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Genome-Wide Association Study of Cell-Mediated Response in Dogs Naturally Infected by *Leishmania infantum*

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A genome-wide association study (GWAS) could unravel the complexity of the cell-mediated immunity (CMI) to canine leishmaniasis (CanL). Therefore, we scanned 110,165 single-nucleotide polymorphisms (SNPs), aiming to identify chromosomal regions associated with the leishmanin skin test (LST), lymphocyte proliferation assay (LPA), and cytokine responses to further understand the role played by CMI in the outcome of natural *Leishmania infantum* infection in 189 dogs. Based on LST and LPA, four CMI profiles were identified (LST^-/LPA^-, LST^+/LPA^-, LST^-/LPA^+, and LST^+/LPA^+), which were not associated with subclinically infected or diseased dogs. LST^+/LPA^+ dogs showed increased interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) levels and mild parasitism in the lymph nodes, whereas LST^-/LPA^+ dogs, in spite of increased IFN-γ, also showed increased interleukin-10 (IL-10) and transforming growth factor β (TGF-β) levels and the highest parasite load in lymph nodes. Low T cell proliferation under low parasite load suggested that *L. infantum* was not able to induce effective CMI in the early stage of infection. Altogether, genetic markers explained 87%, 16%, 15%, 11%, 0%, and 0% of phenotypic variance in TNF-α, TGF-β, LST, IL-10, IFN-γ, and LPA, respectively. GWAS showed that regions associated with TNF-α include the following genes: *IL12RB1*, *JAK3*, *CCRL2*, *CCR2*, *CCR3*, and *CXCR6*, involved in cytokine and chemokine signaling; regions associated with LST, including *COMMD5* and *SHARPIN*, involved in regulation of NF-κB signaling; and regions associated with IL-10, including *LTBP1* and *RASGRP3*, involved in T regulatory lymphocytes differentiation. These findings pinpoint chromosomic regions related to the cell-mediated response that potentially affect the clinical complexity and the parasite replication in canine *L. infantum* infection.

Visceral leishmaniasis (VL) is a zoonotic disease caused by the intracellular parasite *Leishmania infantum* (syn., *Leishmania chagasi*) in the Americas, northern Africa, southern Europe, areas of the Middle East, and Asia and can be fatal if not treated (1). Brazil has the highest incidence of VL in the Americas, being one of the six countries with the highest incidence around the world (1). The domestic dog (*Canis lupus familiaris*) is an important intermediate host that contributes to the risk of human infection (2–4). The spectrum of clinical manifestations occurring in canine *L. infantum* infection ranges from subclinical infection to severe canine leishmaniasis (CanL) (5).

Innate immunity and adaptive immunity play important roles in the outcome of *L. infantum* infection. The parasite induces an inflammatory response via complement activation and pattern recognition receptor signaling (6, 7). An inflammatory environment enriched by chemokines and proinflammatory cytokines is important to attract, activate, and maturate dendritic cells and macrophages and turn them into antigen-presenting cells (APC). These phagocytes express the major histocompatibility complex (MHC) (8) and costimulatory molecules (9) and release interleukin-6 (IL-6), IL-12, IL-18, and tumor necrosis factor alpha (TNF-α) following parasite internalization (10, 11). Thus, mature APC are able to activate naive T lymphocytes and instruct T helper 1 (Th1) differentiation during antigen presentation. Th1 clones release interferon gamma (IFN-γ) and TNF-α, which attract macrophages, activate antileishmanial mechanisms, and induce more proinflammatory cytokines (12–16). The adaptive response to canine *Leishmania* infection is markedly mixed, since *L. infantum* induces Th1 and T regulatory (Treg) responses. Treg lymphocytes inhibit activity of effector phagocytes and prevent exacerbation of inflammation; however, they also allow the spreading of the parasite (17–19).

The leishmanin skin test (LST), also known as the delayed-type hypersensitivity reaction (DTH), consists of a response that occurs between 48 and 72 h following intradermal inoculation of leishmanin antigen. This response evolves erythematous or nodular injury resulting in a chronic inflammatory infiltrate in the dermis, composed mainly of macrophages and lymphocytes (20, 21). The LST is a marker of effective cell-mediated immunity (CMI). A positive LST is often associated with subclinical infec-

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tion, although it has also been rarely described in sick dogs (12, 22–24). The lymphocyte proliferation assay (LPA) is usually employed to evaluate the adaptive response triggered in dogs infected by *L. infantum* (12, 25). This method enables the evaluation of the proliferation index and the profile of cytokines on peripheral blood mononuclear cell (PBMC) culture stimulated with specific antigen. Both LST and LPA demonstrate the ability to mount a cell-mediated response; however, discordant results between them have been reported (25, 26).

The LST response was associated with a genetic component with high heritability and significant familial aggregation in humans (27–29); nevertheless, the genetic basis for the LST response in dogs has been neglected. Dogs are particularly useful as a genetic model for complex disease, as the greatest extent of haplotype blocks and lower allelic diversity in dogs can favor discovery of new genes (30, 31). Variants of *DLA-DRB1* and *NRAMP1* have both been associated with susceptibility to CanL by use of a candidate gene approach (32, 33). A genome-wide association study (GWAS) estimated 64% heritability for CanL, and the clinical status was correctly predicted in 60% of dogs (34). Recently, a GWAS conducted by our group using a variance component model which takes genetic structure into account (35) identified regions associated with susceptibility to CanL, including the genes *IL2RA*, *IL15RA*, and *TLE1* (36). Nonetheless, the genetics of different immunologic profiles have not been investigated.

