Primary Immune Response and Parasite Dissemination in Canine Visceral Leishmaniasis

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Abstract

Zoonotic visceral leishmaniasis is a re-emerging disease caused by *L. infantum/L. chagasi*. The disease is transmitted by phlebotominae sand flies and dogs are the main urban reservoir of the parasite. In the natural history of *L. chagasi* infection in dog, named canine visceral leishmaniasis (CVL), following transmission, the parasites multiply in macrophages in the skin at the site of infection. From this localized cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of other organs such as the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog. In these naturally infected dogs, the outcome of CVL can vary considerably and probably correlates with the capacity of local skin cells to control parasite infection. CVL clinical manifestations are associated with distinct patterns of immune responses to *Leishmania* parasites. After infection, some dogs develop an impaired cell-mediated immune response that permits parasite dissemination and tissue lesion formation (symptomatic dogs), whereas others control parasite proliferation and dissemination to the different tissues (asymptomatic dogs). These infected dogs present positive lymphoproliferative assay *in vitro* or/and a positive skin test early in infection. However, as the disease progresses in susceptible dogs, these responses diminish. The cellular basis and mechanisms for the development of T-cell unresponsiveness in CVL are not understood fully. In the present review it will be discussed the local immune response in skin, other affected organs, and cellular compartments as well as the possible mechanisms involved in dissemination of the *L. chagasi* infection in the dog model of VL.

Keywords: *Leishmania chagasi*, canine visceral leishmaniasis, macrophage, spleen, parasite dissemination.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is a chronic and frequently lethal disease caused by *L. (L) donovani* and *L. (L.) infantum* in the Old World and *L. chagasi* in the Americas (Desjeux, 2001a, b, 2004; Palatnik-de-Sousa *et al.*, 2001; WHO, 1999). Recent evidence shows that the *L. (L) chagasi* strains could not be distinguished from *L. (L.) infantum*, indicating a recent geographical separation, and that *L. (L) infantum* and *L. (L) chagasi* are two names for the same species (Mauricio *et al.*, 2000). VL is mostly a rural disease, with a domestic or peridomestic occurrence. In Brazil, however, urbanization of visceral disease has been found in towns or in the outskirts of large cities such as Belo Horizonte, Montes Claros, Rio de Janeiro, Salvador, and Fortaleza (Palatnik-de-Sousa *et al.*, 2001).

The disease is lethal if not treated early after the onset of the symptoms. Dogs present several signs, and progressive suppression of the cellular immune response. These parasites live inside monocytes and macrophages of lymphoid organs such as the spleen, lymph nodes, bone marrow, and liver. Their biological cycle alternates between the amastigote form in vertebrate host and the promastigote form in the gut of the insect sand fly vector (Baneth and Aroch, 2008). The present review aim to discuss the local immune response in skin, other affected organs, and cellular compartments as well as the possible mechanisms involved in dissemination of the *L. chagasi* infection in the dog model of VL. These mechanisms probably account to development of T-cell unresponsiveness, and disease progression in susceptible dogs.

EPIDEMIOLOGY

The American and European forms of zoonotic VL show several similarities, as both are canid zoonoses that affect mainly children and young human adults (Palatnik-de-Sousa *et al.*, 2001); however, higher seroprevalences have been reported in South America (Ashford *et al.*, 1998; Zerpa *et al.*, 2000). It is well accepted that canids are the most common reservoirs of viscerotropic species causing zoonotic VL in the Mediterranean region, Asia, North Africa, and South America (Moreno and Alvar, 2002), playing a central role in the transmission cycle to humans by phlebotominae sand flies. CVL is a major veterinary and public health problem, and it has also been suggested as a good model for investigating the pathogenesis of human VL (Quinnell *et al.*, 2001b).

