Veterinary Parasitology 248 (2017) 39–47

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Leishmania infantum-specific IFN-γ production in stimulated blood from dogs with clinical leishmaniosis at diagnosis and during treatment

Pamela Martínez-Orellana[a], Daniel Marí-Martorell[a], Sara Montserrat-Sangrà[a], Laura Ordeix[a,b], Gad Baneth[c], Laia Solano-Gallego[a,*]

[a] Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra, Spain
[b] Fundació Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, Bellaterra, Spain
[c] School of Veterinary Medicine, Hebrew University, Israel

ARTICLE INFO

Keywords:
Leishmania infantum
IFN-γ
Dog
Treatment
Follow-up

ABSTRACT

There is limited data regarding *Leishmania infantum* specific T cell mediated immunity in naturally infected sick dogs at the time of diagnosis and during anti-*Leishmania* treatment. Our aim was to investigate the kinetics of *L. infantum* specific IFN-γ production in dogs with leishmaniosis at the time of diagnosis and during treatment and to correlate it with specific *L. infantum* antibodies, blood parasitemia and clinicopathological findings. Thirty-four dogs were diagnosed with leishmaniosis based on physical examination, routine laboratory tests and *L. infantum*-specific antibody levels by quantitative ELISA. Heparinized whole blood was stimulated with *L. infantum* soluble antigen (LSA) and concanavalin A (ConA) and incubated for 5 days. IFN-γ concentration was evaluated in supernatants of stimulated blood using a commercial sandwich ELISA. *Leishmania* real-time PCR was also performed for assessing blood parasitemia. Dogs were treated with meglumine antimoniate and allopurinol.

Sixteen dogs were classified as IFN-γ non-producers after LSA stimulation (mean ± SD: 0 ± 0 pg/mL) and 18 dogs as IFN-γ producers (mean ± SD: 2885.3 ± 4436.1 pg/mL) at the time of diagnosis ($P < 0.0001$). IFN-γ non-producers were classified in a more severe clinical staging than IFN-γ producers that presented a mild to moderate clinical staging ($P = 0.03$). In the IFN-γ non-producer group, production of IFN-γ after LSA stimulation was significantly increased during treatment especially at day 365 ($P = 0.018$) together with clinical improvement when compared with day 0. In contrast, IFN-γ producers maintained their IFN-γ production after LSA stimulation and no statistically significant changes were found during treatment follow-up. At diagnosis, IFN-γ non-producers showed a significantly higher blood parasitemia *versus* IFN-γ –producers ($P = 0.005$). IFN-γ non-producers drastically reduced blood parasitemia to minimum values at day 365 when compared with day 0 ($P = 0.017$). No significant differences were found at day 365 in blood parasitemia of IFN-γ producers compared to pre-treatment. At diagnosis, *L. infantum* specific antibodies were higher in IFN-γ non-producers than IFN-γ producers ($P = 0.014$). A marked reduction of antibody levels was found at day 365 when compared with day 0 in IFN-γ non-producers ($P = 0.005$) and producers ($P = 0.001$). These results demonstrate that IFN-γ concentration increases with long-term anti-*Leishmania* treatment together with clinical improvement in dogs that do not produce IFN-γ at diagnosis. Together with clinical recovery, reduction in blood parasitemia and *L. infantum* specific antibodies, tracking IFN-γ concentration could constitute an important prognostic tool for immune monitoring in CanL.

Abbreviations: CBC, complete blood cell count; ConA, concanavalin A; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; EU, ELISA units; IFAT, indirect immunofluorescence technique; IFN-γ, interferon-gamma; IL-10, interleukin-10; IL-4, interleukin 4; LSA, *L. infantum* soluble antigen; LST, Leishmanin skin test; NO, nitric oxide; PBMC, peripheral blood mononuclear cells; PBS-Tween, phosphate buffer solution-Tween; PD-1, programmed cell death protein-1; P-MAPA, protein aggregate magnesium-ammonium phospholinoleate-palmitoleate anhydride; RPMI-1640, Roswell Park Memorial Institute 1640 medium; RT-PCR, real time PCR; SD, standard deviation; Th1, type 1 T helper lymphocytes; Th2, type 2 T helper lymphocytes; TNFα, tumor necrosis factor-alpha; UPC, urinary protein/creatinine ratio; WBA, whole blood stimulation assay