A GWAS for the cell-mediated response to canine *Leishmania* infection may provide insights regarding host response and new targets for therapeutic or functional investigations. Therefore, the aim of this study was to identify chromosomal regions potentially affecting LST, LPA, and cytokine responses and contribute to the further elucidation of the role played by CMI profiles in the clinical response and control of *L. infantum* infection.

MATERIALS AND METHODS

Animals and clinical evaluation. One hundred eighty-nine purebred dogs, 102 (54%) female and 87 (46%) male, were evaluated. The dogs were kept for at least 18 months in one of the 11 municipalities where CanL is endemic, distributed among the Brazilian states of Bahia (BA), Minas Gerais (MG), São Paulo (SP), and Federal District (DF) (see Table S1 in the supplemental material). Approximately 37% (70/189) of dogs were 18 months to 2 years old, 33% (63/189) were 3 to 5 years old, 18% (34/189) were 6 to 8 years old, and 12% (22/189) were 9 to 13 years old. We investigated clinical signs consistent with CanL such as dermatitis, alopecia, hyperkeratosis, onychogryphosis, lymphadenomegaly, hepatomegaly, splenomegaly, conjunctivitis, and emaciation. Each dog was evaluated once, at the time that we visited the properties in the area of endemicity. The presentation of at least one clinical sign was the parameter used for classification as a diseased dog (DS group). Dogs that did not show clinical signs were considered subclinically infected dogs (SI group). The control group (CLT) consisted of dogs from the same areas of endemicity that had no clinical signs and tested negative by PCR, LST, LPA, and anti-*L. infantum* IgG enzyme-linked immunosorbent assay (ELISA) as described by Laurenti et al. (37). Given the predicted effects of vaccination on the immune response, dogs that had previous vaccination or immunotherapy against CanL were removed from the CMI profile evaluation. This study was conducted according to the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and approved by the Ethics Committee on the use of animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo, under protocol 2391/2011.

Parasitological diagnosis. Real-time quantitative PCR (qPCR) was performed using primers targeting a 120-bp kinetoplastid DNA (kDNA) sequence (38). For canine *Leishmania* infection diagnosis, popliteal or prescapular lymph node fine-needle aspiration biopsy specimens and buccal and conjunctival swabs were collected, maintained in NET buffer (0.15 M NaCl, 50 mM EDTA, 0.1 M Tris-HCl, pH 7.5), and stored at 4°C. DNA was extracted using a commercial kit (NucleoSpin Tissue; Macherey-Nagel, Germany) according to the manufacturer's instructions. Amplification was performed in a final volume of 15 μl that consisted of 5 μl of total DNA (10 ng/μl), 7.5 μl of Kapa SYBR Fast Universal 2× qPCR master mix (Kapa Biosystems, USA), 0.5 μl of each primer at a final concentration of 300 nM, and 1.5 μl of deionized water. PCR amplification was carried out in a thermocycler using the following conditions: 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing of primers at 58°C for 20 s, and extension at 72°C for 8 s. A standard curve was generated using serial dilutions of *L. infantum* DNA (MHOM/BR/72/strain46) from 10^6 to 10^{-4} parasites/μl. The amplifications were performed in duplicates. The parasite load was estimated only in lymph node samples by regressing threshold cycle (C_T) values onto standardized parasite concentrations in Realplex software (Eppendorf, Germany). The parasite load results were expressed as the number of parasites in the volume of lymph node aspirate which yielded 10 ng of DNA. Amplified samples whose dissociation curves overlapped the dissociation curve of the positive-control *L. infantum* DNA, with a peak around 83°C, were determined positive. Amplified samples whose melting curves overlapped the melting curves of the *Leishmania amazonensis* (MHOM/BR/73/M2269) and *Leishmania braziliensis* (MHOM/BR/1995/M15280) control groups were discarded (39, 40). The specificity of the primers employed in qPCR was assessed as described by Ferraz (40).

LST. An antigenic suspension was prepared from *L. infantum* promastigotes (MHOM/BR/72/strain46) at a concentration of 4×10^8 parasites/ml in merthiolate solution (1:10,000) (leishmanin) (23). A volume of 100 μl was intradermally injected in the abdominal region of each of 189 dogs. After 72 h, the area of erythema or nodules (induration area [IA]) was measured. Dogs that presented erythema or nodules with a diameter of ≥5 mm were determined positive for the leishmanin skin test (LST). As a negative control, merthiolate solution (1:10,000) was applied intradermally at an adjacent point at least 5 cm away from the leishmanin inoculation site.

LPA. The lymphocyte proliferation assay (LPA) (25) was performed with 126 of the 189 dogs that were tested in the LST. A volume of 10 ml of EDTA-blood was mixed with an equal volume of sterile phosphate-buffered saline (PBS) and gently added to 1/3 Histopaque 1077 (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by gradient centrifugation at 406 × *g* for 30 min at 20°C without a break. PBMCs were added to 30 ml of sterile PBS and centrifuged at 406 × *g* for 10 min at 4°C. After washing, PBMCs were agitated in 2 ml of erythrocyte lysing buffer for 45 s, followed by resting for 15 s on ice. After red blood cell lysis, PBMCs were resuspended and washed twice in 30 ml of sterile PBS. After washing, the pellet was resuspended in 2 ml RPMI 1640 medium supplemented with 2 mM L-glutamine (Gibco BRL, NY, USA), 20 mM HEPES, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 g/liter sodium bicarbonate, 10% fetal bovine serum (Gibco BRL, NY, USA), 0.05 mM 2-mercaptoethanol, 1× minimal essential medium (MEM) nonessential amino acids solution, 1× MEM vitamin solution, and 1 mM sodium pyruvate (Gibco BRL, NY, USA). PBMCs were counted and plated in a 96-well plate at a concentration of 1×10^6 cells/ml. PBMCs were cultured in supplemented RPMI medium alone (medium), in RPMI medium plus 2 μg/ml concanavalin A (ConA) for 72 h, and in RPMI medium plus 10 μg/ml crude antigen of *L. infantum* (MHOM/BR/72/strain46) (Leish) at 37°C and 5% CO_2 for 5 days. After incubation, plates were centrifuged; the supernatant was collected and stored at −80°C for subsequent cytokine evaluation. In order to measure cell proliferation, PBMCs were incubated for 18 h with 10 mM 5-bromodeoxyuridine (BrdU) (Cell Proliferation ELISA BrdU kit; Roche Diagnostics GmbH, Germany), and a colorimetric assay was performed according to the manufacturer's protocol. The absorbance was measured with 450-nm filters in

