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Short communication

IFN-γ expression is up-regulated by peripheral blood mononuclear cells (PBMC) from non-exposed dogs upon *Leishmania chagasi* promastigote stimulation *in vitro*

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ABSTRACT

While the response to *Leishmania* spp. is well characterized in mice and humans, much less is known concerning the canine immune response, particularly soon after exposure to the parasite. Early events are considered to be a determinant of infection outcome. To investigate the dog's early immune response to *L. chagasi*, an *in vitro* priming system (PIV) using dog naïve PBMC was established. Until now, dog PIV immune response to *L. chagasi* has not been assessed. We co-cultivated PBMC primarily stimulated with *L. chagasi in vitro* with autologous infected macrophages and found that IFN-γ mRNA is up-regulated in these cells compared to control unstimulated cells. IL-4 and IL-10 mRNA expression by *L. chagasi*-stimulated PBMC was similar to control unstimulated PBMC when incubated with infected macrophages. Surprisingly, correlation studies showed that a lower IFN-γ/IL-4 expression ratio correlated with a lower percentage of infection. We propose that the direct correlation between IFN-γ/IL-4 ratio and parasite load is dependent on the higher correlation of both IFN-γ and IL-4 expression with lower parasite infection. This PIV system was shown to be useful in evaluating the dog immune response to *L. chagasi*, and results indicate that a balance between IFN-γ and IL-4 is associated with control of parasite infection *in vitro*.

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1. Introduction

Most cases of leishmaniasis are zoonotic infections caused by *Leishmania* spp.; however, humans can also be part of the parasite's life cycle. Human disease is endemic and highly prevalent in tropical and subtropical regions of the world. Clinical manifestations range from cutaneous ulcers to a potentially fatal visceral disease. *L. donovani* and *L. infantum* cause visceral leishmaniasis (VL) in Africa, India, and Europe, and *L. chagasi* causes VL in Latin America. Dogs are natural hosts and the main reservoir of the parasite (Moreno and Alvar, 2002). These animals represent an appropriate model for the study of leishmaniasis, as the prevalence of canine disease is very high and

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canine visceral leishmaniasis (CVL) is similar to the human disease in several aspects (Nieto et al., 1999). Less than 50% of infected dogs present with signs of severe disease (Berrahal et al., 1996; Lanotte et al., 1979).

The nature of the dog's cellular immune response is not fully understood. Dogs experimentally infected with intradermal inoculation of promastigotes develop asymptomatic infections, and PBMC from these dogs stimulated *in vitro* with soluble leishmanial antigens (SLA) express both Th1 cytokines such as IL-12, IFN-γ, TNF-α, and IL-18 and Th2 cytokines such as IL-4, IL-6, and IL-10. Despite the fact that PBMC from these asymptomatic dogs present such apparently mixed Th1 and Th2 responses, they predominantly display IL-12 and IFN-γ production. In accordance with a previous observation (Pinelli et al., 1994), these data support the protective immune response observed in these animals (Chamizo et al., 2005). On the other hand, dogs experimentally infected with intravenously inoculated amastigotes develop progressive symptomatic infections. PBMC from these dogs produce reduced levels of both Th1 and Th2 cytokines (IFN-γ, IL-2, IL-12, IL-6, and IL-10) in the active phase of the disease (Santos-Gomes et al., 2002). Finally, PBMC from dogs naturally infected with *L. chagasi* display enhancement of IFN-γ expression, which is positively correlated with the humoral but not with the cell immune response to SLA. These results were observed without an increase in mRNA expression for IL-4, IL-10, and IL-18, although a small proportion of these animals display PBMC IL-4 expression related to disease severity (Quinnell et al., 2001).

In vitro priming of naïve mouse splenocytes and human PBMC has been widely used to characterize the early immune response to *Leishmania* spp. (Shankar and Titus, 1993; Soares et al., 1997; Pompeu et al., 2001; Rogers and Titus, 2004). The analysis of the innate response of naïve dogs to *Leishmania* may contribute to the current understanding of the early cellular events triggered by the parasite infection. The use of this *in vitro* assay, which may be capable of predicting canine response to *L. chagasi* or *L. chagasi* antigen stimulation, would be helpful for vaccine design and development (Holzmuller et al., 2005). In addition, the PIV system will facilitate extensive *in vivo* tests that are used to evaluate the efficacy of a dog vaccine candidate (Gradoni, 2001). The present work aimed to establish a PIV system that can discriminate between protective and non-protective immune responses. We detected that IFN-γ mRNA is up-regulated in these cells compared to control unstimulated cells. IL-4 and IL-10 expression by *L. chagasi*-stimulated PBMC was similar to that by control unstimulated PBMC. Correlation studies showed that both higher IFN-γ and IL-4 expressions correlated with lower parasite infection.

