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Can TLR-2+, COX-2+ and VEGF+ expression contribute to skin disease in canine leishmaniasis?

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ABSTRACT.- Sales ET, Ferreira TMV, Girão-Carmona VCC, Ferreira TC, Nunes-Pinheiro DCS. **Can TLR-2+, COX-2+ and VEGF+ expression contribute to skin disease in canine leishmaniasis?** *Pesquisa Veterinária Brasileira 46:e07670, 2026*. Graduate Program in Veterinary Sciences, Faculdade de Veterinária, Universidade Estadual do Ceará, Av. Dr. Silas Munguba 1700, Fortaleza, CE 60714-903, Brazil. E-mail: tiago.cunha@uece.br

Canine leishmaniasis (CanL), caused by *Leishmania infantum* and transmitted by phlebotomine sandflies, presents a broad clinical spectrum with systemic and dermatological symptoms. This study hypothesized that vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), and Toll-like receptor 2 (TLR-2) play crucial roles in the inflammatory and angiogenic responses driving CanL pathogenesis. An analysis was made of the expression of VEGF+, COX-2+, and TLR-2+ in the skin of 33 naturally infected dogs classified as asymptomatic (AG, n = 10) or symptomatic (SG, n = 23), along with five uninfected controls (CG). The symptomatic group was further divided into SG+ (n = 14) with skin lesions and SG- (n = 9) without lesions. Skin biopsies from the scapular region and ear tip were processed for histological and immunohistochemical analyses using monoclonal antibodies. Symptomatic dogs showed clinical signs such as alopecia, keratoconjunctivitis and dermatitis, with more intense inflammatory infiltrates than asymptomatic animals. VEGF+, COX-2+ and TLR-2+ expression levels were significantly higher in symptomatic dogs, particularly those with skin lesions (SG+), suggesting their role in immune response and disease progression. These findings indicate that VEGF+ and COX-2+, potentially through the TLR-2-mediated nuclear factor kappa B (NF-κB) pathway, contribute significantly to the inflammatory mechanisms underlying CanL.

INDEX TERMS: *Leishmania infantum,* canine leishmaniasis, inflammatory mediators, Toll-like receptors, skin disease.

RESUMO.- [**A expressão de TLR-2+, COX-2+ e VEGF+ podem contribuir para a doença cutânea na leishmaniose canina?**] A leishmaniose canina (LCan), causada por *Leishmania infantum* e transmitida por flebotomíneos, apresenta um amplo espectro clínico com manifestações sistêmicas e dermatológicas. Neste estudo, hipotetizou-se que o fator de crescimento endotelial vascular (VEGF), a ciclooxigenase-2 (COX-2) e o receptor Toll-like 2 (TLR-2) desempenham papéis importantes nas respostas inflamatórias e angiogênicas que contribuem com a patogênese da LCan. A expressão de VEGF+, COX-2+ e TLR-2+ foi analisada na pele de 33 cães naturalmente infectados, classificados como assintomáticos (AG, n = 10) ou sintomáticos (SG, n = 23), juntamente com cinco controles não infectados (CG). O grupo sintomático foi subdividido em SG+ (n = 14) com lesões cutâneas e SG- (n = 9) sem lesões. Biópsias de pele da região escapular e ponta da orelha foram processadas para análises histológicas e imunohistoquímicas usando anticorpos monoclonais. Cães sintomáticos apresentaram sinais clínicos como alopecia, ceratoconjuntivite e dermatite, com infiltrados inflamatórios mais intensos do que os cães assintomáticos. Os níveis de expressão de VEGF+, COX-2+ e TLR-2+ foram significativamente maiores em cães sintomáticos, particularmente naqueles com lesões cutâneas (SG+), sugerindo seu papel na resposta imune e na progressão da doença. Esses achados indicam que VEGF+ e COX-2+, potencialmente através da via fator nuclear kappa B (NF-κB) mediada por TLR-2, contribuem significativamente para os mecanismos inflamatórios subjacentes à LCan.

TERMOS DE INDEXAÇÃO: *Leishmania infantum*, leishmaniose canina, mediadores inflamatórios, receptores tipo-Toll, doença cutânea.

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INTRODUCTION

Canine leishmaniasis (CanL), caused by *Leishmania infantum* and transmitted through the bite of infected phlebotomine sandflies (Dantas-Torres et al. 2019), displays a broad clinical spectrum, ranging from subclinical or asymptomatic stages to severe symptomatic cases with systemic and dermatological manifestations (Ferreira et al. 2017). The skin, as the primary interface with the environment, plays a pivotal role in the host's defense against CanL, serving as the initial site for cellular interactions and immune-inflammatory responses to the parasite (Papadogiannakis & Koutinas 2015).

