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Characterization of macrophage polarization in lesions of dogs inoculated with *Leishmania (Leishmania) infantum* (BH401) strain

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Abstract

The aim of this work was to describe the anatomical pathology of dogs experimentally infected with *Leishmania (Leishmania) infantum* strain MCAN / BR / 2002 / BH401, a Brazilian form of *L. infantum* isolated from a symptomatic dog from an endemic area. For this, five beagles (three months old and both sexes) composed the experimental group. Markers of macrophage subpopulations M1 and M2 (related to resistance and susceptibility to visceral leishmaniasis) and the tissue cytokine transforming growth factor beta 1 (TGF-β1) (one of the main cytokines related to the fibrosis process and anti-inflammatory action) were evaluated in livers, lungs and kidneys. The BH 401 *L. infantum* strain induced classical lesions of the visceral disease where all evaluated organs showed a chronic inflammatory reaction and tissue parasitism associated with a higher expression of CD163 and TGF-β1 markers, might be related to the progression of the disease. In this work it was possible to conclude that the BH 401 strain reproduces canine visceral leishmaniasis that occurs naturally.

Key words: Visceral leishmaniasis, pathologic anatomy, *L. infantum*, macrophages, TGF-β1, CD163, Calprotectin (L1).

Background

Human Visceral Leishmaniasis (HVL) is an important parasitic zoonotic disease, potentially fatal if not treated, responsible for high global morbidity and mortality. Brazil reported 16.08 cases of HVL per 100,000 inhabitants in 2015, the second highest number in the world and highest in the Americas (2,15). Models for study of HVL include dogs, hamsters, mice, non-human primates, and in vitro systems (5,27,28,32). Dogs are generally considered the best model, because they reproduce better the pathology observed in HVL where they develop similar symptoms (except depilation, onychogryphosis, emaciation and severe kidney lesions) and anatomopathological aspects to HVL (8,36, 37,38,43).

There are many published experimental protocols for the development of canine visceral leishmaniasis (CVL). Some studies have evaluated laboratory clinical and parasite virulence aspects in experimental dogs, for examples: hypergammaglobulinemia and enlarged lymph node (3,5,13,35,48), the deposition of immunoglobulins (Ig) (IgG, IgM, IgA) and complement C3 in dogs with renal failure (8), the parasite load in spleen and liver with hepatosplenomegaly (12,20,25), the infectivity of promastigotes and amastigotes and parasite load (10,17), the immune response (4,6,7,30,31,40,41,42,44, 45,47,49) and other aspects. Understanding of these aspects is extremely important to elucidate the mechanisms that cause organ damage during the course of visceral leishmaniasis, caused by different strains of *Leishmania (Leishmania) infantum*. However, in literature, little information is available on the anatomical pathology aspects of experimental CVL. Thus, the aim of this study was to describe histological alterations of dogs experimentally infected with a Brazilian strain of *L. infantum*. Silva et al. (46), working with twenty-

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four mongrel dogs naturally infected with *L. infantum*, described a systemic chronic inflammatory disease associated with major fibrosis mainly in liver, lung and kidney. Based on these results, we decided to investigate microscopic alterations in parallel to macrophage markers, CD168 and calprotectin-L1, in these three organs, as well as transforming growth factor-beta (TGF-β1), a central cytokine involved in this systemic chronic inflammation.

Methods

Animals

Six 3-month-old beagle dogs, of both sexes, were purchased from the Tads Henriques kennel Colombo, Paraná, Brazil, a non-endemic geographic area for CVL. The dogs were kept in the kennels with food and water *ad libitum* and vaccinated for rabies, distemper, hepatitis/adenovirus type 2, leptospirosis, and parvovirus (6). Before the experimental infection, blood samples were collected for serological evaluation and no animals showed detectable levels of anti-*Leishmania* antibodies. All dogs were infected intravenously with $1x10^7$ promastigotes/mL of the *Leishmania* strains suspended in PBS. Seroconversion was evident 90 days post-infection, at which time, the infection was also confirmed by parasitological screening (*Leishmania* DNA by PCR) (6). However, one dog was excluded from the study, due to death of no conclusive occurrence before 90 days of seroconversion.