2. Materials and methods

2.1. Animals

Five male healthy adult dogs, belonging to different breeds and ranging from 3 to 10 years of age, were used as a source of PBMC. In clinical and serological examinations, animals displayed no sign of *Leishmania* infection. All animals were vaccinated yearly against rabies, distemper, parvovirosis infections, hepatitis, leptospirosis, parainfluenza virus, coronavirus, and adenovirus type 2. These dogs were closely monitored by a veterinarian for health problems, as recently published (Rodrigues et al., 2007). All experiments were performed in accordance to the standards of the Oswaldo Cruz Foundation guidelines and the Committee of Ethics on Animal Experimentation (CEUA-CPqGM/FIOCRUZ).

2.2. Parasites

The strain of *L. chagasi* (MHOM/BR00/MER02), originally isolated from a Brazilian patient with VL, was maintained by serial passages through hamsters. Parasite isolation and cultivation to obtain promastigotes *in vitro* were performed as previously described (Rodrigues et al., 2007). Promastigotes at the stationary phase of growth were used for macrophage infection and PBMC stimulation *in vitro*.

2.3. Primary in vitro stimulation

The results presented herein comprise five experiments, and for each experiment, a distinct healthy PBMC dog donor was used ($n = 5$). PBMC were obtained from heparinized peripheral blood, were layered over Ficoll–Paque gradient (Pharmacia Biotech, Uppsala, Sweden) and then resuspended at a concentration of 5×10^6 cells/ml in RPMI-1640 supplemented with 2 mM L-glutamine (Gibco BRL, New York, USA), 20 mM HEPES (N-2-hydroxyethylpiperazine-N0-2-ethanesulphonic acid), 100 UI/ml penicillin, 100 μg/ml streptomycin, 2 g/l sodium bicarbonate, and 10% FBS (Gibco BRL) (RPMI complete medium). PBMC were stimulated with one stationary-phase *L. chagasi* promastigote per PBMC for 6 days. Half of the PBMC culture medium was replaced by fresh RPMI complete medium every day during 6 days of *L. chagasi* stimulation to avoid loss of cell viability (Berger, 1979). In parallel, autologous PBMC from the same five donors were cultivated in 24-well plates containing 12 mm-glass coverslips for 5 days to obtain peripheral blood monocyte-derived macrophages, as previously described (Rodrigues et al., 2007). Five days later, macrophage cultures were infected with 10-stationary-phase *L. chagasi* promastigotes per macrophage (10:1 ratio) and used as APCs in the co-cultures as described next. Twenty-four hours after infection, non-adherent cells from the parallel cultures (*L. chagasi*-stimulated PBMC) were recovered and adjusted to a concentration of 2×10^6 cells/ml, added to the 24-h infected macrophages, and then cultivated in RPMI complete medium supplemented with 20% of PBMC supernatant. Co-cultures were maintained for additional 2 and 4 days without replacement of the culture medium. At the end of the incubation period, only non-adherent PBMC were harvested and centrifuged for 15 min at $300 \times g$, and supernatants were collected and stored at $-70\,^{\circ}$C until tested for cytokine content by ELISA as previously described (Caldas et al., 2005; Rodrigues et al., 2007) and for the concentration of nitrite (NO_2^-) by the Griess reaction (Green et al., 1982). In PIV stimulation

assays, cytokine release in cell supernatant is very low. Therefore, PBMC co-cultured for 4 days were harvested for RNA extraction in order to determine cytokine expression by quantitative real-time RT-PCR (qRT-PCR) (Hein et al., 2001). Cytokine expression was determined in PBMC extract of four out of five dog donors. After 2 and 4 days of co-culture, adherent macrophages grown in quadruplicate were fixed with 100% methanol and stained by hematoxilin and eosin (H&E) for microscopic observations. Control PBMC from each dog donor were cultivated in RPMI complete medium in the presence of ConA (2 μg/ml) for 48 h (positive control) or in RPMI medium alone (negative control) during the 6 days of the stimulation period. In all experiments, PBMC harvested at the end of the first and second courses of stimulation presented 98–99% viability as determined by trypan blue exclusion.