Within the skin, various cellular components — including keratinocytes, mature dendritic cells, monocytes/macrophages and granulocytes — form the first line of defense against invading pathogens (Simpson et al. 2011). These cells detect pathogen-associated molecular patterns (PAMPs) on the surface of infectious agents through pattern recognition receptors (PRRs), particularly Toll-like receptors (TLRs) (Kawasaki & Kawai 2014). The activation of these receptors triggers an inflammatory response, which is crucial for the host's defense. Specifically, TLRs have been identified as key elements in the immune response to CanL (Amorim et al. 2011).

Upon recognition of exogenous antigens by TLRs, multiple downstream signaling molecules are activated, initiating the nuclear factor kappa B (NF-κB) signaling cascade. This activation results in the production of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8, which promote T cell activation and the differentiation of Th1 or Th2 responses (Kawasaki & Kawai 2014, Scott & Novais 2016). Beyond TLR signaling, other inflammatory mediators, such as cyclooxygenase-2 (COX-2) (Souza-Filho et al. 2024) and vascular endothelial growth factor (VEGF) (Ribeiro et al. 2024), may also play important roles in the immune response to *L. infantum*, although their specific contributions to CanL remain incompletely understood.

Given the potential role of COX-2 and VEGF in the pathogenesis of cutaneous leishmaniasis caused by *Leishmania donovani* (Fraga et al. 2012, Das et al. 2014), this study aims to evaluate the expression of VEGF+, COX-2+, and TLR-2+ in the skin of dogs naturally infected with *L. infantum*. By elucidating the role of these mediators, this research seeks to address existing gaps in the body of knowledge about the inflammatory mechanisms that underlie CanL.

MATERIALS AND METHODS

Ethical approval. This study was approved by the Ethics Committee on Animal Use (CEUA) at the "Universidade Estadual do Ceará" (UECE), Brazil (Protocol no. 6508268/2016).

Experimental design and groups. Skin samples were obtained from thirty-three adult male and female dogs of various breeds, between 2 and 8 years old, at the Zoonosis Control Center in Fortaleza, state of Ceará, Brazil, a region with a high prevalence of CanL. The inclusion criteria for the study involved a combination of diagnostic tests to accurately confirm *Leishmania infantum* infection. These diagnostic tests included the dual path platform (DPP) rapid test for CanL (Bio-Manguinhos®, Brazil), enzyme-linked immunosorbent assay (ELISA) (Bio-Manguinhos®, Brazil), and parasitological analysis via bone marrow aspiration. Collectively, these methods enabled a comprehensive assessment of the dogs' infection status

Dogs that tested negative in both serological and parasitological tests were classified as uninfected and designated as the control group (CG, n = 5). Infected dogs (n = 33), confirmed to have *L. infantum*, were further divided into two groups: asymptomatic (AG, n = 10) and symptomatic (SG, n = 23), based on the presence or absence of clinical and dermatological signs. The symptomatic group was then subdivided into dogs without skin lesions (SG-, n = 9) and dogs with skin lesions (SG+, n = 14). Inclusion criteria for symptomatic dogs were based on common clinical manifestations associated with *L. infantum* infection, including onychogryphosis, keratoconjunctivitis, skin ulcer, alopecia, dermatitis, and cachexia. Clinical changes were expressed as percentages.

Skin sampling and histological analysis. Skin biopsies were collected from the scapular region and ear tip of each dog. These biological samples were processed for subsequent histological and immunohistochemical analyses.

Skin samples were obtained post-euthanasia, following the administration of a 10% potassium chloride solution, from dogs that had been previously anesthetized with Thiopental (Thiopentax®, Cristalia). Skin tissue sections measuring 5 mm in diameter were collected from both the scapular region and the ear tip of each dog using a dermatological punch. These samples were taken to the lab, where they were fixed in 10% buffered neutral formalin, stored in 70% ethanol, and subsequently embedded in paraffin blocks for routine histological processing. For histological analysis, the paraffin-embedded blocks were sectioned into five-micrometer-thick slices and stained with hematoxylin and eosin (HE). The sections were examined under a light microscope (Nikon Eclipse E200MV) at 200x magnification, and the degree of inflammatory cellular infiltrate was classified as absent, mild, moderate, or intense. The classification was based on the subjective assessment of two independent observers, whose evaluations were averaged to ensure consistent results.

