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Peripheral blood mononuclear cell supernatants from asymptomatic dogs immunized and experimentally challenged with *Leishmania chagasi* can stimulate canine macrophages to reduce infection in vitro

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Abstract

Leishmania chagasi is the causative agent of visceral leishmaniasis in both humans and dogs in the New World. The dog is the main domestic reservoir and its infection displays different clinical presentations, from asymptomatic to severe disease. Macrophages play an important role in the control of *Leishmania* infection. Although it is not an area of intense study, some data suggest a role for canine macrophages in parasite killing by a NO-dependent mechanism. It has been proposed that control of human disease could be possible with the development of an effective vaccine against canine visceral leishmaniasis. Development of a rapid *in vitro* test to predict animal responses to *Leishmania* infection or vaccination should be helpful. In this study, an *in vitro* model was established to test whether peripheral blood mononuclear cell (PBMC) supernatants from dogs immunized with promastigote lysates and infected with *L. chagasi* promastigotes could stimulate macrophages from healthy dogs in order to control parasite infection. PBMC from a majority of the immunized and experimentally infected dogs expressed IFN-γ mRNA and secreted IFN-γ when stimulated with soluble *L. chagasi* antigen (SLA) *in vitro*. Additionally, the supernatants from stimulated PBMC were able to reduce the percentage of infected donor macrophages. The results also indicate that parasite killing in this system is dependent on NO, since aminoguanidine (AMG) reversed this effect. This *in vitro* test appears to be useful for screening animal responses to parasite inoculation as well as studying the lymphocyte effector mechanisms involved in pathogen killing by canine macrophages.

Keywords: *Leishmania chagasi*; Dog macrophage; Parasite killing; Screening *in vitro* test

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1. Introduction

Most cases of leishmaniasis are zoonotic infections, caused by *Leishmania* spp., however, humans can be part of the parasite life cycle. The 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* are the causative agents of visceral leishmaniasis in Africa, India, and Europe. In the New World, visceral leishmaniasis is predominantly caused by *L. chagasi* in Latin America. *L. infantum* and *L. chagasi* are probably the same parasite since molecular evidence has shown low diversity between these strains (Momen et al., 1987; Grimaldi et al., 1989; WHO, 1990; Mauricio et al., 1999). Dogs are the most important domestic reservoir of these parasites (reviewed by Tesh, 1995).

Dogs represent an appropriate model to study leishmaniasis because the prevalence of canine disease is very high and these animals can present with several signs that are shared with the human disease such as fever, hypergammaglobulinaemia, hepatosplenomegaly, and anemia (Nieto et al., 1999). Less than 50% of infected dogs present with signs of the severe disease (Lanotte et al., 1979; Berrahal et al., 1996). These animals present with depressed T cell-mediated functions and high levels of specific antibodies (Santos-Gomes et al., 2002). On the other hand, asymptomatic dogs show protective immunity which has generally been associated with a strong proliferative response by peripheral blood lymphocytes to leishmanial antigens (Cabral et al., 1992; Pinelli et al., 1994, 1995).

Macrophages play an important role in the control of *Leishmania* infection In murine models, it is a well established fact that macrophages participate in parasite killing by reactive oxygen and nitrogen intermediate-dependent mechanisms. However, mechanisms involved in *Leishmania* killing by canine macrophages have not been investigated as thoroughly. There are data which implicate canine macrophages in parasite killing by a NO-dependent mechanism. It was demonstrated that PBMC from vaccinated dogs were able to reduce macrophage infection by a NO-dependent mechanism when stimulated *in vitro* with both *Leishmania* promastigotes and concanavalin A (ConA). This effect was potentiated by LPS stimulus (Panaro et al., 2001). Using a macrophage cell line, Pinelli et al. (2000) showed that parasite burden was reduced upon cell activation with cytokine-rich supernatants. These supernatants were obtained from a *Leishmania*-specific T cell-line generated from dogs immunized with soluble *Leishmania* antigen (SLA) then later restimulated by SLA *in vitro*. This effect was associated with discrete NO liberation into cell supernatants.

It has been proposed that control of human disease could be possible with the development of an effective vaccine against canine visceral leishmaniasis (Tesh, 1995). Development of a rapid *in vitro* test to predict animal response to *Leishmania* infection or vaccination should be helpful. Herein, we established an *in vitro* model to test whether PBMC supernatants from dogs immunized with promastigote lysates and infected with *L. chagasi* promastigotes were able to stimulate macrophages from healthy dogs to control parasite infection. PBMC of the majority of these immunized and experimentally infected dogs expressed IFN-γ mRNA and secreted IFN-γ. Additionally, the supernatants from stimulated PBMC were able to reduce the percentage of infected healthy donor macrophages. As predicted, parasite killing in this system was dependent on NO, since AMG reversed this effect. This *in vitro* test appears to be useful for screening animal responses to parasite inoculation as well as studying the lymphocyte effector mechanisms involved in pathogen killing by canine macrophages.

