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Research paper

Systemic and compartmentalized immune response in canine visceral leishmaniasis

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ABSTRACT

Human visceral leishmaniasis (VL) and canine visceral leishmaniasis (CVL) are the most important emerging diseases with high prevalence in Latin American countries and are mainly caused by *Leishmania* (*L.*) *chagasi* (Syn = *L. infantum*). CVL has a great impact on Brazilian public health because domestic dogs are the most important VL peri-domicile reservoirs in both urban and peri-urban areas. Our findings highlight the complexity of cellular immunological events related to the natural infection from dogs by *L. chagasi*, additionally correlating major peripheral blood phenotypic markers with clinical status and tissues parasite density. Our main results demonstrated that lower frequency of circulating B cells and monocytes are important markers of severe CVL, whereas increased levels of $CD8^+$ lymphocytes appear to be the major phenotypic feature of asymptomatic disease. Determination of the isotypes patterns during CVL demonstrated that asymptomatic dogs and those with low parasitism are associated with an increase of IgG1, while the symptomatic dogs and those with high parasitism are associated with an increase of IgG, IgG2, IgM, IgA and IgE immunoglobulins. Pioneer findings obtained by our group showed a correlation between clinical status of CVL with degree of tissue parasite density. This data demonstrated that asymptomatic dogs presented low parasitism while symptomatic dogs are associated with high parasite load in various tissues such as skin, bone marrow and spleen. We have also investigated the association between tissue parasitism and CVL clinical forms. Regardless of clinical status, skin and spleen are the major sites of high parasite density during ongoing CVL. Furthermore, we demonstrated that bone marrow and spleen parasite density are the most reliable parasitological markers to decode the clinical status of CVL. In this article, we have reviewed some aspects

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Abbreviations: VL, visceral leishmaniasis; CVL, canine visceral leishmaniasis; WHO, world health organization; AD, asymptomatic dogs; OD, oligosymptomatic dogs; SD, symptomatic dogs; IFAT, indirect immunofluorescence assay; ELISA, enzyme linked immunosorbent assay; LDU, leishman donovan units; Ig, immunoglobulins; LP, low parasitism; MP, medium parasitism; HP, high parasitism; qPCR, quantitative real-time PCR; LST, leishmanin skin test; PBMC, peripheral blood mononuclear cells; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; LN, lymph node; Th, T helper; IL, interleukin; T-bet, Th1-specific T box transcription factor; IP-10, human interferon-inducible protein 10; RANTES, regulated upon activation, normal T-cell expressed and secreted; TGF-β, transformation growth factor-beta.

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of the histopathological and immunological events occurring in natural and experimental *L. chagasi*/*L. infantum* infection, pointing out the main *L. chagasi*-parasitized tissue. We have discussed the importance of the association between parasite density, immunological/histopathological aspects and clinical status of the CVL, their current applications, challenges for the future and potential opportunities in CVL research.

1. Impact of canine control on the epidemiology from canine and human visceral leishmaniasis

Visceral leishmaniasis (VL) is one of the most important emerging diseases with high prevalence in Latin American countries (Tesh, 1995). VL and canine visceral leishmaniasis (CVL) are mainly caused by *Leishmania* (*L.*) *chagasi* (Syn = *L. infantum*) in South America and Europe (Mauricio et al., 2000). Peri-domestic sand flies acquire the etiological agent by feeding on infected wild/domestic reservoirs and transmit it, causing severe disease in humans (Tesh, 1995; WHO, 2000). Nowadays, VL is currently expanding worldwide, mainly in Brazil, where recently, the typically rural outline has shifted to a progressively urbanized profile (Palatnik-de-Sousa et al., 2001). VL and CVL have a great impact on Brazilian public health because domestic dogs are the most important VL peri-domicile reservoirs in both urban and peri-urban areas (Reithinger and Davies, 1999; Palatnik-de-Sousa et al., 2001). The major prophylactic practice to control VL, as recommended by WHO, involves a systematic treatment of human cases, besides vector control by insecticide and *Leishmania*-seropositive dogs euthanasia (Tesh, 1995; Palatnik-de-Sousa et al., 2001). Control of CVL remains not completely established mainly because there is no effective vaccine available to be used for immunoprophylaxis (Gradoni, 2001; Giunchetti et al., 2007, 2008c,d). In the past 5 years, more than two million dogs were screened and more than 160,000 seropositive dogs were eliminated, but the incidence of human VL has not been reduced to an acceptable level (Braga et al., 1998). Moreover, drug treatment of infected dogs is expensive and unfortunately, different treatment strategies have failed to achieve a consistent parasitological cure for CVL owing to the permanence of latent *Leishmania*-infected cells (Baneth and Shaw, 2002; Noli and Auxilia, 2005). Based on either the similarity of clinical signs observed in human and dogs and the evolution of natural history of the disease, CVL has been suggested as a good model to better understand the pathogenesis of the human disease (Sanchez et al., 2004; Reis et al., 2006a).