Immunohistochemical analysis. Immunohistochemical evaluations were performed on 5 *μm*-thick paraffin-embedded skin sections mounted on silanized glass slides. The slides were first kept in an incubator at 36 °C, followed by antigen retrieval through immersion in citrate buffer (pH 9.0) for 30 minutes at 97 °C. To block endogenous peroxidase activity, the slides were treated with 3% hydrogen peroxide (DAKO) for 10 minutes. Subsequently, the sections were incubated with specific monoclonal antibodies: anti-VEGF+ (clone VG1, Dako®-M7273), anti-COX-2+ (clone CX-294, Dako®-M3617-1), and anti-TLR-2+ (clone 3H8, Sigma Aldrich®), for one hour at room temperature.

Following antibody incubation, the slides were washed twice with PBS and incubated with EnVision polymer reagent (EnVision TMþDual connection System/HRP, Dako®) for 30 minutes at room temperature. To visualize immunoreactivity, 3,3'-diaminobenzidine (DAB, Dako®) was applied for 10 minutes. The sections were then counterstained with Mayer's hematoxylin for five minutes to increase contrast. Staining intensity was assessed under a light microscope (Leica DM2000) at 200x magnification. The expression levels of the markers were classified as none (-), mild (+), moderate (++), or intense (+++), based on the subjective evaluation of two

independent observers, and the final classification resulted from the averaged assessments (Oliveira et al. 2014).

For *L. infantum* immunohistochemistry (IHC), skin biopsy sections were prepared from paraffin-embedded tissue, as previously described. The silanized slides were first deparaffinized in xylene and rehydrated using a series of graded alcohols. To block endogenous peroxidase activity, the slides were immersed in a 4% hydrogen peroxide solution, followed by rinsing in PBS (0.01 M, pH 7.2). Non-specific binding was minimized by treating the sections with normal goat serum (1:100 dilution). The primary antibody, generously provided by the Institute of Tropical Medicine of São Paulo, was obtained from rabbits experimentally infected with *L. infantum* and diluted (1:200 in 0.01 M PBS) before being applied to the slides. The sections were incubated for 22 hours at 4 °C in a humid chamber. After washing with PBS, the slides were incubated with a biotin solution, washed again, and subsequently treated with the streptavidin-peroxidase complex for 20 minutes at room temperature. The reaction was visualized by applying 0.024% diaminobenzidine (DAB) and 0.16% hydrogen peroxide.

The *L. infantum* amastigote forms were analyzed under light microscopy (Leica® DM2000) at 200x magnification. Staining intensity was classified as none (-), mild (+), moderate (++), or intense (+++), based on the subjective evaluation of two independent observers. To ensure consistency and reliability, the average score of their assessments was used as the final classification (Freitas et al. 2013). Immunohistochemical data were presented as categorical scores indicating absence, mild (+), moderate (++) or intense (+++) staining, in accordance with the criteria established by Ferreira et al. (2017).

RESULTS

Clinical and dermatological findings

Figure 1 depicts the clinical and dermatological findings of dogs with CanL. The primary symptomatic changes observed in the diseased dogs (n = 23) included onychogryphosis (91.3%), cachexia (21.7%), keratoconjunctivitis (34.7%), and various forms of dermatological injuries (60.8%). Among these dermatological manifestations, the most common ones were alopecia/hypotrichosis (56.5%), ulcerative lesions (17.3%), lichenification (12%), and dermatitis (43.4%), with 21.7% of the cases showing lesions at the ear tip.

Skin histopathology results

The presence of inflammatory cellular infiltrate in naturally infected animals was observed in the scapular region and at the ear tip in 60.6% of cases (20/33), with 40% (4/10) in the asymptomatic group (AG) and 73% (17/23) in the symptomatic group (SG). Among symptomatic animals, inflammatory infiltrate was present in 55.5% (5/9) of those without skin lesions (SG-) and 100% (14/14) of those with skin lesions (SG+). A mild to moderate degree of inflammatory infiltrate was predominantly observed in AG (66.6%), while SG- presented mild to moderate infiltrate in 55% of cases, and SG+ exhibited moderate to severe infiltrate in every case (100%). The infiltrate, which was generally distributed from the most superficial to the innermost layers of the dermis, consisted primarily of neutrophils, macrophages, and lymphocytes (Fig. S1-S8).