2. Materials and methods

2.1. Animals

Five healthy animals, identified as MΦ 1–5 (three males and two females, adults, mixed breed), were used as sources of peripheral blood monocyte-derived macrophages. In clinical, serological, and parasitological examinations, animals displayed no sign of *Leishmania* infection. As the PBMC supernatant source, four dogs (two males and two females, adults, mixed breed, identified as dogs 1–4) were immunized. Briefly, these dogs were initially immunized with two biweekly subcutaneous injections of promastigote lysate (1×10^8 parasites/animal) emulsified in incomplete Freund's adjuvant (IFA). Four weeks later, the same animals were infected via one subcutaneous injection of 1×10^8 live *L. chagasi* metacyclic promastigotes (Froes et al., 2004). These four dogs were positive for *in vitro* lymphoproliferative responses to *L. chagasi* and their serological and splenic parasitological examinations were positive for *Leishmania* (data not shown). During this study, the PBMC donors did not show any clinical signs of leishmaniasis. Both macrophage and PBMC donors were kept in a *Leishmania* insect vector-free kennel (Kennel of the CPqGM/Fiocruz, Salvador, Bahia, Brazil). These dogs had all received their yearly routine

vaccinations and were closely monitored for health problems by a veterinarian. All experiments were performed in accordance with the Oswaldo Cruz Foundation guidelines for animal experimentation and the Committee of Ethics of animal experimentation (CEUA-CPqGM/FIOCRUZ).

2.2. Parasites

A strain of *L. chagasi* (MHOM/BR00/MER02), originally isolated from a Brazilian patient with visceral leishmaniasis, was maintained by serial passages through hamsters. Amastigotes were recovered from hamster spleens and allowed to convert to promastigotes in Schneider's insect medium (Sigma Chemical Co., St. Louis, MO) supplemented with 20% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Utah, USA) and 100 μg/ml of gentamicin (Sigma Chemical Co., St. Louis, MO) or in Novy-Nicolle-Mac Neal (NNN). Promastigotes were maintained at 25 °C in Schneider's insect medium for a maximum of seven passages. Parasites at the stationary phase of growth were used for macrophage infection and for *in vitro* PBMC stimulation.

2.3. Macrophage culture

PBMC were obtained from heparinized peripheral blood layered over Ficoll-Paque (Pharmacia Biotech) then resuspended in RPMI-1640 medium supplemented with 2 mM L-glutamine (Gibco BRL, New York, USA), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid), 100 UI/ml penicillin, 100 μg/ml streptomycin, 2 g/l sodium bicarbonate, and 10% FBS (Hyclone Laboratories, Inc., Utah, USA or Gibco BRL, New York, USA) (complete RPMI medium). Cell suspensions were made (5×10^6 ml^{-1}) and 1 ml of cell suspension was distributed in 24-well culture plates (Costar, Cambridge, USA) containing 13 mm diameter glass coverslips (Glasstécnica Imp., SP, Brazil). Cells were then cultured at 37 °C in 5% CO_2/95% humidified air for 24 h. After this incubation period, non-adherent cells were washed-out three times with RPMI-1640 supplemented with 25 mM HEPES and then incubated in fresh complete RPMI medium for 8–10 days to differentiate monocytes into macrophages.

2.4. PBMC from experimentally infected dogs stimulated in vitro with L. chagasi promastigotes

PBMC from experimentally infected dogs were obtained as described above and resuspended in complete RPMI medium and cultivated at 37 °C in 5% CO_2/95% humidified air. Cells were then stimulated *in vitro* with *L. chagasi* stationary phase promastigotes and after 3 and 7 days of incubation, cellular suspensions were centrifuged for 15 min at $640 \times g$ and cytokine-enriched supernatants were collected and stored at −70 °C until ready for use. These supernatants were used as test supernatants. Cellular extracts were utilized for RNA extraction.

2.5. Macrophage activation and infection

After 8–10 days of culture, monocyte-derived macrophages were infected with stationary phase *L. chagasi* at a 10:1 ratio. After 4 h, cells were washed three times with RPMI-1640 supplemented with 25 mM HEPES to remove non-internalized parasites and resuspended in complete medium or test supernatant for an additional 24 or 72 h after infection. Non-stimulated macrophages cultivated in complete medium alone or in the presence of PBMC supernatants of healthy donor dogs stimulated *in vitro* with *L. chagasi* stationary phase promastigotes were used as negative controls. The cells were fixed with 100% methanol for 10 min and stained with hematoxilin-eosin. The percentage of infected cells and the number of *Leishmania* per macrophage were determined by light microscopy (magnification 1000×) and at least 200 macrophages were counted per coverslip. All experiments were performed in triplicate.

2.6. Determination and inhibition of nitrite accumulation

The concentration of nitrite (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% sulfanilamide, 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 $NaNO_2$ as a reference in successive dilutions from 200 to 1 μM. Results were expressed in μM per number of cells in culture. To inhibit NO production, 100 mM of AMG, a competitive inducible NO synthase (iNOS) inhibitor, was added to the cultures 24 h before macrophage infection and replaced after cell washing to remove non-internalized parasites.

2.7. Detection of canine IFN-γ and IL-4 mRNA

IFN-γ and IL-4 mRNA expression were determined in PBMC from experimentally infected dogs (dogs 1–4).