2. Clinical and biochemical/hematological biomarkers of progression in CVL

Canine visceral leishmaniasis manifests itself as a broad clinical spectrum ranging from asymptomatic infection to patent severe disease, which mostly culminates in death (Reis et al., 2006b). According to Mancianti et al. (1988), asymptomatic dogs (AD) do not show visible clinical signs because they are apparently healthy animals (Fig. 1A), whereas oligosymptomatic dogs (OD) present some signs such as cutaneous ulcerations, frequently observed on the tip of the ears and on the periorbital areas, moderate weight loss and localized alopecia (Fig. 1B and C). Symptomatic dogs (SD) may show one or more typically clinical signs of CVL, such as cutaneous ulcerations all over the body, blindness, severe weight loss (anorexia), onychogryphosis, furfuraceous dermatitis, general alopecia, keratoconjuntivitis, progressing towards general morbidity state and death (Fig. 1D–J). From the localized cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of the bone marrow, lymph node (LN), liver and spleen, as well as kidney and gastrointestinal tract (Tryphomas et al., 1977; Keenan et al., 1984a). Initial clinical signs are hypertrophy of lymph node, dermatitis and periorbital and nasal dermatitis that could be disseminated. The opaque bristles, onychogryphosis and edema of the paws could be also found. Other signs such as fever, apathy, diarrhea, intestinal hemorrhage, weight loss, hepatosplenomegaly, hyperkeratosis, cutaneous ulceration, particularly on the nose, ears, tails and keratoconjunctivitis are frequent, although not necessarily present in all animals (Genaro et al., 1988; Dias et al., 1999).

The CVL presumptive diagnosis is generally performed by serological tests, such as indirect immunofluorescence assay test (IFAT) and enzyme linked immunosorbent assay (ELISA), associated with clinical and epidemiological records. The major problem regarding clinical diagnosis is the fact that CVL signs are very similar to those observed in other infectious diseases (Costa et al., 1991). The chronic aspect of the disease and its long incubation period may generate a delay or failure in clinical diagnosis (Cardoso and Cabral, 1998). Despite their high sensitivity, serological tests present a broad range of cross-reactions with other protozoan (Costa et al., 1991; Grimaldi and Tesh, 1993). However, the parasitological diagnosis presents generally low sensitivity when the tissue parasitism is scarce (Reis et al., 2006a). Considering the clinical forms of the disease, it was possible to define various biomarkers closely related to the parasitological, biochemical and hematological findings observed during clinical progression of the CVL. These findings include normocytic/normochromic anemia and increase of total serum protein levels. It seems that biochemical alterations are linked to a polyclonal humoral immune response, which leads to a raised protein levels in serum (Marzochi et al., 1985; Reis et al., 2006a), characterized by hyperglobulinemia and hypoalbuminemia, besides of decreased albumin/globulin ratio (Cardoso and Cabral, 1998; Almeida et al., 2005; Strauss-Ayali et al., 2007). An impaired hematological status is associated with severe clinical aspects of CVL to be evidenced by marked anemia and leucopenia in decurrence of lymphopenia, eosinopenia and monocytopenia (Reis et al., 2006a,c).