2.4. Effect of PBMC stimulation on macrophage infection

The effect of co-incubation with *L. chagasi*- or ConA-stimulated PBMC compared to unstimulated PBMC from five dog donors on autologous macrophage infection was investigated. To determine parasite load, non-adherent cells from each dog donor ($n = 5$) were washed out, infected with autologous macrophages, plated in quadruplicate, and then fixed and stained by H&E. The percentage of infected cells and the number of parasites per cell were determined by quantitative microscopic observations of at least 400 cells per coverslip. Since the data presented a normal distribution, determined by the Kolmogorov–Smirnov (KS) test, the results were expressed as the individual mean values $\pm$ S.E. of the parasite number per 100 macrophages. This represents the number of parasites per infected cell corrected by the percentage of infected cells (parasite number per 100 macrophages = parasite number per infected macrophage $\times$ % infected cells).

2.5. Determination of nitrite accumulation

The concentration of NO_2^- released by macrophages as determined by the Griess reaction was used as an indicator of nitric oxide (NO) production. Equal volumes of cell culture medium were mixed with Griess reagents (1% sulfanilamidde, 0.1% naphthylethylenedamine, and 2.5% H_3PO_4) (Green et al., 1982). This mixture was distributed in a 96-well plate, and the OD_{570} was estimated in a Molecular Device 96-well microplate reader. The standard curve used $NaNO_2$ at successive dilutions from 200 to 1 μM as a reference.

2.6. Quantification of canine IFN-γ, IL-4 and IL-10 mRNA

The primary *L. chagasi*-stimulated PBMC co-cultivated for 4 days with infected macrophages were harvested to determine IFN-γ, IL-4 and IL-10 mRNA expression by qRT-PCR. Total PBMC RNA from dogs 2 to 5 ($n = 4$) were extracted using Trizol according to the manufacturer's recommendations (Life Technologies, Gaithersburg, USA). After synthesis of complementary DNA (cDNA) as previously described (Rodrigues et al., 2007), qRT-PCR was performed in a 20 μl reaction volume including 10 μl of SYBR® Green PCR Master MIX (Applied Biosystems, California, USA), 200 nM of each primer, and 5 μl of cDNA made up to 20 μl with nuclease free water. All reactions were performed in optical 96-well reaction plates on the ABI Prism 7500 (Applied Biosystems, Foster City, USA). For each gene of interest, the reaction was performed in duplicate, with triplicate negative controls in each plate. The primer pair sequences and the thermal cycle conditions for each gene amplification are described in Table 1. To eliminate the possibility of genomic DNA contamination of RNA samples, a control reverse transcription was performed without enzyme addition (Manna et al., 2006), and these samples did not amplify any of the genes tested. Cycle threshold value was calculated for each sample. Standard curves in triplicate were constructed by serial dilution (1:5) starting from a fivefold concentrated pool of cDNA samples. Cytokine mRNA concentrations of each sample were calculated based on the standard curve, normalized to the concentration of the housekeeping gene 18S rRNA, and expressed as $\log_2$ of control, *L. chagasi*- or ConA-stimulated-PBMC mRNA.

2.7. Statistical analyses

To evaluate the statistical significance of the effect of PBMC stimulation on macrophage infection among experimental and control groups, one-way ANOVA was used followed by Newman–Keuls' multiple comparison post-test for parametric comparisons. Since cytokine expressions are not normally distributed, data were transformed to $\log_2$ and expressed as median and confident intervals. The non-parametric Friedman and Dunn's tests were chosen to test whether differences in cytokine expression occur by chance. For correlation analyses between the percentage of infected cells and the cytokine expression by PBMC, the Pearson's test was used. The tests were performed using GraphPad Prism, version 4.00, for Windows (GraphPad Software, San Diego, CA). Differences were considered significant when $p < 0.05$.

Table 1
Primer sequences, qPCR conditions and sizes of qPCR products.

