Immunologic indicators of clinical progression during canine *Leishmania infantum* infection

Running title: Temporal immunologic features of *L. infantum* infection

Paola M. Boggiatto\(^1,2\), Amanda E. Ramer-Tait\(^3\), Kyle Metz\(^2,5\), Erin E. Kramer\(^2,6\), Katherine Gibson-Corley\(^5\), Kathleen Mullin\(^4\), Jesse M. Hostetter\(^2\), Jack M. Gallup\(^7\), Douglas E. Jones\(^1,2\), Christine A. Petersen\(^*\)\(^1,2\)

\(^{1}\)Immunobiology Program, \(^{2}\)Department of Veterinary Pathology, \(^{3}\)Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, \(^{4}\)Laboratory Animal Resources, Iowa State University, Ames, Iowa 50011

\(^{5}\)Molecular Microbiology and Immunology Program, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205.

\(^{6}\)Charles City Animal Clinic, Charles City, Iowa 50616.

\(^{7}\)Corresponding author: Christine A. Petersen, 1600 University Blvd, Iowa State University College of Veterinary Medicine, Ames, Iowa 50011. Tel: 515-294-9013. Fax: 515-294-5423. kalicat@iastate.edu
Abstract

In both dogs and humans Leishmania infantum infection is more prevalent than disease, as infection often does not equate with clinical disease. Previous studies additively indicate that advanced clinical visceral leishmaniasis (VL) is characterized by increased production of anti-Leishmania antibodies, Leishmania-specific lymphoproliferative unresponsiveness, and decreased production of IFN-γ with concomitant increase of IL-10. In order to differentiate infection vs. progressive disease for better disease prognostication, we temporally evaluated humoral and cellular immunologic parameters of naturally infected dogs. The work presented here describes for the first time the temporal immune response to natural autochthonous L. infantum infection in Foxhounds within the United States. Several key changes in immunological parameters should be considered to differentiate infection versus clinical disease, including a dramatic rise in IgG production, progressive increases in antigen-specific PBMC proliferation, and IFN-γ production. Polysymptomatic disease is precluded by increased IL-10 production and consistent detection of parasite kinetoplast DNA in whole blood. This clinical presentation and immuno-dysregulation mirrors that observed in human patients indicating that this animal model will be very useful for testing immunomodulatory anti-IL-10 or other therapies.
Introduction

Leishmaniasis is a group of vector-borne diseases caused by intracellular protozoan parasites of the genus Leishmania. Disease manifestation can range from localized, self-healing cutaneous ulcers to disseminated disease, referred to as visceral leishmaniasis (VL). VL is fatal if left untreated. It is primarily caused by Leishmania (L.) donovani in Africa and India, and by L. infantum/chagasi in the Mediterranean basin, Asia and Central and South America.

VL, as caused by L. infantum infection, is zoonotic (4). Both dogs and humans are natural hosts (27), and in endemic regions, infected dogs are the primary domestic reservoir for zoonotic VL and the most significant risk factor predisposing humans to infection (9). L. infantum infection often does not equate with clinical disease (18).

Typical clinical signs of VL include fever, weight loss, anemia, lymphadenopathy, and hepato- and splenomegaly (4, 22, 27). Clinical stages of infection can be classified by the severity of clinical signs, humoral and cell-mediated responses, and parasite load (33). We propose that these parameters can also be used to determine the best window for treatment and in some cases predict the appearance of clinical signs and prognosis (24).

Host protection against Leishmania infection requires a proinflammatory, T\textsubscript{H}1 immune response, as characterized by the production of interleukin (IL)-12 by antigen presenting cells and interferon (IFN)-γ by T cells (reviewed in (22)). Advanced clinical VL in human patients is characterized by Leishmania-specific lymphoproliferative unresponsiveness and decreased production of IFN-γ following in vitro Leishmania
antigen restimulation (11, 31). Active disease is associated with elevated IL-10 levels in
serum and enhanced IL-10 mRNA in lesional tissues (reviewed in (22)). Cured or
subclinical individuals are able to mount antigen-specific IFN-γ responses following
Leishmania antigen restimulation in vitro. Cured patients are also resistant to reinfection
and are leishmanin skin test positive, suggesting no inherent defect in the antigen-
dependent T_H1 response (3, 7, 34).