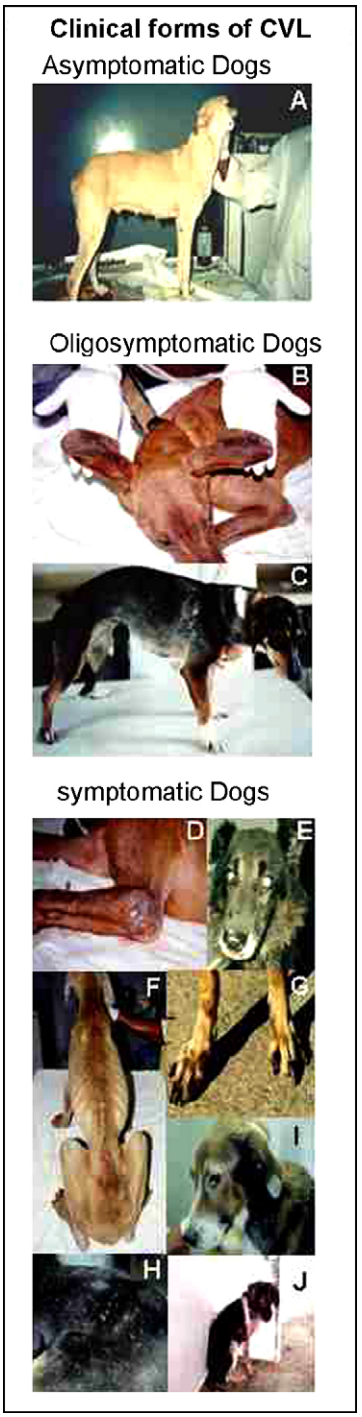

Fig. 1. Clinical status from dogs naturally infected by *L. chagasi*. (A) Asymptomatic dogs do not show visible clinical signs of the disease; (B and C) oligosymptomatic dogs present some signs of the disease such as cutaneous ulcerations on the tip of the ears and periorbital areas (B) as well as moderate weight loss and localized alopecia (C); (D–J) symptomatic dogs have disseminated cutaneous ulcerations (D), blindness (E), severe weight loss (F), onycogriphosis (G), furfuraceous dermatitis (H), general alopecia, keratoconjuntivitis (I) and progress to forward general morbidity state (J); CVL, canine visceral leishmaniasis.

3. Tissues parasite load and clinical progression in CVL

Evaluation of parasite load by "leishman donovan units" (LDU), number of *Leishmania* amastigote by 1,000 nucleated cells (Stauber, 1955 modified by Reis et al., 2006a), and anti-*Leishmania* detection by immunohistochemistry are important parasitological tools to verify parasite density in different lymphoid compartments (Tafuri et al., 2001, 2004; Sanchez et al., 2004; Reis et al., 2006a,b,c; Giunchetti et al., 2006, 2008a,b). In studies performed by our group, tissue parasitism for each compartment was initially classified as low (LP), medium (MP) or high (HP) parasitism based on tissue-specific LDU values statistically categorized into tertiles (Reis et al., 2006a,b). The data demonstrated that AD group presented low parasitism while SD group presented high parasite load in various tissues (skin, bone marrow, spleen, liver and lymph node) (Reis, 2001; Reis et al., 2006a,b,c; Giunchetti et al., 2006, 2008a,b). In this scope, our data showed correlations between AD group with low parasitism and SD group with high parasitism in skin, bone marrow and spleen (Reis et al., 2006b). Previous data obtained by our group has also investigated the association between tissue parasitism in distinct compartments and CVL clinical forms. Despite the clinical status, skin and spleen are the major sites of high parasite density during ongoing CVL (Reis et al., 2006b). Furthermore, we demonstrated that bone marrow and spleen parasite density are the most reliable parasitological markers to decode the clinical status of CVL (Reis et al., 2006b). More recently, Francino et al. (2006) proposed the quantitative real-time PCR (qPCR) to elucidate the status of dogs that are positive for *Leishmania* by conventional PCR, especially in endemic areas. The qPCR turned out to be also very useful to follow-up the parasite load in order to estimate the efficacy of the treatment, vaccines trials or the CVL evolution.

4. Systemic immunological biomarkers of clinical progression in CVL

In the last decade, various research groups have concentrated efforts studying immunopathology of dogs naturally and experimentally infected by *L. chagasi*/*L. infantum* (Cabral et al., 1992; Martinez-Moreno et al., 1993, 1995; Pinelli et al., 1994, 1995, 1999a,b; Brandonisio et al., 1996; Bourdoiseau et al., 1997; Nieto et al., 1999; Alvar et al., 2004; Tafuri et al., 2004; Solano-Gallego et al., 2004, 2005; Giunchetti et al., 2006; Reis et al., 2006a,b,c; Strauss-Ayali et al., 2007; Lage et al., 2007; Cardoso et al., 2007; Rodriguez-Cortes et al., 2007; Giunchetti et al., 2008a,b). After the pioneering studies lead by Pinelli et al., some authors have searched immunological markers according to *in vivo*, *ex vivo* and *in vitro* context to evaluate clinical progression of CVL (Pinelli et al., 1994, 1995).

