

Research Article

FML/QuilA-Vaccinated Dogs Naturally Infected with *Leishmania infantum*: Serum Cytokines, Clinicopathological Profile, and Parasitological Parameters

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Dogs are the main reservoir of *Leishmania infantum* in endemic regions. Canine leishmaniasis, caused by *L. infantum*, can progress to a chronic disease resulting in death. Vaccines have been developed with a certain degree of success. The pathogenesis of this disease is not completely understood, especially in previously vaccinated dogs. We herein described clinical data, parasite load, serum levels of cytokines, and the reservoir potential in vdogs vaccinated with the fucose-mannose ligand (FML)/QuilA saponin vaccine (Leishmune™) naturally infected (Vi) and compared to vaccinated not infected dogs (Vn). Thirty-four dogs from private owners were divided into two groups: vaccinated/infected and vaccinated/uninfected. Clinical evaluation, hematological and biochemical parameters, and serum levels of cytokines were measured by conventional methods. The parasite burden in the bone marrow was measured by quantitative real-time PCR, and the transmissibility of parasites to sand flies was assessed by xenodiagnosis. Clinical, biochemical, and hematological parameters of vaccinated infected dogs were mostly normal. Vi dogs developed mild disease with low clinical scores. Serum levels of IL-10 were higher in Vi dogs, and a strong correlation was observed in IL-4 levels and the A/G ratio in Vi dogs. These results suggest a role of TH2 response in Vi dogs, although more data is needed to better understand the disease in vaccinated dogs.

1. Introduction

Zoonotic visceral leishmaniasis (VL) is a disease caused by the protozoa *Leishmania infantum* and is transmitted to mammalian hosts by the bites of female sand flies. Dogs are the most significant domestic reservoir responsible for its maintenance in endemic foci and are thus one of the strategic targets for disease control [1–3]. The phlebotomine sand fly *Lutzomyia longipalpis* is the main vector of disease in Brazil [4]. While blood feeding on a vertebrate host, females ingest amastigote-infected macrophages in the tissue. Once in the insect gut, amastigotes develop into pro-

mastigotes and, a few days later, into the infective forms known as metacyclic promastigotes. During their next blood meal, the female sand flies regurgitate the metacyclic promastigotes into the host's skin, thus completing the cycle of the transmission [4, 5]. Once inside the host, the parasites replicate inside macrophages and are disseminated throughout the body, infecting various tissues including the liver, spleen, and bone marrow [6].

Control measures focused on dogs include the use of insecticide collars and vaccination [7]. The Leishmune™ vaccine was the first commercial vaccine against canine leishmaniasis to be approved and used in Brazil. It is

composed of the fucose-mannose ligand fraction purified from the promastigote *Leishmania donovani* membrane, along with *Quillaja saponaria* saponin as the adjuvant [8–10]. Leishmune™ was in use for at least ten years, but its sale is now suspended in Brazil as the phase III study was considered insufficient by the Ministry of Agriculture, Livestock, and Food Supply to maintain its license [11]. Additionally, several reports have attempted to demonstrate its efficacy either as a vaccine or as an immunotherapy agent [12, 13].

Clinical signs of canine leishmaniasis that are commonly described include dermatological disorders, lymph node enlargement, and a variable degree of weight loss [14]. However, dogs with no clinical signs, usually called asymptomatic, are prevalent even in experimental studies [15]. Thus, defining the clinical stage of infected dogs is a challenging task. To overcome this problem, the combined use of clinical scores and routine biomarkers such as red and white blood cell counts and clinical biochemistry has been encouraged by several authors to determine the clinical status of individuals [14, 16].

Hematological abnormalities are frequent in diseased dogs. Anemia is observed in 40 to 90% of infected animals [17, 18], being an important marker of disease severity [19]. Abnormal blood counts are positively correlated with the severity of clinical signs and with parasite density in the bone marrow [20, 21]. An increase in the myeloid:erythroid (M:E) ratio also correlates with general bone marrow dysplasia in infected animals [22], while biomarkers specific to hepatic function and to renal function in particular are variable depending on the stage of the disease. Dogs with VL often exhibit hyperproteinemia, characterized by hyperglobulinemia and hypoalbuminemia. Consequently, infected dogs present with albumin/globulin (A/G) ratios below the normal range [14].

