Parasitology Research (2018) 117:1829–1837
https://doi.org/10.1007/s00436-018-5870-2

ORIGINAL PAPER

# Immunodetection of hepatic stellate cells in dogs with visceral leishmaniasis

Natália Cassaro Marques [1] · Pamela Rodrigues Reina Mo reira [1] · Paulo Henrique Leal Bertolo [1] · Fábio Nelson Gava [2] · Rosemeri de Oliveira Vasconcelos [1]

Received: 29 December 2017 / Accepted: 9 April 2018 / Published online: 27 April 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

**Abstract**

Hepatic stellate cells (HSC), or Ito cells, store vitamin A when at rest but undergo phenotypic changes in situations of liver injury, which may induce fibrosis, and they may participate in the immune response in the liver. The objective of the present study was to investigate the role of HSC in the livers of dogs with visceral leishmaniasis (VL). Twenty-eight livers from dogs infected with VL that were living in an area endemic for the disease were evaluated, among which 13 were asymptomatic (A) and 15 were symptomatic (S). A control group (C) was formed by five dogs from an area that was not endemic for VL. These organs were subjected to histopathological analysis (Masson's trichrome for fibrosis) and immunohistochemical analysis (*Leishmania*, smooth-muscle α-actin and TGF-β). In the livers from the symptomatic dogs, a moderate to severe granulomatous inflammatory reaction was observed in the capsule and in the portal, centrilobular and intralobular regions. In the asymptomatic dogs, there was slight to moderate presence of granulomas, and these were even absent in some dogs. The intensity of hepatic fibrosis was predominantly low in the infected dogs (A and S), and fibrosis was absent in the control group. The immunomarking of HSC in the infected groups (A and S) differed significantly ($P = 0.0153$) from that of the control group. The symptomatic dogs presented the largest number of positive cells. This group also presented a larger number of parasitized macrophages, but did not differ statistically from the asymptomatic group ($P > 0.05$). The cytokine TGF-β was only detected at low levels, and only in the infected animals, but this did not differ from the control group. Immunomarking for HSC was observed mainly in the nuclei of cells present in the hepatic granulomas of symptomatic dogs and in the sinusoids of the asymptomatic dogs. It was concluded that in the livers of dogs with VL, the HSC are activated and participate in the hepatic response to the parasite. The cytokine TGF-β may be involved in this activation, but in the chronic phase of the infection, this cytokine was detected at lower proportions. It is possible that HSC may also contribute towards chemotaxis of leukocytes for the hepatic compartment, along with other cell types such as Kupffer cells.

**Keywords** Ito cells · Liver · *Leishmania infantum* · TGF-β · Hepatic fibrosis

## Introduction

Leishmaniasis is a zoonosis caused by protozoa of the genus *Leishmania* and may produce cutaneous, mucocutaneous, and visceral manifestations, according to the species involved. Dogs are considered to be the main urban reservoirs for the human disease. The prevalence of visceral leishmaniasis (VL) is greater in the canine population than in the human population (Figueiredo et al. 2014).

In canine VL, one of the organs affected is the liver (Lima et al. 2004; Reis et al. 2009; Moreira et al. 2016a). Infected livers present increased volume and inflammation in the capsule, portal spaces, and sinusoids, due to the presence of granulomas with variable numbers of parasites inside macrophages, along with hypertrophy and hyperplasia of Kupffer cells (Tafuri et al. 2001; Lima et al. 2007; Giunchetti et al. 2008; Moreira et al. 2016b).

Increased volume of hepatic extracellular matrix is commonly observed in situations of chronic inflammation such as parasitic infections (Andrade 1994), and it has been described

✉ Pamela Rodrigues Reina Mo reira
pamela_rreina@yahoo.com.br

[1] Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista (UNESP), Via de Acesso Prof. Paulo Donato Castellane, s / n , Jaboticabal , SP 14884-900 , Brasil

[2] Universidade do Missisipi Medical Center , Jackson , MS, USA

in dogs infected with *Leishmania infantum* (Melo et al. 2009). In human VL, because of ectasia and persistence of the antigen, chronic stimulation of hepatic Kupffer cells occurs, in association with an interstitial reaction in Disse's space, which causes activation of hepatic stellate cells (HSC) (Corbett et al. 1993). Presence of fibrogenesis is related to interactions between HSC and other components of the hepatic compartment, such as TGF-β, peptides, and extracellular matrix (Andrade 1994; Rockey 2000). In situations of hepatic homeostasis, HSC store vitamin A; while in situations of injury, TGF-β activates HSC such that these lose their intracytoplasmic lipid reserves and change their phenotype to one of myofibroblasts, which stimulates increased extracellular matrix and results in hepatic fibrosis (Friedman 2008).

HSC have also been implicated in induction of hepatic regeneration and immunoregulation. In other words, when activated, HSC can induce chemotaxis of leukocytes to the liver, through production of various types of chemokines, and can present antigens and stimulate proliferation or apoptosis of T lymphocytes. These cells regulate the behavior of T lymphocytes and are influenced by specific subtypes of them. CD8 T lymphocytes may stimulate fibrinogenic activity that leads to fibrosis through HSC more intensely than do CD4 lymphocytes. Thus, diseases in which these lymphocytes predominate in the inflammatory infiltrate may lead to hepatic fibrosis (Friedman 2008). Based on reports in the literature, it is important to investigate the role of HSC in the livers of dogs with VL, in order to evaluate whether they act as stimulators of increased volumes of extracellular matrix, thereby leading to a reparative hepatic response. Thus, the objectives of the present study were to detect HSC and compare immunodetection between these and macrophages and TGF-β and parasite load, and to identify the presence of fibrosis in the livers of dogs with VL that were either symptomatic or asymptomatic for this disease.