Canine visceral leishmaniasis (CVL) in endemic areas mimics both the
immunologic alterations and pathophysiology of human disease. Autochthonous L.
infantum infection in the United States Foxhound population has been recently
described (5, 8). Despite a potentially different means of transmission, i.e. non-vector
borne (12, 24, 29), symptomatic disease and pathologic findings in naturally infected
Foxhounds parallels that observed in both canines and humans endemic regions (12).
For these studies, we hypothesized that the immunopathology of primarily non-vector
mediated L. infantum CVL would reflect the changes observed in humans, including
increased anti-Leishmania antibodies in sera, and decreased lymphoproliferative IFN-γ-
mediated responses with increased IL-10 production. Here we follow a cohort of U.S.
born, naturally-infected canines to determine their immunopathology and clinical
presentation(s) of autochthonous L. infantum infection. Analysis of clinical signs,
serology, and kinetoplast-specific qPCR categorized these animals into four different
groups: 1) non-infected, 2) infected-resistant, 3) infected-susceptible and 4) clinical, as
previously described in (33). Animals in the 4th clinical state had increased production
of IgG1 and IgG2, decreased lymphoproliferative responses and IFN-γ production, and
increased IL-10 production. The appearance of any of these immunological parameters correlated with disease progression.

The work presented here describes for the first time the temporal immune response to natural autochthonous *L. infantum* canine infection in United States. We show that even in the likely absence of vector-mediated transmission (32) clinical presentation and immuno-dysregulation mirrors that observed in endemically-infected dogs and humans (1, 22). The ongoing antigen-specific immune response to *L. infantum* infection wanes as disease progresses and production of anti-*Leishmania* antibodies and IL-10 are key immunologic features of disease manifestation and progression.
Materials and Methods

Description of Animals

Although VL is not endemic in the United States, canine visceral Leishmaniasis (CVL) has recently been described as an epidemic within the Foxhound population in this country. The first report of Foxhound CVL epidemic in the U.S. was in 1999 in a foxhound kennel in New York (8). By 2005, it was reported that 60 kennels in 22 states and two Canadian provinces had *L. infantum*-seropositive Foxhounds, and that autochthonous infection in canines was for the most part limited to Foxhounds (5).

Dogs used in this study, all Foxhounds, ranged in age from six months to seven years of age. These animals or their tissues were donated to Iowa State University College of Veterinary Medicine by two different Midwestern Foxhound kennels. Nine of the dogs were donated based on positive serological indirect immunofluorescence assay test (IFAT) results (>1:64) and presentation of clinical signs. The remaining four dogs were born to an IFAT positive (> 1:256) female. All animals were housed at Iowa State University Veterinary College and the Institutional Animal Care and Use Committee at Iowa State approved all protocols involving animals. Prior to arrival, all dogs were vaccinated for core canine diseases. Once under the care of laboratory animal resources (LAR) at Iowa State University, blood samples were obtained for complete blood count (CBC) and chemistry, and stool samples were collected for enteric parasite assessment. All animals were treated for ecto- and intestinal parasites (*Giardia*, roundworms, and *Coccidia*) via treatment with Strongid (5mg/kg), Baytril (1/4...
tablet), Albon (55mg/kg), Panacur (2ml/kg), Clavamox 13.75mg/kg, and Cephalexin
(25mg/kg).

Clinical Evaluation

Upon arrival to Iowa State University College of Veterinary Medicine, all animals
were clinically assessed via physical examination, complete blood count, chemistry
panel analysis, *L. infantum* kDNA specific qPCR and IFAT serologic analysis. Based on
these parameters, animals were classified into four distinct categories: non-infected,
showing no clinical signs of disease and qPCR and IFAT negative; infected-resistant,
showing none to mild clinical signs and IFAT and qPCR positive/negative; infected-
susceptible, showing mild to moderate clinical signs and qPCR and IFAT positive; and
clinical, showing severe, disseminated disease, and IFAT and qPCR positive.

Parasites

*Leishmania infantum* (LIVT-2) (30) was grown to stationary phase in complete
Grace’s medium (Incomplete Grace’s supplemented with 20% fetal bovine serum,
100U/ml penicillin, 100ug/ml streptomycin and 2mM L-glutamine). Freeze-thawed whole
antigen was prepared as described previously (13).