Primer set	Forwad primer (5′–3′)	Reverse primer (5′–3′)	Annealing temperature (°C)	Cycle numbers	Product size (bp)
IFN-γ	AGCAGCACCAGTAAGAGGGAGGACTT	TCAAATATTGCAGGCAGGATGACCA	55	50	208
IL-4	AAAGAACACAAGCGATAAGGAA	TTCAGTGTACTCTTCTTGATTTCATT	55	50	165
IL-10	GAGCAGGTGAAGAGCGCATTTAGTA	GCCATCCTGGGTGTTTTGTTCTCCA	55	50	152
18S	CACGGCCGGTACAGTGAAAC	CCCGTCGGCATGTATTAGCT	60	40	119

3. Results and discussion

In order to mimic the first events of *L. chagasi* and dog PBMC interaction, the PIV stimulation assay was established in two steps, as previously described in mouse (Shankar and Titus, 1993; Soares et al., 1997) and human (Pompeu et al., 2001; Rogers and Titus, 2004) systems. In this report, PBMC were submitted to a primary course of stimulation by incubating with a low parasite ratio (1:1) followed by a second course of stimulation. Hence, PBMC primarily stimulated *in vitro* were harvested and then co-incubated with autologous infected macrophages (10:1), which served as APCs, as previously described (Pompeu et al., 2001; Rogers and Titus, 2004).

First, experiments were conducted in order to determine the ideal conditions for the PIV assay. Dog PBMC were cultivated at different concentrations: 2.5 and 8×10^6 cells/ml. In response to either ConA or *L. chagasi* stimulation, cultures at 8×10^6 cells/ml displayed a significant reduction in cell viability after 4–6 days in culture, despite periodical reposition of medium. For *in vitro* priming, PBMC from non-exposed dogs were cultivated at 5×10^6 cells/ml and were stimulated with one *L. chagasi* stationary-phase promastigote per cell for a period of 6 days. Cell viability was maintained at 99% as determined by trypan blue exclusion when half of the culture medium was replaced every day during the 6 days of primarily PBMC stimulation. Once the ideal conditions for the PBMC cultures were established, co-culture assays were performed. After 2 and 4 days of co-culture, the parasite load was quantified to determine the effect of PBMC stimulation on macrophage infection. In order to further characterize the nature of the PIV immune response of PBMC from these naïve dogs to *L. chagasi*, cytokine expression levels were determined after a second contact of these PBMC with the infected APCs. In a similar assay using human PBMC and macrophages, the *in vitro* system was capable of discriminating differences in immune responses from several non-exposed individuals to *Leishmania* (Pompeu et al., 2001).

In a previous work, we demonstrated that PBMC from immunized dogs produced factors able to stimulate macrophages to kill intracellular *L. chagasi in vitro* (Rodrigues et al., 2007). We hypothesized herein that the macrophage response to *L. chagasi* co-cultivated with PBMC that had been subjected to primary stimulation *in vitro* would be a useful test to predict the immune responses of non-exposed dogs to *Leishmania* promastigotes or antigens. Hence, PBMC from the five naïve dogs that had been stimulated by *L. chagasi* were assessed for their ability to stimulate autologous macrophages to kill *L. chagasi*. In the present report, at 2 days of infection the parasite load in macrophages co-incubated with *L. chagasi*-stimulated PBMC (133.4 ± 11.8) was 2.3 times higher in comparison to that in cells incubated with unstimulated PBMC (58.3 ± 9.1) (Fig. 1A, one-way ANOVA, $p < 0.01$). This effect seems to be specific since ConA supernatant effect on parasite load (95.0 ± 14.2) was not statistically different from the effect of control unstimulated PBMC supernatant in infected co-cultured macrophages (58.3 ± 9.1). Finally, the enhancement in macrophage infection induced by *L. chagasi*-stimulated PBMC was reversed at 4 days of infection. Fig. 1B shows that, at this time point, macrophage response to infection did not differ among groups. It is possible that stimulated PBMC co-cultivated with infected macrophages produced cytokines that could control parasite infection by an effect dependent on cytokine accumulation in the cell culture supernatant.