## Material and methods

### Selection of animals

In the present study, 33 dogs were evaluated, without regard for sex, breed, or age. The infected dogs were divided into two groups: asymptomatic dogs ($n = 13$), without any apparent clinical signs of VL, and symptomatic dogs ($n = 15$), with clinical signs of the disease (lymphadenopathy, cutaneous alterations, onychogryphosis, keratoconjunctivitis, cachexia), as described by Figueiredo et al. (2014). The infected animals came from the Zoonosis Control Center of the municipality of Araçatuba, in the northwestern region of the state of São Paulo, Brazil, which is a region that is endemic for VL. The animals were put down using an intravenous (IV) overdose of barbiturate, followed by IV administration of potassium chloride (administered in accordance with decree number 51,838 of the Brazilian Ministry of Health and resolution number 714, of June 20, 2002, of the Federal Veterinary Medicine Council).

The control group ($n = 5$) was composed of dogs that came from an area that was not endemic for VL (Oliveira et al. 2008), from the files of the Department of Veterinary Pathology, School of Agrarian and Veterinary Sciences, São Paulo State University (Universidade Estadual Paulista, UNESP), Jaboticabal campus, state of São Paulo, Brazil. A serological test (ELISA) and/or aspiration puncture biopsy of the popliteal lymph node (Lima et al. 2005) was used to determine whether the animals were infected. The control group was selected by histological and immunohistochemical (parasite detection) analysis of the organs and when the cause of death was unrelated to systemic, hepatic, or neoplastic diseases.

### Histopathological and immunohistochemical analyses

For the histopathological analysis, liver fragments that had previously been fixed in a 10% buffered formalin solution (pH 7.6) for 24 h were used. These samples were processed, embedded in paraffin, sliced into sections of thickness 5 μm, and stained using hematoxylin and eosin, for analysis of the hepatic lesions under an optical microscope. Presence of fibrosis in the hepatic tissue was determined by means of Masson's trichrome staining. The severity of the hepatic fibrosis lesions was described using the following scores: 0 = absence, 1 = mild, 2 = moderate, and 3 = severe.

In the immunohistochemical analysis, the general protocol used for all the antibodies consisted of deparaffinization of the sections in a heated chamber at 60 °C for 1 h and then incubation of the sections in xylol for 20 min. Following this, the samples were hydrated in solutions of decreasing alcohol concentration, culminating in a bath of distilled water. Antigen recovery (Table 1) was performed for all antibodies except for the one that enables detection of HSC. Blocking of endogenous peroxidase was performed using a methanol solution (90 mL) and 30 volumes of 10% hydrogen peroxide (10 mL), in a dark chamber at room temperature for 30 min. Blocking of nonspecific reactions was performed using a commercial product (Protein Block, DakoCytomation, code X0909) at room temperature for 30 min. The sections were then incubated with primary antibodies to identify the parasite load, HSC, macrophages, and the cytokine TGF-β (Table 1). Following this, the sections were incubated using different detection methods (Table 1). Between each of the steps described above, the samples were placed in baths of distilled water and in Tris HCl buffer solution (pH 7.4). To view the reaction, the chromogen DAB was used (3,3-diaminobenzidine; DakoCytomation, code K3468-1), with counterstaining using Harris hematoxylin.

**Table 1** Primary antibodies and detection methods used in the liver sections of dogs with Visceral Leishmaniasis

| Primary antibodies | Structures marked | Species of origin | Antigen retrieval | Dilution | Incubation time | Detection methods |
|---|---|---|---|---|---|---|
| *Leishmania*[a] | Amastigotes forms | Canine | Pascal pressure chamber (Dako)[b] | 1:1000 | 18 h (4 °C) | LSAB[d] |
| MCA 874G | Macrophages | Mouse monoclonal (AbD Serotec, cód. Batch 1209) | Pascal pressure chamber (Dako)[b] | 1:3500 | 18 h (4 °C) | LSAB[d] |
| α smooth-muscle actin | Myofibroblasts | Mouse monoclonal (Dako, cód. M3515) | Not performed | 1:700 | 18 h (4 °C) | Advance[e] |
| TGF-β | Cells expressing cytokine TGF-β | Rabbit policlonal (Santa Cruz, cód. SC 146) | Microwave[b,c] | 1:1000 | 18 h (4 °C) | Envision phosphatase alkaline[f] |

[a] Positive dog serum for Visceral Leishmaniasis (Moreira et al. 2010, modified from the protocol described by Tafuri et al. 2004)

[b] 10 mM sodium citrate buffer solution (pH 6.0)

[c] For 2 min at full power and 10 min at minimum power (750 W), opening every 5 min to reset the buffer

[d] Streptavidin-biotin-peroxidase complex (kit LSAB—Dakocytomation, cód. K0690-1)

[e] Polymer complex linked to peroxidase (Kit Advance HRP®, Dako Cytomation, código K406889-2)

[f] Envision Dual Link System-HRP (Dako Cytomation, código K406189-2)

Negative controls for the different immunomarkings were obtained using antibody diluent (DakoCytomation, code S302283-2), instead of the primary antibody. Positive controls were obtained using tissues that were suggested by the manufacturers of each antibody. For the smooth-muscle anti-α-actin antibody (myofibroblast), a sample of canine leiomyoma was used, for macrophages, dog spleen; and for TGF-β, dog lymph node.

