Higher Expression of CCL2, CCL4, CCL5, CCL21, and CXCL8 Chemokines in the Skin Associated with Parasite Density in Canine Visceral Leishmaniasis

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Abstract

Background: The immune response in the skin of dogs infected with *Leishmania infantum* is poorly understood, and limited studies have described the immunopathological profile with regard to distinct levels of tissue parasitism and the clinical progression of canine visceral leishmaniasis (CVL).

Methodology/Principal Findings: A detailed analysis of inflammatory cells (neutrophils, eosinophils, mast cells, lymphocytes, and macrophages) as well as the expression of chemokines (CCL2, CCL4, CCL5, CCL12, CCL17, CCL21, CCL24, and CXCL8) was carried out in dermis skin samples from 35 dogs that were naturally infected with *L. infantum*. The analysis was based on real-time polymerase chain reaction (PCR) in the context of skin parasitism and the clinical status of CVL. We demonstrated increased inflammatory infiltrate composed mainly of mononuclear cells in the skin of animals with severe forms of CVL and high parasite density. Analysis of the inflammatory cell profile of the skin revealed an increase in the number of macrophages and reductions in lymphocytes, eosinophils, and mast cells that correlated with clinical progression of the disease. Additionally, enhanced parasite density was correlated with an increase in macrophages and decreases in eosinophils and mast cells. The chemokine mRNA expression demonstrated that enhanced parasite density was positively correlated with the expression of CCL2, CCL4, CCL5, CCL21, and CXCL8. In contrast, there was a negative correlation between parasite density and CCL24 expression.

Conclusions/Significance: These findings represent an advance in the knowledge about skin inflammatory infiltrates in CVL and the systemic consequences. Additionally, the findings may contribute to the design of new and more efficient prophylactic tools and immunological therapies against CVL.


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Introduction

Visceral leishmaniasis (VL), caused by *Leishmania (Leishmania) infantum* [syn. *Leishmania (Leishmania) chagasi*], is endemic in over 88 countries in Europe and Latin America and is transmitted by the bite of the female sand fly (Phlebotomus) [1]. The skin is considered a key reservoir compartment for amastigotes in both asymptomatic and symptomatic *Leishmania*-infected dogs, and the important role of dogs in VL transmission in urban areas is supported by the high parasitic loads found in the skin of infected animals and their shared habitat with humans [2–4]. Previous investigations have revealed that symptomatic *Leishmania*-infected dogs exhibit an intense diffuse dermal inflammatory infiltrate and a high parasitic burden in comparison with their asymptomatic counterparts [4]. On this basis it was proposed that the immunopathological changes in the skin and the levels of cutaneous parasitism are directly related to the clinical severity of the disease.

Several previous studies correlated immunopathological aspects of canine visceral leishmaniasis (CVL) with tissue parasitic load and/or the clinical status of the disease [5–17]. The typical
Author Summary

Several previous studies correlated immunopathological aspects of canine visceral leishmaniasis (CVL) with tissue parasite load and/or the clinical status of the disease. Recently, different aspects of the immune response in *Leishmania*-infected dogs have been studied, particularly the profile of cytokines in distinct compartments. However, the role of chemokines in disease progression or parasite burdens of the visceralising species represents an important approach for understanding immunopathology in CVL. We found an increase in inflammatory infiltrate, which was mainly composed of mononuclear cells, in the skin of animals presenting severe forms of CVL and high parasite density. Our data also demonstrated that enhanced parasite density is positively correlated with the expression of CCL2, CCL4, CCL5, CCL21, and CXCL8. In contrast, there was a negative correlation between parasite density and CCL24 expression. These findings represent an advance in the knowledge of the involvement of skin inflammatory infiltrates in CVL and the systemic consequences and may contribute to developing a rational strategy for the design of new and more efficient prophylactic tools and immunological therapies against CVL.

histopathological finding in tissues is a granulomatous inflammatory reaction associated with the presence of *Leishmania* amastigotes within macrophages [18]. In the skin of *Leishmania* infected-infected dogs, the histopathological alterations consist of variable degrees of focal or diffuse inflammatory infiltrate in the dermis and variable numbers of plasma cells, macrophages, lymphocytes, and isolated neutrophils [19,20].