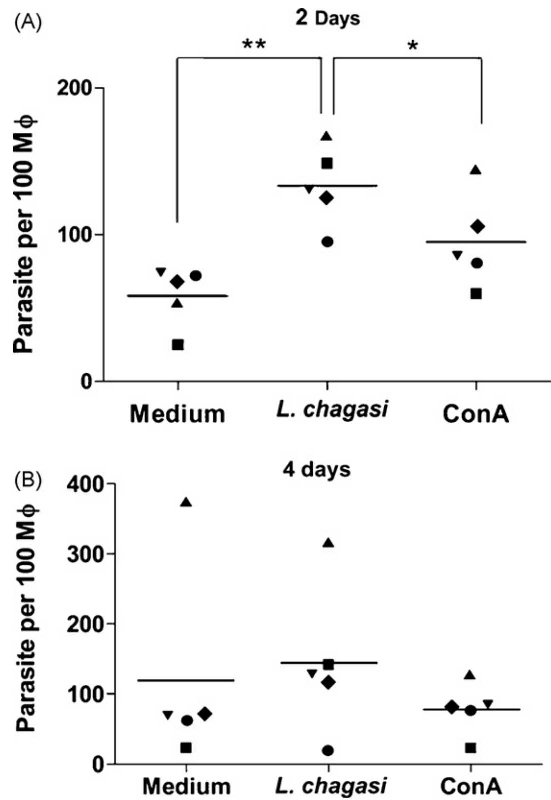

Fig. 1. (A and B) *L. chagasi*-stimulated PBMC modulate parasite load in co-incubated autologous macrophages. The macrophages were obtained as described in Section 2. At day 5 of culture, macrophages were infected with *L. chagasi* stationary-phase promastigotes at a ratio of 10 parasites per macrophage. Twenty-four hours later, PBMC recovered from parallel cultures of non-adherent cells were added to *L. chagasi*-infected macrophages. Adhered macrophages were fixed and stained, and quantitative observations were performed to determine the percentage of infected cells and the number of parasite per cell to calculate the number of parasites per 100 macrophages. Data represent the individual mean values of the number of parasites per 100 macrophages submitted to the co-incubation with unstimulated (medium), *L. chagasi*-stimulated (*L. chagasi*), or ConA-stimulated PBMC at 2 and 4 days of infection. Each symbol represents a distinct dog donor: (■) Dog 1 ($n = 4$), (●) Dog 2 ($n = 4$), (▲) Dog 3 ($n = 3$), (▼) Dog 4 ($n = 4$), (◆) Dog 5 ($n = 4$), line = mean value; $^*p < 0.05$, $^{**}p < 0.01$ (one-way ANOVA).

Previously, we have shown that macrophages co-cultivated with PBMC from immunized dogs were able to produce NO, which correlated with an *in vitro* control of parasite infection (Rodrigues et al., 2007). Different from our previous work (Rodrigues et al., 2007), here we were not able to detect NO in cell culture supernatant. It is possible that in the present study co-incubation of macrophages with *L. chagasi*-stimulated PBMC from naïve dogs was not sufficient to induce detectable levels of NO by Griess reaction.

To further characterize the dog's primary response to *L. chagasi*, the amount of IFN-γ, IL-4 and IL-10 produced by these cells was assessed by ELISA. Since we were not able to detect cytokine in cell culture supernatants, presumably due to low cytokine secretion by primarily stimulated PBMC, qRT-PCR was used to determine cytokine expression levels in these PBMC that had been co-cultivated for 4 days with infected macrophages. Several works have demonstrated that qRT-PCR is a sensitive and reproducible assay that shows only minimal intra-assay variations (Hein et al., 2001; Listvanova et al., 2003; Tassignon et al., 2005), and that it is especially useful to detect cytokine expression in experimental conditions, even when cytokines are not detected in cell supernatant (Hein et al., 2001). Cytokine mRNA was normalized relative to expression of the housekeeping gene 18S rRNA and represented as $\log_2$ of these values.

It is generally accepted that IFN-γ is needed for the control of and the protection from *Leishmania* infections. Evidence supports the notion that IFN-γ is one of the Th1 prototype cytokines responsible for activating macrophages, leading to an efficient killing of the parasites (Murray, 1982; Murray and Nathan, 1999; Holzmuller et al., 2005). Here, we observed that IFN-γ mRNA expression levels by *L. chagasi*-stimulated PBMC co-incubated with infected cells (median = 6.49; 95% CI = 5.08–8.19) (Fig. 2A) are higher compared to the expression levels of unstimulated PBMC co-incubated with infected macrophages (median = 4.10; 95% CI = 1.17–7.29) (Friedman, $p = 0.0417$). This indicates that the enhancement of IFN-γ expression is a specific *L. chagasi*-dependent mechanism. Independent of previous stimulation by *L. chagasi*, PBMC contact with infected macrophages induced higher IFN-γ expression than control unstimulated PBMC co-incubated with uninfected macrophages, in which expression was very low (−0.15; 2.17, $n = 2$). Correlation analyses between IFN-γ expression and the percentage of infected cells have been performed. As expected, IFN-γ expression positively correlated with reduction in the percentage of infected macrophages when they were co-incubated with either unstimulated PBMC ($p = 0.0088$, $r^2 = 0.42$) or *L. chagasi*-stimulated PBMC ($p = 0.0161$, $r^2 = 0.3699$). These data show that the exposure of PBMC from naïve dogs to *L. chagasi* antigens leads to IFN-γ expression levels that are related to the reduction of parasite infection *in vitro*.