CVL has a high prevalence of infection, involving as much as 63%–80% of the dog population, and it is accompanied by a lower rate of apparent clinical disease (Berrahal *et al.*, 1996; Leontides *et al.*, 2002; Solano-Gallego *et al.*, 2004). Previous epidemiologic studies have indicated that about half of the dogs possessing anti-leishmanial antibodies exhibit no clinical signs of disease (Abranches *et al.*, 1991; Acedo-Sanchez *et al.*, 1998; Fisa *et al.*, 1999; Mauricio *et al.*, 1995). Interestingly, in a recent study in Northeast Brazil, a higher prevalence of positive dogs presenting no clinical signs of the disease was detected in the metropolitan when compared to a rural area (Alvar *et al.*, 2004; Berrahal *et al.*, 1996; Queiroz *et al.*, 2008; Solano-Gallego *et al.*, 2001). Cumulative evidence shows that: (1) infection in the canine population in endemic areas is widespread, and the rate of infected dogs is much higher than the fraction that shows clinical illness; (2) infection spreads quickly and

extensively among the dog population when environmental conditions for transmission are optimal (Baneth *et al.*, 2008); and (3) removal or elimination of infected dogs from endemic areas would be followed by immediate substitution of susceptible dogs (Dye, 1996).

Dye (1996) and Burattini and others (1995) have shown that the dog population in endemic areas is composed of four mutually exclusive groups: those susceptible, those resistant, those susceptible that become latent after sand fly bite (asymptomatic), and those infectious to sand flies that emerge from latent dogs at a constant rate (Dye, 1996). Dogs born resistant do not become infectious to sand flies or develop the disease, but do become seropositive after sand fly bite. Such animals include seropositive, non-infectious, asymptomatic dogs from endemic areas that are able to maintain an effective cellular immune response against the parasite. Alvar and others demonstrated that naturally infected asymptomatic seropositive dogs (resistant or latent) are infectious to sand flies (Alvar *et al.*, 1994). Dye also refers to a previous cohort study that used xenodiagnosis to show that infected dogs became infectious to sand flies after a median period of approximately 200 days; although several dogs died of clinical VL, the data indicate that infectiousness was unrelated to the severity of symptoms (Dye, 1996). This model demonstrated that targeting control measures at infectious dogs has a potential impact to reducing transmission. Conversely, infectiousness to sand flies has been shown to be positively associated with antibody titers detected by an enzyme-linked immunosorbent assay (ELISA) and to the intensity of skin disease (dermatitis, alopecia, and chancres) (Courtenay *et al.*, 2002).

In the natural history of *L. chagasi* infection in dog, following transmission the parasites initially multiply in macrophages in the skin at the site of infection. From this localized cutaneous infection, the parasite can disseminate via lymphatic or blood vessels, infecting macrophages of other organs such as the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog (Reis *et al.*, 2006). In these naturally infected dogs, the outcome of CVL can vary considerably and probably correlates with the capacity of local skin cells to control parasite infection. CVL clinical manifestations are associated with distinct patterns of immune responses to *Leishmania* parasites (Cardoso *et al.*, 1998; De Luna *et al.*, 2000; Martinez-Moreno *et al.*, 1995; Pinelli *et al.*, 1994; Pinelli *et al.*, 1999; Santos-Gomes *et al.*, 2002; Solano-Gallego *et al.*, 2000). After infection and before seroconversion, dogs infected with *L. chagasi* present with enlarged lymph nodes and dermatitis, without signs of visceral leishmaniasis or changes in behavior. This phase is followed by dissemination of the infection and clinical findings, including loss of appetite, fever, weight loss, alopecia, skin ulceration, onychogryphosis, keratoconjunctivitis, uveitis, bleeding, diarrhea, neuralgia, polyarthritis, interdigital ulceration, and kidney insufficiency (Abranches *et al.*, 1991; Bettini *et al.*, 1986; Molina *et al.*, 1994).