Furthermore, it has recently been demonstrated that parasite density in the skin, bone marrow, and spleen compartments increases according to the severity of the clinical manifestation of CVL [6,13,16]. Galabrese et al. [21] evaluated histopathological aspects of the skin in naturally infected dogs and showed that low parasite load is associated with an intense inflammatory reaction driven mainly by mast cells, indicating that these cells exert a role in innate immunity and in the resistance against canine *Leishmania* infection.

Recently, different aspects of the immune response in *Leishmania*-infected dogs have been studied, particularly the profile of cytokines in distinct compartments [5,9,12,17,22-24]. However, the role of chemokines in disease progression or parasite burdens of the visceralising species represents an important approach for understanding immunopathology in CVL.

Chemokines are chemotactic factors that coordinate recruitment of leukocytes that are involved in homeostasis as well as innate and adaptive immune responses. In the context of experimental or natural infection in CVL, an up-regulation of the chemokines in the skin has been described, although only CXCL10 and CCL5 were markedly elevated in oligosymptomatic dogs [24]. In addition, the increased levels of chemokines suggested an accumulation of infiltrating monocytes attracted by CCL3 and CCL2, CD4⁺Th1 and CD8⁺ cells also accumulated and may have been recruited by CXCL10, with further expression induced through IFN-γ secretion [24].

Considering the importance of chemokines on the pattern of CVL and the lack of studies on this topic, understanding the chemokine profile during ongoing *L. infantum* infection in dogs is a prerequisite for identifying the mechanisms for resistance or susceptibility in this experimental model. In the present study, the immunopathology of CVL was investigated by performing detailed analyses of the RNA expression of different chemokines (CCL2, CCL4, CCL5, CCL13, CCL17, CCL21, CCL24, and CXCL12) and the occurrence of inflammatory cells (neutrophils, eosinophils, mast cells, lymphocytes, and macrophages). We focused on selected chemokines in order to characterize their role in recruiting particular cell types to the inflammatory infiltrate in skin from *L. infantum*-infected dogs. Thus, we found that the chemokines CCL2, CCL4, CCL5, and CCL21 attract macrophages; CCL5 and CCL4 attract inflammatory lymphocytes, particularly Th1-type cells; CCL24 attracts eosinophils; and CXCL12 attracts neutrophils, monocytes, and lymphocytes. The chemokine CCL17 helps to establish the inflammatory infiltrate, a characteristic feature of various inflammatory skin conditions, by attracting CCR4-bearing cells, which are especially polarized to Th2-type cells and regulatory T cells [25]. This study was carried out using the skin from 35 dogs that were naturally infected with *L. infantum*.

Materials and Methods

Study population and clinical evaluation

The study was approved by the Committees of Ethics in Animal Experimentation of the Universidade Federal de Ouro Preto (protocol no. 003/2007) and of the Universidade Federal de Minas Gerais (protocol no. 020/2007) and the City Council of Belo Horizonte (protocol no. 001/2008). All procedures in this study were according to the guidelines set by the Brazilian Animal Experimental College (COBEA), Federal number 11794. The study population comprised 51 adult dogs (aged between 2 and 6 years) of both sexes that had been captured by the Center of Zoonosis Control in Belo Horizonte (Minas Gerais, Brazil), a region with a high prevalence of CVL and human VL. The animals were maintained under quarantine at the kennels of the Instituto de Ciências Biológicas (Universidade Federal de Minas Gerais) prior to tissue collection for 10 days and treated for intestinal helminthic infections (Endal Plus; Schering-Plough Coopers, São Paulo, SP, Brazil). We treated kennels with pyrethroid insecticide monthly during the quarantine and throughout the experiments. Experimental animals were categorized on the basis of serological results from an indirect immunofluorescence assay test, the “gold standard” immunological test in Brazil for the diagnosis of CVL. Scooted dogs with negative immunofluorescence assay test results from serum samples diluted 1:10 and negative results for *Leishmania* in tissue smears bone marrow, ear skin, spleen, liver, and popliteal lymph node were considered to be non-infected and were used as the control group (CD, n = 16). Thirty-five animals with positive immunofluorescence assay titers ≥1:10 were considered CVL positive and comprised the infected animal groups. The infected animal groups were subdivided on the basis of the presence or absence of signs of infection according to Manzucchi et al. [25] as follows: an asymptomatic group (AD, n = 16), in which indicative signs of the disease were absent; an oligosymptomatic group (OD, n = 10), in which a maximum of three clinical signs of the disease were present, including opaque bristles, localized alopecia, and/or moderate weight loss; and a symptomatic group (SD, n = 15), in which characteristic clinical signs of the disease were present, including cutaneous lesions, onychogryphosis, opaque bristles, severe loss of weight, atrophy, and keratoconjunctivitis.