The control dogs comprised four uninfected 3 -month-old beagles of both sexes, acquired from the same kennel. The dogs were kept in the ICB kennels in the manner described above. These dogs also served as sentinels in the kennel during the experimental period, with all being negative for *Leishmania* DNA by PCR (6). These dogs were euthanized at the same period of time as the infected group.

All the experimental procedures adopted in this project followed the standards of the Ethics Committee on Animal Experimentation in Research and have been approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Minas Gerais, under protocol number 204/2018.

Parasites

Promastigotes of *L. (L.) infantum* MCAN/BR/2002/BH401 (BH401) strain, previously obtained from a naturally infected dog, were isolated from the spleen of infected hamsters. This strain was cultured at 25°C in α-Mem medium (Cultilab) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Cultilab), 0.4 g/L NaHCO3, 4 g/L HEPES, 200 U/mL penicillin (Cultilab), and 100 µg/mL streptomycin (Cultilab), pH 7.4. Culture conditions with respect to exponential growth phase (7–10 days), temperature, parasite concentration, and culture medium were identical for the strains (6).

Euthanasia and histology

Twenty-four months post-infection, all five infected dogs were anesthetized with 0.5 ml/kg ketamine and xylazine and euthanized with intravenous thiopental (2.5%). After necropsy, fragments of liver, lung, kidney, spleen, cervical lymph nodes, bone marrow, skin, and the gastrointestinal tube were collected and fixed in 10% neutral buffered formalin and prepared conventionally for histology. The paraffin blocks were cut in 3-4 µm sections and mounted on ethanol-ether-degreased slides. For this work, sections from liver, lung and kidney were stained with hematoxylin-eosin and slides were prepared for immunohistochemical, and examined by light microscopy (Olympus BX40 microscope).

Immunohistochemical detection of the expression CD163, calprotectin (L1), TGFβ1, and Leishmania amastigotes.

Sections of liver, lung, and kidney were dewaxed and pre-treated for antigenic recovery using a 0.1% citrate retriever buffer solution (pH 6.0) (DAKO®, Vila Real Carpinteria, CA, code S1699)incubated in a water bath for 30 minutes at 97°C. Monoclonal antibodies were used as primary antibodies as follows: (1) (MΦ) anti-calprotectin (L1 antigen) clone MAC387 (DAKO), dilution 1:100 (9,26); (2) monoclonal antibody anti-TGF-β1 cytokine clone V9, (BIO-RAD, 1:100) (26); (3) anti-macrophage (MΦ) anti-CD163 clone ED2, (Santa Cruz Biotechnology), 1:100 (31). To detect *Leishmania* in tissue, we used as cross-reactive primary antibodies heterologous immune sera from dogs naturally infected with *L. infantum* diluted 1:100 in 0.01M PBS (24,50). All slides were incubated 18-22 h at 4°C in a humid chamber, washed in PBS, incubated with biotinylated goat anti-mouse and anti-rabbit antibodies (LSAB2 kit; Dako), washed with PBS, and incubated with streptavidin-peroxidase complex (LSAB2 kit; Dako) for 20 min at room temperature. The reaction was developed with 0.024% diaminobenzidine (DAB; Sigma, St. Louis, MO) and 0.16% hydrogen peroxide (40% vol/vol). Slides were counterstained with Harris's hematoxylin, dehydrated, cleared, and mounted with coverslips.

Morphometrical Analysis

Morphometrical analysis of immunolabeled *Leishmania* amastigotes (tissue parasite load); immunolabeled macrophages (macrophage cell markers) and the cytokine TGF-β1 were carried out. Twenty images of each slide were captured at 400x magnification (Olympus, USA). The method of imaging, segmentation of images, and the definition of morphometry conditions has been previously described (6,14,26).

Statistical analysis

The analyses were carried out using the program GraphPad Prism 5.0. One-way ANOVA followed by the Tukey's post test and/or unpaired t test was used for groups

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with parametric distribution, and the Kruskal-Wallis test followed by Dunn's post-test was used for groups with nonparametric distribution. The differences were considered significant when $p \leq 0.05$.

