manuscript.

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References
1. Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis. 2004;27:305–18.
2. Deane LM, Deane MP. Visceral leishmaniasis in Brazil: geographical distribution and transmission. Rev Inst Med Trop Sao Paulo. 1962;4:198–212.
3. Costa CHN. How effective is dog culling in controlling zoonotic visceral leishmaniasis? A critical evaluation of the science, politics and ethics behind this public health policy. Rev Soc Bras Med Trop. 2011;44:232–42.
4. Coura-Wal W, Marques MJ, Veloso VM, Roat BM, Aguiar-Soares RDO, Reis LE, Braga SL, Moraes MH, Reis AB, Carneiro M. Prevalence and factors associated with Leishmania infection of dogs from an urban area of Brazil as identified by molecular methods. PLoS Negl Trop Dis. 2011;5(8):e1291.
5. Dye C. The logic of visceral leishmaniasis control. Am J Trop Med Hyg. 1996;55:125–30.
6. Reis AB, Giunchetti RC, Carrillo E, Martins-Filho OA, Moreno J. Immunity to and the rational search for vaccines against canine leishmaniasis. Trends Parasitol. 2010;26:341–9.
7. Giunchetti RC, Correa-Oliveira R, Martins-Filho O, Teixeira-Carvalho A, Roat BM, Aguiar-Soares RDO, de Souza JV, de Dores Moreira N, Malaquias LC, Mota e Castro LL, de Lana M, Reis AB. Immunogenicity of a killed Leishmania vaccine with saponin adjuvant in dogs. Vaccine. 2007;25:764–72.
8. Giunchetti RC, Reis AB, da Silveira-Lemos D, Martins-Filho OA, Corrêa-Oliveira R, Bethony J, Vale AM, da Silva Quetz J, Bueno LL, França-Silva JC, Nascimento E, Mayrink W, Fujisawa R. Antigenicity of a whole parasite vaccine as promising candidate against canine leishmaniasis, Res Vet Sci. 2008;85:106–12.
9. Reat BM, Aguir-Sooares RD, Vitorino-Souza J, Coura-Vital W, Braga SL, Correa-Oliveira R, Martins-Filho OA, Teixeira-Carvalho A, de Lana M, Figueiredo-Gontijo N, Marques MJ, Giunchetti RC, Reis AB. Performance of LBPsap vaccine after intradermal challenge with L. infantum and saliva of Lu. longipalpis: immunogenicity and parasitological evaluation. PLoS One. 2012;7(11):e49780.
10. Selvapandian A, Dey R, Nylen S, Duncan R, Sacks D, Nakhasi HL. Intracellular replication-deficient Leishmania donovani induces long lasting protective immunity against visceral leishmaniasis. J Immunol. 2009;183:1813–20.
11. Giunchetti RC, Correa-Oliveira R, Martins-Filho OA, Teixeira-Carvalho A, de Lana M, Figureiredo-Gontijo N, Marques MJ, Giunchetti RC, Reis AB. Setting the proportion of CD4+ and CD8+ T-cells co-cultured with canine macrophages infected with Leishmania chagasi. Vet Parasitol. 2015;198:62–71.
12. Viana KF, Aguiar-Soares RDO, Roat BM, Resende LA, Souza-Enguandem EM, Dutra-WO, Fujikawa RT, da Silveira-Lemos D, Wardini AB, Araújo MS, Martins-Filho OA, Reis AB, Giunchetti RC. Setting the proportion of CD4+ and CD8+ T-cells co-cultured with canine macrophages infected with Leishmania chagasi. Vaccine. 2014;33:280–8.
13. Viana KF, Aguiar-Soares RDO, Roat BM, Resende LA, Silveira-Lemos D, Corrêa-Oliveira R, Martins-Filho OA, Moura SL, Zaninni MS, Araújo MS, Reis AB, Giunchetti RC. Analysis using canine peripheral blood for establishing in vitro conditions for monocyte differentiation into macrophages for Leishmania chagasi infection and T-cell subset purification. Vet Parasitol. 2013;196:62–71.
14. Giunchetti RC. Analysis using canine peripheral blood for establishing in vitro conditions for monocyte differentiation into macrophages for Leishmania chagasi infection and T-cell subset purification. Vet Parasitol. 2013;196:62–71.