PBMC were stimulated *in vitro* with *L. chagasi* promastigotes, as described in Section 2.4. Negative control cells were cultivated in complete RPMI medium alone or, as a positive control, in the presence of Con A. Total PBMC RNA was extracted according to the manufacturer's recommendations using Trizol (Life Technologies). Briefly, for the synthesis of complementary DNA (cDNA), 1 μg of RNA was reverse transcribed with 2.5 μM oligo-dT primers (Perkin-Elmer), 2.5 U/μl MMLV-RT enzyme (Perkin-Elmer), 1 U/μl RNAse out (Perkin-Elmer), 0.25 mM of dNTPs (Amersham-Pharmacia), 5 mM $MgCl_2$ (GibcoBRL), and 1X PCR buffer (GibcoBRL), according to the manufacturer's instructions. PCR reactions contained 0.2 mM of dNTPs (Amersham-Pharmacia), forward and reverse primers (20 pmol per reaction for IL-4 and 40 pmol per reaction for IFN-γ and HPRT), 1X PCR buffer (GibcoBRL), 2.5 mM $MgCl_2$ (GibcoBRL), 6 μl of cDNA, and 0.625 U/μl Taq polymerase (Roche Molecular Biochemicals) made up to 25 μl with distilled water. The same master mix of reagents was used for each sample. Previous experiments were carried out in order to determine the number of cycles (25–40 cycles) at which HPRT, IFN-γ, and IL-4 mRNA were in a linear phase of amplification. A plateau was reached using 40 cycles of amplification. Reaction conditions were as follows: 95 °C for 1 min, then 35 cycles of denaturation condition at 95 °C for 1 min, annealing at 65 °C (IL-4 and HPRT) or 60 °C (IFN-γ and HPRT) for 2 min, and polymerization at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. Negative controls (water and negative control of RT) and positive controls (cDNA prepared from Con A stimulated PBMC and plasmids containing IFN-γ or IL, 4 DNA sequences) were run in each set of PCRs. PCR products were resolved by agarose gel electrophoresis (1.6%) and stained with ethidium bromide. Primer sequences were as follows: IFN-γ sense, 5′-GGTGGGTCTCTTTTCGTA-3′; IFN-γ anti-sense, 5′-ACTCCTTTTCCGCTTCCT-3′; IL-4 sense, 5′-CACTGCTCCAAAGAACACAAGC-3′; IL-4 anti-sense, 5′- AGGTCTTGTTTGCCATGCTG-3′; hypoxanthine phosphoribosyl transferase (HPRT) sense, 5′-TATGGACAGGACTGAACGTCTTGC-3′; (HPRT) anti-sense, 5′-GACACAAACATGATTCAAATCCCTGA-3′.

2.8. Expression of RT-PCR results

The computer software (Scion Image, Version Beta 4.0.2) attributed a number of pixels to each band in the agarose gel, corresponding to amplified IFN-γ, IL-4 or HPRT cDNA, according to their intensities. Results were expressed as the ratio of IFN-γ/HPRT, IL-4/HPRT pixels originated from each sample. For each stimulus, the determinations were carried out once for each animal and the final data are presented as the mean of arbitrary units ±S.D.

2.9. IFN-γ production

PBMC of three experimentally infected dogs (dogs 1, 2, and 4) were obtained as described in Section 2.4. PBMC (10^7 cells/ml) were resuspended in complete RPMI medium and then plated on 6-well tissue culture plates (Costar Corning Inc., USA). *L. chagasi* soluble antigen (SLA) (20 μg/ml) was added to each well; control cultures contained either no *L. chagasi* soluble antigen (medium) or 10 μg/ml of Con A (positive control). Supernatants of the cultures were collected at 48 h and maintained at −20 °C until use. PBMC of two healthy dogs were used as controls. IFN-γ was measured by Sandwich ELISA, according to the manufacturer's recommendations (R&D Systems, Minneapolis, USA) using specific anti-dog IFN-γ (1.0 μg/ml), biotinylated anti-dog IFN-γ (100.0 ng/ml), and streptavidin conjugated to horseradish peroxidase (1:200). Absorbance values were read at OD_{450} in an automatic Microplate Reader EL 3311 (Boehring Mannheim, Germany). A standard curve for IFN-γ was performed in successive dilutions from 32,000 to 31 pg/ml of recombinant canine IFN-γ. Data are expressed in picograms per milliliter. Standard curve linearity varied from 500 to 8000 pg/ml.