Sample collection and assessment of skin parasite load

Animals were euthanized with sodium thiopental (Abbott Laboratories, Abbott Park, IL, USA; 30 mg/kg body weight)
Real-time PCR and cloning and sequencing of amplicons
q-PCR was performed on an ABI Prism 7000 DNA Sequence
Detection System using SYBR Green PCR Master Mix (PE
Applied Biosystems, Foster City, CA, USA), with 100 mM of each
primer and cDNA diluted to 1:5. The samples were incubated
at 95°C for 10 min and then subjected to 40 cycles of 95°C for 15 s
and 60°C for 1 min, during which time fluorescence data were
collected. The efficiency of each pair of primers was evaluated
by serial dilution of cDNA according to the protocol developed by PE
Applied Biosystems. In order to evaluate gene expression of the
chemokines CCL2, CCL4, CCL5, CCL13, CCL17, CCL21,
CCL24, and CXCL8, three replicate analyses were performed,
and the amount of target RNA was normalized with respect to the
eukaryotic control (housekeeping) gene GAPDH. Data were
expressed according to the 2^{ΔΔCt} method using the mean value
of the ΔΔCt of the control group as the calibrator [26]. After
normalization, the expression levels of chemokines in the infected
groups were considered upregulated or downregulated compared to
expression levels in the control group. PCR products were cloned
with pGEM-T Easy Vector (Promega) and sequenced to
check specificity by using an ABI 3100 Automated Sequencer (PE
Applied Biosystems) and a Dye Terminator Kit.

Table 2 presents a summary of the different chemokines and
their biological effects during Leishmania infection in dogs, mice,
and humans. These data illustrate how recruitment of specific cells
might influence the pathogenesis of Leishmania infection.

### Histological analysis
For standard histological examination (morphometric analysis
and leukocyte differential counting) sequids were coded and
stained with hematoxylin and eosin and subsequently underwent
blinded analysis under a light microscope (model CH3RF100,
Olympus Optical Co., Tokyo, Japan). The inflammatory cells
(neutrophils, eosinophils, macrophages, mast cells, and lympho-
cyes) that were recruited to the dermis were counted, and the
results are expressed in percentages. Cell types in the cellular
infiltrate in the dermis were quantified by using 20 random images
(total area = 1.56×10^5 μm²) that adequately represented a slide.
Thus, the density and predominance of cells in the inflammatory
infiltrate and their distribution within the skin layers were assessed.
and registered quantitatively. The images displayed in the 106 objective were digitized through a Leica DM5000B microscope with a coupled camera using the program Leica Application Suite version 2.4.0 R1, Leica Microsystems Ltd., Heerbrugg, Switzerland. For the analysis of images, Leica QWin V3 (Leica Microsystems Ltd.) was used to count all cell models, excluding the psoas fillettes, skin annexes, and epidural cells.

**Statistical analysis**

Statistical analyses were performed using the GraphPad Prism software package version 5.0 (GraphPad Software, San Diego, CA, USA). Normality of the data was established using the Kolmogorov-Smirnoff test. The Kruskal-Wallis test was used for comparative studies between groups, followed by Dunn’s test for multiple comparisons. Spearman’s rank correlation was also computed in order to investigate the relationship between the expression of chemokine mRNAs with clinical forms and skin parasite density as well as cell counting. In all cases, differences were considered significant when the probabilities of equality (p values) were ≤0.05.