Although some hematological and biochemical abnormalities are frequently found in infected dogs, there are no definitive standards to help with clinical staging. Biomarkers that are consistently stable during disease and that correlate with the clinical stage would be a remarkable tool. The levels of some cytokines produced during the disease are among the parameters that could be used to assess the general clinical status of dogs with leishmaniasis, but this is not the usual approach. The production of cytokines in certain tissues may provide some information about the immunopathological processes occurring within them. Several studies have assessed the immunopathological status of dogs by measuring the levels of cytokines produced by stimulated peripheral blood mononuclear cells *in vitro* [23–25]. Furthermore, the direct measurement of cytokines in serum is an approach that may provide some information about the ongoing systemic immunopathological processes; this may correlate with the clinical status and the parasite burden in a given animal [26] and is easier to perform than stimulating peripheral blood mononuclear cells *in vitro*.

Herein, we have described the serum levels of cytokines, clinical score, parasite load, and the reservoir potential of vaccinated noninfected dogs and naturally infected dogs that were previously vaccinated with Leishmune™. We have also

evaluated the putative correlations between analytes and clinical parameters in vaccinated infected dogs.

2. Materials and Methods

2.1. Ethics Statement. All dogs used in this study were handled in accordance with animal practice as defined by the Internal Ethics Committee on Animal Experimentation (CEUA) of Universidade Federal de Minas Gerais, Belo Horizonte, Brazil (Protocol 32/2011). This protocol follows the guidelines of CONCEA/MCT, the animal ethics committee of Brazil. Before participating, all owners signed an informed consent form.

2.2. Animals. Domiciled dogs ($n = 34$) of different breeds and sexes living in the metropolitan region of Belo Horizonte, a VL-endemic area, were included in this study. Dogs were aged from one to five years and had been vaccinated with Leishmune™. The owners of all dogs that came to the hospital for revaccination (annual booster) with Leishmune™ who had a complete and regular history of vaccination were invited to join the study. Vaccination with Leishmune™ was performed according to the manufacturer's recommendations: dogs were first serologically tested for leishmaniasis, and after confirmation of a negative result, an initial three-dose schedule at 21-day intervals was administered, followed by at least an annual booster one year after the first dose [10]. All vaccinated dogs included in this study ($n = 34$) had received at least one annual booster and were sampled up to one year after their last annual booster. Vaccinated dogs were divided into two groups: vaccinated infected ($n = 21$, Vi) and vaccinated noninfected ($n = 13$, Vn) dogs.

Evidence of infection was assessed in the groups by xenodiagnosis, cytology, and quantitative polymerase chain reaction (qPCR) of the bone marrow. Dogs with clinical or laboratory evidence of concurrent disease were not included in this study. All dogs lived in the metropolitan region of Belo Horizonte, according to the owner's statements from anamnesis.

2.3. Disease Staging. Vaccinated infected dogs were categorized according to the disease stage, following a previously established protocol with some adaptations [27]. Briefly, stage I dogs have discrete signs of disease and no laboratory abnormalities, stage II dogs have moderate clinical signs and laboratory abnormalities, stage III dogs have severe clinical signs associated with vasculitis and evidence of renal injury, and stage IV dogs have severe clinical signs and chronic renal disease. Dogs with no clinical signs or laboratory abnormalities were assigned a clinical score of 0.

2.4. Sample Collection. Blood samples were collected from each animal after clinical examination, distributed equally into tubes with and without ethylenediaminetetraacetic acid (EDTA). Bone marrow sampling was performed under sedation with intramuscular injections of 0.05 mg/kg acepromazine (0.2%; Acepran, Univet, Louveira, SP, Brazil) and 0.1 mL/kg fentanyl citrate (0.05 mg/mL; Fentanest, Cristalia, Belo Horizonte, MG, Brazil). Samples from the *manubrium*

sterni were collected under local anesthesia with 2% lidocaine (Hiplabor, Belo Horizonte, MG, Brazil). Glass slide smears were prepared with an aliquot immediately after collection, and the remaining sample was stored at -20°C for qPCR.

2.5. Blood Count and Serum Biochemistry. The whole blood collected in EDTA was processed in an automated hematology analyzer (Abacus Junior Vet, Diatron® Group, Budapest, Hungary) to obtain total leukocyte (WBC), erythrocyte (RBC), hemoglobin, and platelet counts. Packed cell volume (PCV) was evaluated by centrifugation of whole blood for 10 min using a microcapillary centrifuge (Microline micro-centrifuge, Laborline, Barueri, SP, Brazil). Other red blood cell indices including mean corpuscular volume (MCV) and hemoglobin concentration (MCHC) were derived from the PCV, hemoglobin, and erythrocyte counts.