2.10. Statistical analyses

Statistical significance between experimental and control groups was analyzed by Student's *t*-test to compare two groups or by one-way ANOVA (Newman–Keuls as post-test) to compare three or more groups with Gaussian distribution (% of infected cells and NO determination). Cytokine mRNA expression of control and stimulated cells were compared using Mann–Whitney test. A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Reduction of macrophage infection upon stimulation with PBMC supernatants from immunized and experimentally infected dogs stimulated in vitro with L. chagasi

The effect of PBMC supernatants from immunized and experimentally infected dogs stimulated *in vitro*

with *L. chagasi* (test supernatants) on canine macrophage infection was evaluated. Monocyte-derived macrophages were infected in the presence of the test supernatants and the percentage of infected cells was evaluated at 24 and 72 h after infection. PBMC supernatants from healthy donors, stimulated *in vitro* with *L. chagasi*, were used as controls. As expected, similar to medium alone, PBMC supernatants from healthy dogs had no effect on macrophage *Leishmania* infection (data not shown). At 24 h after infection of macrophages from healthy donors treated with test supernatants, the percentage of infected cells was reduced (MΦ 1–5) varying from 12.5 to 42.5% (Fig. 1A, $n = 5$, $p = 0.0009$). Supernatant-mediated reduction of macrophage infection varied from 13.7 to 42.0% at 72 h after infection (Fig. 1B, $n = 5$, $p = 0.0009$). There was no difference in the number of parasites per macrophage in macrophage cultures treated with or without PBMC supernatants (data not shown).

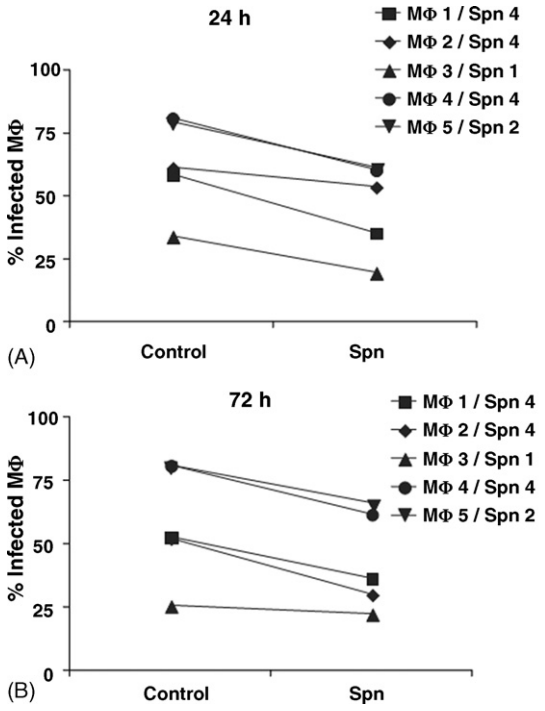

Fig. 1. Test supernatants reduce the percentage of infected macrophages. PBMC were obtained from heparinized peripheral blood layered over Ficoll-Paque, resuspended at a concentration of 5×10^6 ml^{-1} in complete RPMI medium, washed after 24 h to eliminate non-adherent cells, and cultured for 8–10 days to permit macrophage differentiation. The macrophages were infected with *L. chagasi* stationary-phase promastigotes at a ratio of 10:1 for 4 h, and non-internalized parasites were removed by washing and then the cells were reincubated for an additional 24 h (A) and 72 h (B) with test supernatants, obtained as described in Section 2.4. MΦ 1–5: five canine macrophage donors. Control: non-stimulated macrophages. Spn: test supernatants from dogs 1, 2, and 4. These data represent the mean values of five experiments performed in triplicate ($p = 0.0009$, Student's *t*-test).

3.2. IFN-γ and IL-4 mRNA expression in PBMC from experimentally infected dogs upon L. chagasi stimulation

IFN-γ and IL-4 are cytokines related to protective and susceptible responses to *Leishmania* infection, respectively (Mosmann et al., 1986; Mosmann and Coffman, 1989; Locksley and Scott, 1991). We argued whether the effect of the test supernatants on the infected cells was associated with the cytokine profile expressed by these stimulated cells. First, IFN-γ and IL-4 mRNA expression from the PBMC donors (dogs 1–4) was determined. RT-PCR was performed at 3 (Table 1, Fig. 2) and 7 days (data not shown) after stimulation with *L. chagasi*. Negative control cells were cultured in complete RPMI medium alone and positive control cells were cultured in the presence of Con A. IL-4 mRNA expression by *L. chagasi*-stimulated PBMC was similar to the negative control cells cultivated in complete RPMI medium (Table 1, $n = 4$, $p > 0.05$, ANOVA), although Con A enhanced the cytokine mRNA expression (Table 1, $n = 4$, $p < 0.01$, ANOVA). On the other hand, IFN-γ mRNA

Table 1
IFN-γ and IL-4 mRNA expression by PBMC stimulated with *L. chagasi* from experimental infected dogs

	IL-4 (arbitrary units ± S.D.)	IFN-γ (arbitrary units ± S.D.)
Medium		
Dog 1	0.16	0.15
Dog 2	0.17	0.04
Dog 3	0.18	0.07
Dog 4	0.09 (0.15 ± 0.02)	0.02 (0.07 ± 0.03)
L. chagasi		
Dog 1	0.10	0.07
Dog 2	0.26	0.10
Dog 3	0.19	0.17
Dog 4	0.19 (0.18 ± 0.03)	0.21 (0.14 ± 0.03)
Con A		
Dog 1	0.30	0.25
Dog 2	0.50	0.14
Dog 3	0.50	0.23
Dog 4	0.36 (0.41 ± 0.05)**	0.23 (0.21 ± 0.02)*

RT-PCR was performed with *L. chagasi*-stimulated PBMC from the immunized and experimentally infected dogs after 3 days of simulation. Negative control (medium) was cultured in RPMI medium alone and positive control was cultured in the presence of Con A. *$p < 0.05$ compared to PBMC cultured in complete medium; **$p < 0.01$ compared to PBMC cultured in complete medium and stimulated with *L. chagasi* (ANOVA).