The *in vivo* analysis of the intradermal delayed hypersensitivity test, the leishmanin skin test (LST) (Montenegro, 1926), is an useful tool for both clinical diagnosis and epidemiological studies of human VL. The test is negative during the acute stage but it becomes and remains positive following the resolution of clinical symptoms (Reed et al., 1986; Badaró et al., 1986, 1996; Carvalho et al., 1992). The LST was also used for the

diagnosis from a dog population in an endemic region of VL (Cardoso et al., 1998; Cabral et al., 1998). These authors showed that the prevalence of the infection increases considerably using LST to detect *Leishmania*-specific cellular immunity, in comparison with the prevalence obtained only by culture and serology, confirming that the prevalence and incidence of CVL have still been underestimated (Dye et al., 1993; Tesh, 1995). The use of the LST to evaluate *in vivo* immune status to distinguish asymptomatic and symptomatic dogs is also controversial, because the SD group might react in a similar manner to the AD group (Reis, 2001). Baleeiro et al. (2006) tested various antigens obtained by *L. chagasi*, *Leishmania braziliensis* and *Leishmania amazonensis* in VL endemic area in Brazil. These authors showed that the use of antigens from different *Leishmania* species might interfere with the results of the immunological tests performed in dogs naturally infected by *L. chagasi*. Further investigations will be necessary in an endemic area to define the role of the LST in natural and experimental *Leishmania* infections and its applications in vaccine trials to CVL.

In symptomatic CVL, cellular immune response is impaired, as indicated by studies showing that peripheral blood mononuclear cells (PBMC) from dogs fail to respond to parasite antigens both *in vitro* and *in vivo*. Protective immunity has generally been associated with a distinct cellular immune response, manifested by a strong proliferative response of PBMC to leishmanial antigens (Cabral et al., 1992; Carvalho et al., 1981; Sacks et al., 1987; Pinelli et al., 1999a,b) accompanied by the IFN-γ and TNF-α production, which are required for macrophage activation and killing of intracellular parasites (Kemp et al., 1993; Liew and O'Donell, 1993; Nacy et al., 1991; Pinelli et al., 1995).

The role of anti-leishmanial cellular and humoral immunity during systemic and compartmentalized immune response underlying the susceptibility/resistance during CVL has been recognized throughout *ex vivo* and *in vitro* investigations (Pinelli et al., 1994, 1995; Bourdoiseau et al., 1997; Solano-Gallego et al., 2001b, 2005; Reis et al., 2006a,b,c; Giunchetti et al., 2006, 2008a,b; Lage et al., 2007). In order to assess the cellular immune response, we used the immunophenotypic approaches by flow cytometry to study immunological features of circulating leucocytes as immunological markers for clinical status and bone marrow parasite density in dogs naturally infected by *L. chagasi* (Fig. 2) (Reis et al., 2006c). T cells analysis in the peripheral blood showed high levels of $CD5^+$ T cells in AD and OD groups as compared to SD group. On the other hand, the SD group presented low levels of $CD4^+$ T cells as compared to AD group. AD and OD groups presented an increase of $CD8^+$ T cells as compared to SD