Peripheral blood mononuclear cell (PBMC) Isolation and Carboxyfluorescein succinyl
ester (CFSE) Staining
All animals were allowed to acclimate for one week prior to immunological studies. PBMC were isolated from heparinized blood samples using Ficoll-Histopaque 1077 (Sigma, St. Louis, MO) gradient centrifugation. Red blood cells were removed using ACK lysis buffer (0.15M NH₄Cl, 1.0mM KHCO₃, 0.1mM Na₂EDTA, pH 7.4). PBMC were labeled with CFSE (Molecular Probes, Eugene, OR) as described previously (14). PBMC were washed twice in phosphate-buffered saline (PBS) and resuspended in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100ug/ml streptomycin, 2mM L-glutamine, and 25mM HEPES buffer). PMBC were counted and adjusted to 4x10⁶/ml for further analysis.

**PBMC Proliferation Assay**

CFSE-labeled PBMC (4x10⁵/well) were plated on 96-well plates and incubated with media alone, stimulated with concavanalin A (ConA) (5ug/ml) for 4 days or with freeze-thawed, whole *L. infantum* antigen (10ug/ml) for 7 days, or Distemper vaccine (Vanguard Plus 5, Pfizer) control for 10 days, at 37°C with 5% CO₂. Cells were harvested, washed in FACS buffer (0.1% albumin, 0.1% sodium azide in PBS) and labeled with PE-conjugated anti-canine CD4 antibody (Serotec, Raleigh, NC). Cells were fixed in 1% paraformaldehyde and analyzed using the FACSCanto flow cytometer (BD Pharmingen, San Diego, CA). Data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

**IFN-gamma and IL-10 ELISA**
Unlabeled PMBC (4x10⁵) were plated and incubated as described above. Supernatants were collected at the indicated time points and stored at -20°C until analysis. IFN-γ and IL-10 production were measured using R&D ELISA kits (Minneapolis, MN), according to manufacturer’s recommendations.

Serology and Real Time qPCR

Serum samples were collected from all animals, stored at -20°C and sent to the Centers for Disease Control and Prevention for IFAT testing for antibodies to *Leishmania* spp. as previously described (5). DNA from whole blood samples collected in heparinized tubes (BD Pharmingen, San Diego, CA) was isolated using the Qiagen blood DNA isolation kit according to manufacturer’s instructions. DNA quality and quantity was measured using a NanoDrop spectrophotometer ND1000 (Wilmington, DE). *L. infantum* kinetoplast DNA (kDNA)-specific primers and probe F 5’-CCGCCCGCCTCAAGAC, R 5’-TGCTGAATATTGGTGGTTTTGG, (Integrated DNA Technologies, Coralville, IA), Probe 5’-6FAM-AGCCGCGAGGACC-MGBNFQ (Applied Biosystems, Foster City, CA) were used. (FAM: laser-activated reporter dye; MGBNFQ: 3'-minor-groove binder non-fluorescent quencher). Blood DNA samples were assayed via qPCR in duplicate of three dilutions (straight, 1:10, 1:20) using a Stratagene Mx3005P® qPCR System via a 96-well format and Platinum qPCR SuperMix-UDG Master Mix (Invitrogen, Carlsbad, CA). Primers were used at 775 nM and probe at 150 nM with thermocycling at 50°C for 2 minutes, 95°C for 2 minutes, and 50 cycles of [95°C
Results were analyzed via MxPro™ QPCR software version 4.01 in conjunction with Microsoft Excel.