**Results**

Clinical progression in CVL was correlated with increased parasite density and the presence of mononuclear cells in the skin of dogs naturally infected by *L. infantum*

In order to investigate the relationship between clinical forms of CVL and skin parasite density as well as cellular infiltrate, correlation analyses were conducted with these parameters in *L. infantum*-infected animals (*r = 0.9535*) (Fig. 1A). The main histopathological findings are shown in photomicrographs (Fig. 1A). Histopathological examination of the skin showed no histological changes within the CD group (Fig. 1A, panels 1 and 2). In the LP and AD groups (Fig. 1A, panels 3 and 4), there was a mild inflammatory infiltrate, composed mainly of mononuclear cells, while in the OD and MP groups, this infiltrate was mild to moderate, as shown in Fig. 1A (panels 5 and 6). In panels 7 and 8 of Fig. 1A, which represent sections of ear skin in the AD group, an intense cellular infiltrate composed mainly of mononuclear cells was observed.

The intensity and predominance of cells in the inflammatory infiltrate and their distribution within the skin layers were assessed (Fig. 1B, 1C). Our results demonstrated a positive correlation between cellular infiltrate and clinical status (*r = 0.5400, p = 0.0001*) (Fig. 1B) and skin parasite density (*r = 0.7352, p < 0.0001*) (Fig. 1C). Significant increases in the inflammatory infiltrate in the skin samples were observed in the AD and SD groups as compared with CD animals (Fig. 1B). The HP group had a significant increase in inflammatory infiltrate compared with the CD and LP groups (Fig. 1C). Moreover, the inflammatory infiltrate within the MP group was significantly increased as compared with the CD group (Fig. 1C).

The results also indicated positive correlation among clinical evolution of CVL and the increase in parasite density in the skin (*r = 0.4109, p = 0.0080*) (Fig. 1D). Additionally, an increase in parasite density (*p < 0.05*) was detected in the skin of dogs showing the maximum clinical score (SD) when compared with the AD group (Fig. 1D).

**Assessment of the inflammatory cell profile in the skin**

The study of skin tissue cellularity included an assessment of the percentage of cell types (neutrophils, eosinophils, mast cells, lymphocytes, and macrophages) present in the inflammatory infiltrate in the skin of dogs that were naturally infected by *L. infantum* and categorized by clinical status and dogs that were uninfected (Fig. 2A). In this context, we observed a reduction in the percentage of eosinophils in the SD group compared with the CD group (*p < 0.05*), and a negative correlation between this cell population (*r = −0.4775, p = 0.0059*) and clinical status. Additionally, there was a decrease (*p < 0.05*) in the percentage of mast cells in the OD and SD groups when compared with the CD group. Similarly, a negative correlation was observed in the percentage of mast cells (*r = −0.0018, p = 0.0002*) compared with the clinical form of CVL. For lymphocytes, we observed an increased (*p < 0.05*) percentage in the AD group in comparison with the SD group and control dogs. Furthermore, we also observed an increase (*p < 0.05*) in the OD group as compared with the SD group. The analysis of correlation between lymphocyte counts and clinical status showed a negative correlation between the increase of lymphocytes versus the clinical outcome in CVL (*r = −0.0283, p = 0.0001*) (Fig. 2A). Significant increases (*p < 0.05*) were observed in the OD and SD groups in the population of macrophages in comparison to the CD group, and a positive correlation was observed (*r = 0.5553, p = 0.0010*) between the percentage of macrophages and degree of disease.

**Enhanced skin parasite density was correlated with an increase of macrophages and decreases of eosinophils and mast cells in the skin of dogs naturally infected by *L. infantum***

An assessment of cellular infiltrate in the skin of dogs naturally infected by *L. infantum* and uninfected dogs was performed by categorizing them according to skin parasite density (Fig. 2B). Although neutrophil and lymphocyte subsets did not have significant changes, a shift in the cell profiles related to the innate immune response was observed. The percentage of eosinophils decreased (*p < 0.05*) in the MP and HP groups when compared with the CD group. Associated with these observations, a negative correlation between the percentage of eosinophils and skin parasite density was found (*r = −0.3815, p = 0.0025*). The percentage of mast cells was lower in the LP, MP, and HP groups when compared with the CD group (*p < 0.05*). Accordingly, a significant increase (*p < 0.05*) in the percentage of macrophages in the HP and LP groups in comparison with the CD group was found. Furthermore, a positive correlation between the percentage of macrophages and skin parasite density (*r = 0.4153, p = 0.0010*) was observed.