Results and Discussion

Histology

All experimentally infected dogs showed classic lesions of CVL as previously described in naturally infected dogs (46,51). A general histological alteration was: (1) Spleen was characterized by a hypertrophy and hyperplasia of macrophages mainly of the red pulp, no rare parasitized with numerous amastigotes forms of *Leishmania*. Granuloma formation in the red pulp was also observed consisted mainly of epithelioid cells and also vacuolated parasitized macrophages. In parallel, numerous of conspicuous plasma cells (plasmacytosis) with were observed; (2) Lymph nodes exhibited hypertrophy and hyperplasia of the medullary cords and medullary sinuses characterized by edema and a chronic cell inflammatory infiltrate of macrophages, lymphocytes and numerous plasma cells, as well; (3) Bone marrow showed hypertrophy and hyperplasia of the myeloid lineage cells and presence of fibropoesis; (4) Skin, the cellular inflammatory infiltrate was diffuse in the upper dermis and around vessels, glands and pylus follicles in the deep dermis . Gastrointestinal tract hypertrophy and hyperplasia of cells of the *lamina propria*, chiefly macrophages and plasma cells from the stomach to the rectum.

Liver samples from infected dogs showed intense chronic hepatitis characterized by the presence of mononuclear cells (plasma cells, lymphocytes and macrophages) located in the subcapsular, portal, and periportal area. Inside the hepatic lobules, this chronic cellular infiltrate formed nodular structures called intralobular granulomas (51) composed of epithelioid macrophages with and without parasites, plasma cells and lymphocytes with rare presence of neutrophils polymorphonuclear cells. Other histological anomalies such as hyperplasia and hypertrophy of Kupffer cells, sinusoidal congestion, and fatty and hydropic degeneration in hepatocytes were frequently observed.

Lung of infected dogs displayed chronic and diffuse interstitial pneumonitis characterized by alveolar septa thickening as a consequence of an inflammatory mononuclear cell infiltrate. This cellular infiltrate was predominantly composed of plasma cells, macrophages, and lymphocytes, with the rare presence of neutrophils and/or eosinophils. Not rare, numerous macrophages showed a peculiar morphology as epithelioid cells with large shapeless nuclei and pale cytoplasm with irregular borders.

Kidney of all infected dogs showed chronic membranoproliferative, membranous, and sclerosing glomerulonephritis. The main glomerular lesions were characterized by glomeruli containing either non-cellular amorphous material with an acidophilic appearance glomerulosclerosis, or amorphous acidophilic material accompanied by hypertrophic podocytes and the presence of mononuclear inflammatory cells such as macrophages. The glomeruli also showed a reduction in Bowman's space. We observed proliferation of parietal cells associated with migration of monocytes and macrophages into the urinary space characteristic of crescentic glomerulonephritis, indicating rapidly progressing glomerulonephritis.

The search for canine experimental protocols that reproduce clinical, immunological, and anatomopathological aspects homogeneously throughout the infection and effort of several experimental protocols. Some factors involved are (1) breed of animal or genetic differences among individual dogs; (2) dose and route of *inoculum* with or without insect saliva; (3) phases and virulence of *Leishmania* parasites (promastigotes and amastigotes) maintained in the laboratory *in vitro* and *in vivo*; (4) variability of the response of each animal, that is, the same inoculum prepared for a certain group of dogs can promote different clinical forms of the disease (3,4,5,36,43). Therefore, some animals may develop manifestations of disease while others remain asymptomatic or show transient clinical signs or even spontaneous cure showing natural resistance (1,5,22,29,41,44,47).

The intravenous route seems to be preferred as the best way to obtain symptomatic dogs in a shorter pre-patent period, making clinical trials more economically viable (1,52,53). As discussed by Abbehusen et al (1), the ideal and most natural way possible would be the intradermal route using vector insects (sandflies), but this implies in maintaining a robust colony of sandflies in the laboratory, which is often not feasible. In addition, according to these authors and others (37,38), the phlebotomine saliva does not seem to significantly enhance or modulate the course of experimental infection in dogs. Infections with *Leishmania* amastigote or promastigote forms do not seem to interfere much in the course of infection when considering the intravenous route. In fact, average 86% rates of infection are described using amastigote forms of *Leishmania* (3, 12, 38) very similar to the average rates of infection 90% when using promastigote forms (3, 29, 32). Intravenous inoculum of 5 x 107 promastigotes of *Leishmania*, promote 100% success for experimental infection in beagles according to several authors (1,35,41,42). Thus, a similar protocol was carried out in this work.