One blood smear per sample was stained with a routine hematologic (Romanowsky type) stain and evaluated by light microscopy (Olympus, CH 30, Tokyo, Japan) under an oil immersion objective (1000x) for differential leukocyte counts, as well as morphological evaluation of erythrocytes, leukocytes, and platelets.

The serum was separated by centrifugation of whole blood at 3000 rpm for 10 min and transferred to Eppendorf-type vials for automated analysis (Cobas Mira®, Roche, Basel, Switzerland). Biochemical parameters were evaluated, including blood urea nitrogen, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin, and globulin concentrations, as well as the A/G ratio. Each component was evaluated using a specific kit (SynerMed, Monterey Park, California, USA) according to the manufacturer's instructions.

Bone marrow smears were stained with a Romanowsky-type stain and subjected to cytological evaluation of the bone marrow smears and direct parasitological examination. The smears were examined under 10x magnification for the presence of particles, the percentage of cells, estimative iron concentration, and megakaryocyte counts. Under an oil immersion objective (1000x), 500 nucleated cells were enumerated to derive the M:E ratio and to verify the cellular distribution. Direct parasitological evaluation was initially performed on three slides per sample, with evaluation of additional slides as required for the identification of the parasite. Parasite density (mild, moderate, or severe) was determined according to the number of parasites per field under the oil immersion objective [28].

2.6. Quantitative PCR: Extraction and Amplification of DNA. *Leishmania* DNA was extracted according to a previously described protocol [29] and evaluated by qPCR using TaqMan probes followed by the construction of a standard qPCR curve for parasites of the genus *Leishmania* [30]. Customized primers (Applied Biosystems, Belo Horizonte, MG, Brazil) were used to amplify a conserved region in the *Leishmania* kinetoplast DNA minicircle [31]. The number of copies of *Leishmania* DNA/mL of bone marrow material was determined, and the results were plotted on the calibration standard curve.

2.7. Serum Cytokines. Serum samples were stored at -80°C for later analysis. Serum cytokines were measured by the Luminex bead-based multiplex assay for canine cytokines, following the manufacturer's instructions (Milliplex Canine Cytokine, Merck Millipore).

2.8. Xenodiagnosis. Dogs were exposed to approximately forty laboratory-raised 55th generation *L. longipalpis* females. The protocol used for the feeding of insects on dogs has been described elsewhere [17]. Subsequently, the insects were kept in Center for Disease Control minitraps for seven days and fed a 10% sucrose solution. Seven days after blood feeding, the sand flies were dissected on a drop of 0.9% saline under a stereoscope (Labmex, PZO, Monterey, Mexico). Using a needle attached to an epoxy rod, the gut was removed through the posterior portion of the abdomen and covered with a coverslip. The guts of *L. longipalpis* females were examined for the presence of promastigotes under light microscopy (400x). Finally, the numbers of non-infected and infected sand flies per total number of dissected sand flies were determined for each dog in the study.

2.9. Statistical Analysis. Gaussian distribution was inferred using the Shapiro-Wilk normality test. Normally distributed data were evaluated by unpaired Student's *t*-test, while non-normally distributed data were evaluated by the Mann-Whitney test. Correlations between parameters were established using the Spearman method, and associations were assessed by Fisher's exact test, both with 95% confidence intervals. Correlations were interpreted according to values of the correlation coefficient, as follows: negligible (0.0 to 0.2), weak (0.2 to 0.4), moderate (0.4 to 0.6), strong (0.6 to 0.8), and very strong (0.8 to 1.0). Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Staging and Clinical Signs of Canine VL. All dogs were tested by different methods to determine the evidence of infection with *L. infantum* parasites. Table 1 shows the number of dogs among the vaccinated infected group in each test. Out of the 34 vaccinated dogs, 13 (38.2%) had no evidence of infection in any test and were therefore allocated to the Vn group. Of the 21 dogs in the vaccinated infected group (Vi): 13/21 (61.9%), 12/20 (60.0%), and 1/18 (5.5%) tested positive by xenodiagnosis, bone marrow qPCR, and bone marrow cytology, respectively. Only one dog was positive in all tests, while 3 were positive in both xenodiagnosis and BM-qPCR. Seven dogs were positive only in BM-qPCR and 6 only in the xenodiagnoses (Table 1). Bone marrow cytology was not performed in three dogs from the Vi group due to rapid coagulation of the sample during collection, and xenodiagnosis was not performed in one dog. Xenodiagnosis was performed in only 10 dogs of the Vn group, due to owner refusal.