INITIAL IMMUNE RESPONSE

L. chagasi infection initiates when the parasite is inoculated into the skin by a female phlebotominae that probes the skin for blood (Rogers *et al.*, 2004). Incoming and resident phagocytes exit the blood vessels and become infected with the parasites (Moll *et al.*, 1993; Santos-Gomes *et al.*, 2000; Wilson *et al.*, 1987). Based on *in vitro* and *in vivo* animal models of visceral leishmaniasis, it is widely accepted that macrophages play a central role in the control of *Leishmania* infection. Most of these studies have involved human or murine

monocytes/macrophages (Bodman-Smith *et al.*, 2002; Gomes *et al.*, 2000; Murray, 2001), and only a few *in vitro* studies used canine macrophages and *L. chagasi* (Bueno *et al.*, 2005; Goncalves *et al.*, 2005; Sampaio *et al.*, 2007). Previously, Gonçalves and collaborators (2005) demonstrated that the frequency of peritoneal macrophages from naturally infected dogs expressing the monocyte surface molecules CD11b or CD18 significantly drops upon interaction with *L. chagasi*. More recently, Sampaio and collaborators (2007) showed that monocytes from naturally-infected animals compared to those from experimentally-infected ones are significantly more capable of binding to *Leishmania* promastigotes. Using peripheral monocytes from these naturally *L. chagasi*-infected dogs, the authors demonstrated that these cells display a higher frequency of CD11b-positive monocytes when obtained from peripheral blood. Similar to the previous study performed by Gonçalves and collaborators (2005) the frequency of macrophages expressing CD11b or CD18 has been shown to drop significantly upon interaction with *Leishmania,* and this decrease is more accentuated when *Leishmania* is incubated with exogenous serum (Sampaio *et al.*, 2007). The authors propose that downregulation of these receptors may be related to two mechanisms, they can be occupied by *Leishmania*, or the receptor complexes can be internalized after *Leishmania*-macrophage interaction (Sampaio *et al.*, 2007).

After phagocytosis, *Leishmania* promastigotes transform into amastigotes, which can survive inside macrophages. Some genetic alterations have been related to this event. The Slc11a1 (NRAMP) protein acts as a proton/divalent cation antiporter, which controls the replication of intracellular parasites by altering the intravacuolar environment of the microbe-containing phagosome (Gruenheid *et al.*, 1997). The *Slc11a1* gene also regulates macrophage function – including upregulation of chemokine and cytokine genes such as TNF and interleukin-1β and increased expression of inducible nitric oxide synthase (iNOS) (Blackwell *et al.*, 2001). Polymorphisms in the *Slc11a1* gene have been associated with CVL in dogs of different breeds (Sanchez-Robert *et al.*, 2005). In 164 dogs, 24 polymorphisms were found in the *Slc11a1* gene and 3 polymorphisms were associated with an increased risk for CVL (Sanchez-Robert *et al.*, 2008). Among these, two were single nucleotide polymorphisms (SNP) in the *Slc11a1* promoter region that disrupted putative transcription factor binding sites. These types of SNPs in the canine *Slc11a1* gene promoter suggest a possible role of differential *Slc11a1* gene expression that can interfere with Slc11a1 function and/or its interaction with many other genes, contributing to CVL susceptibility (Sanchez-Robert *et al.*, 2005; Sanchez-Robert *et al.*, 2008).

COMPARTMENTAL IMMUNE RESPONSE

Skin

From the original site of infection in the skin, amastigotes disseminate throughout the body, causing lesions in different tissues such as the lymph node, liver, spleen, gut, bone marrow and, in dogs, mainly other sites of the skin (Barrouin-Melo *et al.*, 2004; dos-Santos *et al.*, 2004; Reis *et al.*, 2006). In the last few years, a growing number of systematic works provided important contributions to our understanding of the histopathological alterations that occur in these target organs (Brachelente *et al.*, 2005; Giunchetti *et al.*, 2007; Giunchetti *et*

al., 2008a; Giunchetti *et al.*, 2008b; Lage *et al.*, 2007; Santana *et al.*, 2008; Solano-Gallego *et al.*, 2007; Strauss-Ayali *et al.*, 2007).