To determine the number of immunomarked cells, five microscope fields were selected (Nikon Eclipse E200) per slide, using the × 40 objective lens, which presented an area of 0.19625 $\mu m^2$ (Moreira et al. 2013). These fields were photographed and the percentage of immunomarked cells per animal was determined by means of the Micrometrics SE Premium image analysis software (version 2.8; 2009).

The immunomarkings were evaluated using the nonparametric Kruskal-Wallis test and Dunn's multiple comparison test, between the groups for each antibody analyzed. The values were deemed to be significant when $P < 0.05$. The analyses were developed using the GraphPad Prism statistical software (version 5.00; 2007). For the fibrosis scores, the frequency (%) of each score within the groups was considered.

# Results

## Histopathological analysis

In the symptomatic dogs, a moderate to severe granulomatous inflammatory reaction was observed. This was characterized by well-organized granulomas that were observed in the portal, centrilobular and intralobular regions (Fig. 1a). The granulomas contained epithelioid macrophages (both parasitized and non-parasitized), along with lymphocytes and plasmocytes. Hydropic degeneration of hepatocytes and hepatic congestion were frequently seen. Kupffer cells were evident and hypertrophic in the hepatic sinusoids. In the asymptomatic dogs, the inflammatory alterations ranged from mild to moderate (Fig. 1b), or were not observed in some animals. The control animals did not present any noteworthy microscopic alterations in the hepatic tissue (Fig. 1c). Hepatic fibrosis could be observed in places where inflammatory infiltrate was present in the portal, centrilobular and intralobular regions, and it was characterized by proliferation of thin delicate collagen fibers in small quantities (Fig. 1d).

## Immunohistochemical analysis

Immunomarking of HSC occurred in the cytoplasm of cells present in the sinusoids. In the symptomatic group, the immunomarking was limited to granulomas and was observed slightly in the sinusoids (Fig. 2a, b). In the asymptomatic group, positivity for HSC was diffuse and evident in the sinusoids (Fig. 2c). In the control group, positivity was slight and multifocal (Fig. 2d). Presence of the parasite was observed in the cytoplasm of macrophages that were present in the hepatic granulomas (Fig. 3a). The immunomarking of the Kuppfer cells and the macrophages of the granulomas was cytoplasmic, with predominance of immunomarked cells in the granulomas and sinusoids (Fig. 3b). The cytokine TGF-β presented immunomarking in the cell membrane of cells that were present in the inflammatory infiltrate (Fig. 3c).

The proportion of HSC differed significantly ($P = 0.0153$) between the infected groups and the control group (Fig. 4a). The highest percentage of HSC detection occurred in the symptomatic dogs. This same group also presented a higher percentage of parasitized macrophages than in the asymptomatic group, but without any statistical difference ($P > 0.1252$)