**Enhanced parasite density was positively correlated with higher expression of chemokines *CCL2, CCLA, CCL5, CCL21*, and *CCL18* and lesser expression of *CCL24* in the skin of dogs naturally infected by *L. infantum*. The involvement of chemokines in recruiting cells to the skin and developing a protective response against *Leishmania* infection was evaluated according to skin parasitism. These results are described in Figure 3. In this study, we also performed correlation analysis between the levels of chemokine expression and the clinical status, but significant differences did not exist between the groups (data not shown). The mRNA expression of *CCL2* was increased (5.6-fold; *p < 0.05*) in the HP group as compared with the LP group. Furthermore, the correlation analysis showed that *CCL2* was positively associated with an increase in parasite load in the skin of these animals (*r = 0.5329, p = 0.0010*). *CCLA* was up-regulated in all groups in relation to the CD group and highly expressed in the HP group in comparison to the LP and MP groups (3.5-fold and 2.8-fold, respectively; *p < 0.05*). Additionally, a positive correlation (*r = 0.5774, p = 0.0005*) with an increase in skin parasite density was detected. Similarly, CCL5 expression indicated a significant
Figure 1. Histopathological and parasite density analyses of skin of dogs naturally infected with *L. infantum*. Animals were categorized according to their clinical status into asymptomatic (AD, *n* = 10), oligosymptomatic (OD, *n* = 10), or symptomatic (SD, *n* = 15) or categorized according to skin parasite density into low (LP, *n* = 12), medium (MP, *n* = 11), or high (HP, *n* = 12) parasite density. The control group is represented by CD (*n* = 16). Photomicrographs of cutaneous cellular infiltrates from dogs naturally infected by *L. infantum* stained by hematoxylin and eosin (A). Representative cellular infiltrates of study groups are depicted: Control dogs (1 and 2); AD or LP (3 and 4); OD or MP (5 and 6); SD or HP (7 and 8).

**Left panels:** Slides shown at 10x magnification; bar, 100 µm. **Right panels:** Slides shown at 40x magnification; bar, 25 µm. Correlation between quantitative analysis of cutaneous cellular infiltrate with clinical status (B) or skin parasite density (C) are presented. Correlation between clinical groups and skin parasite density is also presented (D). The results are expressed as the mean number of cells present in cutaneous cellular infiltrates evaluated at 20 fields plus standard deviation. In (D), the results are expressed as the mean of the log number of skin parasite density plus standard deviation. Significant differences (*p* < 0.05) compared with CD and AD or LP groups are indicated by symbols * and #, respectively. Spearman's correlation indexes (r and *p* values) are shown on the graphs when applicable.

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up-regulation occurred in all infected groups when compared to CD, and increased levels were observed in the HP group in comparison with the LP and MP groups (2.1-fold and 1.7-fold, respectively; *p* < 0.05). Moreover, a positive correlation could be established between the expression of CCL5 and skin parasite density (*r* = 0.5400, *p* = 0.0004).

CCL13 and CCL17 were down-regulated in all groups in comparison to the CD group; however, significant differences were not found between experimental groups. For the chemokine CCL21, we observed increased expression in all groups compared with the CD group, and high levels were found in the HP group compared with the LP and MP groups (1.4-fold and 1.3-fold, respectively; *p* < 0.05). In addition, a positive correlation was observed between CCL21 expression and parasite density (*r* = 0.6000, *p* = 0.0004).

The expression of CCL24 was up-regulated in the LP and MP groups and down-regulated in the HP group compared to the CD group. In addition, higher CCL24 expression was observed in the MP group when compared with the HP group (0.9-fold; *p* < 0.05). Furthermore, a negative correlation was observed between CCL24 expression and skin parasite density (*r* = -0.3368, *p* = 0.0479). With regard to CXCL8, we observed an increase in the target transcript levels in the LP and HP groups and down-regulation in the MP group compared with CD. In addition, CXCL8
expression was significantly higher in the HP group compared with the LP and MP groups (6.3-fold and 0.3-fold, respectively; \( p < 0.005 \)). Positive correlation was observed between CCL12 expression and skin parasite density (\( r = 0.4190, p = 0.0153 \)).