Among Vi dogs, 8 (38.1%) had no clinical signs compatible with leishmaniasis, while 13 (61.9%) had clinical signs compatible with lower clinical scores (Table 2). A high

TABLE 1: Distribution of subjects in Vi group by infection status for each diagnostic method.

| Test | Result | # | % |
|---------------|----------|----|-------|
| Xenodiagnosis | Positive | 13 | 61.9% |
| | Negative | 7 | 33.3% |
| BM-qPCR | Positive | 12 | 60% |
| | Negative | 8 | 40% |
| BM-cytology | Positive | 1 | 5.5% |
| | Negative | 17 | 94.4% |

BM: bone marrow.

TABLE 2: Distribution of subjects between groups and corresponding clinical score.

| Clinical score | Infected (Vi) | | Not infected (Vn) | |
|----------------|---------------|-------|-------------------|------|
| | # | % | # | % |
| 0 | 8 | 38.1% | 13 | 100% |
| I | 9 | 42.9% | 0 | 0% |
| II | 4 | 19.0% | 0 | 0% |
| III | 0 | 0% | 0 | 0% |
| IV | 0 | 0% | 0 | 0% |
| Total | 21 | 100% | 13 | 100% |

percentage of Vi dogs (17/21, 81%) had mild signs of clinical disease, mainly consisting of scarce dermatologic lesions, lymph node enlargement, and/or splenomegaly. No clinical signs were observed in Vn dogs. Importantly, among Vi dogs, nine (40.9%) were positive on xenodiagnosis, but negative on bone marrow qPCR.

3.2. Hematological and Biochemical Parameters. No significant difference in red blood cell counts was observed between Vi and Vn dogs. Moreover, no differences were observed in the M:E ratio between Vi and Vn dogs. Anemia (defined as PCV below the reference value) was seen in 4/21 (19.0%) in the Vi group (Fig. S1). Serum levels of blood urea nitrogen, creatinine, AST, and ALT were also similar across all groups (Fig. S2).

3.3. Reservoir Potential. Reservoir potential is defined here as the capacity of a host to infect sand flies and is measured as the percentage of infected sand flies per dog. Xenodiagnosis was successfully performed in 30 dogs, and among those infected with *L. infantum*, the percentage of infected female sand flies ranged from 0 to 32%. Among the 13 Vi dogs with positive xenodiagnoses, 4 had clinical score 0, thus considered asymptomatic reservoir. No significant correlation was observed between clinical scores and reservoir potential. Also, there was no correlation between the rate of infected sand flies and the parasite load in the BM when considering the Vi group. Interestingly, 9 Vi dogs were positive in xenodiagnosis while negative in BM-qPCR. Of the ten dogs in the Vn group, none were positive by xenodiagnosis.

3.4. Serum Cytokine Profile. Levels of IFN- γ were undetectable in most samples (below 9.4 pg/mL); however, 12 dogs from the vaccinated groups (Vn and Vi) had IFN- γ levels ranging from 12.0 pg/mL to 10.9 ng/mL. Among tested cytokines, only IL-10 was significantly higher in Vi dogs than in Vn (Figure 1).

A strong positive correlation was found between serum levels of cytokines TNF- α , IL-18, GM-CSF, and IL-2 ($r > 0.6$; Table 3) when compared in pairs. A very strong positive correlation was found between GM-CSF and IL-18 ($r > 0.8$; Table 3). A strong correlation was observed between serum levels of IL-4 and A/G ratio in the Vi group ($r = -0.723$, CI = -0.883 to -0.412, $p = 0.0002$, Figure 2). No correlation was observed between serum levels of cytokines and any other variables evaluated.