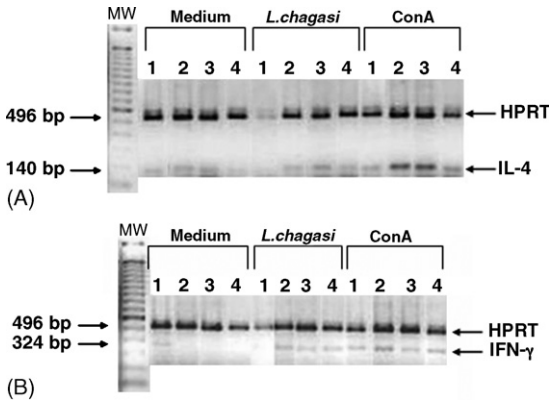

Fig. 2. Gel electrophoretic of RT-PCR products for IFN-γ, IL-4, and HPRT from PBMC of immunized and experimentally infected dogs stimulated with Con A, *L. chagasi* stationary-phase promastigotes (*L. chagasi*), or cultivated in complete RPMI medium alone (medium). The molecular weight of the RT-PCR products is shown. Lanes, MW: 100 base pair ladder DNA molecular weight marker. Lanes 1–4 represent the four canine PBMC donors: (1) dog 1; (2) dog 2; (3) dog 3; (4) dog 4.

expression by *L. chagasi*-stimulated PBMC in three out of four dogs was 2.5–12.1 times higher when compared to negative control cells, although the difference between these two groups displayed no statistical significance (Table 1, $n = 4$, $p > 0.05$, ANOVA). The positive control Con A-stimulated cells expressed three times more IFN-γ mRNA in comparison to non-stimulated cells (Table 1, $n = 4$, $p < 0.05$). At 7 days, IFN-γ and IL-4 mRNA expression was evaluated in PBMC from dogs 2 and 4 and was similar to the same cells cultivated in medium alone (data not shown).

3.3. IFN-γ production by PBMC from immunized and experimentally infected dogs stimulated in vitro with soluble L. chagasi antigen (SLA)

Since mRNA expression does not always reflect cytokine production by stimulated cells, we evaluated IFN-γ release into PBMC supernatants from three dog donors by sandwich ELISA (dogs 1, 2 and 4). IFN-γ concentrations in supernatants of PBMC from healthy dogs stimulated with *L. chagasi* antigen were below the detection limit of the assay (data not shown). PBMC from both healthy and experimentally infected dogs stimulated with Con A were able to release high levels of IFN-γ (12,000–60,000 pg/ml). However, only the PBMC from the experimentally infected dogs released high levels of IFN-γ (2,500–16,000 pg/ml) upon SLA stimulation, which was different from cells cultured in medium alone (≤380 pg/ml) (Fig. 3).

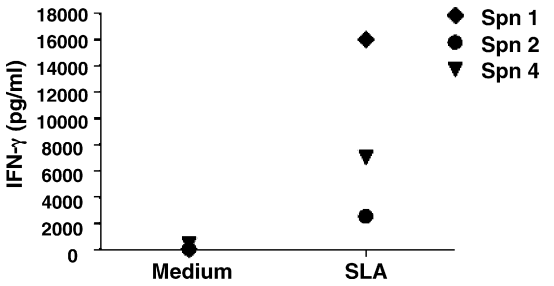

Fig. 3. IFN-γ production by PBMC from asymptomatic, previously immunized and experimentally infected dogs stimulated *in vitro* with antigens of *L. chagasi*. PBMC of the three experimentally infected dogs were obtained as described in Section 2.4 and, after 48 h of stimulation with SLA or cultured in complete RPMI medium (medium), IFN-γ release into culture supernatants was determined by ELISA. Spn: test supernatants from dogs 1, 2, and 4. Standard curve linearity varied from 500 to 8000 pg/ml.

3.4. NO production by macrophages was modified by stimulation with test supernatants

We next evaluated whether the induced effect of test supernatants on macrophage infection was related to NO production. NO production by infected macrophages then stimulated with test supernatants was compared to nitrite accumulation in control cells cultured in medium alone (Fig. 4). All macrophage cultures produced NO in response to test supernatant stimulation, although control non-stimulated cells produced undetectable levels of NO. At 24 h after infection, only test supernatant 1 (Spn 1) induced higher NO release by macrophages (MΦ 3) when compared to the same cells cultured in medium alone (Student's *t*-test, $n = 3$, $p = 0.006$). In addition, after 72 h of infection, three out of five macrophage cultures, when stimulated with test supernatants were able to produce higher NO when compared to control non-stimulated macrophages (Student's *t*-test, $p \leq 0.001$).