a spectrophotometer (Multiskan EX; Labsystems). The LPA outcome was determined by calculation of the proliferation index (PI), which represents the absorbance of the Leish group subtracted from the absorbance of the medium group and this difference multiplied by 100. The threshold was determined by the average PI for uninfected dogs (CTL) plus two standard deviations.

Cytokine quantification. To further characterize T cells which proliferated under specific antigen stimulation (Leish) in the profiles LST^-/LPA^-, LST^+/LPA^-, LST^-/LPA^+ and LST^+/LPA^+, a sandwich enzyme-linked immunosorbent assay (DuoSet ELISA development kit; R&D Systems, USA) was performed according to the manufacturer's instructions to quantify Th1 (IFN-γ and TNF-α) and Th2/Treg (IL-4, IL-10, and transforming growth factor β [TGF-β]) cytokines released in the supernatants of PBMC cultures. Supernatants of medium and ConA were used as negative and positive controls, respectively. Cytokine concentration values for unstimulated cultures (medium) were subtracted from those for stimulated ones (Leish). Thresholds were determined by the average of the cytokine concentration values for uninfected dogs (CTL) plus two standard deviations. The positive and negative dogs were then tested in GWAS for each cytokine.

Statistical analysis for phenotypic data. To assess whether the data distribution was Gaussian, the D'Augustine-Pearson normality test was employed. The nonparametric Kruskal-Wallis test was used to compare more than two groups with a non-Gaussian distribution. Parametric one-way analysis of variance (ANOVA) was employed to compare more than two groups with a Gaussian distribution. For comparison between two groups with a non-Gaussian distribution, the nonparametric Mann-Whitney test was used. The chi-square test was employed to assess association between groups. All tests were implemented in Prism v.5 (GraphPad Software Inc.).

Genotyping. DNA was isolated from whole blood samples of 189 dogs living in areas of endemicity by use of a commercial kit (NucleoSpin Tissue; Macherey-Nagel, Germany) according to the manufacturer's instructions and was genotyped for 173,662 single-nucleotide polymorphism (SNP) markers with the Illumina CanineHD BeadChip assay according to the manufacturer's protocol (Illumina Inc., San Diego, CA, USA).

GWA analysis. Prior to the genome-wide association (GWA) analysis, the following multivariate logistic model for binary traits was fitted using the glm procedure in R v3.2.3 (available at https://www.r-project.org/): $y \sim$ mean + origin + sex + age + repellent collar + anti-CanL vaccination + anti-CanL treatment. Then, significant covariates were used to correct associations between genotypes and phenotypes in a mixed linear model controlled for polygenic effects and sample structure (EMMAX) (35) in SNP & Variation Suite (SVS) v.8 software (Golden Helix, Inc., Bozeman, MT, USA). Genotypes were filtered in order to remove SNPs with a call rate below 95%, a minor allele frequency (MAF) below 5%, and a P value in an exact test for Hardy-Weinberg equilibrium (HWE) less than 10^{-5}. Samples with a call rate of less than 90% were also removed. Markers were prioritized for investigation based on a P value of $<1 \times 10^{-5}$ (36). Gene coordinates in the CanFam v3.1 assembly were obtained from Ensembl genes 84 using the BioMart tool (available at http://www.ensembl.org/biomart/martview/). Genes mapping to a maximum distance of 1 Mb from associated markers were considered positional candidates. Log odds ratios (ORs) for significant markers were estimated based on the slopes from a generalized linear model, assuming a binomial distribution and a logit link function in SVS. The inflation factor and variance explained by the markers were obtained in SVS.

RESULTS

Naturally infected dogs respond in four different ways to LST and LPA. Contact with the parasite was assumed to be a crucial condition for the triggering of specific CMI. Therefore, for this analysis, only nonvaccinated and untreated dogs that were positive in at least one of PCR, LST, or LPA ($n = 100$) tests were selected. In this set, LST and LPA were concordant in 51% of the dogs (30 LST^-/LPA^- and 21 LST^+/LPA^+) and discordant in 49% (9 LST^+/LPA^- and 40 LST^-/LPA^+). The frequency of dogs that had active CMI (LST^+ or LPA^+) was higher (70%) than the frequency of dogs that showed no CMI (LST^-/LPA^-) (30%). Among dogs with active CMI, the number of LPA-positive dogs (61) was twice the number of LST-positive dogs (30). These data showed that triggering of an effective LST seems to be a complex event that does not depend only on lymphocyte proliferation. Briefly, of the 100 infected dogs, 30 were LST^-/LPA^-, 9 were LST^+/LPA^-, 40 were LST^-/LPA^+, and 21 were LST^+/LPA^+. These groups were designated CMI profiles in this study. Seventeen dogs that tested negative for LST, LPA, and diagnosis by parasitology or anti-*L. infantum* IgG constituted the control group (CTL) (Fig. 1).