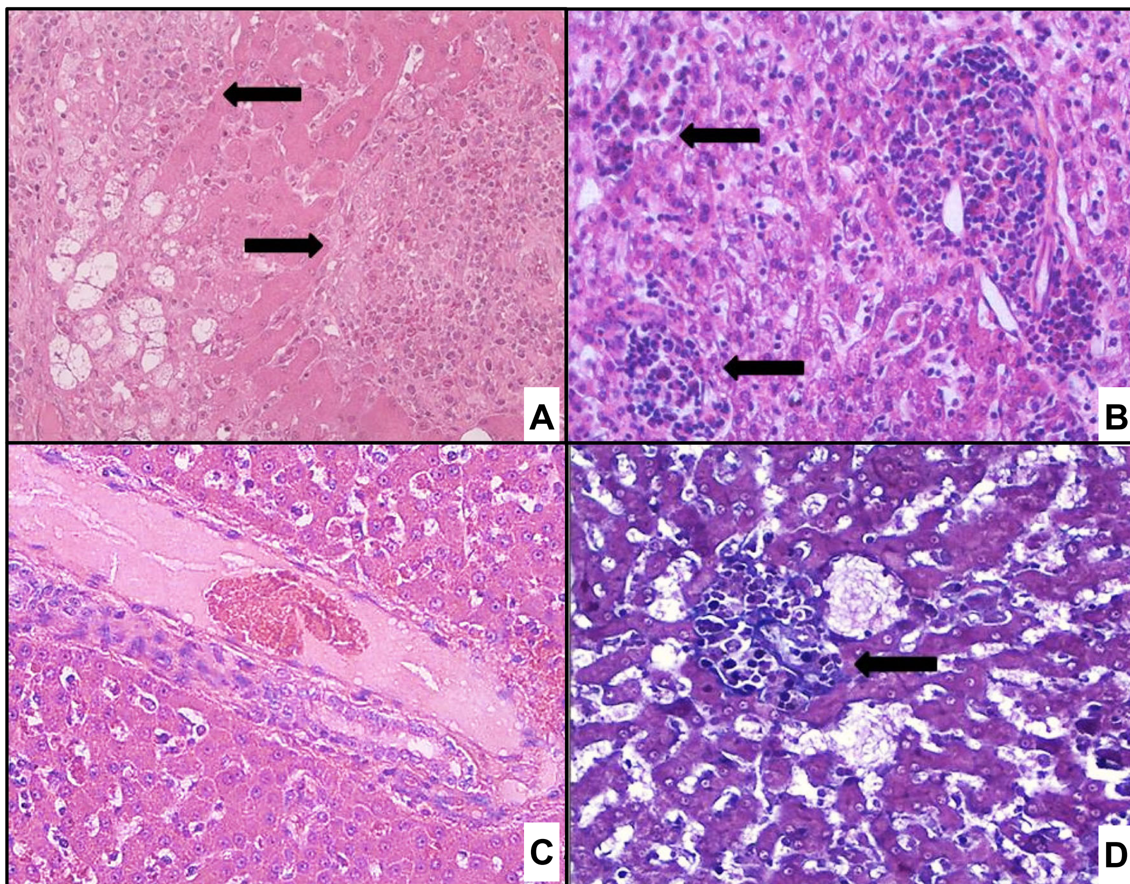



**Fig. 1** Photomicrographs of the livers of dogs in the control group or with visceral leishmaniasis. **a** Symptomatic dog with the presence of granulomas (arrows) and vacuolized hepatocytes (fatty degeneration). **b** Asymptomatic dog with foci of intralobular inflammation (arrows). **c** Note the absence of lesions in the liver of a control animal. **d** Liver of asymptomatic dog with slight proliferation of collagen fibers associated with foci of hepatic inflammation (arrow). Hematoxylin and eosin (**a**–**c**). Masson's trichrome (**d**). × 40 objective lens

(Fig. 4b). The immunomarking of macrophages was higher in the symptomatic dogs than in the asymptomatic and control dogs, with a significant difference ($P = 0.0044$) between the S and A groups (Fig. 4c). The cytokine TGF-β was only detected at a low rate, and although it was only seen in the infected dogs, its presence in these animals did not differ ($P > 0.3484$) from that of the control group (Fig. 4d). Hepatic fibrosis was only observed at low levels and only in the infected dogs (S and A), while it was absent in the control group. However, there was only a significant difference for this variable ($P = 0.0117$) between the symptomatic dogs and the control dogs (Fig. 5).

The correlations between all the variables within each group of infected dogs only differed significantly regarding the percentages of HSC and tissue fibrosis in the asymptomatic dogs ($P = 0.034$; $r = 0597$). The other variables did not differ between each other (Fig. 6).

## Discussion

The findings relating to hepatic lesions in the infected animals (A and S) coincided with those in reports from other authors (Lima et al. 2007; Giunchetti et al. 2008; Moreira et al. 2016b), who observed well-organized intralobular granulomas in the livers of all the animals studied, independent of the clinical form. However, the results from the present study differed from those of Sanchez et al. (2004) and Melo et al. (2009), who reported that the proportion of granulomas was greater in the dogs of the asymptomatic group than in those of the symptomatic group.

The liver has been considered to be the organ that is most resistant to multiplication of the parasite *Leishmania infantum*, possibly because of the cell composition of the granulomas, which are rich in T lymphocytes. These conclusions were based on comparison of this organ with the spleen (Lima et al. 2007; Moreira et al. 2016b), and with the peripheral lymph nodes and different regions of the skin (Moreira et al. 2016a). These other organs commonly present parasite loads that are higher than that of the liver (unpublished data).

Regarding HSC, it has been highlighted in the literature that the main role of these cells, when activated, relates to production of extracellular matrix after liver injury (Friedman 2008). In the present study, these cells were more

