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Immunologic Indicators of Clinical Progression during Canine *Leishmania infantum* Infection

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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 is characterized by increased production of anti-Leishmania antibodies, Leishmania-specific lymphoproliferative unresponsiveness, and decreased production of gamma interferon (IFN-γ) with a concomitant increase of interleukin-10 (IL-10). In order to differentiate infection versus 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 when differentiating infection versus clinical disease, including a dramatic rise in IgG production, progressive increases in antigen-specific peripheral blood mononuclear cell 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 the immuno-dysregulation mirror those observed in human patients, indicating that this animal model will be very useful for testing immunomodulatory anti-IL-10 and other therapies.

Leishmaniasis is a group of vector-borne diseases caused by intracellular protozoan parasites of the genus Leishmania. Disease manifestations 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 transmitted by the bite of infected sandflies to humans and other mammalian species. L. infantum (or L. chagasi) is the causative agent of visceral leishmaniasis in both humans and dogs, and infection is more prevalent than disease, as infection often does not equate with clinical disease. Previous studies additively indicate that advanced clinical visceral leishmaniasis is characterized by increased production of anti-Leishmania antibodies, Leishmania-specific lymphoproliferative unresponsiveness, and decreased production of gamma interferon (IFN-γ) with a concomitant increase of interleukin-10 (IL-10). In order to differentiate infection versus 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 when differentiating infection versus clinical disease, including a dramatic rise in IgG production, progressive increases in antigen-specific peripheral blood mononuclear cell 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 the immuno-dysregulation mirror those observed in human patients, indicating that this animal model will be very useful for testing immunomodulatory anti-IL-10 and other therapies.

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autochthonous *L. infantum* infection. Analysis of clinical signs, serology, and kinetoplast-specific quantitative PCR (qPCR) categorized these animals into four different groups: (i) noninfected, (ii) infected resistant, (iii) infected susceptible, and (iv) clinical, as previously described in reference 33. Animals in the fourth 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 the United States. We show that even in the likely absence of vector-mediated transmission (32), clinical presentation and immuno-disregulation mirror those observed in dogs and humans infected in regions of endemicity (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 has recently been described as an epidemic within the foxhound population in this country. The first report of a foxhound CVL epidemic in the United States 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 6 months to 7 years. These animals or their tissues were donated to Iowa State University College of Veterinary Medicine by two different midwestern foxhound kennels. None of the dogs were donated based on positive serological indirect immunofluorescence assay test (IFAT) results (>1:164) 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 ectoparasites and intestinal parasites (*Giardia*, roundworms, and *Coccidia*) via treatment with Strongid (5 mg/kg of body weight), Baytril (1/4 tablet), Albon (55 mg/kg), Panacur (2 ml/kg), Clavamox (13.75 mg/kg), and Cephalexin (25 mg/kg).

**Clinical evaluation.** Upon arrival at Iowa State University College of Veterinary Medicine, all animals were clinically assessed via physical examination, complete blood count, chemistry panel analysis, *L. infantum* kinetoplast DNA (kDNA)-specific qPCR, and IFAT serologic analysis. Based on these parameters, animals were classified into four distinct categories: noninfected, showing no clinical signs of disease and qPCR and IFAT negative; infected resistant, showing no to mild clinical signs and IFAT and qPCR positive or negative; infected susceptible, showing mild to moderate clinical signs and qPCR and IFAT positive; 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 (incorporate Grace’s supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine). Freeze-thawed whole antigen was prepared as described previously (13).

**PBMC isolation and CFSE staining.** All animals were allowed to acclimate for 1 week prior to immunological studies. Peripheral blood mononuclear cells (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 (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.4). PBMC were labeled with carboxyfluorescein succinimidyl ester (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, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, and 25 mM HEPES buffer). PMBC were counted and adjusted to 4 × 10⁶/ml for further analysis.