15. Araújo MS, De Andrade RA, Sathler-Avelar R, Magalhães CP, Cavalo RT, Andrade MC, Campolina SS, Mello MN, Vanina LR, Mayrink W, Reis AB, Malaquias LC, Rocha LM, Martins-Filho OA. Immunological changes in canine peripheral blood leukocytes triggered by immunization with first or second generation vaccines against canine visceral leishmaniasis. Vet Immunol Immunopathol. 2011;144:64–75.
16. Araújo MS, De Andrade RA, Sathler-Avelar R, Teixeira-Carvalho A, Andrade MC, Viana KF, Giunchetti RC, Martins-Filho OA, Araújo MS. One-year timeline kinetics of cytokine-mediated cellular immunity in dogs vaccinated against visceral leishmaniasis. BMC Vet Res. 2015;11:92.
17. Costa-Pereira C, Moreira ML, Soares RP, Martelohe BH, Ribeiro VM, França-Dias MH, Cardoso LM, Viana KF, Giunchetti RC, Martins-Filho OA, Araújo MS. One-year timeline kinetics of cytokine-mediated cellular immunity in dogs vaccinated against visceral leishmaniasis. BMC Vet Res. 2015;11:92.
18. Fernandes CB, Junior JT, de Jesus C, Souza BM, Laranjeira DF, Fraga DB, Tavares VD, Parouin-Melo SM. Comparison of two commercial vaccines against visceral leishmaniasis in dogs from endemic areas, IgG, and subclasses, parasitism, and parasite transmission by xenodiagnosis. Vaccine. 2014;32:1287–95.
19. Reis AB, Teixeira-Carvalho A, Giunchetti RC, Roat BM, Coura-Vital W, Nicolato Rde C, Silveira-Lemos D, Correa-Oliveira R, Martins-Filho OA. Cellular immunophenotypic profile in the splenic compartment during canine visceral leishmaniasis. Vet Immunol Immunopathol. 2014;157:190–4.
20. Bourdouze C, Huguet C, Goncalves RB, Vézilier F, Petit-Didier E, Papierok G, Lemere JL. Effective humoral and cellular immunoprotective response in Leishmania infantum vaccinated protected dogs. Vet Immunol Immunopathol. 2009;128:71–8.
21. Martin V, Vouloudakis I, Moreno J, Mc Gahe D, Gueguen S, Cusinier AM. The protective immune response produced in dogs after primary vaccination with the LIESP/QA-21 vaccine (CanLeish®) remains effective against an experimental challenge one year later. Vet Res. 2014;45:69.
22. Pinelli E, Gonzalo RM, Boog CJ, Rutten VP, Giebhard D, del Real G, Ruitenbier EJ. Leishmania infantum-specific T cell lines derived from
asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner. Eur J Immunol. 1995;25:1594–600.

23. Resende LA, Roatt BM, Soares RDA, Viana KF, Mendonça LZ, Lanna MF, Silveira-Lemos D, Correa-Oliveira R, Martins-Filho OA, Fujiwara RT, Carneiro CM, Reis AB, Giunchetti RC. Cytokine and nitric oxide patterns in dogs immunized with LBSap vaccine, before and after experimental challenge with Leishmania chagasi plus saliva of Lutzomyia longipalpis. Vet Parasitol. 2013;198:371–81.

24. Holzmüller P, Cavaleyra M, Moreaux J, Kovaric R, Vincendeau P, Papierok G, Lemesre JL. Lymphocytes of dogs immunised with purified excreted-secreted antigens of Leishmania infantum co-incubated with Leishmania infected macrophages produce IFN gamma resulting in nitric oxide-mediated amastigote apoptosis. Vet Immunol Immunopathol. 2005;106:247–57.

25. Lemesre JL, Holzmüller P, Gonçalves RB, Bourdoiseau G, Hugnet C, Cavaleyra M, Papierok G. Long-lasting protection against canine visceral leishmaniasis using the LiESAp-MDP vaccine in endemic areas of France: double-blind randomised efficacy field trial. Vaccine. 2007;25:4223–34.

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