**L. infantum-specific IgG ELISA**

High-affinity plates were coated overnight at 4°C with 10μg/well of freeze-thawed *L. infantum* antigen in 50mM carbonate-bicarbonate buffer. Plates were blocked with 200μl of blocking buffer for 1 hour at room temperature and washed. Serum samples (100μl) were diluted 1:100 and incubated for 2 hours at room temperature. Plates were developed with HRP-conjugated anti-canine IgG1 or IgG2 (1:20,000) (Bethyl Laboratories, Montgomery, TX) for 1 hour and Absorbance was read at 405nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

**Statistical analysis**

Statistical significances were analyzed using Prism4 (GraphPad Software Inc., La Jolla, CA). Differences between groups were determined using Mann-Whitney U-test. *P*-values below 0.05 were considered significantly different.
Results

Clinical evaluation

Clinical assessment included a complete blood (cell) count (CBC) and chemistry panel (Table 1, fourth column). Following euthanasia, necropsy was performed by a veterinary pathologist and a complete set of tissues was collected for each animal and evaluated histologically (Table 1, last two columns). Lymphocytosis (elevated lymphocyte numbers in the blood) was consistently present in all dogs tested. Persistent lymphocytosis is indicative of chronic antigen stimulation, which we would attribute to the presence of *Leishmania* parasites in infected animals. However, since non-infected dogs also show lymphocytosis, we cannot rule out the possibility of increased circulating lymphocyte number due to other infections including gastrointestinal or ecto-parasitism, which has been observed in these Foxhounds previously (data not shown). In all infected animals we observed a moderate to marked hyperglobulinemia. Serum chemistry and histopathologic findings in the infected-susceptible dogs indicated the onset of systemic disease consistent with visceral leishmaniasis, including elevated blood urea nitrogen (BUN), creatinine and phosphorous, anemia, lymphoplasmacytic portal hepatitis, histiocytic splenitis, and membranous glomerulonephritis. CBC and serum chemistry evaluation of clinical dogs indicated these animals had signs of nonregenerative anemia, renal compromise (elevated BUN, creatine and phosphorus), and hepatic injury (elevated alanine transferase). Despite their clinical state, lymphocytopenia was not observed in clinical dogs. Histopathologic examination...
confirmed these findings and also showed systemic histiocytic inflammation with a myriad of intracellular *Leishmania* amastigotes.

**Serology and qPCR**

To confirm infection and disease status, each dog was evaluated for *L. infantum* serostatus and qPCR for *L. infantum* kinetoplast (k) DNA. Serum samples from all Foxhounds in the study were sent to the Centers for Disease Control and Prevention (CDC) for IFAT analysis of antibodies against *Leishmania* spp (Table 1). All dogs in the control (non-infected, non-Foxhound), non-infected group and two dogs from the infected-resistant group were seronegative (≤1:16). The two remaining dogs within the infected-resistant group had seropositive titers (1:64). Dogs within the infected-susceptible group (3 dogs) had titers of 1:256, and dogs within the clinical group (4 dogs) had strong sero-reactivity to *Leishmania* antigen (1:512).

*L. infantum*-specific kDNA amplification was observed in all clinical and infected-susceptible dogs, and in two of the infected-resistant group. As expected, no amplification was observed in the non-infected Foxhounds (Table 1) and in the control, non-Foxhound dogs. These data indicate that increased parasitemia is found during later stages of infection.

*L. infantum*-specific IgG1 and IgG2 production

Chemistry findings in serum samples from clinical dogs indicated these animals had pan-elevation of immunoglobulins (Ig): IgA >500mg/dl, IgG >5000mg/dl, IgM
respectively. Hypergammaglobulinemia has been associated with CVL disease progression pathophysiology (12) and suppression of the immune response to *L. infantum* (26). However, a relationship between immunoglobulin (Ig) G isotype profile and disease resistance versus susceptibility remains to be established. Conflicting reports fail to provide a clear role for IgG1 or IgG2 production in disease development (25, 28). Based on our findings of detectable circulating parasites as disease progressed, we wanted to determine if this observation correlated with detection of specific antibody levels. Using whole parasite antigen we found that sera from control and non-infected groups contained minimal IgG1 and IgG2 antibodies when measured by ELISA, as OD values observed were similar to background readings (OD ~0.01). Highest levels of anti-*L. infantum* IgG1 (Figure 1A) and IgG2 (Figure 1B) were produced by the infected susceptible and clinical groups. Overall, IgG levels increase as disease progresses, however, we did not observe a direct correlation between either IgG isotype and clinical status. Other, non-antibody, effector functions may therefore be more predictive of disease progression.