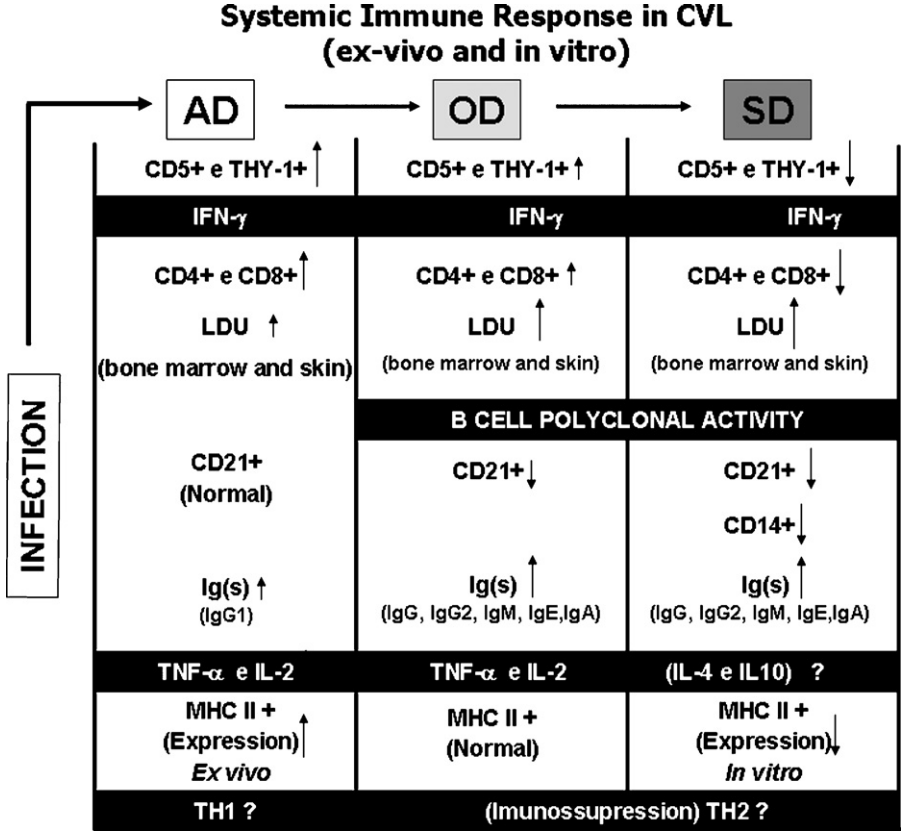

Fig. 2. Clinical status *versus* cellular and humoral immune response profile in the peripheral blood, bone marrow and skin from dogs naturally infected by *L. chagasi*; CVL, canine visceral leishmaniasis; AD, asymptomatic dogs; OD, oligosymptomatic dogs; SD, symptomatic dogs; LDU, leishman donovan units; Ig, immunoglobulins.

and control groups. Interestingly, increased levels of $CD8^+$ lymphocytes appear to be the major phenotypic feature of asymptomatic disease. A decrease in the number of B cells in SD group as compared to AD and control groups were also observed (Reis et al., 2006c). Moreover, a decrease in the number of circulating $CD14^+$ monocytes in SD group as compared to control group was detected (Fig. 2). Furthermore, bone marrow parasite density is related closely to major phenotypic changes reported for peripheral blood leucocytes and pointed out as hallmarks of the clinical status of CVL. We observed a lower number of $CD5^+$ T cells, their subpopulations ($CD4^+$ and $CD8^+$) as well as a lower number of B cells and monocytes mainly in dogs with high parasitism (Reis et al., 2006c). Several reports have focused on the relationship between distinct clinical forms of CVL, disease progression and the IgG isotype levels, in both experimental and natural *L.* (*L.*) *infantum*/*L.* (*L.*) *chagasi* infections (Solano-Gallego et al., 2000, 2001a,b; Leandro et al., 2001; Vercammen et al., 2002; Quinnell et al., 2003; Cordeiro-da-Silva et al., 2003). Although the majority of these investigations have been performed based on well-established ELISA and Western-blot protocols, controversial data on immunoglobulin isotype profiles are frequently documented. Increased levels of IgG and IgG2 have been indiscriminately reported for AD and SD groups as described by Bourdoiseau et al. (1997) and Vercammen et al. (2002). However, according to Deplazes et al. (1995), Nieto et al. (1999) and Solano-Gallego et al. (2001a,b), SD group showed considerably higher anti-*Leishmania* IgG1 antibodies in comparison to asymptomatic carriers. Additionally, Courtenay et al. (2002) and Quinnell et al. (2003) reported that higher levels of anti-*Leishmania* IgG/IgG1 and lower levels of IgG2 were also observed in SD group (Day, 2007). However, Leandro et al. (2001) and Cordeiro-da-Silva et al. (2003) have documented increased levels of IgG2 in sera samples from infected animals, particularly in the case of SD group. Despite these controversial findings regarding the immunoglobulin isotype profile associated with CVL, it is clear that during canine *Leishmania* infection a dichotomous humoral immune response is triggered, similarly with the human infection (Anam et al., 1999). Recently, our group has studied the isotype patterns of immunoglobulins as hallmarks for clinical status and tissue parasite density from Brazilian dogs naturally infected by *L. chagasi* (Reis et al., 2006b). We have observed that AD and LP groups are associated with an increase of IgG1, while the SD and HP groups are associated with an increase of IgG, IgG2, IgM, IgA and IgE immunoglobulins (Fig. 2).