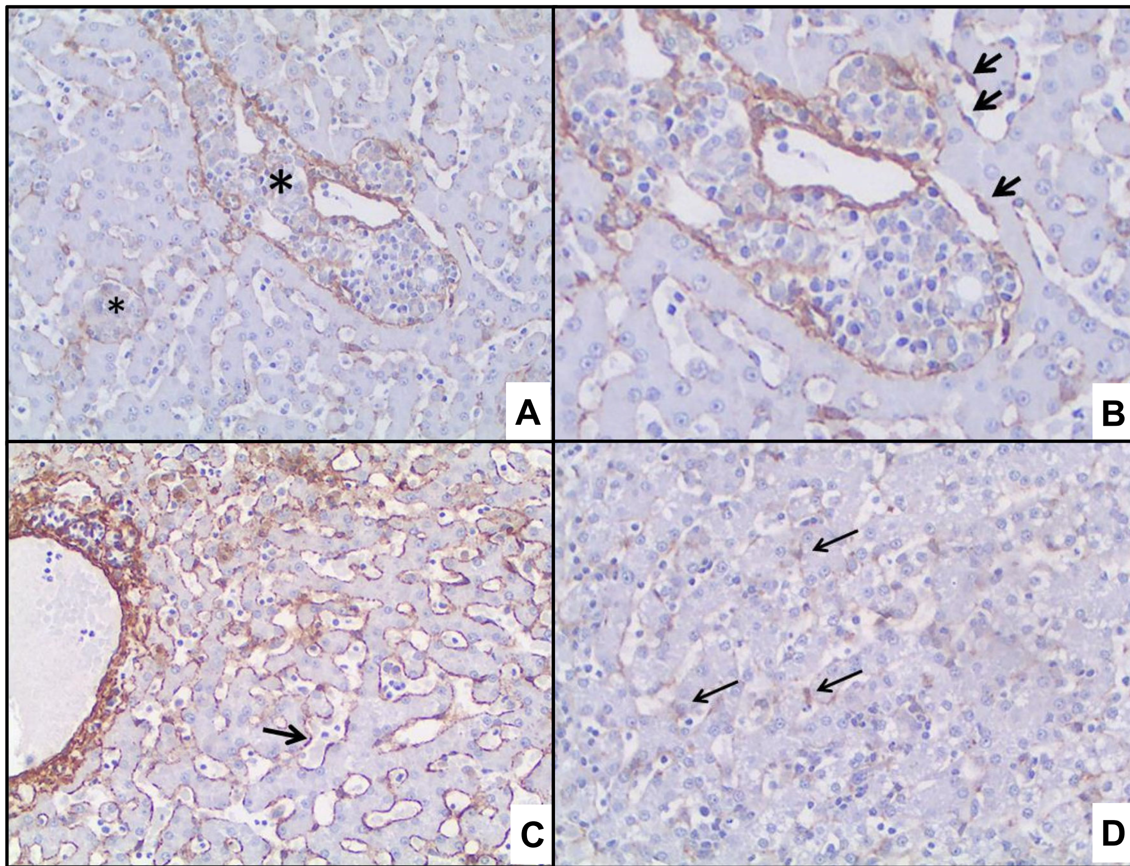


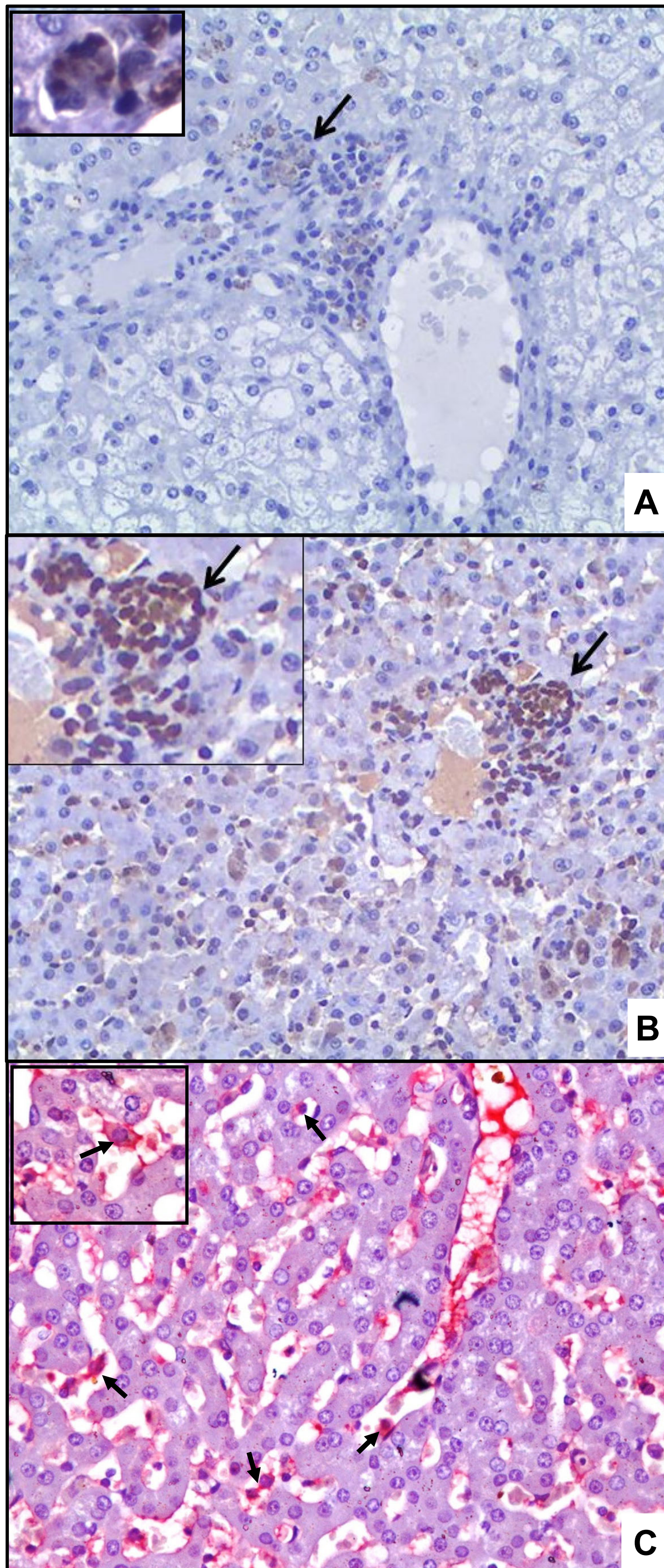

**Fig. 2** Photomicrographs of livers with immunomarking of hepatic stellate cells (HSC). **a** Symptomatic dog with evident immunomarking of HSC surrounding the hepatic granulomas (*,× 20 objective lens). **b** In the inset of (**a**), note the hypertrophy of HSC in the sinusoids (arrows, × 40 objective lens). **c** Liver of asymptomatic dog with positive delimitation of sinusoids for HSC (arrow, × 20 objective lens). **d** Liver of control animal. Note the delicate marking of HSC in the sinusoids (arrows, × 20 objective lens). Peroxidase-linked polymer complex

evident in the groups of infected dogs (A and S) than in the control group. The severity of hepatic fibrosis was low in both groups (A and S), but only the symptomatic dogs differed statistically from the control group. This finding was concordant with the report from Melo et al. (2009), who observed that in symptomatic dogs, the density of hepatic fibrosis was greater. However, in the present study, the hepatic fibrosis observed in the symptomatic dogs was mild. Melo et al. (2009) reported that in the symptomatic dogs, collagen fibers were distributed diffusely in the hepatic parenchyma and that there was a positive correlation between hepatic fibrosis and parasitism.

