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Review article

Canine visceral leishmaniasis biomarkers and their employment in vaccines

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ABSTRACT

The natural history of canine visceral leishmaniasis (CVL) has been well described, particularly with respect to the parasite load in different tissues and immunopathological changes according to the progression of clinical forms. The biomarkers evaluated in these studies provide support for the improvement of the tools used in developing vaccines against CVL. Thus, we describe the major studies using the dog model that supplies the rationale for including different biomarkers (tissue parasitism, histopathology, hematological changes, leucocytes immunophenotyping, cytokines patterns, and *in vitro* **co**-culture systems using purified T-cells subsets and macrophages infected with *L. infantum*) for immunogenicity and protection evaluations in phases I and II applied to pre-clinical and clinical vaccine trials against CVL. The search for biomarkers related to resistance or susceptibility has revealed a mixed cytokine profile with a prominent proinflammatory immune response as relevant for *Leishmania* replication at low levels as observed in asymptomatic dogs (highlighted by high levels of IFN-γ and TNF-α and decreased levels in IL-4, TGF-β and IL-10). Furthermore, increased levels in $CD4^+$ and $CD8^+$ T-cell subsets, presenting intracytoplasmic proinflammatory cytokine balance, have been associated with a resistance profile against CVL. In contrast, a polyclonal B-cell expansion towards plasma cell differentiation contributes to high antibody production, which is the hallmark of symptomatic dogs associated with high susceptibility in CVL. Finally, the different studies used to analyze biomarkers have been incorporated into vaccine immunogenicity and protection evaluations. Those biomarkers identified as resistance or susceptibility markers in CVL have been used to evaluate the vaccine performance against L. *infantum* in a kennel trial conducted before the field trial in an area known to be endemic for visceral leishmaniasis. This rationale has been a guiding force in the testing and selection of the best vaccine candidates against CVL and provides a way for the veterinary industry to register commercial immunobiological products.

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1. Introduction

Canine visceral leishmaniasis (CVL) presents a high diversity of clinicopathological manifestations, ranging from different clinical signs, biochemical/hematological and histological changes to distinct levels of histopathological changes. The parasite load varies in different infected dogs and in accordance with target organs, such as skin, bone marrow, spleen, liver, and lymph nodes. In addition, the presence of signs such as alopecia, dry exfoliative dermatitis, lesions of the ear tip, onychogryphosis, weight loss, and splenomegaly occur in at least 50% of infected dogs (Giunchetti et al., 2006; Ordeix et al., 2017; da Silva et al., 2018).

As such, the direct relationship between clinical manifestation and/or parasite load and pathological progression in CVL has been the object of different studies in recent decades (Abranches et al., 1991; Reis et al., 2009; Menezes-Souza et al., 2011; Nascimento et al., 2013; Ali et al., 2014; Torrecilha et al., 2016; Abbehusen et al., 2017; Bagues et al., 2018). Aiming to investigate the polarized response in CVL treatment, Mancianti et al. (1988) grouped dogs for the first time according to distinct clinical forms, such as: (i) asymptomatic (AD), which do not show any signs of the disease, (ii) oligosymptomatic (OD), which exhibit few clinical signs, and (iii) symptomatic dogs (SD), which have some or all of the signs of the disease. This approach favored the categorization of clinical forms and their correlation with immunopathological features during ongoing CVL. Complementarily, AD displayed a resistance profile response while SD presented the susceptibility pattern throughout the different biomarkers analyzed. In doing so, several strategies were used to identify and cluster these biomarkers in accordance with the profile of resistance or susceptibility in dogs during ongoing CVL. The levels of parasitism were classified as low (LP), medium (MP), or high parasite (HP) burden based on tissue-specific LDU (Leishman Donovan Units) values (Giunchetti et al., 2006; Reis et al., 2006a; Giunchetti et al., 2008a, b; Reis et al., 2009). This approach revealed a direct correlation between AD and LP or SD and HP groups in different compartments (skin, bone marrow, spleen, liver, and lymph nodes), showing that the susceptibility profile is associated with a high parasitic load (Giunchetti et al., 2006; Sant'Ana et al., 2007; Giunchetti et al., 2008b; Reis et al., 2009).

Therefore, the immune response against parasites in CVL is highly associated with the pathogenic profile during infection (Reis et al., 2010). The clustering of biomarkers associated with resistance or susceptibility during CVL could provide additional support for developing or improving diagnostic methods and useful when choosing a therapeutic protocol for infected dogs. In this sense, the resistance profile was shown to be associated with the development of a specific anti-*Leishmania* cell-mediated response, causing the production of proinflammatory cytokines (such as IFN-γ and TNF), which lead to an increase in leishmanicidal macrophage activity by means of nitric oxide (NO) production and reactive oxygen species (ROS) (Zafra et al., 2008; de Almeida et al., 2014). A susceptible profile is associated with parasite dissemination and an increased parasite load in combination with elevated antibody levels and a suppressed cellular immune response (Reis et al., 2006a, b, c; Reis et al., 2010; Zafra et al., 2008; Ali et al., 2014; de Almeida et al., 2014; Matralis et al., 2016; Solcà et al., 2016), along with higher concentrations of anti-sand fly saliva antibodies (Solcà et al., 2016).

The role of immune response in visceral leishmaniasis (VL) progression and parasite burdens represents an important approach for understanding immunopathology in CVL. Dogs presenting severe clinical forms of CVL and high parasite density showed an increase of inflammatory infiltrate in the skin, which was mainly composed of mononuclear cells (Giunchetti et al., 2006; Menezes-Souza et al., 2011). In addition, parasite density is positively correlated with the expression of specific chemokines, such as CCL2, CCL4, CCL5, CCL21, and CXCL8 (Menezes-Souza et al., 2011). Moreover, the increase of CVL severity was associated with high parasite loads, gradual decrease of leukotriene (LTB4) and prostraglandin (PGE2), and a gradual increase in CXCL1 and CCL2 (Solcà et al., 2016).