Some studies evaluated histological alterations that occur in dog skin in response to *Leishmania* infection (dos-Santos *et al.*, 2004, Brachelente *et al.*, 2005; Solano-Gallego *et al.*, 2007). In a previous study, the histological pattern and parasite load were investigated in clinically normal skin of *Leishmania*-infected dogs (Solano-Gallego *et al.*, 2004). Two groups of *Leishmania*-infected dogs, symptomless animals that, although seronegative or only mildly seropositive, provided positive PCR results for *Leishmania* in the skin and a group of clinically affected dogs that were highly seropositive and PCR-positive were compared. The muzzle skin of symptomless dogs had no demonstrable microscopic lesions or amastigotes. This, together with the positive PCR results for *Leishmania*, indicates that the number of parasites in skin samples from the muzzle must have been very low. The most severe lesions and the greatest parasite loads were located around hair follicles, mainly around the isthmus, associated with the middle vascular plexus of the dermis. This finding suggested hematogenous dissemination of the parasite and tropism for the skin (Solano-Gallego *et al.*, 2007). In conclusion, the results of this study cast doubt on the relevance of infected but symptomless dogs in the epidemiology of canine leishmaniasis (Solano-Gallego *et al.*, 2007).

In another recent study (Brachelente *et al.*, 2005), the question of whether a correlation exists between the number of parasites, the histological response, and the expression of cytokines produced by $CD4^+$ Th (Thelper)-2 and Th-1 lymphocytes in lesional skin of naturally infected dogs was assessed. To achieve this objective, the authors evaluated the mRNA expression of canine cytokines such as IL-4, IL-13, TNF-α, and IFN-γ by real-time RT-PCR (qRT-PCR) to determine the cellular immune response in lesional skin biopsies from naturally infected dogs. qPCR was used to determine the number of *Leishmania* in paraffin-embedded tissue sections, and this was compared with cytokine expression in tissue lesions . In *Leishmania*-infected dogs, IL-4, TNF-α, and IFN-γ mRNA production were significantly higher than in controls. Furthermore, dogs with a high *Leishmania* burden demonstrated significantly higher IL-4 expression, whereas no difference was noted with regard to expression of other cytokines. By comparing the pattern of inflammation and cytokine expression, a clear trend became evident, that levels of IL-4, TNF-α, and IFN-γ were elevated both in biopsies with a periadnexal nodular pattern and in biopsies where the severity of the periadnexal infiltrate was equivalent to that of the perivascular interstitial infiltrate. Expression of IL-4, IL-13, and TNF-α was slightly increased in biopsies in which plasma cells prevailed over lymphocytes, whereas expression of IFN-γ was moderately higher when lymphocytes were predominant. In summary, this study demonstrates that the local immune response in naturally occurring leishmaniasis includes Th-1 as well as Th-2 cytokine subsets. Furthermore, the increased expression of the Th2-type cytokine IL-4 associated with both severe clinical signs and a high parasite burden in skin lesions connects severity of the disease to a Th-2-type of immune response (Brachelente *et al.*, 2005).

From Skin to other Organs

A variety of adhesion molecules are involved in phagocyte adherence to the extracellular matrix and cells of the connective tissue (Carlos and Harlan, 1994). Using adhesion blocking assays, our group observed that adherence of non-infected mononuclear phagocytes to the

inflamed connective tissue is mediated by beta-1 and beta-2 integrins (Carvalhal *et al.*, 2004). Flow cytometry experiments showed no consistent changes in the expression of several integrins on the surface of infected murine phagocytes (Carvalhal *et al.*, 2004; Pinheiro *et al.*, 2006). These data suggest that infected and non-infected phagocytes expressed similar amounts of integrins, even though adherence of the former cells to the connective tissue was diminished (Carvalhal *et al.*, 2004). CCR1 and CCR7 have been shown to be upregulated about two-fold compared to the control group (Steigerwald and Moll, 2005) and, after infection, there is a decrease in CCR4 and CCR5 expression on phagocytes infected with *L. amazonensis* (Pinheiro *et al.*, 2006). Another factor that may differentially interfere with the migration capability of infected cells is parasite burden. There is an inverse relationship between the degree of infection and the adhesive capability of infected cells. Although infection with small numbers of *Leishmania* does not reduce phagocyte adhesion to connective tissue, connective-tissue adhesion by infected phagocytic cells reaches levels of 20-30% that observed for non-infected cells under conditions of high parasite burden (Pinheiro *et al.*, 2006). These data are in accordance with evidence suggesting that heavily *Leishmania*-infected phagocytes present a wide spectrum of suppressive changes, including changes in B7 expression, impaired integrin function, and inhibition of the expression of a variety of genes in mononuclear phagocytes (Buates and Matlashewski, 2001).