In the dogs of the present study, the effects of the inflammatory cells possibly influenced the low proportion of collagen fibers in the liver, since activated macrophages can synthesize enzymes (matrix metalloproteinases) that degrade mature collagen and other components of the extracellular matrix, which thus facilitates leukocyte migration to the injury site. Greater density of these cells was observed in the group of symptomatic dogs than in the group of asymptomatic dogs. It is possible that Melo et al. (2009) may have used animals that were at a more advanced stage of VL than those of the present study.

In the asymptomatic group, attention was drawn to the diffuse immunomarking pattern of HSC in the sinusoids, in comparison with the symptomatic group. This difference suggested that when the lesions are not so severe, activation of HSC in the hepatic parenchyma occurs diffusely. At this stage, the HSC may be contributing towards hepatic clearance, along with the Kupffer cells, since these dogs had lower numbers of parasitized macrophages than did the symptomatic dogs. The immunoregulatory role of HSC has already been described in the literature (Friedman 2008), along with the greater parasite load in hepatic granulomas in the symptomatic group (Lima et al. 2007; Giunchetti et al. 2008; Reis et al. 2009; Moreira et al. 2016b).

Cells producing the cytokine TGF-β were only detected at low levels, but they were more evident in the infected dogs. Friedman (2008) highlighted that the presence of this cytokine is important for activation of HSC, which results in fibrosis of the organ. Corrêa et al. (2007) observed higher levels of TGF-β in asymptomatic dogs than in symptomatic dogs. In


A
B
C

◀ **Fig. 3** Photomicrographs of immunomarking of parasites, macrophages, and TGF-β in the livers of dogs with VL. **a** Immunodetection of amastigote forms of the protozoa *Leishmania* spp. inside macrophages in the portal spaces (arrow, × 20 objective lens) and in the inset (× 40 objective lens), note the immunomarking of the parasites. Streptavidin-biotin-peroxidase complex. **b** Presence of immunomarked macrophages in the hepatic granulomas (arrow and inset) and in the sinusoids (× 20 objective lens in figure and × 40 in inset). Streptavidin-biotin-peroxidase complex. **c** Note the positive immunostaining for TGF-β in the sinusoids (arrows) and, in the inset, immunostaining in membrane of Kupffer cells (arrow) (× 40 objective lens). Alkaline phosphatase (Envision)

the present study, this cytokine and HSC were detected at higher levels in the infected dogs (A and S) and fibrosis also occurred in these groups, albeit only mildly. Also in the present study, there was a positive correlation among the asymptomatic dogs between the HSC levels and the density of

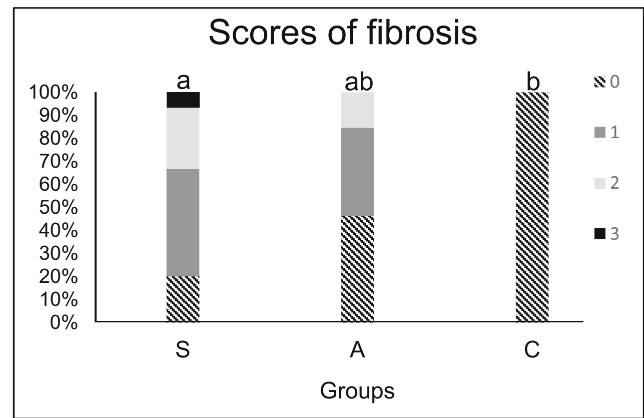



**Fig. 5** Scores proportions in the areas with fibrosis hepatic in dogs with VL (S and A) and control (C). Note the significant difference ($P = 0.0117$) between the symptomatic dogs (S) and control dogs (C). Kruskal-Wallis and Dunn tests