With this in mind, the need to develop new strategies to identify polarized patterns of biomarkers becomes evident. The cluster of biomarkers associated with resistance or susceptibility in dogs during ongoing VL, and its relationship with clinical forms, could benefit different aspects associated with the diagnosis, treatment, and vaccine immunogenicity analysis, in addition to differentiation in vaccinated and L. *infantum*-infected dogs.

2. The polarized patterns of clinical and parasitological conditions on canine visceral leishmaniasis as a prerequisite to associate with pathological progression

2.1. Histopathological features in canine visceral leishmaniasis

Histopathological investigations during CVL constitute a strong point for a better understanding of events related to the development of severe clinical forms and its relation to disease transmission. The role of dogs in VL transmission is supported by high skin parasite loads favoring the infection of vectors (Deane and Deane, 1962; Giunchetti et al., 2006; Borja et al., 2016). In this context, histopathological changes in the skin of *Leishmania*-infected dogs may result in a focal or diffuse inflammatory infiltrate in the superficial or/and deep dermis, represented by plasma cells, with smaller populations of macrophages and lymphocytes (Santos et al., 2004; Giunchetti et al., 2006; Carvalho et al., 2017). The reorganization of the extracellular matrix (ECM) in the skin of infected dogs is also remarkable, with a reduction of collagen type I and an increase of collagen type III (reticular fibers) (Giunchetti et al., 2008a). These histopathological changes may be related to the degree of tissue destruction produced by inflammatory processes and parasite burden, as previously reported by Giunchetti et al., 2008a. It has also been demonstrated that the lymph node from *L. infantum*-infected dogs displays chronic lymphadenitis, with hypertrophy and hyperplasia of cortical and medullary zones without specific lymph node lesions (Giunchetti et al., 2008b). The most striking changes have been the depletion of follicular structures and lymphocytes, which are replaced by macrophages in the cortical zone during CVL (Giunchetti et al., 2008a,b).

The histopathological evaluation of the hepatic compartment is essential for understanding the genesis of hepatomegaly in CVL (Tryphonas et al., 1977; Tafuri et al., 1996; Giunchetti et al., 2008b; Silva et al., 2013; Madeira et al., 2016). Changes are typically related to the presence of portal inflammation, comprising mononuclear cells and hypertrophic and hyperplastic Küpffer cells (many of which are parasitized), and the occurrence of granulomas. In fact, *L. infantum*-naturally infected dogs presented intralobular and intravascular granulomas, consisting of parasitized and non-parasitized macrophages, epithelioid cells, and a small number of lymphocytes together with rare granulocytes (neutrophils) (Tafuri et al.;, 1996; Tafuri et al., 2001; Alvar et al., 2004; Giunchetti et al., 2008b; Abbehusen et al., 2017). The intense capsule and portal inflammation associated with CVL is most likely related to hepatomegaly (Giunchetti et al., 2008b; Reis et al., 2009). Moreover, clinical ongoing CVL has been associated with systemic deposition of collagen in the liver and other organs, including the spleen, cervical lymph nodes, lungs, and kidneys of *L. infantum*-infected SD (Silva et al., 2013). Hepatic fibropoiesis has been correlated with parasite tissue load and the overexpression of TGF-b in *L. infantum*-infected dogs (Madeira et al., 2016).

Of the major target organs of *L. infantum*, the spleen shows a higher parasite density due to the presence of numerous cells related to the phagocytic mononuclear system, frequently parasitized (Alvar et al., 2004; Reis et al., 2006a; Souza et al., 2014). Splenic histopathology in CVL is associated with cellular and structural changes such as hyperplasia and hypertrophy, specifically in the red pulp splenic region, due to the presence of mononuclear cell infiltrates, essentially plasma cells

(Reis et al., 2009; Bagues et al., 2018; da Silva et al., 2018). Due to intense parasitism, a disorganization of the splenic white pulp is frequently observed, which compromises the activity of lymphocytes located in the central arterioles of the organ (Alvar et al., 2004). In fact, this disorganization has been associated with severe clinical signs, in addition to higher laminin and collagen deposition, higher MMP-9 expression, and lower $CD4^+$ T-cells counts (da Silva et al., 2018). Finally, it indicated that ongoing CVL leads to immunosuppression that contributes to death in *L. infantum*-infected dogs. It is possible that the disruption of the splenic microarchitecture could lead to immunosuppression and contribute to the early death of dogs infected with *L. infantum* as a result of secondary infections caused by bacteria, viruses, and protozoa.

Although hepatic and splenic manifestations are of great importance in CVL, renal disease is considered the main cause of mortality in dogs with this disease and it occasionally presents without typical skin abnormalities (Koutinas and Koutinas, 2014). CVL frequently causes nephropathies, such as glomerulonephritis and chronic kidney disease, due to the formation and deposition of immunocomplexes, histologically classified as: mesangial glomerulonephritis, membranous glomerulonephritis, membranoproliferative glomerulonephritis, and focal segmental glomerulonephritis (Zatelli et al., 2003). Furthermore, the staging of chronic kidney disease is frequently associated with proteinuria levels (da Costa et al., 2003; Koutinas and Koutinas, 2014). Finally, the signs of end-stage renal failure can be present depending on glomerular and tubulointerstitial disease development (Zatelli et al., 2003).

The biomarkers traditionally recommended by the International Renal Interest Society (IRIS) to evaluate and monitor renal damage/dysfunction are serum creatinine (sCr) and urinary protein to creatinine ratio (UPC) (Elliott et al., 2013). Other more sensitive biomarkers, such as immunoglobulin G (IgG) and acute phase proteins (C-reactive protein – CRP) and ferritin, have high molecular weight proteins and can be used to monitor glomerular barrier permeability (D'Amico and Bazzi, 2003; García-Martínez et al., 2015).

2.2. Humoral immune response during canine visceral leishmaniasis

Many aspects of CVL pathogenesis are attributed to antibody production, which forms immune complexes that are deposited in various tissues, generating inflammatory lesions. SD generally present high titers of anti-*Leishmania* antibodies, while AD exhibit low levels or the absence of these antibodies (Oliveira et al., 1993; Desplazes et al., 1995; Martinez-Moreno et al., 1995; Carrera et al., 1996; Solano-Gallego et al., 2001; Reis et al., 2006c; Coura-Vital et al., 2011).