4. Discussion

In this study, we assessed the clinical data, parasitological parameters, and the serum cytokine profile of vaccinated dogs with the first-generation vaccine against leishmaniasis developed in Brazil. This was a transversal descriptive study with relevant data relating to naturally infected dogs that were previously vaccinated against canine VL. We focused on comparing the data available between vaccinated dogs that were infected with those that were not (Vi and Vn, respectively). Data from vaccinated dogs that become infected are often neglected. Here, we present data that represent the dogs' parameters at some point during infection. While this may not predict the outcome of canine VL, our results will hopefully inform future studies to shed further light on this disease.

Sixty-two percent of the vaccinated dogs (21/34) were positive on at least one of the parasitological tests performed in the study. Dermatologic lesions and lymphadenopathy were the most common clinical signs observed in this study and are in accordance with previous findings [16]. Vi dogs had mild clinical signs which agreed with previous findings that report a lower incidence of clinical signs in FML-vaccinated dogs that became infected (5 to 8%) compared to unvaccinated dogs (25 to 33%) [32, 33]. This is an important characteristic of vaccines used against diseases that result in severe infections, such as canine VL. It has been shown that vaccination with Leishmune™ interferes with the development of *L. infantum* in sand flies due to impairment of promastigote adhesion in the sand fly midgut by anti-FML antibodies [13], which is of importance for public health. The transmission blocking activity is an interesting feature of a vaccine against leishmaniasis, which has also been shown by others [34].

Anemia is the most common hematological disorder in dogs with leishmaniasis [14, 35, 36]. Vi dogs commonly displayed neither anemia nor medullary dyspoiesis. Although reported by others [18, 35, 36], alterations in the absolute counts of different leukocytes were not present between the groups in our study. In the present study, dogs within the Vi group did not display a decreased A/G ratio compared to Vn dogs. Increased levels of serum proteins and globulins are commonly detected in dogs with leishmaniasis, while

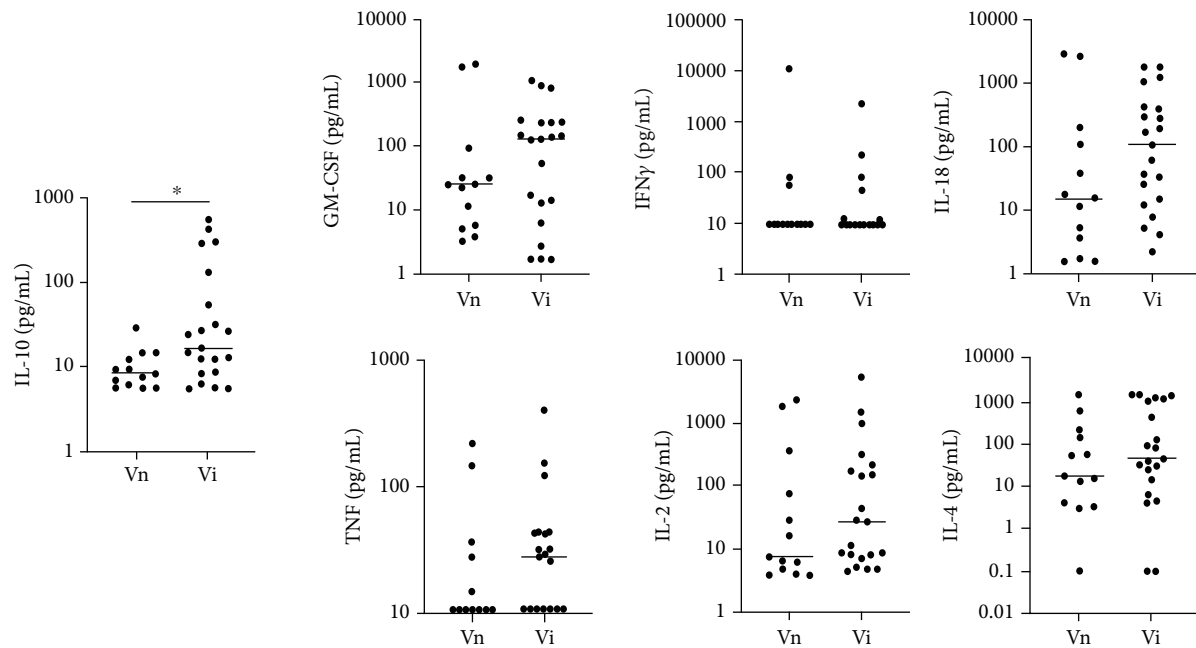


FIGURE 1: Serum levels of cytokines in dogs vaccinated with Leishmune™ infected or not by *Leishmania infantum*. The Y-axis is transformed as \log_{10} . Dots represent the individual values of each dog. Lines represent the median of each group. Vn: vaccinated not infected dogs; Vi: vaccinated infected dogs. Mann-Whitney *U* test. * $p < 0.05$.