We still have a long way to go to understand the mechanisms that control parasite dissemination in dogs. It appears, however, that animals exhibiting more strict control of parasite burden develop a more limited pattern of differential dissemination of infected cells. As recently shown by Reis and collaborators (Reis *et al.*, 2006), higher parasite burden is found in the skin and spleen than in the bone marrow, liver, and lymph nodes of infected healthy animals (Reis *et al.*, 2006). The parasite distribution tends to be more uniform among tissues of unhealthy animals, and parasitism tends to be more intense in the spleen of animals with more severe disease.

In some visceral leishmaniasis-endemic areas of Brazil, the skin of dogs is prone to be in an almost permanent inflammatory state, not always related to *Leishmania* infection. In a series based on the study of stray dogs from the streets of Jacobina (Bahia state, Brazil), 81% of the dogs without evidence of *Leishmania* infection had inflammatory infiltrates in the ear skin (dos-Santos *et al.*, 2004). Pucheu-Haston and collaborators (Pucheu-Haston *et al.*, 2006) showed that inflammation causes dog skin to function as a source of chemoattractants and favors phagocyte adherence (Carvalhal *et al.*, 2004) to the connective tissue. This may, therefore, constitute an important incentive to infected phagocytes remaining or disseminating to the skin of dogs during different phases of visceral leishmaniasis. A pattern of parasite dissemination to inflammatory sites has been confirmed in an experimental model of cutaneous leishmaniasis (Bertho *et al.*, 1994).

In fact, it is not well established how *Leishmania* parasites are transported from the original infection site in the skin to other organs. Transport of amastigotes by cutaneous Langerhans cells from the skin to the draining lymph nodes was demonstrated by Moll and collaborators (1993) in murine models of infection, and Fiorini and collaborators (2002) detected myeloid cells containing *Leishmania* in human blood (Fiorini *et al.*, 2002). In all lesions caused by *Leishmania*, the parasite is found inside mononuclear phagocytes, which maintain close contact with the extracellular matrix and cells of the connective tissue (Abreu-Silva *et al.*, 2004). In only a few instances have parasites been found free in the tissues (Santos-Gomes *et al.*, 2000; Wilson *et al.*, 1987). These data suggest that the main interface

between *Leishmania* and the host may be the mononuclear phagocyte cell surface. It also suggests that the relevant information for *Leishmania* to remain within or to leave tissues may be expressed on the surface of these phagocytes.

Another interesting aspect of cell migration that affects the tissue in leishmaniasis is related to the loss of lymphoid tissue structure in the spleen, as reported by some authors for visceral leishmaniasis. Such alterations have been described in human beings by Veress and collaborators in 1977 (Veress *et al.*, 1977), and have recently been re-examined in a number of important studies performed by the group led by Paul Kaye. These studies have shown that the observed changes in the lymphoid tissue of the spleen are due to impaired leukocyte migration into the white pulp induced by TNF (Engwerda *et al.*, 2002) and IL10 (Ato *et al.*, 2002). Basically, a number of interactions between lymphocytes and mononuclear phagocytes may be disrupted in the marginal zone of the spleen, disturbing the entry of cells into the white pulp and follicle organization (Ato *et al.*, 2002; Engwerda *et al.*, 2002). We recently observed a similar pattern of lymphoid tissue disorganization in the spleens of dogs with visceral leishmaniasis. Such changes were more prominent in animals with a susceptibility pattern in response to *L. chagasi* infection (negative leishmanin skin test and positive spleen culture for *Leishmania*) than in non-infected animals or in animals with a positive leishmanin skin test (Santana *et al.*, 2008). These alterations of the white pulp appear to be associated with the disappearance of a population of marginal zone macrophages defined by an HI1 monoclonal antibody staining (Aguiar *et al.*, 2004). Whether this represents a cause or consequence of tissue disorganization remains unclear. Such a loss of lymphoid tissue structure may, however, underlie the increased susceptibility of these animals to bacterial infection and enhanced dissemination of *Leishmania* during late stages of the disease.