Skin parasite density was most strongly correlated with chemokines that induce macrophage migration during CVI.

In order to better identify the association between inflammatory cells present in skin and chemokine levels, we performed additional correlation analyses between distinct cell types and cutaneous chemokine expression (Fig. 4). Interestingly, our results indicated that macrophages were the cell type that was most likely to be recruited by chemokines CCL2 (\( r = 0.3514, p = 0.0406 \)), CCL4 (\( r = 0.3600, p = 0.0596 \)), CCL5 (\( r = 0.3453, p = 0.0159 \)), and CCL21 (\( r = 0.3410, p = 0.0459 \)) (Fig. 4). Negative correlation was observed between CCL21 levels and neutrophils (\( r = -0.3502, p = 0.0419 \)).

**Discussion**

The analysis of chemokine expression in lymphoid compartments is crucial for assessing central regulation and pathophysiological processes, including traffic homeostasis, inflammation,
and hematopoiesis [27,28]. In this context, few studies have investigated the levels of chemokines in ongoing CVL. In one of these studies, Strauss-Ayali et al. [21] evaluated the expression of CCL2, CCL4, CCL5, and CXCL10 in the spleen of dogs naturally or experimentally infected by L. infantum and found an increase of CCL2 and CCL5 in the experimentally infected dogs.

Herein, increased levels of CCL2, CCL4, and CCL5 in dogs with high parasitemia were observed, and these chemokines were positively correlated with parasitic density. These results indicate a preferential migration of macrophages into the skin, suggesting a host strategy to control parasitism during ongoing CVL. It has been proposed that in leishmaniasis, chemokines CCL2, CCL4, and CCL5 generally play a role not only as chemotactic factors but also as co-activators of macrophages and consequently have a part in the elimination of parasites [29–32].

After stimulation with CCL2, human macrophages experimentally infected with L. infantum produced levels of nitric oxide that were similar to those obtained by stimulation with IFN-γ, which increased the ability of these cells to eliminate the parasite [33]. In addition, CCL2 and CCL3 may induce leishmanial activity in vitro in human macrophages infected by L. infantum and can control the growth and multiplication of intracellular L. donovani via regulatory mechanisms mediated by nitric oxide [34]. In the present work, we demonstrated an increase in the percentage of macrophages in dogs with clinical signs (OD and SD) or with moderate to high parasitemia (MP and HP). There was also a positive correlation between the percentage of macrophages and expression of CCL2, CCL4, and CCL5. Previous data published by our group demonstrated a decrease in absolute values of circulating monocytes as a hallmark found in the symptomatic group and in the group with the higher parasite load [6]. These data may suggest the recruitment of monocytes to other tissues during active CVL, where they might play an important role in immunological connections throughout antigen presentation and parasite clearance. However, the presence of macrophages in the skin infiltrates does not guarantee their ongoing function since histological analysis of skin during CVL described in this and other studies showed an intense cell infiltrate composed of mononuclear cells in animals with high parasitemia that were clinically symptomatic [19]. The finding that expression of macrophage-chemoattractants was associated with parasite burden contradicts previous in vivo data demonstrating that these chemokines have a macrophage-activating protective effect. This would suggest that the chemokines are recruiting immature or unresponsive macrophages. Moreover, the levels of CXCL8 observed in HP animals, despite inducing macrophage recruitment, seemed to favor the persistence of the parasite in the skin compartment. In addition, high levels of macrophages in the skin of dogs with active CVL (OD and SD) and in dogs with MP and HP density were demonstrated and highlighted the inability of these cells to control parasitism.