Hypergammaglobulinemia is the dominant characteristic of CVL, primarily in SD, with an elevated level of total IgG (Cooper et al., 1946; Chaves and Ferri, 1996; Pateraki et al., 1983; Giunchetti et al., 2008b). This overactive humoral immunity (Koutinas and Koutinas, 2014) may lead to the production of nonspecific immunoglobulins, such as autoantibodies (Pateraki et al., 1983). Autoantibodies in CVL have already been described, *e.g.*, anti-actin, anti-tubulin, anti-nuclear, and anti-transferrin (Pateraki et al., 1983; Smith et al., 2004; Ginel et al., 2008; Chaabouni et al., 2018).

The immunophenotypic features and humoral immune response of AD with negative serology, yet presenting *Leishmania* positivity, is similar to that of non-infected dogs (de Almeida et al., 2014). These findings suggest that AD are in the initial CVL phase and most likely within an "immunological window" that occurs prior to seroconversion. During the initial period, the B lymphocytes do not secrete polyclonal antibodies and, consequently, serological methods are less sensitive at this stage of *L. infantum*-infection (Coura-Vital et al., 2011).

Accordingly, many studies aim to assess a prognostic value of anti-*Leishmania* IgG subclasses to identify a pattern of immune response in infected dogs. However, in contrast with the murine model of *Leishmania major*, there is no consensus in the literature regarding anti-*Leishmania* immunoglobulin subtypes that could be associated with a resistance or susceptibility profile in CVL (Day, 2007). Some authors correlate the IgG1 subtype with the asymptomatic form of the disease, while IgA, IgE, and IgG2 displayed a stronger association with the symptomatic form (Oliveira et al., 1993; Reis et al., 2006c). They also observed a positive correlation between tissue parasite density with IgG, IgG2, IgM, and IgA levels (Reis et al., 2006c). These data re-enforce the anti-*Leishmania* IgG, but with IgA reactivity as a better marker for overall tissue parasitism. Conversely, some authors have shown that SD present high concentrations of anti-*Leishmania* IgG1 antibodies as compared to AD (Nieto et al., 1999; Quinnell et al., 2003; Lima et al., 2017). Similarly, a European report that analyzed the serological IgG subclass concluded that dogs produced IgG1 and IgG2 isotypes, with IgG2 being associated with subclinical infections and IgG1 with disease expression (Desplazes et al., 1995). Moreover, Lima et al. (2017) demonstrated a relationship of the IgG1 subclass with the susceptibility and with higher levels in SD as compared to AD. Chaabouni et al. (2018) demonstrated that the IgG2 subclass was associated with the presence of SD. De Freitas et al. (2012) reported an increase in immunoglobulins, especially IgG1 and IgG2 subclasses, in asymptomatic and symptomatic infected animals as compared to healthy dogs.

Such inconsistent results may partially reflect a low specificity of *Leishmania*-antigenic preparations and/or commercial antibodies used in these studies. The lack of consensus prevents a conclusion as to what type of IgG subclass would actually be related to resistance or susceptibility in CVL.

2.3. Cellular immune response and cytokine pattern according to clinical progression in L. Iinfantum-infected dogs

In humans and dogs, the skin is the first target of the *Leishmania infantum* during blood meal by infected sand flies (Brasil, 2016). The resulting cutaneous lesion is an inflammation in the dermis induced by parasitized macrophages (Solano-Gallego et al., 2001; Giunchetti et al., 2006; Jacintho et al., 2018). Macrophages play an important role in the immune response against *Leishmania* as they are the parasites' target cells and can trigger an adaptive immune response.

Several immunological approaches have been used to improve the cellular immune response analysis in CVL. Flow cytometry is the tool currently used to search for new biomarker relationships with resistance or susceptibility (de Almeida et al., 2014; Reis et al., 2009, 2010). Analysis of circulating T-cells in *L. infantum*-naturally infected dogs has shown low levels of $CD4^+$ and $CD8^+$ T-cells in SD, along with $CD21^+$ B-cells (Reis et al., 2006b; de Almeida et al., 2014). Moreover, a decrease in circulating $CD14^+$ monocytes was described in SD (Giunchetti et al., 2006; Reis et al., 2006b). Similar results were demonstrated in dogs with high parasitism displaying lower numbers of $CD5^+$ T-cells and T-cells subsets ($CD4^+$ and $CD8^+$), and lower counts of $CD21^+$ B-cells and $CD14^+$ monocytes (Reis et al., 2006b, 2009, 2010). Furthermore, M2 macrophages, associated with immunosuppressive action, have been related to parasite load in the skin, lymph nodes, and spleen (Moreira et al., 2016). In contrast, M1 macrophages and prostaglandin E2 may contribute to an increase in TNF-a and, therefore, control *L. infantum* parasitism in CVL (Venturin et al., 2016).

An *in vitro* analysis using *Leishmania* antigens as a stimulus revealed a high frequency of intracytoplasmic IFN-γ in neutrophils in both AD and SD, and decreased IL-4 levels in AD. Moreover, $CD8^+$T-cells exhibited the highest IFN-γ and IL-4 frequencies in AD, displaying seronegative yet positive molecular results for *L. infantum* (de Almeida et al., 2014). Similarly, subclinically infected dogs were hallmarked by low percentage of $CD4^+IL\text{-}4^+$ and $CD8^+IL\text{-}4^+$ and high $CD4^+IFN\text{-}\gamma^+$ after *Leishmania* antigen stimulation (Matralis et al., 2016).