**L. infantum-specific PBMC proliferative response**

A key immunologic feature of late clinical VL is the inability of PBMC to generate a protective, *L. infantum*-specific immune response (31). This is characterized by the loss of the antigen-specific lymphoproliferative response and the loss of IFN-γ production. To identify if this lack of antigen-responsiveness as disease progresses...
occurs in our canine cohort, we analyzed the antigen-specific proliferative response of PBMC CD4$^+$ T cells from all four groups. Blood samples were collected every four weeks during a period of at least three months for each dog. PBMC were isolated from whole blood samples, stained with CFSE, and stimulated with concavanalin A (ConA), L. infantum antigen, distemper vaccine or left untreated. PMBC were then analyzed for CD4$^+$ T cell proliferation via flow cytometry. CD4$^+$ T cells from all dogs proliferated in response to stimulation with ConA, indicating that the CD4$^+$ T cell compartment was not mitogenically deficient (Figure 2B). In response to distemper vaccine stimulation all groups except for the clinical dogs had a proliferative response indicating that although mitogenically competent, clinical dogs were not capable of initiating antigen-specific proliferative responses (Figure 2A, B). In response to L. infantum-antigen stimulation, control (uninfected, non-Foxhound) and non-infected dogs showed a minimal level of proliferation in response to antigen restimulation (Figure 2). While a significantly greater percentage of CD4$^+$ T cells from infected-resistant dogs proliferated in response to antigen restimulation as compared to non-infected dogs, infected-susceptible dogs demonstrated the greatest percentage of proliferative CD4$^+$ T cells, significantly higher than infected-resistant animals (Figure 2A). In contrast, the antigen-specific CD4$^+$ T cell proliferative response from clinical animals was significantly decreased as compared to that of infected-susceptible dogs. These data suggest that as disease progresses, there is an initial increase in antigen-specific lymphoproliferative responsiveness of CD4$^+$ T cells that eventually dwindles. Appearance of clinical disease correlates with the loss of the antigen-specific lymphoproliferative response. Based on this observed loss of
proliferative response in late disease, we wished to determine if cytokine production, specifically IFN-γ and IL-10, could be correlated with this lympho-suppressive change.

Disease progression and antigen-specific PBMC IFN-γ and IL-10 production

Treated individuals develop a cell-mediated immune response capable of offering protection from reinfection, as characterized by antigen-specific IFN-γ responses (7, 34). In contrast, individuals with advanced VL show a decrease in antigen-specific IFN-γ production and elevated levels of the immunoregulatory cytokine IL-10 in serum and increased IL-10 mRNA expression in lesional tissue (6, 10). The correlation between VL disease progression and IL-10 production in humans is now well established (22). In CVL, IFN-γ-mediated responses seem to predominate in *L. infantum*-infected but asymptomatic dogs (23). Similar to human disease, IL-10 mRNA expression has been positively correlated with parasitic load and progression of clinical disease in naturally infected dogs (15). In order to determine the correlation between disease and cytokine production in our cohort, culture supernatants from PBMC restimulated with *L. infantum* antigen were assayed for IFN-γ (Figure 3A) and IL-10 (Figure 3B) production.

Production of IFN-γ and IL-10 from PBMC in the control group (Figure 3A and B) was below the detection limit of the assay (16pg/ml and 10pg/ml respectively). PMBC from infected-resistant and infected-susceptible animals produced comparable levels of IFN-γ (Figure 3A). PBMC from infected-resistant dogs produced significantly higher levels of IFN-γ as compared to non-infected animal PMBC. PBMC from clinical dogs, however,
produced significantly lower amounts of IFN-γ as compared to infected-susceptible and infected-resistant dogs.

Analysis of IL-10 production from culture supernatants indicated a significant increase in the production of this cytokine with disease progression. PBMC from dogs in the clinical group produced the greatest amount of IL-10 as compared to all other groups (Figure 3B), with decreasing amount detected from infected-susceptible and then infected-resistant dogs. All three groups were significantly different from one another. PBMC from non-infected dogs produced levels of IL-10 that were below the detection limit of the assay (10pg/ml). These data demonstrate that clinical progression and loss of antigen-specific T cell proliferation in our cohort were associated with decreased levels of antigen-specific IFN-γ production, and increased production of IL-10 in response to *L. infantum* antigen restimulation.
Discussion

During CVL, susceptibility to symptomatic infection has been associated with increased antibody production and loss of *L. infantum*-specific CD4^+ T cell function with a concomitant increase in immunosuppressive mechanisms. However, little is known regarding the mechanisms that control the balance between resistance to infection and susceptibility. Characterization of measurable immunopathological endpoints may provide a means to better predict disease development in infected dogs. Our studies using a cohort of naturally infected dogs show how changes in IgG production, lymphoproliferative responses, and effector cytokine production correlate with the appearance of clinical signs and disease progression.