Skin immunohistochemical results

Table 1 and Figure 2-13 summarize the major findings of the immunohistochemistry analysis. All the groups showed expression of COX-2+ and VEGF+, albeit in varying intensities. In the symptomatic group with skin lesions (SG+), COX-2+ and VEGF+ expressions were far more intense (Fig. 11-12) than in the asymptomatic group (AG) (Fig. 5-6) and in the symptomatic group without skin lesions (SG-) (Fig. 8-9), which showed expressions ranging from mild to moderate. Additionally, moderate to intense COX-2+ labeling was detected at the ear tips of both SG- and SG+ animals.

TLR-2+ expression ranged from mild to moderate in the skin of AG (Fig. 7) and SG- (Fig. 10), unlike the control group (CD) (Fig. 4). In both AG and SG, TLR-2+ was predominantly localized in the peripheral regions of the epithelium and around blood vessels, whereas in SG+, TLR-2+ was distributed multifocally and intensely throughout the epithelium, with stronger expression found in areas of inflammatory infiltrate.

DISCUSSION

Canine leishmaniasis is an immunoparasitic disease capable of disrupting the cutaneous environment, which is reflected in the variety of clinical alterations observed in the present study. The presence of the protozoan *Leishmania infantum* induces diffuse inflammatory alterations, characterized by the infiltration of neutrophils, macrophages, lymphocytes, and plasma cells throughout the dermis (Esteve et al. 2015,

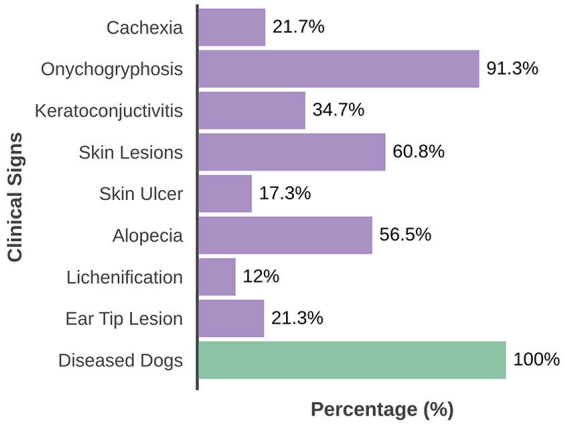

Fig. 1. Clinical and dermatological findings of dogs naturally infected by *Leishmania infantum.*

Table 1. Expression levels of COX-2, VEGF, TLR-2, and *Leishmania infantum* in the skin of dogs naturally infected with *L. infantum*

Markers	CG	AG	SG-	SG+
COX-2+	+	++	++	+++
VEGF+	+	+	++	+++
TLR-2+	+	++	++	+++
L. infantum	-	+	++	++

Study groups: CG = control group, AG = asymptomatic group, SG- = symptomatic group without skin lesions, SG+ = symptomatic group with skin lesions; COX-2 = cyclooxygenase-2, VEGF = vascular endothelial growth factor, TLR-2 = Toll-like receptor 2; Expression scores: (-) none, (+) mild, (++) moderate, (+++) intense.

Ferreira et al. 2017). In our study, these inflammatory cells were observed upon histopathological examination, and notably, their presence was evident even in the group of asymptomatic animals. This finding suggests that, despite the absence of clinical lesions, the tropism of *L. infantum* for cutaneous tissue (Scorza et al. 2021) may trigger the migration of inflammatory cells to the skin.

The interaction between the parasite and immune system cells, as well as keratinocytes, via the surface receptor TLR-2, stimulates signaling cascades such as NF-κB, leading to the production of pro-inflammatory and chemotactic cytokines (Kar et al. 2011). Although some studies report the association of TLR-2 expression with parasite control (Gatto et al. 2015), in our study, animals with higher tissue expression of this