The IL-4 release in murine and human *Leishmania* infection models is related to Th2 response, and IL-4 production often occurs during disease progression (reviewed by Pompeu et al., 2001). However, using a PIV murine model, we have shown that mouse PBMC stimulated with either *L. major* or *L. amazonensis* did not express IL-4 levels different from control cells, although these PBMC released IFN-γ into the cell culture supernatant upon *L. major* stimulation (Veras et al., 2006). The role IL-4 plays in the regulation of immunity in CVL is still poorly understood (Quinnell et al., 2001). Previously, we have not detected differences in IL-4 expression by PBMC from immunized dogs, whether they were stimulated with *L. chagasi in vitro* or not (Rodrigues et al., 2007). Similarly, we observed herein that, upon *L. chagasi* stimulation, PBMC

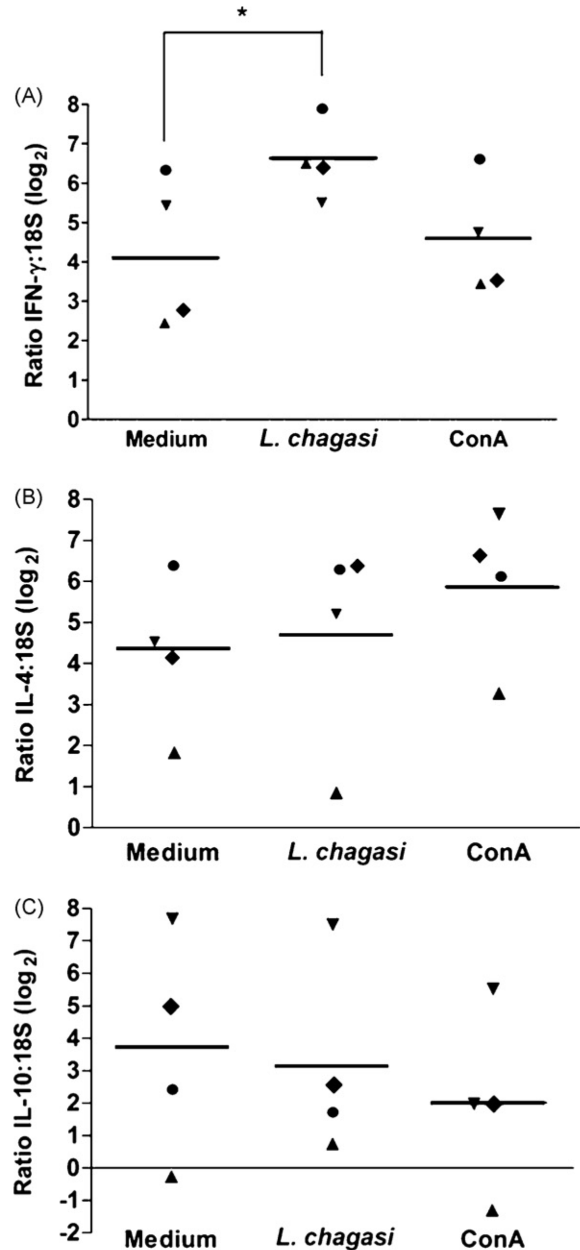

Fig. 2. IFN-γ expression is up-regulated by PBMC from naïve dogs upon primary *in vitro* stimulation with *L. chagasi*. Quantitative mRNA of cytokine was determined by qRT-PCR of primary *L. chagasi*-stimulated PBMC and control unstimulated PBMC cultivated for 6 days and co-cultivated for an additional 4 days with infected macrophages as described in Section 2. Each symbol represents a distinct dog donor: (●) Dog 2, (▲) Dog 3, (▼) Dog 4, (◆) Dog 5, line = median value of IFN-γ (A), IL-4 (B) and IL-10 (C) mRNA concentrations normalized to the concentration of the housekeeping gene 18S RNA expression represented as $\log_2$. To test statistical differences Friedman test followed by the Dunn's multiple comparisons post-test were used: *L. chagasi*-stimulated × medium = $^*p < 0.05$.