Recently, the cellular response in spleen was investigated (Lages *et al.*, 2008; Strauss-Ayali *et al.*, 2007). In both works, the mRNA expression levels for a wide panel of cytokines, transcription factors, and chemokines were examined. Both studies clearly show that Th1-1 and Th-2 immune responses occur simultaneously in the spleen during canine *L. infantum* infection (Lage *et al.*, 2007; Strauss-Ayali *et al.*, 2007). The frequency of IL-12 and IFN-γ expression within symptomatic dogs was significantly different from that of the uninfected group, although there were no significant differences between the symptomatic groups with respect to the expression of these cytokines (Lage *et al.*, 2007). In accordance, the other study identified higher IFN-γ, T-bet, IP-10, and RANTES mRNA levels in infected dogs during both oligosymptomatic and polysymptomatic stages of the disease (Strauss-Ayali *et al.*, 2007). These results agree with those reported by Quinnell and collaborators (Quinnell *et al.*, 2001b), who suggested that IFN-γ expression is not an appropriate indicator of resistance since asymptomatic and polysymptomatic dogs accumulated similar levels of this cytokine in tissues, as is the case in humans, mice, and hamsters (Lage *et al.*, 2007). On the other hand, the Th-2 immune response in dogs was differentially described by these works. In one study, positive correlations between the levels of IL-10 expression with respect to the progression of the disease were observed. The other authors identified increased IL-4 and IL-5 expression during oligosymptomatic disease instead of enhanced expression levels of the Treg (T regulatory)-associated cytokines, IL-10 and TGF-β (Strauss-Ayali *et al.*, 2007).

Blood Compartment

The results of the serological analysis of VL-infected dogs also identified mixed Th-1 and Th-2 responses in the serum of infected dogs, with detectable expression levels of IFN-γ, TNF-α, and IL-12 together with IL-4 and IL-10. However, when clinical indications are considered alongside the biochemical data, the Th-2 response appears to be predominant, since the expression of IL-4 increased within the symptomatic group while the expression of IL-12 increased within the asymptomatic group (Santos-Gomes *et al*., 2002).

The nature of the dog's PBMC responses to *Leishmania* is not completely understood. Asymptomatic dogs show protective immunity, which has generally been associated with a strong proliferative response of peripheral blood lymphocytes to leishmanial antigens (Cabral *et al*., 1992; Pinelli *et al*., 1995; Pinelli *et al*., 1994). However, development of a Th-1 and Th-2 mixed response by antigen-stimulated PBMCs from asymptomatic dogs expressing IL-2, IFN-γ, and IL-10 mRNA transcripts has also been reported. Although, in these studies, IL-2 and IFN-γ predominated in asymptomatic dogs, the development of symptomatic infections could not be related to IL-10 expression (Carvalho *et al*., 1994; Chamizo *et al*., 2005). Thus, in contrast to what occurs in human visceral leishmaniasis, the role played by PBMC-expressed IL-10 in *L. chagasi*-infected dogs is not well established (Carvalho *et al*., 1994).

Dogs with symptomatic CVL (Berrahal *et al*., 1996) present with depressed T cell-mediated functions and high levels of specific antibodies (Abranches *et al*., 1991; Barbieri, 2006; Killick-Kendrick *et al*., 1994; Oliva *et al*., 2004; Santos-Gomes *et al*., 2002). These animals present immunological changes involving T cells, including absence of delayed type hypersensitivity (DTH) to *Leishmania* antigens (Berrahal *et al*., 1996; Oliva *et al*., 2004; Quinnell *et al*., 2001b), decreased T cell numbers in the peripheral blood (Cabral *et al*., 1998; Killick-Kendrick *et al*., 1994; Oliva *et al*., 2004), and absence of IFN-γ and IL-2 production by PBMCs *in vitro* (Alvar *et al*., 2004; Oliva *et al*., 2004; Pinelli *et al*., 1994). Interestingly, the Th-1 cytokine profile in bone marrow aspirates positively correlates with humoral, but not with lymphoproliferative responses to *Leishmania* antigen. It is noteworthy that increased accumulation of IL-4, IL-10, and IL-18 mRNA was not observed in infected dogs, and the mRNA for these cytokines did not correlate with antibody or proliferative responses. However, infected dogs with detectable IL-4 mRNA display significantly more severe symptoms (Quinnell *et al*., 2001b). These data suggest that clinical symptoms are not due to a deficiency in IFN-γ production.