In our study increases in serologic titer were associated with disease progression (Table 1). The highest titers (1:256 and 1:512) were observed in dogs displaying mild to severe clinical disease within the infected-susceptible and clinical groups. Moreover, high antibody titers also correlated to the detection of *L. infantum* parasites in peripheral blood samples via qPCR (Table 1), indicating an increase in circulating parasites later in disease. Analysis of antigen-specific IgG1 and IgG2 in sera of the four groups of dogs showed an increase in both isotypes with disease progression (Figure 1A and B). Infected-susceptible and clinical dogs exhibited the highest O.D. values indicating increased IgG1 and IgG2 levels as compared to non-infected and infected-resistant dogs but there was no clear difference between isotypes regarding clinical state or progression.
During human VL increased levels of anti-\textit{Leishmania} IgG have been shown to have a negative correlation with delayed-type hypersensitivity (DTH) responses \cite{17}. Here we show that along with increased IgG in sera, \textit{L. infantum}-antigen responsiveness of PBMC CD4$^+$ T cells significantly decreased in the clinical group of animals (Figure 2). This loss of lymphoproliferation has been described as “immune exhaustion” due unchecked levels of pathogen antigen \cite{2, 19}. Infected-susceptible animals showed the most robust proliferative response compared to all other groups. Proliferation in the non-infected Foxhound group may be attributed to non-specific proliferation or perhaps a dwindling recall response. Animals in this group were donated as part of a litter of puppies born to a seropositive, qPCR-positive female. It is therefore possible they may have been exposed to \textit{L. infantum} parasites \textit{in utero} at a very low dose, leading to exposure and some T cell activation but perhaps not patent infection. Altogether, our data shows that PBMC CD4$^+$ T cells from \textit{L. infantum}-infected dogs respond to antigen stimulation during the earlier stages of infection, but lose that ability as they progress to clinical disseminated disease, negatively correlating with the increased levels of IgG in sera.

Antibody production is an important contributor to VL pathology due to antigen-antibody complex deposition. B cell activation and increased IgG production are observed in conjunction with IL-10 overproduction during VL \cite{22}. To determine what effector cytokines were produced by the dampened T cells with limited antigen-responsiveness in our cohort, we assessed IFN-$\gamma$ and IL-10 production in cultured PMBC. We found that decreased proliferative responses in the clinical group were...
accompanied by significantly decreased IFN-γ production (Figure 3A) and significantly increased IL-10 production (Figure 3B). This profile matches observed changes in cytokine production in endemic human cohort studies (10, 20, 21, 31) and dogs (23). Our infected-resistant and infected susceptible groups produced similar levels of IFN-γ, however, the infected-susceptible group showed significantly increased production of IL-10 compared to the infected-susceptible group. The observed increase in IL-10 production, along with increased blood parasite burden, may be specific factors which promote clinical disease.

The factors that determine disease progression in CVL remain poorly understood. It is clear that no one clinical parameter can be used to predict which infected dogs will likely become clinically ill. Our studies using our canine cohort of progressive CVL indicate that several key changes in clinical parameters should be considered, including a rise in IgG production, a progressive increase of antigen-specific PBMC proliferation followed by a decreased IFN-γ-mediated response, a dramatic increase in IL-10 production, and consistent detection of parasite kDNA in whole blood. Further studies are needed to fully understand the relationship between increased IgG, IL-10 production and parasite load. While it has been shown that all three of these events precede clinical disease (16, 17, 23), the causal relationship between them is yet to be determined. Understanding which event drives the others may provide insight into the mechanisms leading to VL and future immuno-therapies.

Acknowledgments
This work was supported by the American Kennel Club (AKC) CHF ACORN grants 799-A and 1220-A. The authors would like to thank Marie Bockenstedt, Jenna Bjork, Kevin Esch, Alex Osanya and Clara Haydée Quevedo Salazar for their technical assistance. We thank the ISU LAR staff and the collaborating Foxhound Hunts for their support.