3.5. The effect of test supernatants on macrophage infection was reversed by NO blocking

First, we tested whether AMG was able to reverse NO production by test supernatant-stimulated dog macrophages. As expected, at 72 h after infection, AMG was able to reduce macrophage (MΦ 5) NO production by 33.5% following test supernatant (Spn 2) stimulation (Fig. 5, $n = 3$, $p < 0.01$). Furthermore, NO production by these cells was not statistically different from control non-stimulated *L. chagasi*-infected cells (Fig. 5, $n = 3$, $p > 0.05$).

Since AMG reduced NO production by supernatant-stimulated macrophages, we then tested whether this

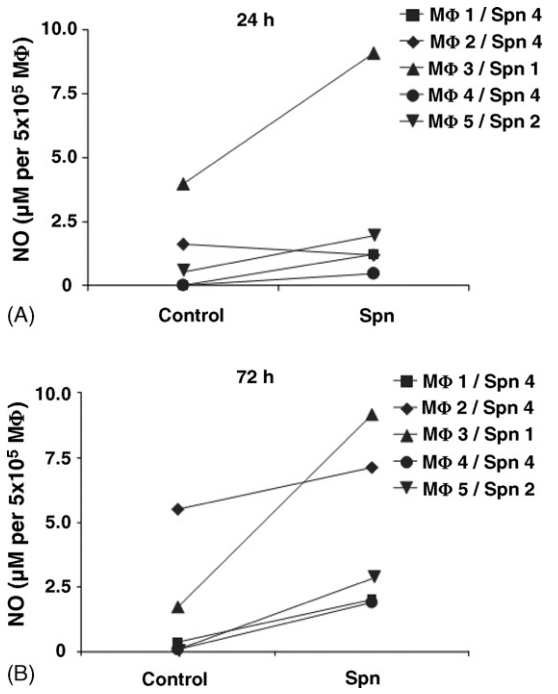

Fig. 4. Nitric oxide production by monocyte-derived dog macrophages was enhanced upon test supernatant stimulation. Macrophages were cultured and infected as described in Fig. 1. After infection, macrophages were cultured either in medium alone (Control) or with test supernatant (Spn). Supernatants were harvested 24 h (A) and 72 h (B) after infection and nitrite concentration was determined as an indicator of NO production using Griess reaction. These data represent the mean values of five experiments performed in triplicate. MΦ 1–5: five dog macrophage donors. MΦ 3, 24 h ($p = 0.006$); MΦ 3, 72 h ($p = 0.0012$), MΦ 4 and MΦ 5, 72 h ($p < 0.001$) (Student's *t*-test). Standard curve linearity varied from 1.56 to 200 μM.

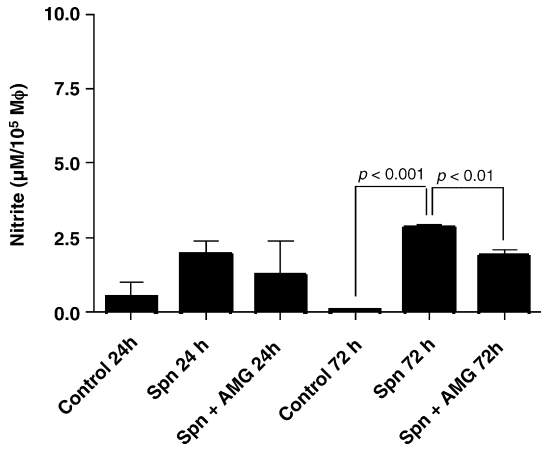

Fig. 5. AMG reversed NO production induced by test supernatant stimulation on macrophage infection. The macrophages (MΦ 5) were cultured and infected as described in Fig. 1 and incubated in medium alone (Control) or with test supernatant (Spn2) in the absence or presence of AMG. Nitrite levels were measured by Griess reaction as described in Fig. 4. These data are representative of one of two similar experiments ±S.D., done in triplicate. *p* values are indicated in the figure (ANOVA).

treatment was able to enhance the percentage of infected macrophages (MΦ 5) treated with test supernatants (Spn2). Indeed, the percentage of infected macrophages stimulated with test supernatant then treated with AMG increased from 60.3 ± 0.68% to 75.9 ± 8.7% (Fig. 6, $n = 3$, $p < 0.05$) at 24 h after infection. At 72 h after infection, AMG addition to macrophage culture induced an enhancement in the percentage of infected cells from 61.6 ± 3.2% to 78.8 ± 6.4% (Fig. 6, $n = 3$, $p < 0.01$). It is noteworthy that the percentage of infected and supernatant-stimulated macrophages once treated with AMG was similar to that from control non-stimulated *L. chagasi*-infected cells.