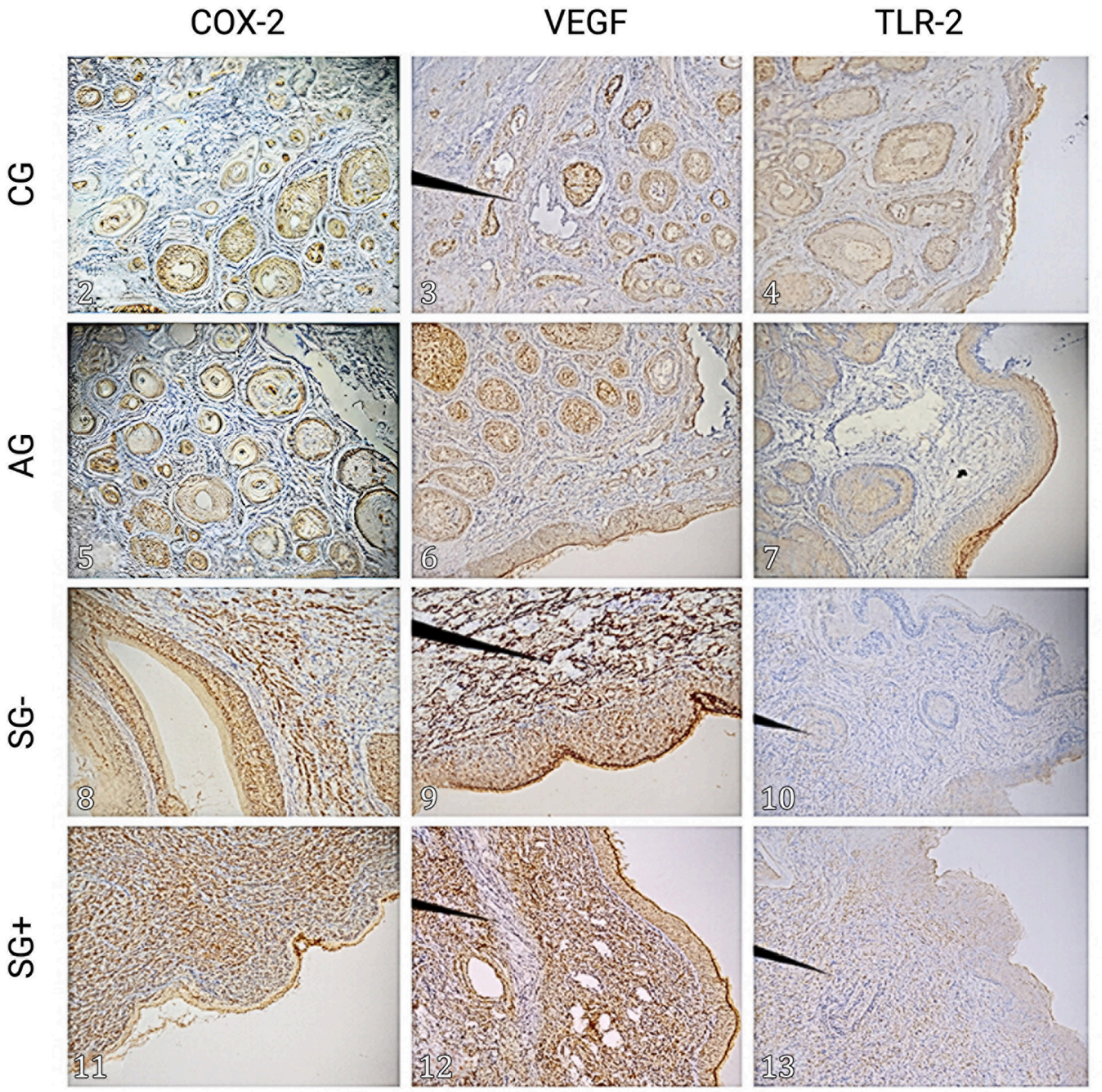

Fig. 2-13. Immunohistochemical analysis of skin from dogs naturally infected with *Leishmania infantum*. Dogs were categorized based on their dermatological clinical status into asymptomatic (AG), symptomatic without skin lesions (SG-), symptomatic with skin lesions (SG+), and control group (CG). COX-2+ expression was classified as (**2**) mild, (**5** and **8**) moderate, and (**11**) intense. VEGF+ expression was categorized as (**3** and **6**) mild, (**9**) moderate, and (**12**) intense (K). TLR-2+ expression was classified as (**4**) mild, (**7** and **10**) moderate, and (**13**) intense. All images at 200x magnification.

receptor exhibited greater inflammatory infiltrate density and tissue damage. Considering that the activation of TLR-2 promotes an inflammatory response (Kar et al. 2011, Esteve et al. 2015), the expression of this receptor is expected to be increased in cutaneous tissue with a higher degree of lesions. Additionally, recent studies suggest that the protozoan exploits TLR-2 as a mechanism to evade immune clearance, facilitating its persistence in the tissue, and hence, eliciting a non-protective inflammatory response (Roy et al. 2025).