Figure Legends

Table 1. Summary of *L. infantum* kDNA qPCR, IFAT serology, CBC and blood chemistry, gross- and histopathology findings of *L. infantum*-infected dogs.

Figure 1. Anti-*L. infantum* IgG1 and IgG2 responses increase with CVL disease progression. *L. infantum*-specific IgG responses were measured using sera from control (1 dog, ■), non-infected (2 dogs, □), infected-resistant (4 dogs, ♦), infected susceptible (2 dogs, ○) and clinical (3 dogs, ●) animals via ELISA. Blood samples were collected and centrifuged to clarify serum. Shown are O.D. values from antigen-specific (A) IgG1 and (B) IgG2 ELISA. Lines indicate mean value for each group.

Figure 2. Decreased lymphoproliferative response in PBMC from *L. infantum*-infected, clinical dogs. (A) PBMC proliferative responses from control (1 dog, ■), non-infected (2 dogs, □), infected-resistant (4 dogs, ♦), infected susceptible (2 dogs, ○) and clinical (2 dogs, ●) animals repeated monthly over three to six months. PBMC were isolated, stained with CFSE, restimulated with freeze-thawed *L. infantum* antigen, and incubated for seven days at 37°C. Cells were then harvested and stained with a PE-conjugated anti-CD4 antibody. PMBC CD4+ T cell proliferation was assessed via CFSE dilution using flow cytometry. Each point is indicative of a blood draw from each animal over a three to six month period and subsequent proliferation assay. At least four separate proliferation assays were carried out over time on each dog in every group.
Lines indicate the mean response for each group. (*) Indicates significant difference, p< 0.05. (B) PBMC proliferative response to *L. infantum* (black), Distemper vaccine (DHPP) (gray), and ConA (white) stimulation for control (1 dog), infected-susceptible (3 dogs) and clinical dog (2). PBMC were isolated and processed as in (A) and stimulated with *L. infantum* Ag for seven days, DHPP for 10 days and ConA for 4 days. CD4⁺ T cell proliferation was assessed via CFSE dilution using flow cytometry. At least 3 separate experiments were carried out for each dog in every group. Bars indicate average proliferation for each group, ±SEM.

**Figure 3. Disease progression correlates with decreased IFN-γ and increased IL-10 production.** Shown are PBMC effector cytokine responses from control (1 dog, ■), non-infected (2 dogs, □), infected-resistant (4 dogs, ♦), infected susceptible (2 dogs, ○) and clinical (2 dogs, ●) animals. Culture supernatants were collected from PBMC cultures stimulated with *L. infantum* antigen for seven days and analyzed via ELISA for (A) IFN-γ and (B) IL-10. Each point is indicative of one experiment. At least three separate experiments were carried out for each dog in every group. Lines indicate the mean response for each group. (*) Indicates significant difference, p< 0.05.
Table 1

<table>
<thead>
<tr>
<th>Clinical state</th>
<th>Number of dogs</th>
<th>PCR +</th>
<th>IFAT</th>
<th>CBC and chemistry</th>
<th>Body condition</th>
<th>Gross pathologic findings</th>
<th>Histopathologic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected</td>
<td>2</td>
<td>0/2</td>
<td>-</td>
<td>Lymphocytosis (2/2)</td>
<td>Adequate</td>
<td>Mild mesenteric and popliteal lymphadenomegaly (2/2)</td>
<td>No significant findings (2/2)</td>
</tr>
<tr>
<td>Infected</td>
<td>4</td>
<td>2/4</td>
<td>1:64</td>
<td>Lymphocytosis (4/4) Hyperglobulinemia (2/4)</td>
<td>Adequate</td>
<td>Moderate systemic lymphadenomegaly (4/4) Mild splenomegaly (2/4)</td>
<td>No significant findings (2/4) Mild lymphoplasmacytic portal hepatitis (2/4) Rare Leishmania amastigotes identified via splenic impression smear 1/4</td>
</tr>
</tbody>
</table>