* Corresponding author.
E-mail addresses: pamela.martinez@uab.cat (P. Martínez-Orellana), dani_205_3@hotmail.com (D. Marí-Martorell), sara.montserrat@uab.cat (S. Montserrat-Sangrà), laura.ordeix@uab.cat (L. Ordeix), gad.baneth@mail.huji.ac.il (G. Baneth), laia.solano@uab.cat (L. Solano-Gallego).

http://dx.doi.org/10.1016/j.vetpar.2017.10.018
Received 20 August 2017; Received in revised form 26 October 2017; Accepted 27 October 2017

1. Introduction

Canine leishmaniosis (CanL) is caused by protozoa *Leishmania infantum* (Baneth et al., 2008), which is an intracellular parasite of the mononuclear phagocyte system being transmitted by *Phlebotomus* sand fly bites (Akhoundi et al., 2016). This infection is endemic and affects dogs and humans in the Mediterranean basin (Baneth et al., 2008; Gramiccia and Gradoni, 2005), China (Zhao et al., 2016) and South America (Dantas-Torres, 2009), and is an emergent disease in North America (Duprey et al., 2006) and in the northern latitudes of Europe (Espejo et al., 2015; Ready, 2010). CanL can manifest as chronic subclinical infection, self-limiting disease, or non-self-limiting disease which can be fatal. In addition, the severity of this disease ranges from mild to very severe. According to this variability, a clinical staging system has been proposed by the LeishVet group (Solano-Gallego et al., 2017, 2009).

The most common clinical signs in dogs with moderate to very severe disease include cutaneous lesions such as exfoliative dermatitis, onychogryphosis, ulcerations, generalized lymphadenomegaly and lesions derived from immune-complexes deposition such as uveitis, vasculitis or glomerulonephritis. Frequent clinicopathological abnormalities are mild non-regenerative anemia, hypoalbuminemia, hyperglobulinemia and mild proteinuria. Mild disease is commonly diagnosed with mild cutaneous lesions such as papular dermatitis (Lombardo et al., 2014; Ordeix et al., 2005) or with solitary lymphadenomegaly without clinicopathological abnormalities (Solano-Gallego et al., 2011).

The diagnosis of clinical leishmaniosis is challenging due to the wide spectrum of its clinical and immunological manifestations. Laboratory diagnostic methods commonly used in the clinical practice for this disease include serology to detect *L. infantum* antibodies. Serological methods include the indirect immunofluorescence technique (IFAT) and enzyme-linked immunosorbent assay (ELISA) currently considered the most sensitive tests. Cytology, histopathology and real-time polymerase chain reaction (RT-PCR) are used to demonstrate infection in tissues (Maia and Campino, 2008).

The most standardized treatment for the disease consists of using several drugs with different mechanisms of action, which reduce *Leishmania* infection. The combination of meglumine antimoniate or miltefosine, and long term treatment with allopurinol, is the most used and effective treatment protocol in clinical practice (Noli and Saridomichelakis, 2014).

It is well known that the clinical manifestations and outcome of infection in CanL is a consequence of complex interactions between the parasite and the genetic and immunological background of the dog (Hosein et al., 2017). The progression of infection to active disease in susceptible dogs is characterized by a marked humoral response, a depression of the cellular immune response against the parasite and the appearance of a full array of clinical signs and/or clinicopathological abnormalities. In the opposite spectrum, resistant dogs lack clinical signs or clinicopathological abnormalities, develop low levels of anti-*Leishmania* antibodies and parasite loads, and a strong *in vitro* lymphocyte proliferative response (Carrillo and Moreno, 2009; Solano-Gallego et al., 2016b). The resistant profile in healthy infected dogs is due to the onset of a strong T helper 1 (Th1)-like response against the parasite, mediated principally by a high expression of interferon gamma (IFN-γ) and tumor necrosis factor-α (TNF-α). Conversely, a susceptible profile and the establishment of clinical disease is mediated by a Th2-like immune response associated with anti-inflammatory cytokines such as interleukin- 4 (IL-4) and IL-10 together with different degrees of detection of IFN-γ in a low proportion of dogs (Baneth et al., 2008; Barbieri, 2006; Boggiatto et al., 2010; Esch et al., 2013).