TABLE 3: Correlations between serum cytokine levels.

| | GM-CSF | IFN- γ | IL-2 | <i>p</i> value IL-10 | IL-18 | TNF- α | IL-4 |
|---------------|---------|---------------|---------|-------------------------|---------|---------------|--------|
| GM-CSF | | 0.2501 | <0.0001 | 0.8915 | <0.0001 | <0.0001 | 0.5471 |
| IFN- γ | -0.2167 | | 0.2731 | 0.1575 | 0.4634 | 0.5105 | 0.8808 |
| IL-2 | 0.7311 | -0.2067 | | 0.9547 | <0.0001 | <0.0001 | 0.8761 |
| IL-10 | 0.0260 | 0.2647 | 0.0108 | | 0.2918 | 0.2980 | 0.1250 |
| IL-18 | 0.8361 | -0.1391 | 0.6850 | 0.1990 | | <0.0001 | 0.7828 |
| TNF- α | 0.7588 | 0.1250 | 0.6770 | -0.1965 | 0.7975 | | 0.6376 |
| IL-4 | 0.1144 | 0.0286 | 0.0297 | 0.2863 | 0.0525 | 0.0896 | |

levels of albumin and the A/G ratio are frequently decreased [36, 37]. The A/G ratio has been extensively used by veterinary practitioners to monitor the clinical course of the disease [20, 38, 39]. An increase in serum levels of BUN and creatinine only occurs several months after infection, and chronic kidney disease is a frequent finding in dogs chronically infected with *Leishmania*, representing the final stage of the disease [14]. Other abnormalities on laboratory tests are highly variable in canine leishmaniasis and may be present due to concurrent diseases and conditions [36]. Interestingly, nine dogs, all belonging to the Vi group, were positive on xenodiagnosis, but negative on BM-qPCR. This is relevant to public health since all of these dogs were asymptomatic or were assigned the lowest clinical score. However, this is a transversal study, and since dogs were not followed up, we cannot assume the progression of the disease or their reservoir potential in chronic disease.

Taken together, these results suggest the potential role of the vaccine as a means of protecting dogs from being infected or developing severe disease, which is corroborated by others [8, 17]. This potential has been explored as an immunotherapeutic option with a certain degree of success and should be considered in future studies [12, 40]. However, considering that some vaccinated dogs may become infected, it is critical to determine whether clinical scores remain low during chronic infection and if chronically infected vaccinated dogs are a poor source of infection to sand flies. Also, until now, there is no solid scientific evidence of reduced transmission of *Leishmania infantum* from infected vaccinated dogs to sand flies to a level that would significantly reduce the risk of infection of visceral leishmaniasis in humans [41].

The strong correlations between levels of TNF- α , IL-18, GM-CSF, and IL-2 might be explained by the concomitant expression of these cytokines during inflammation. While

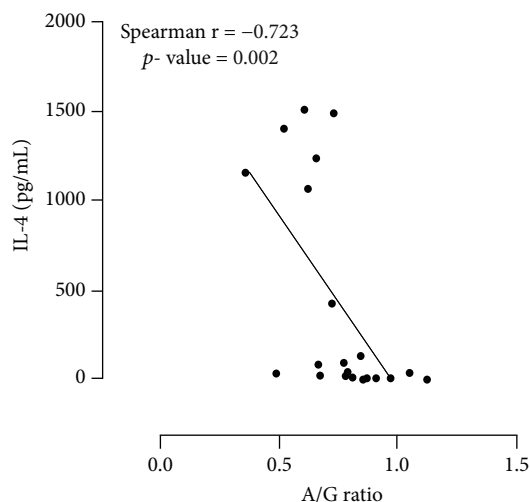


FIGURE 2: Correlation between IL-4 and A/G ratio among Vi dogs. Dots represent individual values of each dog. Lines represent the linear regression model of presented data. Statistical significance is plotted as Spearman's rank correlation coefficient (Spearman r) and p value.