It is well established that early events are considered to be a determinant of infection outcome in humans and mice (Gomes *et al*., 2000; Rogers and Titus, 2004; Shankar and Titus, 1993; Veras *et al*., 2006). Prediction of dog immune responses *in vivo* early after exposure to *L. chagasi* is a difficult task (human models). We have established an *in vitro* priming system (PIV) using naïve canine PBMCs in order to assess dog PIV immune response to *L. chagasi* (Rodrigues *et al*., 2008). We co-cultivated PBMCs primarily stimulated with *L. chagasi in vitro* with autologous infected macrophages and found that IFN-γ mRNA is upregulated in these cells compared to control unstimulated cells. IL-4 and IL-10 mRNA expression in *L. chagasi*-stimulated PBMCs was similar to control unstimulated PBMCs when incubated with infected macrophages. Surprisingly, correlation studies showed that a lower IFN-γ/IL-4 expression ratio correlates with a lower percentage of infection. We proposed that the direct correlation between the IFN-γ/IL-4 ratio and parasite load is

dependent on the positive 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 the results indicate that a balanced expression of IFN-γ and IL-4 by these naïve cells is associated with control of parasite infection *in vitro* (Rodrigues *et al*., 2008).

In experimental infections, intradermal inoculation of promastigotes triggers asymptomatic infections, and PBMCs from these dogs stimulated with soluble leishmanial antigens (SLA) *in vitro* express both Th-1 cytokines, such as IL-12, IFN-γ, TNF-α, and IL-18, and Th-2 cytokines, such as IL-4, IL-6, and IL-10. Despite the fact that PBMCs from these asymptomatic dogs present such apparently mixed Th-1 and Th-2 responses, they predominantly produce IL-12 and IFN-γ. 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). We recently observed that PBMCs from immunized dogs and than subcutaneously challenged with *L. chagasi* promastigotes are still asymptomatic. PBMCs from these apparently protected dogs liberate IFN-γ into the cell supernatant upon *L. chagasi* stimulation *in vitro* (Rodrigues *et al*., 2007). Moreover these cells express IFN-γ but not IL-4 mRNA (Rodrigues *et al*., 2007), showing that these dogs display a predominant Th-1 type of immune response. On the other hand, dogs experimentally infected by intravenous inoculation of amastigotes develop progressive symptomatic infections. PBMCs from these dogs produce reduced levels of both Th-1 and Th-2 cytokines (IFN-γ, IL-2, IL-12, IL-6, and IL-10) during the active phase of the disease (Santos-Gomes *et al*., 2002).

In murine models, it is well established that macrophages participate in parasite killing via reactive oxygen and nitrogen intermediate-dependent mechanisms. However, mechanisms involved in *Leishmania* killing by canine macrophages have not been as thoroughly investigated. There are cumulative data implicating canine macrophages in parasite killing by a NO-dependent mechanism. NO produced by macrophages has been found to be the principal effector molecule responsible for mediating intracellular killing of *Leishmania* (Holzmuller *et al*., 2006; Panaro *et al*., 2001; Pinelli *et al*., 2000). A canine macrophage cell line incubated with supernatant (containing IFN-γ, IL-2, and TNF-α) produced significant amounts of NO, sufficient to mediate *L. infantum*-killing (Pinelli *et al*., 2000). PBMCs from vaccinated dogs were also able to reduce macrophage infection via an NO-dependent mechanism upon *in vitro* stimulation with both *Leishmania* promastigotes and concanavalin A (ConA). This effect was potentiated by the addition of LPS (Panaro *et al*., 2001). Using a macrophage cell line, Pinelli and collaborators (Pinelli *et al*., 2000) showed that parasite burden is reduced upon activation of cells with cytokine-rich supernatants. These supernatants were obtained from a *Leishmania*-specific T cell-line generated from dogs immunized with soluble Ag (Panaro *et al*., 2001). Infected canine macrophages incubated with autologous lymphocytes of immunized dogs also induced IFN-γ with increased NO production (Holzmuller *et al*., 2005). The increased IFN-γ production and NO release by macrophages suggest a role for this cytokine in iNOS induction. We recently established an *in vitro* model to test whether PBMC supernatants from asymptomatic dogs immunized with promastigote lysates and infected with *L. chagasi* promastigotes were able to stimulate PBMC-derived macrophages from healthy dogs to control parasite infection (Rodrigues *et al*., 2007). Using our system, we demonstrated for the first time that PBMCs from these asymptomatic dogs stimulated exclusively with *L. chagasi in vitro* reduce macrophage infection by the parasite (Rodrigues *et al*., 2007). Moreover this effect is associated with high IFN-γ, but not IL-4,