4. Discussion

In the present study, we developed an *in vitro* model using PBMC supernatants from asymptomatic dogs that had previously been immunized and infected with *L. chagasi* to stimulate macrophages from healthy dogs. Using our system, we demonstrated for the first time that PBMC from these asymptomatic dogs, stimulated *in vitro* with *L. chagasi*, reduced macrophage *Leishmania* infection. Distinct from other works, in our system PBMC were stimulated *in vitro* with *L. chagasi* alone. In a previous study, supernatants which reduced dog macrophage infection were obtained from PBMC

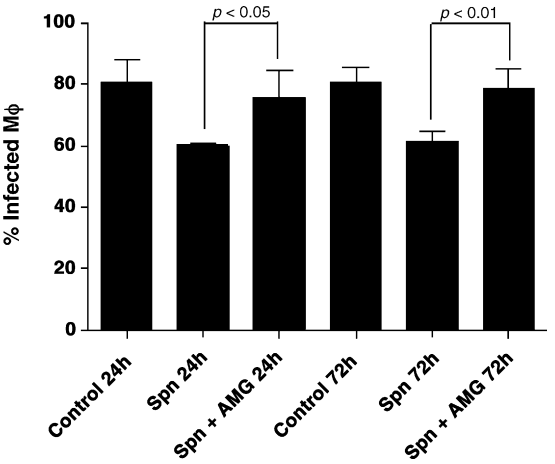

Fig. 6. The effect of test supernatant on macrophage infection was reversed by NO blocking. The macrophages (MΦ 5) were cultured, infected, and treated as described in Fig. 5. These cells, incubated in medium alone (Control) or with test supernatant (Spn2) in the absence or presence of AMG, were fixed at 24 and 72 h after infection. These data are representative of one of two similar experiments ±S.D., done in triplicate. *p* values are indicated in the figure (ANOVA).

stimulated *in vitro* with nonspecific Con A stimuli (Panaro et al., 2001). In another experimental study, a reduction in infection was observed using a macrophage cell line infected with *L. infantum* and stimulated with supernatant from a *Leishmania*-specific T cell line generated *in vitro* (Pinelli et al., 2000).

In the present study, the dogs we used as PBMC donors were asymptomatic (Froes et al., 2004) and were immunized and challenged with subcutaneous parasite inoculation. PBMC from these dogs exhibited a positive *in vitro* proliferative response to *Leishmania* antigens (data not shown). This suggests that their lymphocytes produce cytokines related to a protective immune response. The protective *Leishmania*-specific cellular immunity in dogs has been described, with resistance to *L. infantum* being associated with low titers of *Leishmania*-specific serum antibodies and production of IL- 2, TNF-α, and IFN-γ (Cabral et al., 1992; Pinelli et al., 1994, 1995; Moreno et al., 1999). Recently, it was demonstrated that, although Th1 and Th2 cytokines are produced in asymptomatic *Leishmania*-infected dogs, there is a prevalent Th1 cytokine response that confers immunity against the parasite (Chamizo et al., 2005). In this study, we evaluated the mRNA expression of the Th1/IFN-γ and the Th-2/IL-4 cytokines in the *L. chagasi*-stimulated PBMC from these immunized and challenged dogs. It is noteworthy that *L. chagasi in vitro* stimulation of PBMC, in three out of four dogs, was able to enhance IFN-γ mRNA expression from 2.5 to 12.1 times compared to parallel cultures from parasite-free PBMC, although these results did not reach statistical significance. Interestingly, we detected IFN-γ production by ELISA in the supernatants of *Leishmania* soluble antigen-stimulated PBMC from the same three immunized and experimentally infected dogs (Fig. 3).

In most of the experiments performed and reported herein, macrophages from healthy dogs produced significantly higher NO levels when activated *in vitro* with test supernatants at 72 h after stimulation, as compared to control supernatants. Reports indicate that dog macrophages produce lower levels of NO compared to murine macrophages (Pinelli et al., 2000; Gantt et al., 2001). As previously discussed for humans (Gantt et al., 2001), the low NO detection in our system can be related to either low production or to efficient intracellular scavenging of this molecule. AMG addition to the culture enhanced the percentage of infected macrophages and reversed the leishmanicidal capacity of these cells at 72 h after infection. Other groups have also observed a statistical difference in the reversion of parasite destruction by PBMC supernatant-stimulated macrophages in the presence of the NO synthase inhibitor L-N monomethylargine (Panaro et al., 1998, 2001) or the L-arginine analogue L-N^G-nitroargininemethyl ester (Pinelli et al., 2000). Finally, our data suggest that the leishmanicidal effect is dependent on NO, since three experiments demonstrated that H_2O_2 production by test supernatant-stimulated macrophages was similar to those from non-stimulated control cells or *L. chagasi*-stimulated *in vitro* PBMC supernatants from healthy donors (data not shown).

This study may provide a useful model for the characterization of a protective immune response, including the effector mechanisms involved in the killing of this pathogen. Finally, this experimental model opens the possibility for use as an *in vitro* screening test to predict animal responses to vaccination or parasite infection.

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References

Berrahal, F., Mary, C., Roze, M., Berenger, A., Escoffier, K., Lamouroux, D., Dunan, S., 1996. Canine leishmaniasis: identification of asymptomatic carriers by polymerase chain reaction and immunoblotting. Am. J. Trop. Med. Hyg. 55, 273–277.