Different methods for the evaluation of specific T cell mediated immunity have been investigated in CanL although standardization is lacking. One of the most sensitive methods is the leishmanin skin test (LST) based on a delayed hypersensitivity reaction. LST specifically detects cellular immunity against the parasite *in vivo* in the dog, especially when illness is not present and is frequently used in epidemiological investigations in CanL endemic regions (Cardoso et al., 1998; Fernandez-Bellon et al., 2005; Solano-Gallego et al., 2000). Other methods like the IFN-γ cytopathic effect inhibition bioassay, detection of IFN-γ transcripts and lymphocyte proliferation assays have been tested, with impaired results (Fernandez-Bellon et al., 2005; Hosein et al., 2017).

Another example of measurement of T cell-mediated immunity in leishmaniosis is quantifying specific IFN-γ production in *Leishmania* stimulated whole blood or peripheral blood mononuclear cells (PBMC). This assay is commonly used to evaluate the immunogenicity of *Leishmania* vaccines (Carson et al., 2009; Costa-Pereira et al., 2015). However, limited information is available regarding *L. infantum* specific IFN-γ production in stimulated blood in sick dogs with different degrees of disease at the time of diagnosis and during treatment and clinical cure (Boggiatto et al., 2010; Strauss-Ayali et al., 2005). A recent study performed by our group, demonstrated that IFN-γ producers sick dogs were associated with lower antibody levels, parasite load and milder disease when compared with IFN-γ non-producers (Solano-Gallego et al., 2016b). We hypothesize that IFN-γ non-producer dogs might recover IFN-γ production when effective anti-*Leishmania* treatment is provided and clinical improvement is achieved. In contrast, it is likely that IFN-γ producer sick dogs might maintain or slightly increase their initial IFN-γ concentration.

The aim of this study was to investigate the kinetics of *L. infantum* specific IFN-γ production in blood from dogs with clinical leishmaniosis (IFN-γ producers *versus* IFN-γ non-producers) at the time of diagnosis and during long term standard anti-*Leishmania* treatment, and to correlate with clinicopathological, parasitological and serological data.

2. Materials and methods

2.1. Dogs

Five mixed breed and 29 pure breed sick dogs (age range between 5 and 153 months) from an endemic area of CanL (Catalonia, Spain) were prospectively enrolled in this study from January 2014 to January 2016. Breeds of sick dogs are described in Supplementary Table 1. All sick dogs attended three veterinary centres: *Hospital Clinic Veterinari* of Autonomous University of Barcelona (Bellaterra, Spain), ARS Veterinària (Barcelona, Spain) and Consultori Montsant (Tarragona, Spain). A full physical examination and routine laboratory tests were performed in all dogs. All dogs were diagnosed with leishmaniosis based on two-fold serial dilution ELISA antibody levels as described previously (Solano-Gallego et al., 2016a) and/or by observation of amastigotes on cytological and/or histopathological evaluation in skin and/or lymph nodes.

Ten mL of blood sample were taken by jugular or cephalic venipuncture and in ethylenediaminetetraacetic acid (EDTA), heparinized and serum sterile tubes for routine haematology [Siemens Advia 120 Haematology System (Siemens Healthcare GmbH, Germany)], serum biochemistry [Olympus AU400 Chemystry Analyzer (CLIAwaived, USA)] and serum electrophoresis [Hydrasys® (Sebia Electrophoresis, USA)]. Blood parasite quantification by *Leishmania* RT-PCR and serology for the detection of *L. infantum* antibodies at the time of diagnosis (day 0) and during treatment at days 30, 180 and 365 were also performed as previously described (Solano-Gallego et al., 2016a).

Urine samples from all dogs were mostly taken by cystocenthesis for urinalysis and for urinary protein creatinine ratio (UPC). All dogs were classified into clinical stages following the Leishvet guidelines according to their clinicopathological findings and serological results at the time of diagnosis (Solano-Gallego et al., 2009).

We prospectively evaluated the possibility of coinfection in the dogs that presented clinical signs and/or clinicopathological abnormalities that were highly compatible with other infectious diseases at discretion