TNF- α and IL-18 are mainly produced by macrophages, GM-CSF is produced by several cell types including macrophages, which is the predominant cell type in lesions caused by VL [42]. While TNF- α and IL-18 are cytokines directly involved in effector mechanisms within the inflammatory milieu, GM-CSF and IL-2 are involved in cell proliferation and thus indirectly involved in inflammation [43]. TNF- α and IL-18 are frequently associated with resistance against *Leishmania* due to their contribution to the resistance phenotype in murine models [44].

The levels of cytokines are commonly evaluated by stimulation of peripheral blood mononuclear cells with *Leishmania* antigens. This approach allows us to detect an antigen-specific immune response against *Leishmania* in an individual and is considered the gold standard for evaluation of the immune response in these animals [45]. Direct measurement of cytokine levels in serum is an approach that has been used extensively and is evolving to deliver more accurate results [46–49]. Whether the cytokine levels in serum accurately portray the systemic immune response in an individual or not is yet to be determined. However, it is an interesting and practical tool that is worthy of further study.

The levels of cytokines might be altered depending on the time point evaluated, varying greatly during disease. Additionally, they might be strongly determined by the organ samples, methodological approach, and animals used in the study. Our approach in measuring cytokines directly from serum samples is a practical and straightforward strategy to, in effect, assess a general immunologic index of a patient. New studies are needed to verify the value of this approach and the use of cytokines as potential biomarkers for disease progression or even for the assessment of treatment efficacy.

Data on serum levels of cytokines during VL are mostly absent, but one study reported no significant differences in

levels of TNF- α between infected and uninfected dogs [50]. This result was corroborated by another approach [25, 51]. The levels of IL-10 were higher in Vi dogs compared to Vn dogs (Figure 1). Levels of IL-10 were also found elevated in infected dogs by different approaches compared to uninfected dogs [25, 51]. IL-10 has been associated with the suppression of TH1 cytokines, skewing the immune response towards a TH2 immune response, especially in cases of human visceral leishmaniasis [47].

It is worth noting that the levels of cytokines vary immensely between individuals, with some individuals having extremely high levels of some cytokines. This finding suggests the presence of a mixed-type immune profile, as infected dogs displayed higher levels of cytokines with no clear correlation with the polarized immune response observed in murine models [44, 45]. The lack of a polarized TH1/TH2 immune response in canine leishmaniasis has been suggested by others [24, 41, 47] as has a compartmented immune response with different cytokine profiles in certain tissues [52]. In both cases, serum levels of cytokines may represent a systemic view of the immune responses occurring in the tissues.

We found a strong correlation between the levels of IL-4 in the serum and the albumin/globulin ratio in Vi dogs (Figure 2). A low A/G ratio is commonly observed in infected dogs as a result of the increase of gamma-globulin due to polyclonal activation of B cells and is commonly related to a worsening in the disease [20, 36–39]. In murine models, the production of IL-4 is increased in susceptible mice, but not in resistant mice [44]. However, in dogs, levels of IL-4 are not directly related to susceptibility, as dogs commonly display a mixed TH1/TH2 immune response [52]. Others have shown that the number of T cells expressing IL-4 in dogs vaccinated with Leishmune™ is comparable to those in uninfected dogs [51]. Levels of IL-4 are increased in the skin and bone marrow of some infected dogs [53, 54] and in the serum of infected humans [55]. Taken together, our results suggest a role of the TH2 response in Vi dogs with a possible correlation with disease development. Again, this is a transversal study, and since dogs were not followed up, we cannot make confident assumptions on how the disease developed in Vi dogs. More data is needed to verify whether Vi dogs develop mild disease compared to nonvaccinated dogs and the role of their immunity in the disease outcome.

In conclusion, we observed that although vaccinated dogs become infected, they may display discreet clinical signs and still be infective to sand flies. However, vaccination might also impact reservoir potential by limiting the percentage of sand flies that are infected after blood feeding. The value of serum cytokine measurements alone is limited, but in association with other parameters, they might be useful for evaluating disease progression in some individuals.

Data Availability

Complementary data can be obtained from the authors by request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Gregório Guilherme Almeida and Fernanda Morcatti Coura contributed equally to this work.

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Supplementary Materials

Fig. S1: hematological features of vaccinated infected (Vi) and vaccinated not infected (Vn) dogs. Fig. S2: serum biochemistry features of vaccinated infected (Vi) and vaccinated not infected (Vn) dogs. (*Supplementary Materials*)

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