5. Compartmentalized immune response in different lymphoid organs in CVL

Analysis of the immune response in different compartments during chronic infection is a useful new scientific strategy for the study of the immune response in parasitic/infectious diseases. This approach allows simultaneous investigation of the immunological events observed in the peripheral blood and may indicate whether they are representative of those occurring in the lymphoid tissues (Teixeira-Carvalho et al., 2002).

Although CVL is known to be a severe systemic disease, there are a few studies describing detailed histopathological features of distinct host compartments affected by the parasite. Aiming to better understand events related to compartmentalized immunopathology of CVL, several research groups have performed a broad investigation focusing on histopathological, parasitological and immunological aspects of skin, spleen, liver and lymph nodes (LN) in dogs naturally and experimentally infected by *L. chagasi*.

The skin is the first point of contact with *Leishmania* from sand fly vectors, and recent investigations demonstrated that in endemic regions a large population of parasitized asymptomatic dogs is living (Berrahal et al., 1996; Cabral et al., 1998; Solano-Gallego et al., 2000, 2001a,b). Earlier reports of CVL described various macroscopical aspects of skin lesions (desquamation, alopecia, pustular dermatitis, ulcerative dermatosis and nodular disease), which may be associated with immune response (Adler and Theodor, 1932; Cunha, 1938; Torres, 1941; Ferrer et al., 1988). The histopathologic evaluation of the skin biopsies revealed that in all *Leishmania*-infected dogs the predominant inflammatory cells were macrophages. Lymphocytes and plasma cells were the second most frequent cell types.

Dos-Santos et al. (2004) showed that inflammatory infiltrates are a common histological finding in the skin of dogs regardless of the presence of parasites or evidence of infection with *Leishmania*. However, skin parasitism in CVL was always associated with inflammation. Macrophages infiltrates were more frequently associated with the presence of *Leishmania* and a higher parasite burden than pleomorphic focal or diffuse inflammatory infiltrate was found.

We have described that SD group presented intense dermal inflammatory infiltrate characterized as diffuse with a high parasitic burden, decrease in the levels of collagen type I and increase in collagen type III (Giunchetti et al., 2006) (Fig. 3A and B). Furthermore, sections stained with hematoxylin and eosin demonstrated a higher intensity of inflammatory changes in SD group than AD group. Correlation between cellular phenotypes and histological changes seemed to reflect cellular activation and leucocytes migration from peripheral blood to the skin, mediated by antigenic stimulation. The results suggested that chronic dermal inflammation and cutaneous parasitism were directly related to the severity of clinical disease (Giunchetti et al., 2006) as related by Martinez-Moreno et al. (1995), Tarantino et al. (2001) and Solano-Gallego et al. (2004).

Brachelente et al. (2005) demonstrated that the local immune response in CVL includes Th1 as well as Th2 cytokine subsets suggesting that increased expression of the Th2 cytokine-IL-4 is associated with both severe clinical signs and a high parasite burden in the skin lesions. In addition, Guarga et al. (2000) demonstrated that dogs naturally infected by *L. infantum* presenting lower count of $CD4^+$ cells were more capability to infect sand fly vector.

Considering the spleen as one of the major affected organs during CVL, significant alterations in its morphology and immunological aspects become a relevant