The activation of TLR-2 may also be associated with COX-2 expression, given that this enzyme is upregulated via NF-κB signaling pathways (Shi et al. 2015). The role of COX-2 in leishmaniasis and its activation pathways has been investigated in experimental models (Das et al. 2014). In *Leishmania donovani* infections, studies have suggested that proteases secreted by the parasite play a significant role in the inflammatory response by enhancing COX-2 expression, which subsequently leads to increased prostaglandin production. This increase in prostaglandins can inhibit pro-inflammatory cytokines such as IL-12, and reduce nitric oxide production in infected macrophages, which promotes the persistence of the protozoan in the tissue (Das et al. 2014). These findings are consistent with those of our study, in which dogs with higher COX-2 expression also showed increased TLR-2 expression, as well as greater lesion severity, indicating a possible relationship between COX-2 and TLR-2.

Likewise, VEGF expression, which is also associated with TLR-2 activation (Varoga et al. 2006), was most pronounced in symptomatic animals with skin lesions (SG+), in line with the findings of studies on *Leishmania amazonensis*-infected mice that link skin lesions, hypoxic environments, and VEGF synthesis (Arrais-Silva et al. 2005). In human tegumentary leishmaniasis, dysregulated angiogenesis and elevated VEGF levels are similarly observed in symptomatic skin (Fraga et al. 2012), reinforcing the findings of our study. VEGF is a molecule that promotes angiogenesis and, within the context of CanL, may facilitate the dissemination of parasites to various tissues (Ribeiro et al. 2024). Elevated VEGF expression in dogs exhibiting dermatological symptoms (SG+) may also correlate with an increased influx of inflammatory cells into the skin, thereby intensifying cutaneous manifestations of the disease. Moreover, VEGF has been shown to polarize tissue macrophages toward the M2 subtype, which exhibits immunomodulatory activity and potentially enhances protozoan persistence within the skin of infected dogs (Lai et al. 2019). This mechanistic insight may help explain the higher parasite load observed in symptomatic groups (SG) compared to asymptomatic ones (AG) in this study.

Taken together, our findings suggest a synergistic role of COX-2 and VEGF in promoting protozoan survival, both potentially arising from the activation of a shared surface receptor (Fig. 14). However, the main limitations of our study are the relatively small sample size, which may limit the broader applicability of the findings, and the lack of longitudinal data to track the progression of inflammatory markers over time. Moreover, our study did not explore other immune mediators that could further elucidate the complex interactions involved in CanL pathogenesis.

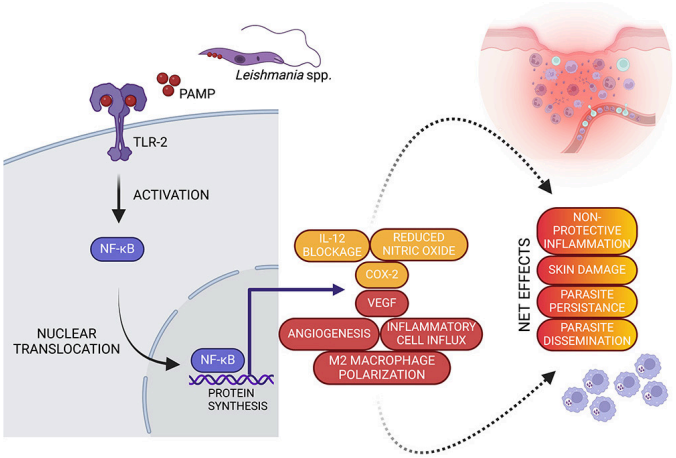

Fig. 14. Synergistic activation of COX-2 and VEGF through TLR-2 signaling in the skin of dogs infected with *Leishmania* spp., contributing to inflammation and parasite survival.

CONCLUSION

Toll-like receptor 2 (TLR-2) expression is involved in canine leishmaniasis (CanL), and suggest activation of the nuclear factor kappa B (NF-κB) pathway for vascular endothelial growth factor (VEGF+) and cyclooxygenase-2 (COX-2+) expression on the skin immune system in response to *Leishmania infantum*.

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Conflict of interest statement.- The authors declare that there are no conflicts of interest.

Credit author statement.- Emmanuel T. Sales, Társsila M.V. Ferreira, and Tiago C. Ferreira were responsible for biological sample collection and laboratory processing. Virgínia C.C. Girão-Carmona and Diana C.S. Nunes-Pinheiro conducted the histological and immunohistochemical processing and analyses. Emmanuel T. Sales, Tiago C. Ferreira, and Diana C.S. Nunes-Pinheiro contributed to the writing, translation, formatting, and critical revision of the manuscript.

Data availability statement.- The data supporting the findings of this study are available from the corresponding authors upon reasonable request.

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