Experimental infections in dogs have been carried out since the beginning of the 20th century. However, only few manuscripts describe histological aspects (pathology) of experimentally infected dogs (20,22,23,27,28,29). From these works, only Keenan et al. (23) describes an anatomical pathological aspect of three dogs experimentally infected with *L. chagasi* (syn *L. infantum*) and three dogs experimentally infected with *L. donovani*. Thus, these authors described only liver, spleen and skin histological alterations. Adding to that, our histopathological results of liver, lung and kidney could give a contribution where some histological aspects have not been explored so far, such as: (1) Liver: a chronic hepatitis defined by a conspicuous

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and defined intralobular epithelioid granulomas was seen in all infected animals. Previous works describe it as only small aggregates of large macrophages; (2) Lung: a diffuse chronic pneumonitis characterized by an intense interstitial thickening of alveolar septa. No rare notable epithelioid granulomas and numerous fibrosis focus were also seen; (3) Kidney: all forms of chronic glomerulonephritis were found in all animals in the following frequency: membranoproliferative glomerulonephritis (MPGN), membranous glomerulonephritis (MGN), focal segmental glomerulonephritis (FSGN), and (4) sclerosing glomerulonephritis (SGN). Chronic interstitial nephritis characterized by multifocal or diffuse infiltration of plasma cells (plasmacytosis) is also a frequent lesion. These reports were not found in the literature (experimental infection), even though the manuscript published by Keenan et al. (23). Besides all histological analysis described in liver, lung, kidneys, we could say that analysis of spleen, lymph node, bone marrow, skin (pina) and gastrointestinal tract was also examined along characterizing the systemic chronic inflammatory reaction. In addition, amastigotes forms of *Leishmania* were easily found in all three organs described. Important to call attention especially considering lungs and kidneys parenchyma where it is not so easy to observe parasites in naturally infected dogs as well (4).

Immunohistochemistry

Immunohistochemistry to detect amastigotes in all organs showed similar tissue parasite load in all three organs as liver, lung, and kidney. Thus, there was no statistical difference among the three organs. (One way-ANOVA unpaired test; Liver P =0.4518; Lung P= 0,9849; Kidney P=0.7518) (data not shown). Calprotectin (L1) and CD163 positive cells were those that make up the hepatic intralobular granulomas, in the interalveolar septa in the lungs, glomeruli and kidney tubules. After morphometrical analysis, the expression of calprotectin (L1) in the liver, lung and kidney did not show any difference between the BH 401 group and the uninfected control group. However, CD 163 antigen expression was higher in all tissues evaluated in BH 401 animals compared to controls (Fig. 1, Fig. 2). TGFβ-1 expression was observed in the liver parenchyma along