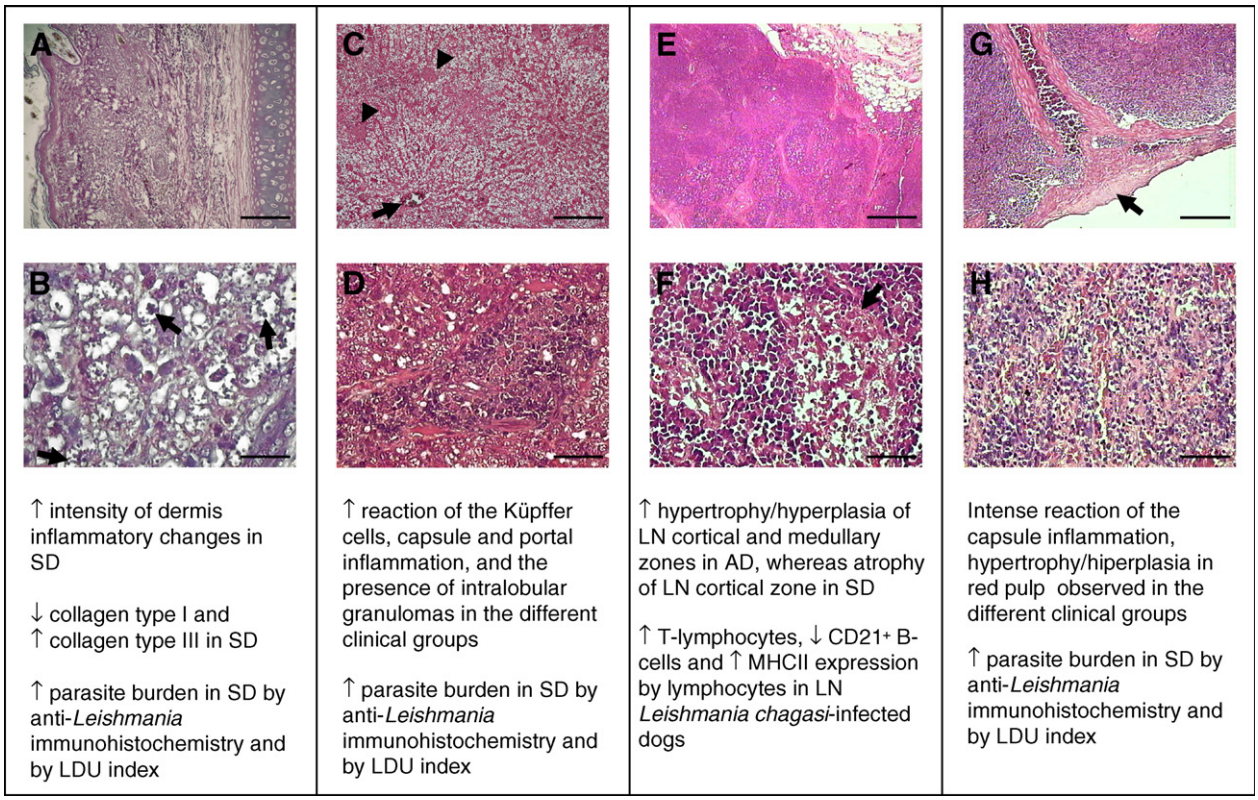

Fig. 3. Photomicroscopes of hematoxylin–eosin stained specimens highlighting the major histopathological features of *Leishmania chagasi*-infected dogs considering ear skin (A and B), liver (C and D), popliteal lymph node (E and F) and spleen (G and H). (A) Chronic inflammation of the dermis showing sparse cellular infiltrates in the superficial and deep dermis (bar = 200 μm); (B) high parasite burden presenting amastigotes forms (arrowed) inside vacuolated macrophages in the ear skin (bar = 50 μm); (C) granulomatous inflammatory reaction with intralobular granulomas dispersed in the hepatic parenchyma (arrow heads) and centrilobular vein (arrow) (bar = 200 μm); (D) inflammation of portal tract showing cellular mononuclear infiltrate characterized by plasmocytes, macrophages and lymphocytes (bar = 50 μm); (E) hypertrophy/hyperplasia of cortical and medullary regions observed in lymph node (bar = 500 μm); (F) chronic inflammation in the lymph node capsule with amastigotes forms (arrowed) inside vacuolated macrophages (bar = 50 μm); (G) inflammation of the splenic capsule showing capsule thickened (arrowed) suggesting collagen deposition (bar = 500 μm); (H) hypertrophy/hyperplasia of red pulp showing cellular mononuclear infiltrate characterized by plasmocytes, macrophages and lymphocytes in spleen (bar = 50 μm). AD: asymptomatic dogs; SD: symptomatic dogs; LN: lymph node.

investigation, because it is a site where cell activation might occur during the infection. Since the contribution of the immune response in the genesis of splenomegaly during CVL is still unclear, we have developed a morphological and phenotypic analysis of spleen biopsies from dogs with CVL. In Fig. 3G and H, the splenic tissue presents capsule inflammation more frequently in infected dogs as compared to control group. These alterations are more intense in OD and SD groups as compared to AD group. We also observed hypertrophy and hyperplasia in red pulp from spleen in all infected groups, characterized by mononuclear infiltrate cells, mainly plasmocytes. The white pulp presented the substitution of macrophage by lymphocytes in decurrence of hypertrophy and hyperplasia of this region (Fig. 3G and H).