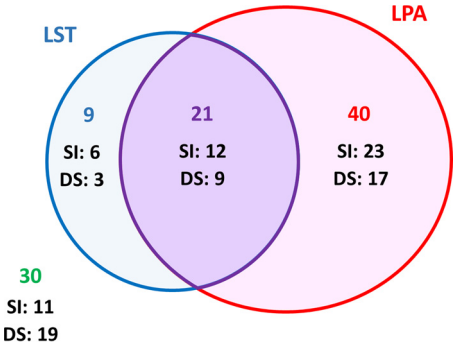

FIG 1 Cell-mediated immunity (CMI) profiles of dogs naturally infected by *L. infantum*. CMI profiles were based on LST and LPA outcomes. LST, number of positive dogs in leishmanin skin test; LPA, number of positive dogs in PBMC proliferation assay (Leish stimulated PBMCs); SI, number of subclinically infected dogs; DS, number of diseased dogs. No significant association between clinical response and CMI profiles was observed (chi-square test).

Lymphocyte proliferation is dependent on parasite load. In order to assess whether the nonresponsiveness of LST^-/LPA^- and LST^+/LPA^- dogs to the LPA was related to parasitism, we compared the parasite loads in lymph nodes (PL) and the levels of proliferation among CMI profiles and uninfected dogs (CTL). Surprisingly, the PL of the LPA^+ dogs (LST^-/LPA^+ and LST^+/LPA^+) ranged between 1.8 and 576 parasites and was significantly higher ($P = 0.0004$) than those of the LPA^- dogs (LST^-/LPA^- and LST^+/LPA^-), which ranged between 1.3 and 9 parasites in the volume of lymph node aspirate which yielded 10 ng of DNA (Fig. 2). When proliferation in the Leish group was compared to that in the medium group, a significant increase ($P < 0.001$) was observed only in groups with the highest PL (LST^-/LPA^+ and LST^+/LPA^+). In addition, comparison of Leish groups between LST^-/LPA^+ and LST^+/LPA^+ showed a higher proliferation in LST^+/LPA^+ dogs ($P < 0.05$), suggesting dominance of the proinflammatory response in LST^+ dogs. Dogs with low parasitism (LST^-/LPA^- and LST^+/LPA^-) and uninfected dogs (CTL) were nonresponsive to *L. infantum* antigen, although the PBMCs of all dogs had a strong response to the unspecific mitogen ConA ($P < 0.001$). This result suggests that *L. infantum* was not able to induce an effective T cell response under a low parasite load.

The cytokine response is predominantly Th1 in LST^+/LPA^+ dogs and mixed Th1/Treg in LST^-/LPA^+ dogs. To evaluate the predominant T cell subsets expanded in LPA, we quantified IFN-γ, TNF-α, IL-4, IL-10, and TGF-β levels in PBMC supernatants from the Leish group corrected by the values for medium.

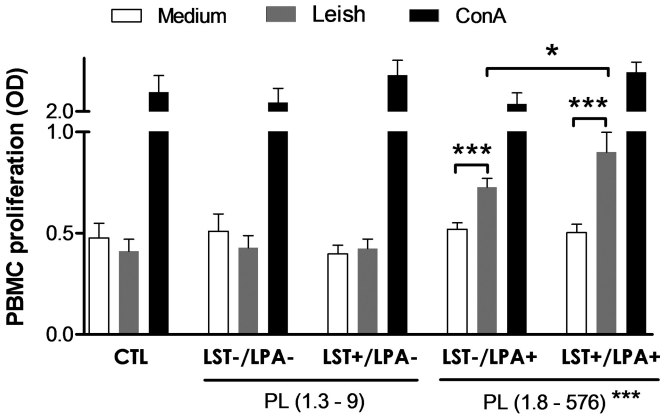

FIG 2 Comparison of ex *vivo* PBMC proliferation among cell-mediated immunity profiles. PBMCs were stimulated by *L. infantum* antigen (Leish) for 5 days or with concanavalin A (ConA) or medium alone (Medium) for 72 h. Proliferation was then measured by BrdU colorimetric assay. CTL, uninfected dogs; PL, parasite load (number of parasites in the volume of lymph node aspirate which yielded 10 ng of DNA); OD, optical density. *, $P < 0.05$; ***, $P < 0.001$ (ANOVA parametric test for proliferation and nonparametric Mann-Whitney test for PL).

The IFN-γ level was increased in LST^-/LPA^+ dogs ($P < 0.05$) and even higher in LST^+/LPA^+ dogs ($P < 0.001$) compared to control dogs. Similarly, there was an increase of IFN-γ levels in LST^+/LPA^+ dogs compared to LST^-/LPA^- dogs ($P < 0.001$) (Fig. 3A). TNF-α levels were higher in LST^+/LPA^+ dogs ($P < 0.001$) than in both LST^-/LPA^- and control dogs (Fig. 3B). IL-4 levels were significantly suppressed in LST^+/LPA^+ dogs ($P < 0.05$) compared to control dogs (Fig. 3C). Although also present in LST^+/LPA^+ dogs, IL-10 and TGF-β levels were significantly increased only in LST^-/LPA^+ dogs compared to control ($P < 0.05$) (Fig. 3D) and LST^-/LPA^- ($P < 0.05$) (Fig. 3E) dogs, respectively. Despite the diversity of cytokines in supernatants of LPA^+ dogs, increased IFN-γ and TNF-α and suppression of IL-4 point to triggering of a predominantly Th1 response in LST^+/LPA^+ dogs, whereas a mixed response of Th1/Treg was observed in LST^-/LPA^+ dogs.