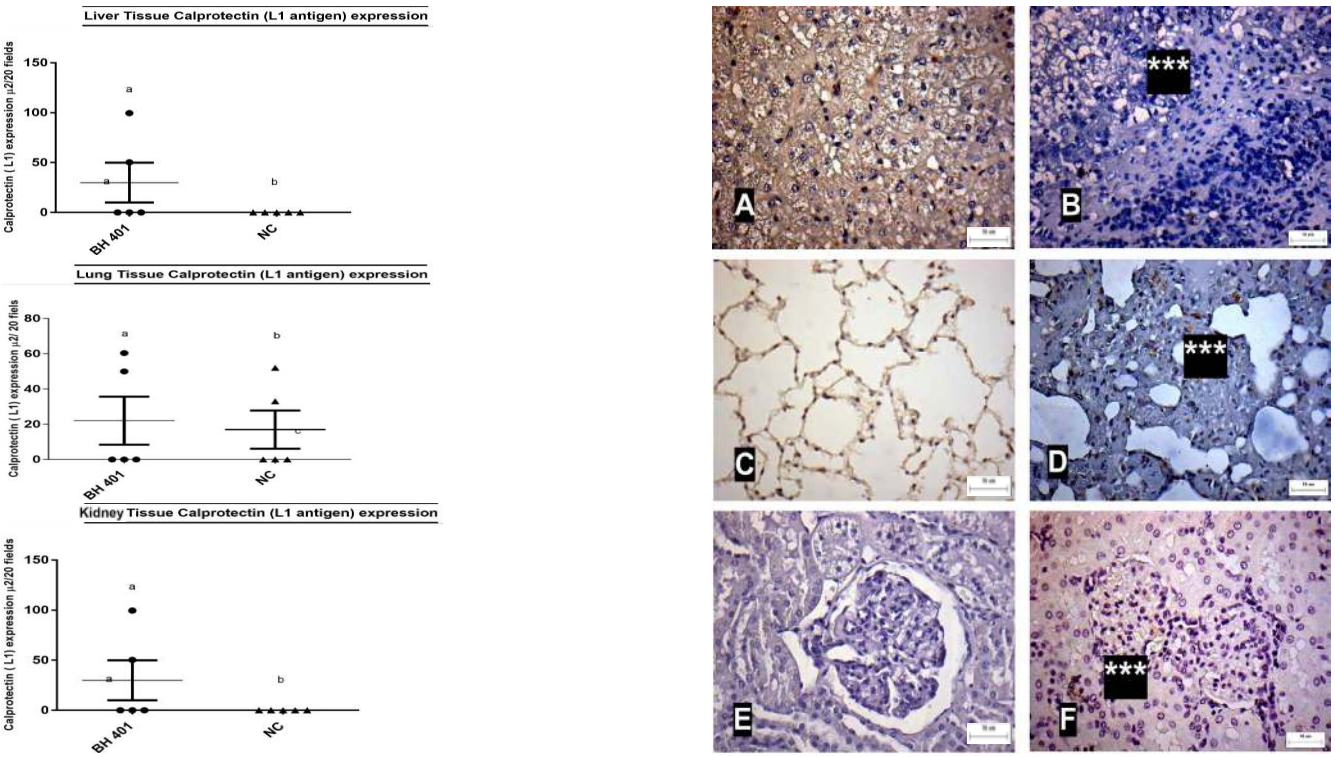

Figure 1. Calprotectin (L1) antigen marker immunohistochemical study. On the left side observe three graphs showing a quantitative of the expression of L1 antigen in liver, lung and kidney, respectively control group (Liver) ; B. *** calprotectin (L1) positive cells in the liver parenchyma in the BH 401 group . C. Negative control group (Lungs); D.*** calprotectin (L1) positive cells in the interalveolar septa in the lungs in the BH 401 group; E. Negative control group (Kidneys); F. *** calprotectin (L1) positive cells in the and tubules in the kidneys of BH 401 group. Statistical analysis : Dogs experimentally infected with strain BH401 group and negative controls (NC). Results are displayed as mean ± SEM; n = 5 dogs in the BH401 group, 4 in the NC groups respectively. There was no statistical difference when compared (a) to (b) . Paired T Test. Liver: **P < 0.2071; Lungs: ***P< 0.7215; kidneys ***P < 0.5023. ANOVA "one-way" seguido do pós-teste de Tukey. On the right side, liver, lungs and kidneys sections expressing calprotectin (L1) antigen stained by immunohistochemistry. **A.** Negative control group (Liver) ; **B.** *** calprotectin (L1) positive cells in the liver parenchyma in the BH 401 group . **C.** Negative control group (Lungs); **D.**** calprotectin (L1) positive cells in the interalveolar septa in the lungs in the BH 401 group; **E.** Negative control group (Kidneys); **F.** *** calprotectin (L1) positive cells in the and tubules in the kidneys of BH 401 group.

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the sinusoids (Kuppfer cells), in the cells that make up the hepatic intralobular granulomas, without exception. In the lung, the expression of this marker occurred in pneumocytes, epithelial cells of terminal bronchioles and alveolar sacs. In the kidney, it was seen in the proximal glomeruli and tubules and rarely in areas of interstitial nephritis. All infected dogs of group BH 401 strain showed a higher average of TGFβ expression in relation to the negative control group, for all organs studied (Fig. 3).