Recently, studies referring to the evaluation of cytokines profile in spleen cells from dogs naturally/experimentally infected by *L. chagasi*/*L. infantum* were conduced (Lage et al., 2007; Strauss-Ayali et al., 2007). Lage et al. evaluated the cytokines expression in splenocytes from dogs naturally infected by *L. chagasi* and did not detected differences on the TNF-α, IL-12, IL-4, IFN-γ and IL-10 levels when the dogs were classified by different clinical forms. However, when the dogs were categorized by different parasite load, the authors observed that hundred percent of dogs with high parasitism expressed IL-10 as compared to those with low and medium parasitism. These data suggest that CVL is marked by a balanced production of Th1/Th2 cytokines, with a predominant accumulation of IL-10, probably as a consequence of an increase in parasite load and progression of the disease. These data still showed a possible relationship between intensity of splenic parasitism and evolution of the clinical manifestations of CVL (Lage et al., 2007).

Strauss-Ayali et al. developed a study investigating the immune response in splenocytes of *L. infantum*-infected dogs following experimental and natural infection. Increased levels on IFN-γ, T-bet, IP-10 and RANTES were observed in all evaluated groups. IL-4 levels increased as early as one month after experimental infection, while IL-5 was high at later stages. IL-10 and TGF-β did not change during the infection. The study indicated that both type-1 and type-2 immune responses occur in the spleen during CVL and suggested that the early elevation of IL-4 might

have a role in the persistence of parasites in the presence of high IFN-γ expression.

The immunopathological evaluation of the hepatic compartment associated with parasitism and biochemical findings have been also performed in order to better understand the genesis of hepatomegaly in CVL (Giunchetti et al., 2008b). Intense reaction of the Kupffer cells, capsule and portal inflammation and the presence of intralobular granulomas were observed in the different clinical groups (Fig. 3C and D). SD group presented a higher frequency of parasitism as compared to AD group. Inflammatory alterations were more intense in the SD group and were associated with parasitism. Moreover, the results indicated an association between histological liver changes (inflammation of the hepatic capsule, portal inflammation, and hypertrophy/hyperplasia of the Kupffer cells) and the enhancement of biochemical alterations (plasmatic globulin) according to progression of clinical forms of CVL.

Despite LN is one of the most relevant lymphoid tissues involved in the parasite-host interface during *L. chagasi* infection, the cellular and molecular basis of the compartmentalized immune response as well as histopathology into LN is not completely established. There are only few studies focusing on the LN during CVL (Keenan et al., 1984a,b; Martinez-Moreno et al., 1993; Tafuri et al., 2001; Lima et al., 2004; Giunchetti et al., 2008a). A detailed histopathologic analysis was performed by our group aim to increment information about parasite load and major immunophenotypic features of the LN in *L. chagasi*-infected dogs (Giunchetti et al., 2008a). Our major histopathological findings highlighted that hypertrophy/hyperplasia of LN cortical and medullary zones were the principal characteristics observed in AD group, whereas atrophy of LN cortical zone was predominant feature in SD group (Fig. 3E and F).

In CVL, lymphadenopathy is usually defined as an increase in LN size (enlargement of LN), usually described as a regional or generalized alteration (Rogers et al., 1993). It has been demonstrated that all LN from *L. chagasi*-infected dogs display a chronic lymphadenitis, regardless of the anatomical region analyzed, with hypertrophy/hyperplasia of cortical and medullary zones (Lima et al., 2004). However, the clinical status or the tissue parasitism load might not be directly related to the intensity of the lesions, once previous data demonstrated that AD group presented higher LN parasitism than OD or SD groups (Giunchetti et al., 2008a).

6. Conclusion and new challenges

Our findings highlight the complexity of cellular immunological events related to natural infection from dogs by *L. chagasi*, correlating major peripheral blood phenotypic markers with clinical status and tissues parasite density. Our data suggest that the sustained T cell compartment (both $CD4^+$ and $CD8^+$ T cells) observed in AD and LP groups may be resultant from the high activity of the host immune system to perform antigen presentation and to remove parasites from affected sites. Lower frequency of circulating B cells and monocytes are important markers of severe CVL, whereas increased levels of $CD8^+$ lymphocytes appear to be the major phenotypic feature of asymptomatic disease. Despite the clinical status, skin and spleen are the major sites of high parasite density during ongoing CVL. Furthermore, we demonstrated that bone marrow and spleen parasite density are the most reliable parasitological markers to decode the clinical status of CVL.

Additional studies on the specificities of the activated cells and their both cytokine and chemokine profiles may provide important information that will lead to a better understanding of the immunological/inflammatory events that take place in the distinct lymphoid compartments during canine visceral leishmaniasis.

Conflict of interest

None.

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