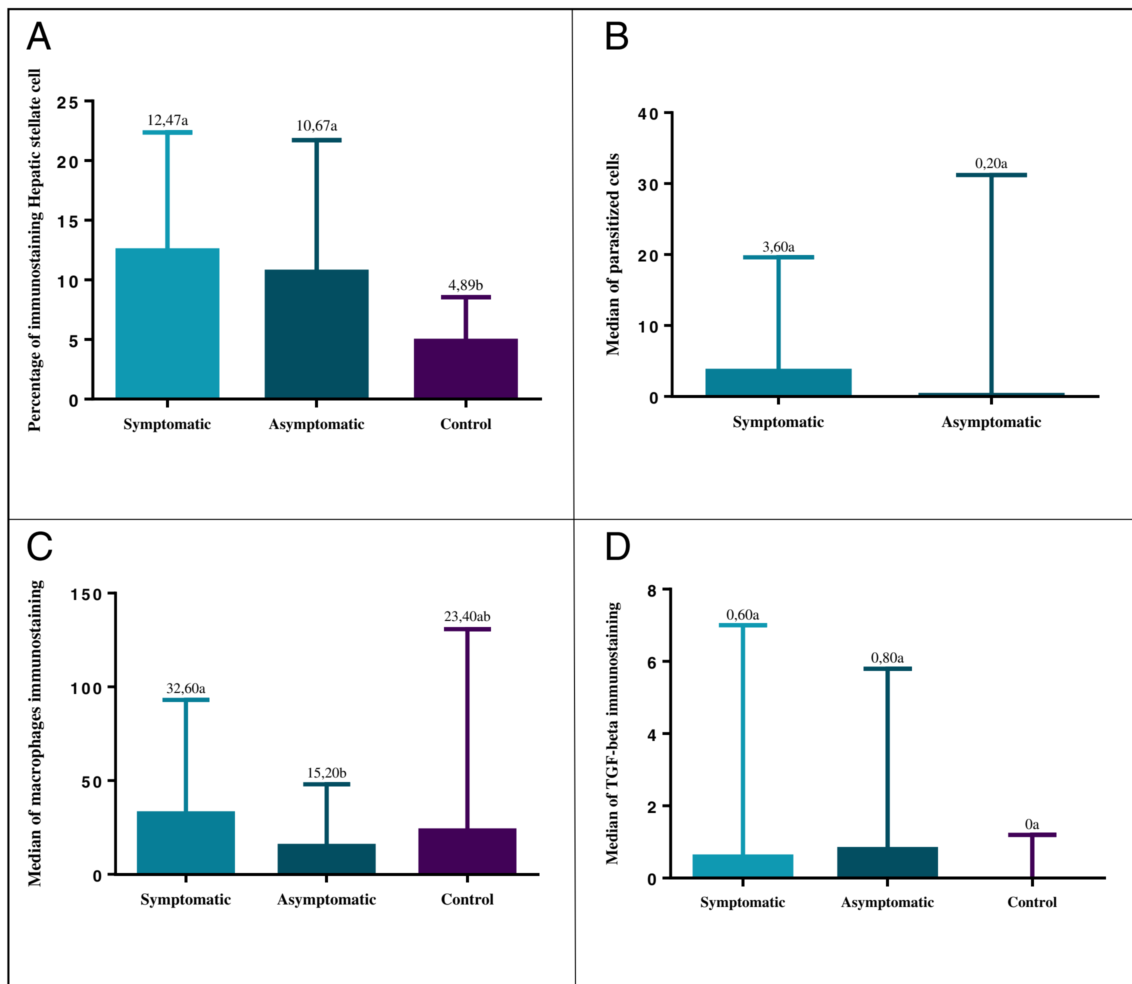



**Fig. 4** Medians and standard errors for cell immunomarking in the livers of dogs with and without visceral leishmaniasis. **a** Note the higher proportion of Ito cells (hepatic stellate cells) in the infected groups (both asymptomatic and symptomatic), which differed from the control group (Kruskal-Wallis and Dunn tests; $P = 0.0153$). **b** Among the dogs in the symptomatic group, there was a higher proportion of parasitized macrophages in the liver, but not statistically different from the proportion in the asymptomatic group (Mann-Whitney test; $P = 0.1252$). **c** Median level of immunomarking of macrophages, with a difference between the infected dogs (Kruskal-Wallis and Dunn tests; $P = 0.0044$). **d** The density of cells immunomarked for TGF-beta was low and there was no statistical difference between the groups (Kruskal-Wallis and Dunn tests; $P = 0.3484$)

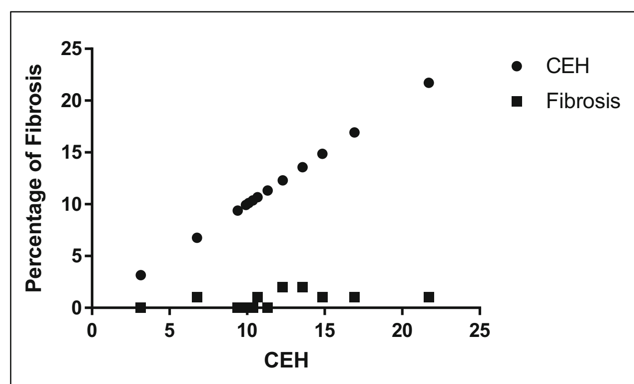



**Fig. 6** Correlation between the percentage of HSC and fibrosis in the livers of asymptomatic dogs, as determined using linear regression analysis and Spearman correlation coefficients (*r*)

fibrosis, i.e., as the percentage of HSC increased, the proportion of hepatic fibrosis also increased. Therefore, it can be suggested that production of the cytokine TGF-β may be related to activation of HSC, although the low number of immunomarked cells might have been related to the phase of development of the infection and might have been influenced by the lower proliferation of collagen fibers, thus differing from the findings from the study by Melo et al. (2009). The animals of the present study presented spontaneous disease with chronic evolution and, for this reason, it can be suggested that the levels of this cytokine might have been higher in the initial stages of the disease and might have regressed over time, as the disease became chronic.

From the results of this study, it can be concluded that HSC are activated and participate in the hepatic response to the parasite in dogs with VL. The cytokine TGF-β is also involved in activation of these cells, but it was detected in lower proportions in the chronic phase of the infection. It is possible that HSC may also contribute towards chemotaxis of leukocytes to the hepatic compartment, along with other cell types such as Kupffer cells.

**Acknowledgements** The authors wish to acknowledge the Zoonosis Control Center of Araçatuba for providing the animals of this study.

**Funding information** Financial assistance was provided by FAPESP, Pamela Rodrigues Reina Moreira (Fundação de Amparo à Pesquisa do Estado de São Paulo; Procedural number 2013/00763-4). Paulo Henrique Leal Bertolo (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). Natália Cassaro Marques (Conselho Nacional de Pesquisa e desenvolvimento, *PIBIC-CNPq*).

## Compliance with ethical standards

**Ethical approval** The design for this study was approved by the Ethics and Animal Welfare Committee (CEBEA no. 11976/15) of FCAV/UNESP, Jaboticabal, state of São Paulo, Brazil.