Macrophages expressing the calprotectin (L1) antigen have been described as an M1 profile cell (9,11). Brandtzaeg et al. (11) in a study in humans, observed Küpffer cells negative for leucocyte calprotectin (L1 antigen). It was supposed that labeled cells represented only monocytes/ macrophages attracted to the liver parenchyma and not resident cells (Küpffer cells). Moreover, other studies have shown L1 positive cells (macrophages) as effector cells. Mozos et al (34), for example, in a study of in a study with 29 dogs with cutaneous leishmaniasis, showed L1 positive cells with no intracellular *Leishmania* amastigotes. In visceral model, Castro et al. (14), working with naturally infected dogs treated with liposome encapsulated meglumine antimoniate and allopurinol, showed lower numbers of L1 positive cells in these treated dogs. Authors implied lower chemotaxis for monocytes/macrophages L1 positive cells in parallel with a lower parasite as result of the treatment. However, in this work, after the analysis of L1 expression, the animals in the BH 401 group showed no statistical difference from the negative control group. Thus, BH 401 group showed an intense systemic chronic inflammatory response with no higher numbers of L1 positive cells associated with a higher parasite load reported.

As we know, macrophages were first recognized for their role in host immunity and phagocytosis followed by evidence of their importance in development, tissue homeostasis, metabolism, and tissue regeneration (21,33). They express a variety of receptors which are upregulated in response to inflammation. However, the activation of these receptors can result in a pro-inflammatory response (M1 classical macrophages) or an anti-inflammatory response (M2 alternative macrophages) (33). A variety of scavenger receptors expressed by M2 macrophages including CD163,