mRNA expression and release of this Th1 cytokine into the PBMC supernatant via an NO-dependent mechanism, as AMG reversed this effect (Rodrigues *et al*., 2007). In contrast to other works (Panaro *et al*., 2001), PBMCs in our system were exclusively stimulated with *L. chagasi in vitro*. Additionally, the protective response of these dogs to *L. chagasi* was demonstrated by the positive proliferative response to *Leishmania* antigens exhibited by PBMCs from these dogs *in vitro* (Rodrigues *et al*., 2007). In addition, PBMCs from the majority of these immunized and experimentally infected dogs expressed IFN-γ mRNA and released IFN-γ upon LSA stimulation. These data suggest that lymphocytes from apparently protected dogs produce cytokines related to a protective immune response (Rodrigues *et al*., 2007). A recent study demonstrated that, although Th-1 and Th-2 cytokines are produced in asymptomatic *Leishmania*-infected dogs, there is a prevalent Th-1 cytokine response that confers immunity against the parasite (Chamizo *et al*., 2005). Finally, our data reinforce the notion that the leishmanicidal effect of canine macrophages is NO-dependent (Rodrigues *et al*., 2007).

It was recently demonstrated that there are some differences in iNOS expression in lesion macrophages *in situ*. iNOS-negative dermal and splenic macrophages contain numerous *Leishmania* amastigotes. In contrast, dermal and splenic macrophages, which present high iNOS expression, contain few or no amastigotes, suggesting that iNOS-positive activated macrophages are able to destroy and/or do not allow multiplication of intracellular amastigotes (Zafra *et al*., 2008). PBMC-derived macrophages infected with *L. infantum* produce a significantly higher amount of NO than uninfected macrophages *in vitro* (Panaro *et al*., 2008; Rodrigues *et al*., 2007). In a comparison between infected dogs, the levels of NO in supernatants of *Leishmania*-infected macrophages were significantly higher in symptomatic than in asymptomatic animals. However, four months after diagnosis, the addition of autologous lymphocytes significantly decreased NO production only in symptomatic dogs, while NO production by macrophages co-cultured with autologous lymphocytes was significantly reduced eight months after diagnosis in *Leishmania*-infected macrophages from both asymptomatic and symptomatic dogs (Panaro *et al*., 2008). These higher levels of NO observed during follow-up of symptom-free (only 8 months) animals may suggest a protective role for this molecule in long-term asymptomatic parasitism.

CONCLUSION

The nature of the dog's cellular immune response is not completely understood. Evidence points that although CVL dogs develop a mixed Th-1 and Th-2 cellular immune response, asymptomatic dogs present positive lymphoproliferative assay *in vitro* or/and a positive skin test early in infection, as well as predominance of Th-1 cytokines. On the other hand, as the disease progresses in susceptible dogs, the protective responses diminish with involvement of either IL-4 or IL-10 in uncontrolled infection. Disease progression occurs together with parasite dissemination. *Leishmania*-infected mononuclear phagocyte may stay or leave inflammatory sites disseminating the parasite through the host tissues. The migration of these cells depends upon the leukocyte phenotype and is modulated by parasite burden that results in changes in integrin function and in the expression of chemokine receptors.

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