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

Andrade ZA (1994) Extracellular matrix degradation in parasitic diseases. Braz J Med Biol Res 27:2273–2281

Corbett CEP, Duarte MIS, Bustamante SE (1993) Regression of diffuse intralobular liver fibrosis associated with visceral leishmaniasis. Am J Trop Med Hyg 49:616–624

Corrêa APFL, Dossi ACS, Vasconcelos RO, Munari DP, Lima VMF (2007) Evaluation of transformation growth factor-ß1, interleukin-10, and interferon-γ in male symptomatic and asymptomatic dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi*. Vet Parasitol 143:267–274. https://doi.org/10.1016/j.vetpar.2006.08.023

Figueiredo MM, Deoti B, Amorim IF, Pinto AJW, Moraes A, Carvalho CS, Silva SM, Assis ACB, Faria AMC, Tafuri WL (2014) Expression of regulatory T cells in jejunum, colon, and cervical and mesenteric lymph nodes of dogs naturally. Infected with *Leishmania infantum*. Infect Immun 82(9):3704–3712. https://doi.org/10.1128/IAI.01862-14

Friedman SL (2008) Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 88:125–172. https://doi.org/10.1152/physrev.00013.2007

Giunchetti RC, Mayrink W, Carneiro CM, Oliveira RC, Martins-Filho OA, Marques MJ, Tafuri WL, Reis AB (2008) Histopathological and immunohistochemical investigations of the hepatic compartment associated with parasitism and serum biochemical changes in canine visceral leishmaniasis. Res Vet Sci 84:269–277. https://doi.org/10.1016/j.rvsc.2007.04.020

Lima WG, Michalick MSM, Melo MN, Tafuri WL, Tafuri WL (2004) Canine visceral leishmaniasis: a histopathological study of lymph nodes. Acta Trop 92:43–53. https://doi.org/10.1016/j.actatropica.2004.04.007

Lima VMF, Biazzono L, Silva AC, Correa APFL, Luvizotto MCR (2005) Serological diagnosis of visceral leishmaniasis by an enzyme immunoassay using protein A in naturally infected dogs. Pesq Vet Bras 25(4):215–218

Lima WG, Oliveira PS, Caliari MV, Gonçalves R, Michalick MSM, Melo MN, Tafuri WL, Tafuri WL (2007) Histopathological and immunohistochemical study of type 3 complement receptors (CD11b/CD18) in livers and spleens of asymptomatic and symptomatic dogs naturally infected with *Leishmania* (*Leishmania*) *chagasi*. Vet Immunol Immunopathol 117:129–136. https://doi.org/10.1016/j.vetimm.2007.02.012

Melo FA, Moura EP, Ribeiro RR, Alves CF, Caliari MV, Tafuri WL, Calabrese KS, Tafuri WL (2009) Hepatic extracellular matrix alterations in dogs naturally infected with *Leishmania* (*Leishmania*) *chagasi*. Int J Exp Path 90:538–548. https://doi.org/10.1111/j.1365-2613.2009.00681.x

Moreira PRR, Vieira LM, Andrade MMC, Bandarra MB, Machado GF, Munari DP, Vasconcelos RO (2010) Immune response pattern of the popliteal lymph nodes of dogs with visceral leishmaniasis. Parasitol Res 107:605–613. https://doi.org/10.1007/s00436-010-1902-2

Moreira PRR, Bandarra MB, Magalhães GM, Munari DP, Prandini MM, Alessi AC, Vasconcelos RO (2013) Influence of apoptosis on the cutaneous and peripheral lymph node inflammatory response in dogs with visceral leishmaniasis. Vet Parasitol 192:149–157

Moreira PRR, Fernando FS, Montassier HJ, André MR, Vasconcelos RO (2016a) Polarized M2 macrophages in dogs with visceral leishmaniasis. Vet Parasitol 226:69–73. https://doi.org/10.1016/j.vetpar.2016.06.032

Moreira PRR, Franciscato DA, Rossit SM, Munari DP, Vasconcelos RO (2016b) Influence of apoptosis on liver and spleen resistance in dogs with visceral leishmaniasis. Braz J Vet Parasitol 25(3):341–347. https://doi.org/10.1590/S1984-29612016054

Oliveira TMFS, Furuta PI, Carvalho D, Machado RZ (2008) A study of cross-reactivity in serum samples from dogs positive for *Leishmania* sp, *Babesia canis* and *Ehrlichia canis* in enzyme-linked immunosorbent assay and indirect fluorescent antibody test. Braz J Vet Parasitol 17:7–11

Reis AB, Martins-Filho AO, Teixeira-Carvalho A, Giunchetti RC, Carneiro CM, Mayrink W, Tafuri WL, Corrêa-Oliveira R (2009) Systemic and compartimentalized immune response in canine visceral leishmaniasis. Vet Immunol Immunopathol 128:87–95. https://doi.org/10.1016/j.vetimm.2008.10.307

Rockey DC (2000) Hepatic fibrogenesis and hepatitis C. Semin Gastrointest Dis 11:69–83

Sanchez M, Diaz NL, Zerpa O, Negron E, Convit J, Tapia FJ (2004) Organ-specific immunity in canine visceral leishmaniasis: analysis of symptomatic and asymptomatic dogs naturally infected with *Leishmania chagasi*. Am J Trop Med Hyg 70(6):618–624

Tafuri WL, Oliveira MR, Melo MN, Tafuri WL (2001) Canine visceral leishmaniosis: a remarkable histopathological picture of one case reported from Brazil. Vet Parasitol 96:203–212

Tafuri WL, RL de Santos, RME de Arantes, Gonçalves R, Melo MN, MSM de Michalick, Tafuri WL (2004) Um método immunohistochemical alternativo para detectar amastigotas de *Leishmania* em tecidos caninos embebidos em parafina. J Immunol Methods 292: 17-23. https://doi.org/10.1016/j.jim.2004.05.009