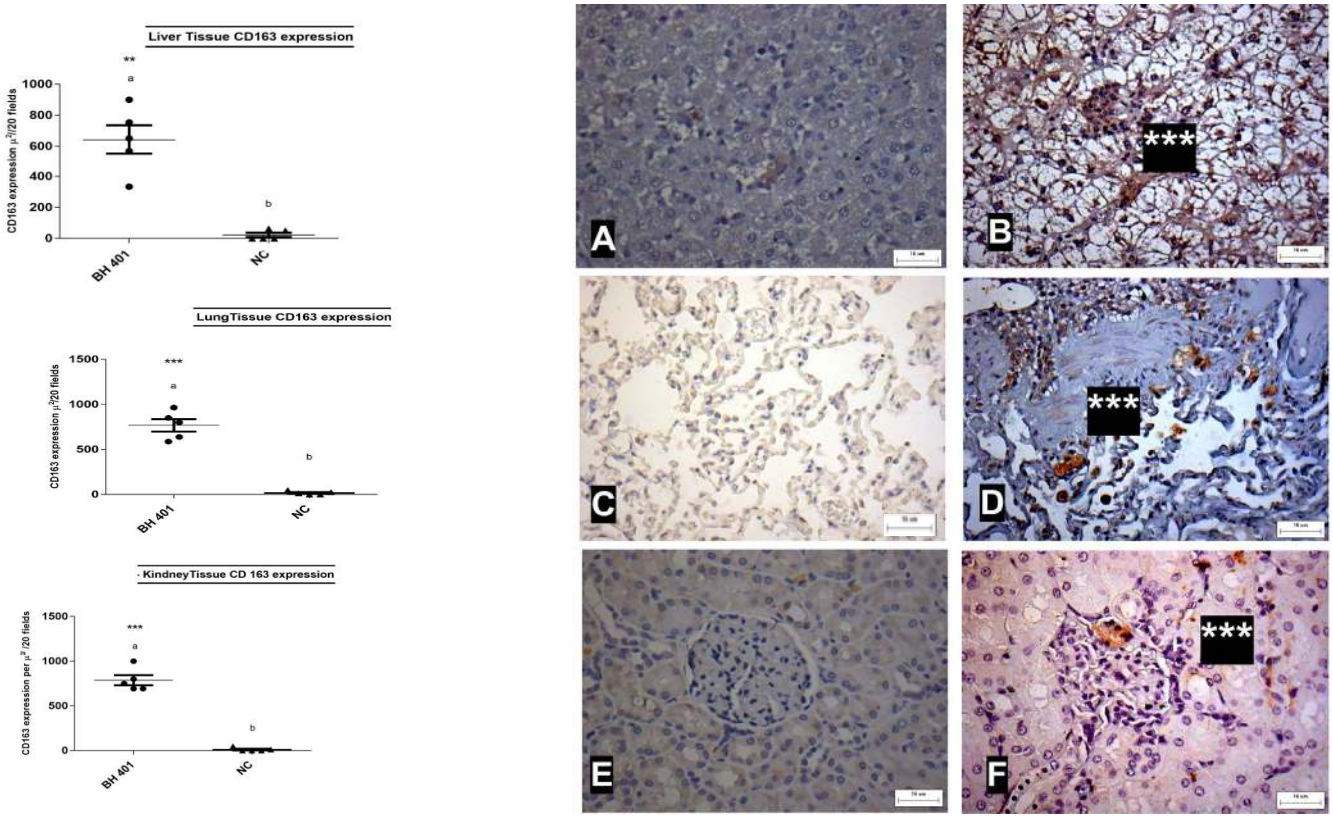

Figure 2. CD163 macrophage marker immunohischomical study: On the left side observe three graphs showing a quantitative of the expression od CD163 antigen in liver, lung and kidney, respectively. Dogs experimentally infected with strain BH401 (Group BH401) and negative controls (NC). Statistical analysis : Results are displayed as mean ± SEM; n = 5 dogs in the BH401 group, 4 in the NC groups respectively. Statistical difference was observed in all cases; Liver: **P < 0,0018; Lungs: ***P< 0,0005; kidneys ***P < 0.0001. ANOVA "one-way" seguido do pós-teste de Tukey. On the right side, liver, lungs and kidneys sections expressing CD163 antigen stained by immunohistochemistry. A. Negative control group (Liver), B *** CD163 positives cells in the liver parenchyma and in the hepatic intralobular granulomas; C. Negative control group (Lungs); D. *** CD163 positives cells in the interalveolar septa; E. Negative control group (Kidney); F. *** CD163 positives cells in the glomeruli and tubules in the kidneys.

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which are upregulated in response to inflammation, was explored in this study. CD163 is a 130 kDa protein exclusively expressed by monocytes and macrophages. The main function of CD163 is to remove hemoglobin-haptoglobin complexes from the blood circulation during intravascular hemolysis (6,39). However, CD163 is also involved in the production of anti-inflammatory and anti-oxidative substances (IL-10, ferritin, bilirubin, CO) (16). Herein, animals inoculated with the BH 401 strain showed greater expression of CD 163 compared to the negative control groups. As we reported before, it was associated with an intense systemic chronic inflammatory response, but in parallel to a highest tissue parasitic load. These findings corroborate the studies carried out by Roy et al. (44) that demonstrated that in the human visceral disease caused by *L. donovani* and *L. infantum* there is an increase in M2 macrophages and a decrease in M1 macrophages, characterized by increased levels of CD163, IL-10 and CXCL14. In addition, Moreira et al. (31) in CVL, found predominance of CD163 positive cells (M2 macrophages) in organs with higher parasite load. These authors also discussed that the predominance of the M2 response in the initial stage of the disease is responsible for the visceralization of the disease.

TGFβ1 plays an important role in the progression of leishmaniasis by suppressing the expression of inducible NO synthase, IFN-γ and the development of Th1 and Th2 cells (18). In studies using rodents, several authors report the increase of this cytokine in infection by *L.infantum* (7,19). In dogs, naturally infected with *L.infantum*, Alves et al. (7) observed higher expression of IFN-γ and TNF-α that the of asymptomatic dogs with a low parasitic load, indicating these cytokines as protective against infection. On the other side, a highest expression of IL-10 and TGF-β and high parasitic load was observed in symptomatic dogs, suggesting a role for these cytokines in disease progression. Herein, the immunohistochemistry study in all BH401 group showed greater intensity of lesions and tissue parasitism associated with a higher expression of CD163 and TGF-β1, which might be related to the progression of the disease. Thus, the increase in TGF-β1expression observed in this study may be related to the decrease in the expression of calprotectin (L1), since the increase in this cytokine is related to a regulatory action of the M1 response and a central role in the pathogenesis of CVL.

