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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*.

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 °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