**PBMC proliferation assay.** CFSE-labeled PBMC (2 × 10⁶/well) were plated on 96-well plates and incubated with medium alone or stimulated with concanavalin A (ConA; 5 µg/ml) for 4 days, with freeze-thawed whole *L. infantum* antigen (10 µg/ml) for 7 days, or with distemper vaccine (Vanguard Plus 5; Pfizer) control for 10 days, at 37°C with 5% CO₂. Cells were harvested, washed in fluorescence-activated cell sorter (FACS) buffer (0.1% albumin, 0.1% sodium azide in PBS), and labeled with phycoerythrin (PE)-conjugated anti-canine CD4 antibody (Serotec, Raleigh, NC). Cells were fixed in 1% paraformaldehyde and analyzed using a FACS Canto flow cytometer (BD Pharmingen, San Diego, CA). Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

**IFN-γ and IL-10 ELISA.** Unlabeled PMBC (2 × 10⁶) 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 levels were measured using enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN) according to the 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 the manufacturer’s instructions. DNA quality and quantity were measured using a NanoDrop ND1000 spectrophotometer (Wilmington, DE). *L. infantum* kDNA-specific primers and probe (F, 5'–CCCGCCGCCTCAAGAC; R, 5'–TGCCGTTATTTGGTGCTTGGTGG [Integrated DNA Technologies, Coralville, IA]; probe, 5'–6-carboxyfluorescein [6-FAM]–AGCCCGGAGGACC–3' minor groove binder nonfluorescent quencher [Applied Biosystems, Foster City, CA]) were used. (FAM is a laser-activated reporter dye.) DNA blood samples were assayed via qPCR in duplicates of three dilutions (straight, 1:10, and 1:20) using a Stratagene MX3005P qPCR system via a 96-well format and Platinum qPCR SuperMix-UDQ master mix (Invitrogen, Carlsbad, CA). Primers were used at 775 nM and probe at 150 nM, with thermocycling at 50°C for 2 min, 95°C for 2 min, and 50 cycles of 95°C for 30 s, 57°C for 1 min, and 60°C for 1 min. 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 10 µg/well of freeze-thawed *L. infantum* antigen in 50 mM carbonate-bicarbonate buffer. Plates were blocked with 200 µl of blocking buffer for 1 h at room temperature and washed. Serum samples (100 µl) were diluted 1:100 and incubated for 2 h at room temperature. Plates were developed with horseradish peroxidase-conjugated anti-canine IgG1 or IgG2 (1:20,000; Bethyl Laboratories, Montgomery, TX) for 1 h, and absorbance was read at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

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

**RESULTS**

**Clinical evaluation.** Clinical assessment included a CBC and chemistry panel (Table 1). 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). 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 noninfected dogs also showed lymphocytosis, we cannot rule out the possibility of an increased circulating lymphocyte number due to other infections, including gastrointestinal or ectoparasitism, which had 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,
Clinical (4) 3/3 1:256 Lymphocytosis (3/3),
Infected resistant (4) 2/4 1:64 Lymphocytosis (4/4),

Histopathologic examination confirmed these findings and also found during later stages of infection.