Identification of the cytokine profile related to the resistance or susceptibility of dogs with CVL plays an important role in the clinical follow-up of disease progression. During the course of *L. infantum*

infections, a mixed immune response profile Th1/Th2 is observed. Infected dogs generally have reduced IL-12, IL-17, and TGF-β with an increase in TNF-α, IFN-γ, IL-2, IL-4, IL -5, IL-6, IL-10, IL-12, and IL-13 (Santos-Gomes et al., 2002; Strauss-Ayali et al., 2007; Cavalcanti et al., 2015; Hosein et al., 2015; Rodríguez-Cortés et al., 2016; Tonin et al., 2016; Lima et al., 2017). Moreover, different cytokine profiles are observed in the compartmentalized immune response during CVL, especially in view of the organs *L. infantum* uses for its replication, such as the spleen, liver, lymph nodes, bone marrow, skin, and blood (Maia and Campino, 2012). As previously shown, the presence or absence of symptoms affects the dog differently, in a way that modifies the cytokine expression profile. In this sense, distinct organ-specific cytokine patterns in SD have been described in the liver (low IL-10, IL-17, TGF-β, and IFN-γ), bone marrow (low IL-4), skin (low IL-13), lymph nodes (high TGF-β and low IL-4), and spleen (high IL-4 and low levels IL-10 and IL-17) (Correa et al., 2007; Alves et al., 2009; de Lima et al., 2010; Barbosa et al., 2011; Menezes-Souza et al., 2011; Nascimento et al., 2015).

The search for biomarkers related to resistance or susceptibility has revealed a mixed cytokine profile with a prominent proinflammatory immune response as being relevant for *Leishmania* replication at low levels, as observed in AD (high levels of IFN-γ, TNF-α with decreased levels in IL-4, TGF-β and IL-10 – Fig. 1). Furthermore, increased levels in $CD4^+$ and $CD8^+$ T-cell subsets, showing intracytoplasmic proinflammatory cytokine balance, have been associated with a resistance profile against CVL. In contrast, a polyclonal B-cell expansion towards plasma cell differentiation contributes to high antibody production, which is the hallmark of SD associated with high susceptibility in CVL (Fig. 1). These biomarkers could be used as "target biomarkers" in asymptomatic disease for guiding prediction analysis of canine immune response, providing successful vaccine trials and treatment approaches against CVL.

2.4. Canine visceral leishmaniasis biomarkers used for evaluating immunogenicity parameters in Phase I and II vaccine clinical trials in dogs

The role of anti-*Leishmania* cellular immunity in the systemic response underlying resistance or susceptibility during CVL was initially recognized by hematological status (Reis et al., 2006a) and immune response in peripheral blood cell analysis (Giunchetti et al., 2006; Reis et al., 2006b). Different hematological disorders between symptomatic and asymptomatic clinical forms have been demonstrated in dogs naturally infected by *L. infantum*. SD present severe anemia with a prominent decrease in the number of red blood cell, hemoglobin, and hematocrit as compared to AD and OD (Reis et al., 2006a; de Almeida et al., 2014). SD also manifest a pronounced decrease in leukocyte populations (leukopenia) associated with eosinophilia, monocytopenia, and highlighted lymphopenia (Reis et al., 2006a; Coura-Vital et al., 2011; de Almeida et al., 2014).

Analysis of the immune system has demonstrated a polarized response since SD display (i) decreased levels of circulating $CD14^+$ monocytes, $CD4^+$ and $CD8^+$ T-cell subsets, and $CD21^+$ B-cells, and (ii) a mixed cytokine pattern with prominent immunoregulatory cytokines: levels of IFN-γ, TNF-α with IL-4, TGF-β and IL-10 (Giunchetti et al., 2006; Reis et al., 2006b, 2009; Reis et al., 2010).

Consequently, biomarker studies in the clinical evolution of CVL associated with resistance or susceptibility profiles may guide the development of new vaccines for controlling the disease. That being so, biomarkers attributed to asymptomatic disease maintenance have been applied to evaluating immunoprotection in vaccines and treatment approaches.

Immunization against infectious agents requires the participation of innate (neutrophils, dendritic cells, NK cells, macrophages), and adaptive immune (T-cells, B-cells) responses; therefore, determining the kinetics of cell migration in the inoculation area is extremely relevant (Teixeira et al., 2005), providing information on the immune response in the microenvironment of the inoculation site (Vitoriano-Souza et al., 2008). The adjuvant characteristics play an important role in vaccine formulation by inducing successive waves of infiltrating cell populations (as macrophage and lymphocyte cell populations) that produce a proinflammatory cytokine microenvironment, resulting in improved immune responses against intracellular pathogens (Vitoriano-Souza et al., 2012).

The development and testing of LBSap (*L. braziliensis* antigens associate with saponin as an adjuvant) and LBSapSal (LBSap vaccine associated with *Lutzomyia longipalpis* saliva antigens) vaccines against CVL illustrate the application of biomarkers used to identify immunological parameters pertaining to resistance against *L. infantum*