LST^-/LPA^+ dogs are the most permissive to *L. infantum* replication. In order to evaluate the role of CMI profiles in control of *L. infantum* infection, we quantified the parasite load in fine-needle aspiration biopsy specimens of lymph node. Comparison among the four CMI profiles showed a significant increase of PL in LST^-/LPA^+ dogs compared to that in LST^-/LPA^- dogs ($P < 0.05$) (Fig. 4). Moreover, a positive correlation between IL-10 levels and the parasite load in the lymph nodes was also observed ($r = 0.416$, $P < 0.01$). These data indicated that participation of Treg subsets, as evidenced by increased IL-10 and TGF-β levels, allowed the increased replication of *L. infantum* in lymph nodes.

CMI profiles are clinically heterogeneous. In order to investigate the role of cell-mediated immunity (CMI) in determining the clinical response to *L. infantum* infection, we evaluated the frequencies of diseased and subclinically infected cases for the four CMI profiles. Of the 30 LST^-/LPA^- dogs, 11 were subclinically infected and 19 diseased. In profile LST^+/LPA^-, 6 dogs were subclinically infected and 3 were diseased, and in profile LST^-/LPA^+, 23 dogs were subclinically infected and 17 were diseased. Lastly, in profile LST^+/LPA^+, 12 dogs were subclinically infected and 9 were diseased. Statistical analysis showed no association between clinical and CMI profiles ($P = 0.23$). Therefore, none of CMI profiles

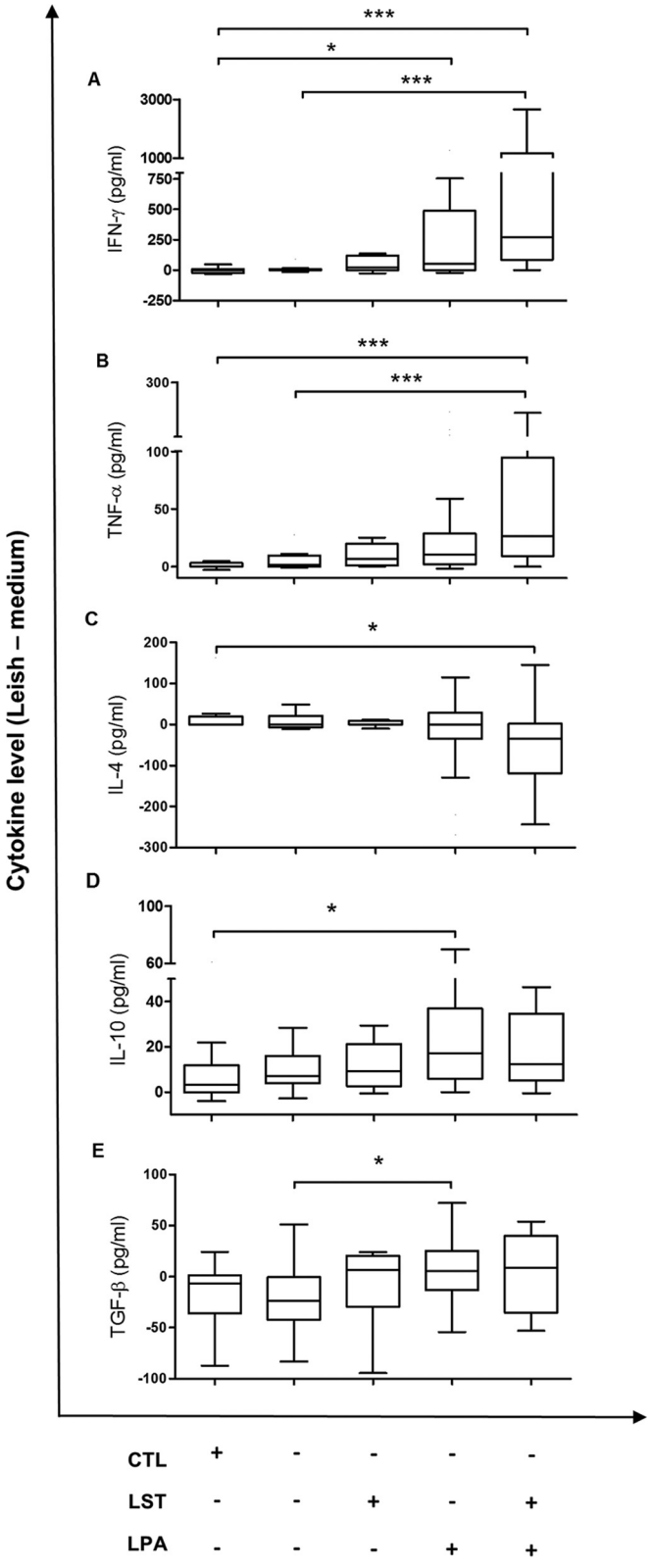

FIG 3 Comparison of concentrations of cytokines released in supernatants of Leish stimulated PBMCs among cell-mediated immunity profiles. Box plots show medians and interquartile ranges for IFN-γ (A), TNF-α (B), IL-4 (C), IL-10 (D), and TGF-β (E) amounts in the Leish group corrected by values for the medium group. LST, leishmanin skin test; LPA, lymphocyte proliferation assay; CTL, control dogs. *, $P < 0.05$; ***, $P < 0.001$ (Kruskal-Wallis test).