Our study represents the first investigation on the involvement of CCL21 in CVL. We also observed increased levels of CCL21 in...
animals with high parasitemia, independent of the positive correlation between the chemokine and cutaneous parasitemia. It has been reported that CCL21 is an important chemokine involved in recruiting antigen-presenting cells (APCs) to lymphoid organs [59]. In murine infection by *L. donovani*, Ato et al. [36] demonstrated that CCL21 is important in the marginal zone of the spleen for maintaining the structure of its cellular composition and capturing blood antigens during *Leishmania* infection. Moreover, mice deficient for the gene encoding CCL21 have greater susceptibility to infection when exposed to *L. donovani* due to the loss of dendritic cell migration [37]. In this context, we hypothesize that increased skin parasitemia has the potential to stimulate the expression of CCL21, resulting in the recruitment of APCs in the skin from the lymphoid organs. However, it is possible that either these cells, like macrophages, do not present a functional profile favoring a Th1-immune response that would be effective against *Leishmania* infection. Alternately, the increase of CCL21 may lead to retention of APCs in the skin and reduce their migration to the regional lymph node where antigens would be presented to T cells.

Several studies have reported the involvement of mast cells in regulating immunity against various *Leishmania* species [38–40]. In the present study, a decrease of this population was observed in the skin of animals presenting severe clinical forms of the disease (OD and SD group) and in all groups categorized according to parasitemia density [LP, MP, and HP] when compared with the control group. This finding could be related to this cell type being involved in attempts to contain the intense skin parasitemia, as described in several studies that evaluated a murine model [41–43]. Calabrese et al. [21] described an intense inflammatory skin reaction formed mainly by mast cells, indicating that these cells may exert a role in innate immunity against *L. infantum* infection. Our data regarding mast cells conflict with this possibility, however. The discrepancy might be explained by *L. infantum* infection causing a diverse range of clinical and histopathological manifestations. Variations in host resistance may help to explain the variations found in the skin parasite load in dogs. Moreover, when dogs from different regions are compared, additional factors must be considered, such as variations in weather conditions (e.g., *Leishmania* infection seems to occur chiefly in dry seasons).

In the present study, decreases in the eosinophil population and CCL21 expression were observed that were related to the clinical progression and skin parasitemia density. CCL21 is a specific agonist for CCR3, attracting and activating eosinophils in parasitic diseases [41]. Some authors have described a microbicidal capability of eosinophils against *L. donovani* and *L. major* parasites [45,46] and suggested this cell type could play an important role in protection against *Leishmania* infection [47]. Moreover, Amastuza et al. [48] reported that eosinophil counts were higher in dogs that presented cutaneous signs, and they suggested that this finding was associated with allergic responses. More studies are necessary to determine the role of eosinophils in the cutaneous immune response in CVL.

The participation of neutrophils in addressing infection by parasites of the genus *Leishmania* has been studied in recent years to understand the mechanism related to the innate immune response [49–51]. We observed that higher CXCL8 levels existed in dogs presenting high cutaneous parasitemia. This chemokine induces neutrophil chemotaxis, and the initial influx of neutrophils seems to be beneficial for the survival of *Leishmania* in the infected tissue [50]. Interestingly, it has been reported that the parasite itself produces a protein with chemotactic properties, called *Leishmania* chemotactic factor, which promotes the migration of neutrophils to the site of infection [19], thereby boosting the phagocytosis of the parasite. Peters et al. [51] evaluated the events that occur in the skin during the initial phase of the transmission of *L. major* by sand flies and observed that a decrease in neutrophils at the infection site is associated with the inability of parasites to establish infection. This hypothesis is strongly supported by a recently published study from our group that showed a mixed cytokine profile during active CVL with predominantly higher cutaneous levels of interleukin II-10 and transforming growth factor II apart from lower expression of II-12. These findings might represent a key condition that allows persistence of parasite replication in the skin [17].

Herein, our data highlight the skin as an important organ in CVL and suggest that increased levels of CCL2, CCL4, CCL5, and CCL21 are associated with the immunopathogenesis of CVL. Our data also suggest that the expression of these cytokines in skin could be used as biomarkers for disease progression in dogs naturally infected by *L. infantum*. Our findings represent an advance in the knowledge of the involvement of skin inflammatory infiltrates in CVL and the systemic consequences and may contribute to developing a rational strategy for the design of new and more efficient prophylactic tools and immunological therapies against CVL.

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Author Contributions

Conceived and designed the experiments: RGS CMC RCG GCO ABR. Performed the experiments: DMS RGS JVS DSL. Analyzed the data: DMS CMC RCG ABR. Contributed reagents/materials/analysis tools: RGS CMC ATC RCO ABR. Wrote the paper: DMS RGS JVS DSL ABR.

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