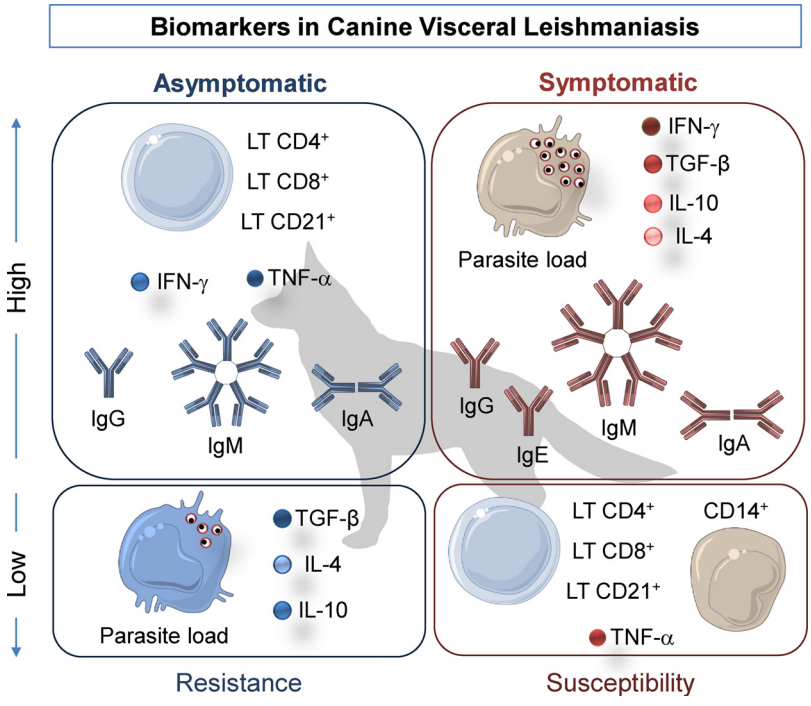

Fig. 1. Biomarkers identified during ongoing canine visceral leishmaniasis classified according to polarized clinical forms. Asymptomatic (left panel) and symptomatic (right panel) dogs. The upper panel shows biomarkers presented in high levels for both asymptomatic and symptomatic dogs. Similarly, the bottom panel illustrates the biomarkers observed in low levels in asymptomatic and symptomatic dogs. These parameters are seen as important biomarkers that could be applied in immunogenicity and efficacy evaluations of canine visceral leishmaniasis vaccine candidates.

infection in dogs. An immunogenicity analysis confirmed by previous studies has shown the importance of the innate immune response induced by LBSap vaccine in hamsters (Moreira et al., 2016), mice (de Mendonça et al., 2016), and dogs (Giunchetti et al., 2007, 2008c; Vitoriano-Souza et al., 2008; Roatt et al., 2012; Resende et al., 2013; Aguiar-Soares et al., 2014; Resende et al., 2016). Our studies verified that the LBSap vaccine was able to induce intense cell migration into the dermis with the participation of neutrophils, eosinophils, and lymphocyte following sensitization, triggering the initial immunogenic events with an increased production of iNOS aimed at controlling *Leishmania* parasites (Vitoriano-Souza et al., 2008).

LBSap and LBSapSal are known to induce a strong immune response by triggering reduced parasite load in dogs (Giunchetti et al., 2007, 2008c; Vitoriano-Souza et al., 2008; Roatt et al., 2012; Resende et al., 2013; Aguiar-Soares et al., 2014; Moreira et al., 2016; Resende et al., 2016). LBSap and LBSapSal vaccines have been shown to be safe for administration, inducing a resistance lymphocyte activation profile in dogs with increased $CD5^+$ T-cells, which results in high $CD4^+$ and $CD8^+$ T-cell subsets (Giunchetti et al., 2007, 2008a, c). Moreover, LBSap induced a prominent type 1 immune response that was characterized by increased levels of interleukin IL-12 and IFN-γ production by peripheral blood mononuclear cells (PBMC) upon *Leishmania* antigen stimulation in dogs (Resende et al., 2013). An increase in the expression of IFN-γ and reduction of IL-10 and TGF-β1 in splenocyte-stimulated *Leishmania* antigens also induced a reduction in the parasite load in the bone marrow and spleen (Roatt et al., 2012; Resende et al., 2013). Similarly, the LBSapSal vaccine triggered a prominent type I immune response with high levels in TNF-α, IL-12, IFN-γ and low IL-4 and TGF-β levels, after an *L. infantum* experimental challenge (Resende et al., 2016). Thus, it was shown that the LBSap vaccine induces high antigenicity with sustained production of anti-*Leishmania* total IgG, IgG1, and IgG2a, and a shift in the cytokine balance towards higher IFN-γ/IL-10, which contributes to the reduction of parasitism in the murine model. This profile resembles the one described for other high-performing vaccines, such as Leish-Tec® and Leishmune®.

Table 1 describes biomarkers analyzed in studies using vaccinal antigens that were tested and formulated in commercial vaccines against canine visceral leishmaniasis. Leish-Tec® (Ceva Saúde Animal Ltda, Brazil), a commercially available vaccine in Brazil, presents rA2 antigen associated with a saponin adjuvant. It has been shown that a mixed Th1/Th2 immune is characterized by the production of anti- and proinflammatory cytokines in mice (Ghosh et al., 2002). Leish-Tec® induced increased levels of IFN-γ in dogs that were associated with protection against *L. infantum* challenge (Fernández et al., 2018). Field trials demonstrated that efficacy for the Leish-Tec® vaccine was 58.1%, according to xenodiagnoses and parasitological findings (Regina-Silva et al., 2016). However, Leish-Tec® was not able to produce a fully sterilizing form of protection since 43% of vaccinated dogs developed the disease during a two-year follow-up period (Grimaldi et al., 2017).

In Brazil, Leishmune® (Zoetis, Brazil) was commercially available for dogs from VL endemic areas up until September 2014. An immunogenicity evaluation of Leishmune® described (i) high phagocytic activity of neutrophils and monocytes, (ii) increased NO production, (iii) enhanced expression of TLR (2, 4, 5, 9), (iv) decreased levels of IL-4 and enhanced IL-8 production by monocytes, and (v) increased IFN-γ and IL-17 levels produced by T-cells (Moreira et al., 2016). Furthermore, Leishmune® vaccination triggered a pro-inflammatory immune response with high IL-8, TNF-α, and IFN-γ with a decrease in IL-10 levels of cytokine secreted by peripheral blood leukocytes after *in vitro Leishmania* stimulation. Moreover, adaptive immunity was highlighted by increased intracytoplasmic TNF-α and IFN-γ by $CD4^+$ T-cells, in addition to an increase in IL-17a and decrease in IL-4 in $CD8^+$ T-cells induced by Leishmune® vaccination (Costa-Pereira et al., 2015). These immunogenicity analyses revealed that the major biomarkers for protection against *L. infantum* infection were identified by the first 6 and 9 months post-Leishmune® vaccination (Costa-Pereira et al., 2015; Moreira et al., 2016). After 12 months post-*L. infantum* challenge, Leishmune® vaccination remained with high amounts of TNF-α and IL-4 in cell cultures stimulated with *Leishmania* antigens, resulting in a mixed Th1/Th2 immune response. After 24 months of experimental *L. infantum*-challenge, Leishmune® provided 63% protection in dogs (Fiuza et al., 2015).