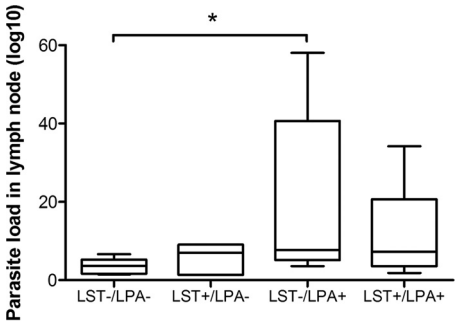

FIG 4 Increased parasite load in lymph nodes from LST$^-$/LPA$^+$ dogs. The parasite load (number of parasites in the volume of lymph node aspirate which yielded 10 ng of DNA) was measured by quantitative real-time PCR. *, $P < 0.05$ (nonparametric Kruskal-Wallis test).

was able to ensure protection against clinical manifestation of CanL. The distribution of clinical outcomes in the immunity profiles is shown in Fig. 1.

GWA for LST response. Analysis was conducted with 110,165 scanned markers and 185 dogs (36 LST$^+$ and 149 LST$^-$) which passed the filtering criteria, regardless of the outcome in qPCR. The mixed linear model analysis was corrected for vaccination. The inflation factor was approximately 1.0, suggesting that confounding due to population structure was duly controlled in the analysis. The phenotypic variance explained by markers was estimated at 15% (Table 1). A single marker on chromosome 13 was significant (BICF2P1154155, $P = 9.46 \times 10^{-6}$) (see Fig. S1 in the supplemental material). This marker mapped to position 38698768, in the vicinity of positional candidates *COMMD5* and *SHARPIN*, both implicated in regulation of NF-κB signaling. The B allele (A/B Illumina code) presented a frequency of 22.2% in LST$^+$ dogs and 3.6% in LST$^-$ dogs, with an estimated odds ratio and range of 7.45 ± 6.68 (Table 2), and was associated with higher proportion of LST$^+$ dogs and increased TNF-α in LPA (see Fig. S2 in the supplemental material). These findings are an indication of a likely association of allele B with the induction of NF-κB.

GWA for LPA response. We scanned 110,165 markers and 102 dogs (65 LPA$^+$ and 37 LPA$^-$), regardless of qPCR outcome (Table 1). One significant SNP was identified ($P < 10^{-5}$) (see Fig. S3 in the supplemental material): marker BICF2P826452 ($P = 7.37 \times 10^{-6}$) located on chromosome 7 at position 47582500. However, the phenotypic variance explained by markers was 0%. This result indicated no genetic control underlying LPA response or low statistical power.

GWA for cytokine response. (i) TNF-α. The GWA analysis for TNF-α showed the highest estimated proportion of phenotypic variance explained by markers (87%) in the present study. One hundred twenty-seven dogs (24 TNF-α$^+$ and 103 TNF-α$^-$) and 110,165 markers were scanned. An inflation factor of 1.36 indicated a possible sample structure effect on associations (Table 1). Twenty-seven markers were declared significant, including the top-scoring SNP BICF2S22949424 ($P = 8.25 \times 10^{-9}$) located on chromosome 20 at position 45037155 (see Fig. S4 in the supplemental material). Positional candidate genes included *JAK3*, *IL-12RB1*, *IFI30*, *CCRL2*, *CCR2*, *CCR3*, *XCR1*, and *CXCR6*. Other positional candidate genes in the vicinity of less significant markers included *ITGA4*, *FCRL1*, *FCRL4*, *CD5L*, and *TP53BP2*. Allele B (A/B Illumina code) of the top SNP was present in 64% of TNF-α$^+$ dogs and 20% of TNF-α$^-$ dogs (odds ratio of 7.33 ± 5.41) (Table 2). Coherently, allele B was associated with increased TNF-α levels in supernatants of Leish stimulated PBMCs (see Fig. S5 in the supplemental material).

(ii) IFN-γ. One hundred twenty-seven dogs (30 IFN-γ$^+$ and 97 IFN-γ$^-$) and 110,165 markers were scanned (Table 1). Two markers were significant: BICF2G630445990 ($P = 3.84 \times 10^{-6}$) on chromosome 20 at position 54536589 and BICF2P417230 ($P = 6.12 \times 10^{-6}$) on chromosome 28 at position 29131923 (see Fig. S6 in the supplemental material). Nevertheless, the phenotypic variance explained by the markers was 0%, which suggested no genetic control underlying the IFN-γ response of PBMCs in LPA or low statistical power to detect significance.

(iii) IL-10. For IL-10, we scanned 127 dogs (20 IL-10$^+$ and 107 IL-10$^-$) and 110,165 markers (Table 1). Three markers were declared significant ($P < 10^{-5}$), all located in the same region of chromosome 17 (see Fig. S7 in the supplemental material). The top-scoring SNP BICF2G630213974 ($P = 1{,}04 \times 10^{-6}$) was located 255 kb apart (chromosome 17, position 26778592) from *LTBP1*, which is involved in the activation of TGF-β. In the same region we found *RASGRP3*, which is implicated in downregulation of proinflammatory cytokines. Allele A (A/B Illumina code) of the top SNP was associated with increased release of IL-10 by Leish stimulated PBMCs (see Fig. S8 in the supplemental material), with an estimated odds ratio of 5.27 ± 4.42 and frequencies of 40% in IL-10$^+$ dogs and 11% in IL-10$^-$ dogs (Table 2). This finding points to the likely induction of TGF-β activation and its effect on Treg differentiation, which would favor the release of IL-10.