*Only 2/4 dogs were assessed via CBC, chemistry and necropsy.
<table>
<thead>
<tr>
<th>Clinical state</th>
<th>Dog number</th>
<th>PCR+</th>
<th>IFAT</th>
<th>CBC and chemistry</th>
<th>Body condition</th>
<th>Gross pathologic findings</th>
<th>Histopathologic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Non-infected</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>Lymphocytosis</td>
<td>Adequate</td>
<td>Mild mesenteric and popliteal lymphadenomegaly</td>
<td>No significant findings</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>Lymphocytosis; normochromic anemia</td>
<td>Adequate</td>
<td>Mild mesenteric and popliteal lymphadenomegaly</td>
<td>No significant findings</td>
</tr>
<tr>
<td>Infected</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>Lymphocytosis</td>
<td>Adequate</td>
<td>Moderate systemic lymphadenomegaly; mild splenomegaly</td>
<td>No significant findings</td>
</tr>
<tr>
<td>Resistant</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td>Lymphocytosis</td>
<td>Adequate</td>
<td>Moderate systemic lymphadenomegaly</td>
<td>No significant findings</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>+</td>
<td>1:64</td>
<td>Lymphocytosis; hyperglobulinemia</td>
<td>Adequate</td>
<td>Moderate systemic lymphadenomegaly; mild splenomegaly</td>
<td>Mild lymphoplasmacytic portal hepatitis; mild membranous glomerulonephritis</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>-</td>
<td>1:128</td>
<td>Lymphocytosis; hyperglobulinemia</td>
<td>Adequate</td>
<td>Moderate systemic lymphadenomegaly</td>
<td>Mild lymphoplasmacytic portal hepatitis; mild membranous glomerulonephritis; rare Leishmania amastigotes identified via splenic impression smear</td>
</tr>
<tr>
<td>Infected</td>
<td>8</td>
<td>+</td>
<td>1:256</td>
<td>Lymphocytosis; hyperglobulinemia</td>
<td>Adequate</td>
<td>Moderate systemic lymphadenomegaly; mild splenomegaly</td>
<td>Lymphohistiocytic portal hepatitis; membranous glomerulonephritis.</td>
</tr>
<tr>
<td>Susceptible</td>
<td>9</td>
<td>+</td>
<td>1:256</td>
<td>Lymphocytosis; hyperglobulinemia</td>
<td>Adequate to thin</td>
<td>Moderate systemic lymphadenomegaly</td>
<td>Lymphohistiocytic portal hepatitis; membranous glomerulonephritis; Histiocytic splenitis</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+</td>
<td>1:256</td>
<td>Lymphocytosis; normochromic anemia; Elevated blood urea nitrogen (BUN), creatinine and phosphorus; hyperglobulinemia</td>
<td>Thin</td>
<td>Moderate systemic lymphadenomegaly; splenomegaly; hepatomegaly</td>
<td>Lymphohistiocytic portal hepatitis; membranous glomerulonephritis; rare Leishmania amastigotes identified via splenic impression smear</td>
</tr>
<tr>
<td>Clinical</td>
<td>11</td>
<td>+</td>
<td>1:512</td>
<td>Lymphocytosis; normochromic anemia; Elevated BUN, creatinine and phosphorus; elevated alanine transaminase, hyperglobulinemia</td>
<td>Thin to emaciated</td>
<td>Marked systemic lymphadenomegaly; splenomegaly; hepatomegaly</td>
<td>Lymphohistiocytic portal hepatitis; membranous glomerulonephritis; Histiocytic splenitis; systemic histiocytic inflammation with myriad intracellular Leishmania amastigotes</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>+</td>
<td>1:512</td>
<td>Normochromic anemia; Elevated BUN, creatinine and phosphorus; elevated alanine transaminase, hyperglobulinemia</td>
<td>Thin to emaciated</td>
<td>Marked systemic lymphadenomegaly; splenomegaly; hepatomegaly</td>
<td>Lymphohistiocytic portal hepatitis; membranous glomerulonephritis; Histiocytic splenitis; systemic histiocytic inflammation with myriad intracellular Leishmania amastigotes</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>+</td>
<td>1:512</td>
<td></td>
<td>Thin to emaciated</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>+</td>
<td>1:512</td>
<td></td>
<td>Thin to emaciated</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>