Recently, our research group standardized a new method for evaluating the immune system using the dog model to analyze vaccine treatment. We propose a co-culture system using canine *L. infantum*-infected macrophages and purified $CD4^+$ and $CD8^+$ T-cell subsets to examine the parasitological and immunological status of dogs (Viana et al., 2013, 2015; Viana et al., 2016). This co-culture system has been validated as a methodology applied to canine immune system analysis, specifically for studying changes induced by vaccine candidates, therapeutic schemes, or as a tool for prognosis follow-up (Viana et al., 2016). In actuality, we have demonstrated the ability of this co-culture system to assess immunoprotection biomarkers. In this sense, the Leishmune® vaccine triggered high levels of IFN-γ and IL-12, reduced levels of IL-4 and IL-10, and a high IFN-γ/IL-10 ratio in the co-culture systems. These immune profiles were able to control the *in vitro L. infantum* parasite load analyzed by co-culture systems using *L. infantum* infected macrophages and $CD4^+$ and/or $CD8^+$ T-cells (Viana et al., 2016).

Interestingly, the comparative performance of Leishmune® and Leish-Tec® vaccines in pre-clinical trials using the mice model showed 64% protection (Leishmune®) and 36% (Leish-Tec®) in the spleen, and 71% (Leishmune®) and 48% (Leish-Tec®) in the liver, using qPCR parasite analyses (de Mendonça et al., 2016). In the field study, no differences were reported for Leishmune® and Leish-Tec® vaccines with respect to seroconversion rates, clinical signs, parasitism, and parasite transmission by xenodiagnosis during an eleven-month investigation in a CVL endemic area (Fernandes et al., 2014).

The LiESP/QA-21 (excreted-secreted proteins of *L. infantum* and the QA-21 saponin adjuvant) vaccine (CaniLeish®, Virbac, France) is commercially available in Europe. The immunogenicity before and after experimental *L. infantum*-challenge of CaniLeish® was associated with: (i) ELISpot detection of IFN-γ secreting lymphocytes, (ii) *in vitro* leishmanicidal activity (ability of macrophages to control parasite replication in co-culture system using autologous lymphocytes), and (iii) NO production in the supernatant from the co-culture system (Martin et al., 2014; Moreno et al., 2014). The immunogenicity status revealed that CaniLeish® was able to provide protection in 50% of the dogs that presented at least one positive result by parasitological analysis after 48 weeks of experimental *L. infantum*-challenge (Martin et al., 2014). A randomized double-blind controlled trial of CaniLeish® revealed that 66.66% of control dogs and 50.22% of CaniLeish® vaccinated dogs presented as positive for *Leishmania* in parasitological analyses during a 24-month period in a CVL endemic area (Oliva et al., 2014).

The recombinant protein formed by the genetic fusion of five *L. infantum* intracellular antigen fragments, known as the Q protein, was examined in dogs at 330 days post-*L. infantum* infection. The parasitological analysis revealed that the cultures were 71% positive in lymph nodes and 57% in the skin in dogs vaccinated with a single Q protein dose, whereas after two Q protein doses, the *L. infantum* parasites were isolated in 86% of the lymph nodes and 57% in the skin (Carcelén et al., 2009). Recently, a single dose of Q protein was licensed throughout the European Union under the trade name of LetiFend® (LETI, Spain). A field trial of LetiFend® revealed 9.4% *L. infantum* positivity in vaccinated dogs after 730 days of follow-up without statistical difference as compared to 16.1% in the control group. However, LetiFend® was able to provide clinical protection in dogs and reduce the clinical signs of CVL by 9.8 times as compared to unvaccinated dogs (Fernández et al., 2018).

Table 1
Biomarkers described in studies that analyzed vaccinal antigens that were tested and formulated in commercial vaccines against canine visceral leishmaniasis.