(iv) TGF-β. Analysis for TGF-β was conducted with 110,165 markers and 127 dogs (18 TGF-β$^+$ and 109 TGF-β$^-$) (Table 1). The most significant markers were BICF2G630236868 ($P = 6.31 \times 10^{-8}$) on chromosome 5 at position 86823011, BICF2G630326124 ($P = 1.54 \times 10^{-7}$) on chromosome 22 at position 25981879, and BICF2S23124311 ($P = 2.49 \times 10^{-7}$) on chromosome 2 at position 63049391 (see Fig. S9 in the supplemental material). Neighboring candidate genes included *CCR8*, *CX3CR1*, *TNFSF4*, *TNFSF18*, *SELE*, *SELL*, and *SELP*. The results indicated that a baseline TGF-β response is ensured by many genetic variants. Allele B (A/B Illumina code) of the top SNP was associated with increased production of TGF-β by Leish stimulated PBMCs (see Fig. S10 in the supplemental material), with an estimated odds ratio of 8.91 ± 8.90. The allele B frequencies in TGF-β$^+$ and TGF-β$^-$ dogs were 36% and 6%, respectively (Table 2).

TABLE 1 Inflation factor, variance explained by the markers, covariates used in the mixed linear model, and number of significant markers for each phenotype

Phenotype	Inflation factor	Explained variance (%)	Covariate(s)	No. of significant markers
LST	1.01	15	Vaccine	1
LPA	1.11	0	Origin	1
IFN-γ	1.10	0	Origin, age	2
TNF-α	1.36	87	Origin, repellent collar	27
IL-10	1.08	11	Origin	3
TGF-β	1.07	16	Origin, sex	22

TABLE 2 Position, frequency, estimated effect, and significance of the top SNPs and positional candidates potentially associated with phenotypes

Phenotype	Top SNP	Chromosome:position	Frequency	Odds ratio ± range (*P*)	Genes
LST	BICF2P1154155	13:38698768	22.2% LST^+, 3.6% LST^-	7.45 ± 6.68 (9.46×10^{-6})	*COMMD5*, *SHARPIN*
TNF-α	BICF2S22949424	20:45037155	64% TNF-α^+, 20% TNF-α^-	7.33 ± 5.41 (8.25×10^{-9})	*JAK3*, *IL12RB1*, *IFI30*, *CCRL2*, *CCR2*, *CCR3*, *XCR1*, *CXCR6*
IL-10	BICF2G630213974	17:26778592	40% IL-10^+, 11% IL-10^-	5.27 ± 4.42 (1.04×10^{-6})	*LTBP1*, *RASGRP3*
TGF-β	BICF2G630236868	5:86823011	36% TGF-β^+, 6% TGF-β^-	8.91 ± 8.90 (6.31×10^{-8})	*CCR8*, *CX3CR1*, *SELE*, *SELL*, *SELP*, *FASLG*, *AKTIP*, *RAB39A*, *ATM*, *CAMK1G*

DISCUSSION

Upon solving bottlenecks of stratifications and confounding factors on the phenotypes (35), we finally employed a tool capable of scanning thousands of markers to map loci explaining variance in the immune response against *L. infantum*. Thus, we further investigated the complex role of the cell-mediated response to CanL. This effort began with the combination of *in vivo* and *ex vivo* approaches. Afterward, data were explored as phenotypic evidence of the functional relevance of the identified loci to the outcome of *L. infantum* infection.

Thirty percent of the infected dogs were LST positive in the present study. This is a positivity slightly higher than that observed in mongrel dogs from other areas of endemicity in Brazil, which was approximately 22% (22, 23). Our findings suggest genetic advantages in LST-positive dogs which favor an inflammatory response. The *COMMD5* gene encodes the protein for copper metabolism Murr1 domain-containing 5, which belongs to a conserved group of factors that have been related to the binding of nuclear factor κB (NF-κB) to a multimeric ubiquitin ligase, thereby promoting their ubiquitination and degradation (41). NF-κB is a pleiotropic transcription factor involved in multiple processes, including transcription of proinflammatory cytokines such as TNF-α, IL-1, and IL-6 and cell survival and proliferation (41, 42). Increased TNF-α levels in carriers of allele B of SNP BICF2P1154155 suggested association of this marker with the suppression of *COMMD5*. This result seems to have been reflected in the cytokine response of CMI profiles. In fact, LST^+/LPA^+ dogs were clearly prone to a Th1 response, whereas LST^-/LPA^+ dogs tended to a Treg response. *COMMD5* also may be involved with the highest proliferative response observed in LST^+/LPA^+ dogs. Increased TNF-α levels in LST^+/LPA^+ dogs but not in those with other CMI profiles corroborates the hypothesis of the involvement of NF-κB signaling in the LST response. *SHARPIN* also appeared as a potential candidate affecting the LST response. This gene encodes a SHANK-associated RH domain-interacting protein, which is a regulatory subunit crucial to the catalytic activity of the complex LUBAC (linear ubiquitin assembly complex), which plays a critical role in the activation of NF-κB (43). A spontaneous mutation in the gene *SHARPIN* was shown to lead to multiorgan inflammation, including dermatitis in mice (44). All four CMI profiles failed to protect dogs against the manifestation of clinical signs of CanL, even in LST-positive dogs exhibiting a predominantly Th1 response. This clinical heterogeneity observed in CMI profiles is compatible with the existence of a genetic component involved in exacerbation of inflammation and development of CanL.