expressed similar levels of IL-4 mRNA (median = 5.78; 95% CI = 0.53–8.86) compared to unstimulated PBMC (median = 4.38; 95% CI = 1.25–7.17) (Fig. 2B). However, we have observed that IL-4 expression was higher when compared to the expression by the control unstimulated PBMC co-incubated with uninfected macrophages (0.68; 2.61, $n = 2$). In this PIV system, IL-4 seems to collaborate with IFN-γ in the control of parasite infection *in vitro*, since higher IL-4 expression positively correlates with control of parasite infection by macrophages co-incubated with either unstimulated PBMC ($r^2 = 0.7973$, $p < 0.0001$) or *L. chagasi*-stimulated cells ($r^2 = 0.7167$, $p < 0.0001$). This idea is also supported by the observation that a lower IFN-γ/IL-4 ratio correlates with a lower percentage of infected cells when co-incubated with *L. chagasi*-stimulated PBMC ($r^2 = 0.5997$, $p = 0.0007$). It is plausible to propose that this direct correlation between the IFN-γ/IL-4 ratio and parasite load is dependent on the higher correlation of both IFN-γ and IL-4 expression with lower parasite infection. Several years ago, it was clearly demonstrated that IL-4 in the presence of low concentrations of IFN-γ provides a strong stimulus for the killing of intracellular *L. major* (Bogdan et al., 1991). The synergism of IFN-γ and IL-4 is apparently based on the ability of these lymphokines to induce the endogenous production of TNF-α (Stenger et al., 1991). Recently, it has been demonstrated that extremely susceptible golden hamsters once immunized with a kinetoplastid membrane protein-11-encoding construct were protected in both pentavalent antimony responsive and antimony resistant virulent-*L. donovani* challenges. These animals were protected by a surge in IFN-γ, TNF-α, and IL-12 levels, along with extreme down-regulation of IL-10. Expression of IL-4 was found to have a positive correlation to protection (Basu et al., 2005).

Recently, IL-10 has been clearly implicated in immune-modulation of murine and human models of VL (Nylen and Sacks, 2007). The role IL-10 plays in CVL still is not very well defined (Lage et al., 2007; Santos-Gomes et al., 2002; Quinnell et al., 2001). Here, we observed that IL-10 mRNA from *L. chagasi*-stimulated PBMC (median = 2.14; 95% CI = −1.69–8.00) was similarly expressed in unstimulated PBMC (median = 3.71; 95% CI = −1.72–9.18) (Fig. 2C) either co-incubated with infected macrophages. This was similar to the IL-10 mRNA expression by un-stimulated PBMC co-incubated with uninfected macrophages (3.71; 4.10). Interestingly, the co-incubation of infected macrophages with *L. chagasi*-stimulated PBMC neutralized the positive correlation of IL-10 expression with a decrease in infection that we observed in unstimulated PBMC that had been co-incubated with infected macrophages. These data indicate that IL-10 seems not to participate in PIV stimulation of dog splenocytes. Similar to our work, no correlation with disease progression and IL-10 expression was observed in bone marrow cells from naturally infected dogs (Quinnell et al., 2001) or PBMC from experimentally infected dogs (Santos-Gomes et al., 2002).

In summary, we have established a PIV system with canine PBMC that allowed us to examine the early canine immune responses to *L. chagasi* infection. Briefly, when we co-cultivated *L. chagasi*-stimulated PBMC with infected macrophages, we found that IFN-γ mRNA was up-regulated in these cells, compared to control unstimulated cells. IL-4 and IL-10 expression by *L. chagasi*-stimulated PBMC was similar to control unstimulated PBMC. Both higher IFN-γ and IL-4 expressions by *L. chagasi*-stimulated PBMC and a lower IFN-γ/IL-4 expression ratio correlated with reduction in the percentage of infection. Since this system allows the identification of canine primary immune responses, it could potentially be used as a screening test for the *in vitro* prediction of animal responses to vaccination or parasite infection.

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