Vaccine	Vaccinal formulation	Vaccinal protocol and *L. infantum* challenge	Type of trial	Biomarkers analyzed	Efficacy evaluation	References
Leish-Tec®	Recombinant A2 protein plus saponin as adjuvant	3 doses with 21-day intervals 7 vaccinated and 7 control dogs Experimental *L. infantum* challenge (5×10^7promastigotes by iv route) 4 weeks after days of vaccination protocol	Kennel trial	After each vaccine dose and infection, and 1 month after vaccination protocol: ↑IgG, ↑ IgG2, ↑IFN-γ 7 months after *L. infantum* challenge: ↑IFN-γ and ↑IL-10	After 9 months post-challenge: 57.14% positivity in bone marrow aspirates (Culture) in vaccinated dogs; 28.5% positivity in blood (*Leishmania* DNA) in vaccinated dogs	Fernandes et al. (2008)
		3 doses with 21-day intervals 429 vaccinated and 418 control dogs	Field trial in VL endemic area (Porteirinha, Minas Gerais State, Brazil)	73 days after vaccination protocol: ↑IgG, ↑IgG2, ↑IgG1	After 497 days from vaccination protocol: Xenodiagnosis: 46.6% reduction in transmission to sand flies from vaccinated animals 63.7% of vaccinated dogs were asymptomatic and remained seronegative	Regina-Silva et al., 2016
		3 doses with 21-day intervals and a booster after 12 months after vaccination protocol 151 vaccinated and 78 control dogs	Field trial in VL endemic area (Porteirinha, Minas Gerais State, Brazil)	1 month after vaccination protocol: ↑IgG, ↑IgG2	After 24 months from vaccination protocol: 31% of vaccinated dogs converted to a seropositive status 40/151 (26.49%) vaccinated and 33/78 (42.31%) control dogs converted to a seropositive status after 24 months of follow-up	Grimaldi et al., 2017
LetiFend®	Protein Q plus BCG as adjuvant (preliminary study using antigen present in LetiFend® formulation)	Vaccine doses inoculated at days 0, 21, and 44. 10 vaccinated and 10 control dogs Experimental *L. infantum* challenge (5×10^6 promastigotes) after 108 days of first immunization	Kennel trial	634 days after *L. infantum* challenge: ↑ DTH (9/10 vaccinated dogs)	After 634 days post-challenge: 90% of vaccinated dogs remain normal considering parasite positivity (lymph nodes culture), clinical and anatomo-pathologic analysis	Molano et al. (2003)
	Protein Q without adjuvant (preliminary study using antigen present in LetiFend® formulation)	Vaccinated groups: Q = 1 dose at day 0; Q + Q = 2 doses at days 0 and 21. 14 vaccinated (7 dogs for Q and 7 dogs for Q + Q) and 10 control dogs Experimental *L. infantum* challenge (5×10^5 promastigotes) 60 days after the first dose	Kennel trial	327 months after challenge: ↑ NO production (group Q > Group Q + Q > group control) ↑ DHT (control: 1/7; Q: 4/7; Q + Q: 3/7)	After 11 months post-challenge: Positivity in PCR (skin): control (6/7); Q (1/7); Q + Q (4/7) Positivity in PCR (lymph node): control (5/7); Q (1/7); Q + Q (1/7) Positivity in PCR (spleen): control (6/7); Q (0/7); Q + Q (2/7)	Carcelén et al. (2009)
	Protein Q without adjuvant	1 dose at day 0 with annual booster at day 365 275 vaccinated and 274 control dogs Dogs were kept in open kannel for natural *L. infantum* infection	Open Kannel trial (Auvergne-Rhône-Alpes, Corsica and Provence-Alpes-Côte d'Azur, France; and Extremadura, Spain)	14 days after vaccination: ↑IgG2 anti-Protein Q	After 730 days from the first vaccination: 72% efficacious in the prevention of clinical cases of visceral leishmaniasis	Cotrina et al. (2018)
Leishmune®	Fucose-Mannose-ligand (FML antigen) plus saponin as adjuvant (preliminary study using antigen present in Leishmune® formulation)	3 doses with 21-day intervals 58 vaccinated and 59 control dogs	Field trial in VL endemic area (São Gonçalo do Amarante, Rio Grande, Brazil)	7 months after vaccination protocol: ↑total IgG anti-FML	After 24 months from vaccination protocol: Clinical exam: 8% symptomatic dogs post vaccination	Da silva et al., 2000
	FML antigen plus saponin as adjuvant (preliminary study using antigen present in Leishmune® formulation)	3 doses with 21-day intervals 45 vaccinated and 41 control dogs	Field trial in VL endemic area (São Gonçalo do Amarante, Rio Grande do Norte, Brazil)	41 months after vaccination protocol: ↑total IgG anti-FML	After 12–48 months from vaccination protocol: Clinical exam: 5% symptomatic dogs post vaccination; 25% symptomatic dogs in control dogs	Borja-Cabrera et al. (2002)
	FML antigen plus saponin as adjuvant (preliminary study using antigen present in Leishmune® formulation)	3 doses with 21-day intervals 550 vaccinnated and 588 control dogs	Field trial in VL endemic area Araçatuba, Andradina, Valparaíso, Guararapes, Bauru (São Paulo state) and Belo Horizonte, Nova Lima, Sete Lagoas (Minas Gerais state),	24 months after vaccination protocol: ↑$CD8^+$ T-cells, ↑$CD4^+$ T-cells, ↑$CD21^+$ B-cells	After 24 months from vaccination protocol: Clinical exam: 98.8% asymptomatic dogs (at the end of first year) and 99% healthy survivors (at the end of the second year) among vaccinated dogs; 79.4% asymptomatic dogs (at the end of first	Borja-Cabrera et al. (2008)

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Table 1 (*continued*)

Vaccine	Vaccinal formulation	Vaccinal protocol and *L. infantum* challenge	Type of trial	Biomarkers analyzed	Efficacy evaluation	References
					year) and 61% healthy survivors (at the end of the second year) among control dogs	
	FML antigen plus saponin as adjuvant	3 doses with 21-day interval with annual booster 20 vaccinated and 20 control dogs	Field trial in VL endemic area (Araçatuba, São Paulo State, Brazil)	12 months after vaccination protocol: ↑total and specific IgG ↑IFN-γ and reduced CD4 + /CD25 + T cell	After 12 months from vaccination protocol: no parasite DNA detected by PCR in the vaccinated group; 45% positivity in control dogs	Lima et al. (2010)
	FML antigen plus saponin as adjuvant	3 doses with 30-day interval and one annual booster. A total of 30 dogs were vaccinated with Leishmune and 30 controls dogs.	Kennel trial (Department of Parasitology (Biological Sciences Institute, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil).	36 months after vaccination protocol: ↑IgG by FML-ELISA (Fucose–mannose ligand-ELISA)	After 36 months post-challenge: Positivity in PCR (skin): 12/19 vaccinated dogs (63%) and 5/9 control dogs (55%).	Amorim et al. (2010)
	FML antigen plus saponin as adjuvant	3 doses with 21-day interval. 40 vaccinated and 9 dogs with borderline results to the ELISA cut off values at the time of the first vaccine injection were used as control group.	Field trial in VL endemic area (Lauro de Freitas,and Camaçari, Bahia State, Brazil)	21 days post third after vaccination dose:↑IgG1 and ↑ IgG2	After 11 months from vaccination protocol: Xenodiagnosis: positivity in 5.1% (2/39) vaccinated dogs	Fernandes et al. (2014)
CaniLeih®	LiESP antigen plus QA-21 as adjuvant	3 doses with 21-day interval 10 vaccinated and 10 control dogs 3 weeks after vaccination protocol: *in vitro L. infantum* infection for Macrophage Leishmanicidal Assay challenge using 1 Macrophage:5 *L. infantum* promastigotes ratio	Kennel trial	3 weeks after vaccination protocol: ↑IFN-γ, ↑iNOS, and ↑ nitrite	3 weeks after vaccination protocol: ↓ *in vitro* macrophage parasitism (˜25%)	Moreno et al. (2012)
	LiESP antigen plus QA-21 as adjuvant	3 doses with 21-day interval and annual booster 23 vaccinated and 22 control dogs per site (Barcelona and Naples) Dogs were kept in open kannel for natural *L. infantum* infection	Open Kannel trial (Barcelona, Spain and Naples, Italy)		After 24 months from vaccination protocol: 22.1% reduction in active infection (PCR) after 2 years 15.8% reduction in symptomatic dogs Reduction in bone marrow parasite load (˜230 parasites/mL)	Oliva et al. (2014)
	LiESP antigen plus QA-21 as adjuvant	3 doses with 21-day interval and annual booster 10 vaccinated and 10 control dogs Experimental *L. infantum* challenge ($10^{8.5}$ promastigotes one year after vaccination)	Kennel trial	1 year after vaccination protocol: ↑IFN-γ, ↑iNOS and ↑nitrite	47 weeks post-challenge: ˜95% reduction in bone marrow parasite load	Martin et al. (2014)
	LiESAp antigen (50 μg; 100 μg; 200 μg) and MDP as adjuvant	2 doses with 21-day interval 6 dogs from control (group 1); vaccinated: 3 dogs from group 2 (50 μg), 6 dogs from group 3 (100 μg), 3 dogs from group 4 (200 μg) 2 months after vaccination protocol dogs from groups 2 and 4 and half of dogs from groups 1 and 3 were challenged. Eight months after vaccination protocol, the remaining dogs from groups 1 and 3 were challenged. All experimental *L. infantum* challenges were performed using 10^8 promastigotes	Kennel trial	8 months after vaccination protocol: ↑IFN-γ/ nitrite	*in vitro* infection of *L infantum* in macrophages: 42 days after immunization: Leishmanicidal effect - 42% group 2; 78.9% group 3; 65.8% group 4 336 days after first challenge: Leishmanicidal effect: 31.6% group 2; 63.8% group 3; 67.2% group 4 84 days after immunization: Nitrite production (nmol/10^5 cells/72 h) – 14 nmol group 2; 20 nmol group 3; 21 nmol group 4 336 days after first challenge: Nitrite production (nmol/10^5 cells/72 h) – 13 nmol group 2; 24 group 3; 20 group 4 336 days after first challenge: IFN-γ production (ng/mL): 1.1 group 2; 1.95 group 3; 1.83 group 4	Lemesre et al. (2005)
	LiESAp antigen (100 μg)	2 doses with 21-day interval and annual booster 205 vaccinated and 209 control dogs	Field trial in VL endemic area 500 km along	7-9 months post basic vaccination and 6-8 months post-booster: ↑IFN-γ; ↑ nitrite	*in vitro* infection of *L infantum* in macrophages: 7–9 months post-vaccination:	Lemesre et al. (2007)