Cabral, M., O'Grady, J., Alexander, J., 1992. Demonstration of Leishmania specific cell mediated and humoral immunity in asymptomatic dogs. Parasite Immunol. 14, 531–539.

Chamizo, C., Moreno, J., Alvar, J., 2005. Semi-quantitative analysis of cytokine expression in asymptomatic canine leishmaniasis. Vet. Immunol. Immunopathol. 103, 67–75.

Froes, A.M., dos Santos, C.V., Penha-Filho, M.L., Teixeira, M.C., Correa Silva, T.M., Oliveira, G.G., dos Santos, W.L., Pontes-de-Carvalho, L.C., Alcantara-Neves, N.M., 2004. Sub-clinical infection as an effective protocol for obtaining anti-*Leishmania chagasi* amastigote antibodies of different animal species. Vet. Immunol. Immunopathol. 99, 135–141.

Gantt, K.R., Goldman, T.L., McCormick, M.L., Miller, M.A., Jeronimo, S.M., Nascimento, E.T., Britigan, B.E., Wilson, M.E., 2001. Oxidative responses of human and murine macrophages during phagocytosis of *Leishmania chagasi*. J. Immunol. 167, 893–901.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Grimaldi Jr., G., Tesh, R.B., McMahon-Pratt, D., 1989. A review of the geographic distribution and epidemiology of leishmaniasis in the New World. Am. J. Trop. Med. Hyg. 41, 687–725.

Lanotte, G., Rioux, J.A., Perieres, J., Vollhardt, Y., 1979. [Ecology of leishmaniasis in the south of France. 10. Developmental stages and clinical characterization of canine leishmaniasis in relation to epidemiology (author's transl)] Ann. Parasitol. Hum. Comp. 54, 277–295.

Locksley, R.M., Scott, P., 1991. Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. Immunol. Today 12, A58–A61.

Mauricio, I.L., Howard, M.K., Stothard, J.R., Miles, M.A., 1999. Genomic diversity in the *Leishmania donovani* complex. Parasitology 119 (Pt 3), 237–246.

Momen, H., Grimaldi Junior, G., Deane, L.M., 1987. *Leishmania infantum*, the aetiological agent of American visceral leishmaniasis (AVL)? Mem. Inst. Oswaldo Cruz. 82, 447–448.

Moreno, J., Nieto, J., Chamizo, C., Gonzalez, F., Blanco, F., Barker, D.C., Alva, J., 1999. The immune response and PBMC subsets in canine visceral leishmaniasis before, and after, chemotherapy. Vet. Immunol. Immunopathol. 71, 181–195.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L., 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136, 2348–2357.

Mosmann, T.R., Coffman, R.L., 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7, 145–173.

Nieto, C.G., Garcia-Alonso, M., Requena, J.M., Miron, C., Soto, M., Alonso, C., Navarrete, I., 1999. Analysis of the humoral immune response against total and recombinant antigens of *Leishmania infantum*: correlation with disease progression in canine experimental leishmaniasis. Vet. Immunol. Immunopathol. 67, 117–130.

Panaro, M.A., Acquafredda, A., Lisi, S., Lofrumento, D.D., Mitolo, V., Sisto, M., Fasanella, A., Trotta, T., Bertani, F., Consenti, B., Brandonisio, O., 2001. Nitric oxide production by macrophages of dogs vaccinated with killed *Leishmania infantum* promastigotes. Comp. Immunol. Microbiol. Infect. Dis. 24, 187–195.

Panaro, M.A., Fasanella, A., Lisi, S., Mitolo, V., Andriola, A., Brandonisio, O., 1998. Evaluation of nitric oxide production by *Leishmania infantum*-infected dog macrophages. Immunopharmacol. Immunotoxicol. 20, 147–158.

Pinelli, E., Gebhard, D., Mommaas, A.M., van Hoeij, M., Langermans, J.A., Ruitenberg, E.J., Rutten, V.P., 2000. Infection of a canine macrophage cell line with *Leishmania infantum*: determination of nitric oxide production and anti-leishmanial activity. Vet. Parasitol. 92, 181–189.

Pinelli, E., Gonzalo, R.M., Boog, C.J., Rutten, V.P., Gebhard, D., del Real, G., Ruitenberg, E.J., 1995. *Leishmania infantum*-specific T cell lines derived from asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner. Eur. J. Immunol. 25, 1594–1600.

Pinelli, E., Killick-Kendrick, R., Wagenaar, J., Bernadina, W., del Real, G., Ruitenberg, J., 1994. Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania infantum*. Infect. Immun. 62, 229–235.

Santos-Gomes, G.M., Rosa, R., Leandro, C., Cortes, S., Romao, P., Silveira, H., 2002. Cytokine expression during the outcome of canine experimental infection by *Leishmania infantum*. Vet. Immunol. Immunopathol. 88, 21–30.

Tesh, R.B., 1995. Control of zoonotic visceral leishmaniasis: is it time to change strategies? Am. J. Trop. Med. Hyg. 52, 287–292.

WHO, 1990. Control of Leishmaniasis. In: Control of Leishmaniasis, Geneva.