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 noninfected foxhounds (Table 1) or in the control, nonfoxhound 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 Igs: IgA, >500 mg/dl; IgG, >5,000 mg/dl; IgM, 400 mg/dl (normal ranges are 20 to 150 mg/dl, 1,000 to 2,000 mg/dl, and 70 to 270 mg/dl, respectively). Hypergammaglobulinemia has been associated with CVL pathophysiology in disease progression (12) and suppression of the immune response to L. infantum (26). However, a relationship between IgG isotype profile and disease resistance versus susceptibility remains to be established. Conflicting reports have failed 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 detec-tion of detectable circulating parasites as disease progressed, we wanted to determine if this observation correlated with deta-
tion of specific antibody levels. Using whole parasite antigen we found that sera from control and noninfected groups contained minimal IgG1 and IgG2 antibodies when measured by ELISA, as optical density (OD) values observed were similar to background readings (OD, ~0.01). The highest levels of anti-\(L.\) \(infantum\) IgG1 (Fig. 1A) and IgG2 (Fig. 1B) were produced by the infected susceptible and clinical groups. Overall, IgG levels increased as disease progressed; however, we did not observe a direct correlation between either IgG isotype and clinical status. Other nonantibody 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-\(\gamma\) production. To identify whether this lack of antigen responsiveness as disease progresses occurred 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 4 weeks during a period of at least 3 months for each dog. PBMC were isolated from whole blood samples, stained with CFSE, and stimulated with ConA, \(L.\) \(infantum\) antigen, or distemper vaccine or left untreated. PBMC 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 (Fig. 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 (Fig. 2A and B). In response to \(L.\) \(infantum\) antigen stimulation, control (uninfected, nonfoehound) and noninfected dogs showed a minimal level of proliferation in response to antigen restimulation (Fig. 2). While a significantly greater percentage of CD4\(^+\) T cells from infected resistant dogs proliferated in response to antigen restimulation than in noninfected dogs, infected susceptible dogs demonstrated the greatest percentage of proliferative CD4\(^+\) T cells, with a level significantly higher than in infected resistant animals (Fig. 2A). In contrast, the antigen-specific CD4\(^+\) T-cell proliferative response from clinical animals was significantly decreased 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 lymphsuppressive 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 (Fig. 3A) and IL-10 (Fig. 3B) production. Production of IFN-γ and IL-10 from PBMC in the control group (Fig. 3A and B) was below the detection limit of the assay (16 pg/ml and 10 pg/ml, respectively). PBMC from infected resistant and infected susceptible animals produced comparable levels of IFN-γ (Fig. 3A), PBMC from infected resistant dogs produced significantly higher levels of IFN-γ than noninfected animal PMBC. PBMC from clinical dogs, however, produced significantly lower amounts of IFN-γ than 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 compared to all other groups (Fig. 3B), with decreasing amounts detected from noninfected animal PMBC. PBMC from infected resistant dogs produced significantly higher levels of IFN-γ than noninfected animal PMBC.

**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 end points 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 with 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 (Fig. 1A and B). Infected susceptible and clinical dogs exhibited the highest OD values, indicating increased IgG1 and IgG2 levels compared to noninfected and infected resistant dogs, but there was no clear difference between isotypes regarding clinical state or progression.

During human VL, increased levels of anti-*Leishmania* IgG have been shown to have a negative correlation with delayed-type hypersensitivity (DTH) responses (17). Here we show that along with increased IgG in sera, *L. infantum* antigen responsiveness of PBMC CD4+ T cells significantly decreased in the clinical group of animals (Fig. 2). This loss

**FIG. 3.** Disease progression correlates with decreased IFN-γ and increased IL-10 production. Shown are PBMC effector cytokine responses from the control (one dog), noninfected (two dogs), infected resistant (four dogs), infected susceptible (two dogs), and clinical (two dogs) animals. Culture supernatants were collected from PBMC cultures stimulated with *L. infantum* antigen for 7 days and analyzed via ELISA for IFN-γ (A) and IL-10 (B). 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. *, significant difference (P < 0.05). ND, not detectable.
of lymphoproliferation has been described as ‘immune exhaustion’ due to unchecked levels of pathogen antigen (2, 19). Infected susceptible animals showed the most robust proliferative response compared to all other groups. Proliferation in the noninfected foxhound group may be attributed to nonspecific 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 that they may have been exposed to L. infantum parasites in utero at a very low dose, leading to exposure and some T-cell activation but perhaps not patent infection. Altogether, our data show that PBMC CD4+ T cells from 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 (22). To determine what effector cytokines were produced by the dampened T cells with limited antigen responsiveness in our cohort, we assessed IFN-γ 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 (Fig. 3A) and significantly increased IL-10 production (Fig. 3B). This profile matches observed changes in cytokine production in human cohort studies (10, 20, 21, 31) and dogs (Fig. 3B). This profile profiles observed changes in cytokine production in human cohort studies (10, 20, 21, 31) and dogs (Fig. 3B). This profile profiles observed changes in cytokine production in human cohort studies (10, 20, 21, 31) and dogs (Fig. 3B). We thank Marie Bockenstedt, Jenna Bjork, Kevin Esch, Alex Otsanay, and Clara Hayde Quevedo Salazar for their technical assistance. We thank the ISU LAR staff and the collaborating Foxhound Hunts for their support.

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