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Table 1 (*continued*)

Vaccine	Vaccinal formulation	Vaccinal protocol and *L. infantum* challenge	Type of trial	Biomarkers analyzed	Efficacy evaluation	References
			[Mediterranean Sea coast between B´eziers and Menton (France)]		Leishmanicidal activity – 51.1%; 7.5% control dogs 6–8 months post-booster: Leishmanicidal activity – 60.7%; 4.3 control dogs 7-9 months post-vaccination: Nitrite production (nmol/10^5 cells/72 h) – 17.5 nmol in vaccinated dogs; ˜5 control dogs 6–8 months post-booster: Nitrite production (nmol/10^5 cells/72 h) – ˜17 in vaccinated dogs; ˜4 control dogs 7-9 months post-vaccination: IFN-γ production (ng/mL) – 1.68 vaccinated dogs; 0.14 control dogs 6–8 months post-booster: IFN-γ production (ng/mL) – ˜1.68 vaccinated dogs; ˜0.14 control dogs	
	LiESAp antigen (100 μg)	2 doses with 21-day interval 10 vaccinated and 10 control dogs *In vitro* experimental *L. infantum* challenge (ratio of *in vitro* infection: 5 *L. infantum* promastigotes per 1 macrophage	Kennel trial	6 weeks after vaccination protocol: ↑IFN-γ; ↑ nitrite	*in vitro* infection of *L infantum* in macrophages 6 weeks post-vaccination: Leishmanicidal effect: ˜75% vaccinated dogs; ˜5% control dogs Nitrite production (nmol/10^5 cells/72 h): ˜20 vaccinated group; ˜7 control dogs IFN-γ production (ng/ml): ˜1 vaccinated dogs; ˜0.5 control dogs	Holzmuller et al. (2005)

DTH: delayed type of hypersensitivity response; iv: intravenous; MDP: muramyl dipeptide; iNOS: inducible nitric oxide synthase; Protein Q plus BCG as adjuvant: quimeric multi component antigenic protein formed by genetic fusion of fragments from the acid ribosomal proteins Lip2a Lip2b P0 and histone H2A protein associated with BCG (Bacillus Calmete Guérin); VL: visceral leishmaniasis; Symbol "˜": approximate value reported when the precise values were not provided in the study; " ↑ = increase in the biomarker levels when compared to controls groups; ↓ = decrease in the biomarker levels when compared to controls groups.

3. Conclusion

The different studies used to analyze biomarkers for resistance and susceptibility in CVL have been incorporated into vaccine immunogenicity and protection evaluations. The biomarkers identified as resistance markers have been used to evaluate the vaccine performance against *L. infantum* in a kennel trial conducted prior to a field trial in an endemic area for VL. The most relevant biomarkers predictive of protection against CVL were: (i) proinflammatory cytokine pattern (high levels of IFN-γ and TNF-α, in addition to decreased levels in IL-4, TGF-β and IL-10); (ii) a high count of circulating $CD4^+$ and $CD8^+$ T-cell subsets displaying intracytoplasmic proinflammatory cytokine balance after *in vitro Leishmania* stimulus; (iii) low inflammatory and histopathologic damage in different organs; and (iv) high microbicide capacity in *L. infantum*-infected macrophages co-cultured with purified $CD4^+$ and $CD8^+$ T-cell subsets. The presence of these biomarkers can prompt a low parasite density in the spleen, bone marrow, liver, lymph nodes and skin, compatible with a low risk of *L. infantum* transmission. This rationale has been used to test and select the best vaccine candidates against CVL and provide a way for the veterinary industry to register commercial immunobiological products.

Competing interests

The authors declare that they have no competing interests.

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