Previous studies have shown that *L. infantum* downregulates expression of mediators from innate and adaptive immunity in the early stage of infection in phagocytes (6, 10, 16). These findings are consistent with the idea that an early suppression would prevent T cell activation and would allow a silent establishment of infection. These assumptions are in accordance with the unresponsiveness to LPA observed in LST^-/LPA^- and LST^+/LPA^- dogs, which exhibited low parasite burdens. Other authors (45) had already demonstrated low T cell proliferation in the early stage of canine *L. infantum* infection. Furthermore, the fact that nine dogs were positive to the LST even under low parasitic conditions (LST^+/LPA^-) suggests that tissue factors absent in *ex vivo* models, e.g., competent Langerhans dendritic cells able to activate T cells or subsets of dermal Tγδ cells, would provide requirements for a local innate immunity that interconnects with the adaptive immunity. In this case, an enhanced induction of NF-κB would increase maturation of APC able to instruct differentiation of Th1 subsets and consequently to promote the delayed hypersensitivity skin reaction.

GWAS for LPA presented no evidence of genetic control of lymphocyte proliferation in the present study. Therefore, the LPA response appears to depend more on the interaction between the immune system of the host and *L. infantum* than on the host genetic background itself. Likewise, GWAS for the IFN-γ response in LPA resulted in nonsignificant phenotypic variance explained by markers. Further investigation is required to confirm these data or detect epigenetic variations affecting IFN-γ and LPA responses.

The highest proportion of phenotypic variance was attributed to the markers associated with TNF-α. The top SNPs associated with this cytokine map to a position close to the *IL12RB1* gene, which encodes the beta 1 receptor of IL-12 and IL-23. *IL12RB1* signaling triggers the IFN-γ response in natural killer (NK) cells and differentiation of Th1 lymphocytes, which release proinflammatory cytokines such as TNF-α, as well as inducing TNF-α in macrophages (46). The *JAK3* gene also maps near the top SNP. *JAK3* encodes Janus kinase 3, which is a tyrosine kinase involved in cytokine receptor-mediated intracellular signal transduction, including IL-2R, IL-4R, IL-15R, and IL-21R, which are involved in leukocyte activation (47). Other genes relevant for immunity are in the vicinity of significant markers associated with TNF-α, such as the chemokine receptor *CCRL2*, *CCR2*, *CCR3*, and *CXCR6* genes. These receptors may activate monocytes and neutrophils attracted to the inflammation site upon binding of their respective chemokines. Monocytes, in turn, produce TNF-α upon activation and differentiation into macrophages (48).

Regarding regulatory cytokines, especially IL-10, the genome-wide scan identified critically relevant regions for the Treg response. *LTBP1* is localized in the vicinity of the top SNP signifi-

cantly associated with the IL-10 response. *LTBP1* encodes the latent TGF-β binding protein 1 (LTBP1). This protein regulates assembly, secretion, and targeting of TGF-β1 to extracellular matrix, where it is stored or activated (49). An increase of biologically active TGF-β was shown in *L. infantum* infection in human macrophages without altering total TGF-β (50). These findings suggest that the rise of TGF-β availability may favor differentiation of regulatory T cells secreting IL-10. Another gene in the same region, *RASGRP3*, encodes Ras guanine nucleotide-releasing protein 3 (RasGRP3). RasGRP3 is a small GTPase which is a critical molecular GDP/GTP exchanger coupled to multiple receptors on the surface for transduction of extracellular signals in intracellular signaling pathways. This molecule is responsible for the activation of a Ras inductive signal and/or a Rap inhibitory signal. In a recent study, RasGRP3 was shown to play a regulatory role that limits production of proinflammatory cytokines, especially IL-6, induced by Toll-like receptors in human and murine macrophages via Rap1 activation (51). A possible connection between RasGRP3 and increased IL-10 levels in LST^{-}/LPA^{+} dogs may be due to the inhibition of IL-6. Deficient IL-6 would lead to the predominance of TGF-β and Treg instead of Th17 differentiation (52). A protective role of the Th17 response by cooperation with the Th1 response has been described in VL in mice, dogs, and humans, although it is also associated with exacerbated inflammation (53–55). Here, LST^{-}/LPA^{+} dogs showed the highest IL-10 and TGF-β levels compared to the other CMI profiles. Moreover, these dogs were the most permissive to replication of parasites, likely as an aftermath of the suppressive effect of IL-10 and TGF-β on phagocytes (56). Similarly, the increase of parasite load in lymph node, skin, spleen, and intestine associated with the Treg response has been demonstrated in dogs infected by *L. infantum* (17, 18, 57, 58). These results emphasize the important role of Treg in parasite spreading and point to a pivotal chromosomal region for susceptibility to *L. infantum*.

CMI evaluation showed TGF-β production by almost all CMI profiles, mainly LST^{-}/LPA^{+}. This same profile showed a slight increase in parasite load, suggesting an immunosuppressive effect of TGF-β on phagocytes (59). Furthermore, GWAS demonstrated several significant markers and relevant genes for the TGF-β response, including chemokine receptor genes *CCR8* and *CX3CR1*, selectin genes *SELE*, *SELL*, and *SELP*, FAS ligand gene *FASLG*, and signaling genes *AKTIP*, *RAB39A*, *ATM*, and *CAMK1G*. This large number of markers associated with TGF-β indicates the crucial role of this cytokine for homeostasis (56) if we take into account that genetic redundancy may guarantee the expression of TGF-β.

In summary, analysis of the data set presented here allows us to suggest that the canine genetic background related to cell-mediated immunity is involved in the complexity of the clinical response to *L. infantum* infection as well as in the control of parasite spreading. Thus, this study has identified chromosomal regions relevant for the immune functions and provided potential targets for therapeutics of canine leishmaniasis.

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