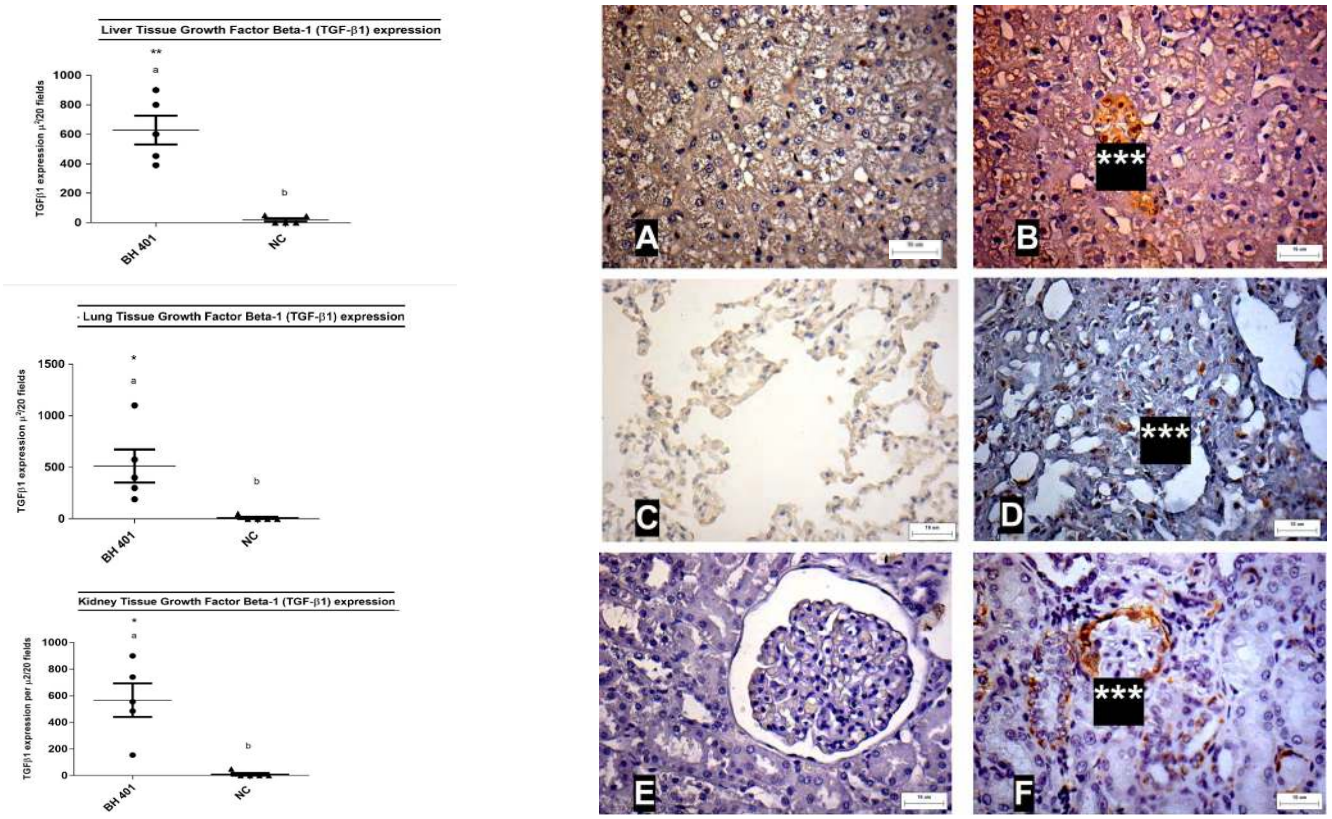

Figure 3. CA-F. TGF-β cytokine fibrosis marker related immunohischomical study: On the left side observe three graphs showing a quantitative of the expression of TGF-β antigen in liver, lung and kidney, respectively. Dogs experimentally infected with strain BH401 (Group BH401) and negative controls (NC). Statistical difference was observed in all cases; Liver: **P < 0,0049; Lungs: ***P< 0,0369; kidneys ***P < 0.0121. ANOVA "one-way" seguido do pós-teste de Tukey. On the right side: Liver, lungs and kidneys sections expressing TGFβ antigen stained by immunohistochemistry. A. Negative control group (Liver), B *** TGFβ positives cells in the intralobular granulomas; C. Negative control group (Lungs); D. *** TGFβ positives cells in the interalveolar septa; E. Negative control group (Kidney); F. ***TGFβ positives cells in the glomeruli and tubules in the kidneys. Statistical analysis: Dogs experimentally infected with strain BH401 and negative controls (NC).

Conclusions

Based on the histopathological findings, associated with the parasitic tissue load, the decrease expression of the L1 and increase expression of CD163 and TGF-β1, we might conclude that the BH 401 strain is considerably pathogenic and reproduces the disease similar to natural visceral canine leishmaniasis.

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