The control of infection by *Leishmania infantum* (syn. *Leishmania chagasi*) in dogs is essential to stop the current spread of zoonotic visceral leishmaniasis. The past few years have seen significant advances in achieving efficient immunization of dogs and, more than ever before, an effective vaccine against canine leishmaniasis can now be considered a feasible goal. This article summarizes experimental data gathered from recent dog trials aimed at identifying immunological mechanisms implicated in protection against canine infection to discuss their potential to serve as quantitative surrogate markers of immunization and, more importantly, its usefulness to evaluate whether the immunity induced by the vaccine candidate is strong enough to protect against canine leishmaniasis.

Threat to public health by *Leishmania*-infected dogs

Zoonotic visceral leishmaniasis (ZVL), caused by *Leishmania infantum* parasites, is a severe infectious disease endemic to Mediterranean countries, the Middle East, Asia and South America. In recent years, ZVL has emerged as an important public health concern owing to the increase in risk factors associated with environmental changes, human migration and the immune status of the host [1,2].

Canines are the main reservoir for the parasite and play a relevant role in transmission to humans. The emergence of human cases of visceral leishmaniasis (VL) in previous non-endemic territories has been preceded by an increase in the incidence of canine infection and the appearance of new foci. Canines have been responsible for the spread of the disease observed in large Brazilian cities where, since the 1980s, the disease has become endemic and epidemic, resulting in the continuous increase of human cases in Brazil [3]. In Italy, the observed northward spread of leishmaniasis is the consequence not only of climate changes, but also of the increased mobility of dogs to and from ZVL-endemic areas [4]. Moreover, the continued importation of infected dogs has expanded the canine reservoir population to the UK and might allow stable transmission if climate conditions allow the competent vector to survive [5]. The risk of introducing parasite-infected dogs in non-endemic areas has become patent in North America, where the possible active transmission of the parasite by indigenous sand flies might have caused the widespread presence of *L. infantum*-infected foxhounds [6]. Furthermore, given the existence of a stable transmission cycle for *L. infantum* in southern Europe, the introduction and spread in endemic areas of new *Leishmania* species could also be more prevalent. This has recently occurred on the island of Cyprus, where several cases of human leishmaniasis caused by *L. donovani* MON-37 have been described. This *Leishmania* parasite was also isolated from infected dogs, which confirms the circulation in a zoonotic cycle of such an ‘imported’ parasite [7]. All these aspects reveal the important threat that both symptomatic and asymptomatic *Leishmania*-infected dogs represent for public health and that its control is of prime urgency [8].

Control of ZVL is a difficult assignment. Many animals remain asymptomatic after infection, but, in some cases, are still capable of transmitting the parasite, as well as those who develop symptoms of canine leishmaniasis. Current chemotherapy reduces or eliminates clinical cure in dogs, and the epidemiological risk persists [9]. The culling of seropositive dogs has not prevented the increasing number of human cases in Brazil [3], whereas other measures such as topical insecticides and impregnated collars are costly and difficult to implement in a national control programme. Additionally, the available field data do not allow an estimate of their epidemiological impact [10]. In the absence of other successful strategies, the development of vaccines against canine leishmaniasis has been promoted as an important tool and a cost-effective strategy for controlling ZVL [11]. There is increasing awareness that induction in dogs of a protective response...
against *Leishmania* is a feasible objective and that it will have a direct impact on human leishmaniasis.

The course of *Leishmania* infection in dogs is linked to the host immune response and to the persistence and multiplication of the parasite. The components of innate and adaptive immunity engage in a range of interactions that is remarkably diverse and complex. The innate immune response has a relevant role in protection against the parasite besides switching on the adaptive response as shown by those experimentally infected animals that are able to control the *Leishmania* infection without developing a specific adaptive immunity [12]. By contrast, the role of anti-leishmanial cellular immunity in systemic immune responses that underlies resistance during canine leishmaniasis is widely recognized throughout *ex vivo* and *in vitro* investigations [8].

The absence of an adequate T-cell response to control the parasite leads to appearance of clinical symptoms, high *Leishmania*-specific antibody levels and high parasite burden in skin, bone marrow, spleen, liver and lymph nodes [13–20]. Several levels of interaction occurred at the different parasite-infected tissues by attack of the host effector mechanisms. Symptomatic dogs (SDs) present dermal inflammatory infiltrates with a high parasitism [16], and a mixed immune response at the skin tissues [21]. High parasite burden has been described in cervical lymph nodes, and high expression of IL-10 and TGF-β were observed in prescapular lymph nodes [22]. Spleen from *L. infantum* infected-dogs is characterized by mononuclear cellular infiltrate in red pulp and the substitution of lymphocytes by macrophages in the white pulp [23]. There is a predominant accumulation of IL-10 in those animals with high parasitism [24], whereas the hepatic compartment presents a high frequency of parasitism and intense inflammatory alterations [20]. Analyses of circulating lymphocytes in SDs have demonstrated low levels of CD4+ and CD8+ T-cells and a decrease in CD21+ B-cells and CD14+ monocytes, changes that are closely related to bone marrow parasite density since a lower frequency of cell subsets are mainly observed in dogs with high levels of parasitism [15]. Furthermore, a direct relationship between decreased levels of circulating CD4+ T-cells and the transmission to sand flies has been established in dogs with canine leishmaniasis [25].

Those animals that remain asymptomatic (asymptomatic dogs, ADs) present an overall low tissue parasitism and can develop a positive leishmanin skin test or an *in vitro* lymphoproliferative response after stimulation with leishmanial antigens, both associated with the development of a parasite-specific cell-mediated immunity. In addition, IFN-γ expression by *Leishmania*-specific T-cells is also associated with disease resistance. Studies in canines have established the predominant role of IFN-γ in the activation of macrophages and, in collaboration with TNF-α, the stimulation of their leishmanicidal activity to control parasite dissemination [26–28]. Quantitative real-time (QRT) PCR analysis of IFN-γ expression in peripheral blood from ADs showed that high levels of the cytokine are related to the absence of symptoms [27], and *in vitro* assays with peripheral blood mononuclear cells (PBMCs) from experimentally infected ADs showed expression of high levels of IFN-γ after stimulation with parasite antigens [28].

The natural history of canine leishmaniasis depends on several factors, e.g. parasite burden, cell mediated immunity, cell subsets, humoral response and cytokine expression. The relative importance of these factors in each individual, and the different balances established between all of them, result in the appearance of a dynamic spectrum of clinical forms which ranges from asymptomatic resistant animals to severe disease in naturally infected, susceptible animals. Furthermore, these parameters have a predictive value for the progress of the infection and should, therefore, be taken into account as hallmarks of resistance and susceptibility to canine leishmaniasis (Figure 1).

The limited number of animals used in dog trials (an inherent aspect to all dog experiments) makes it difficult to obtain statistically consistent conclusions on the efficacy of the vaccine candidates. Our aim is to review the latest advances in this field, considering those factors contributing significantly to the resolution of infection in dogs and discussing their potential to serve as quantitative, general surrogate markers of immunity that will be useful in the

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**Figure 1. Biomarkers of resistance and susceptibility in canine leishmaniasis.** Susceptibility and resistance to development of canine leishmaniasis depends on the immune response elicited in the dog after natural infection. The evaluation of different factors like parasite burden, humoral response, cell mediated immunity or cytokine expression, have a predictive value for the progress of the infection and should, therefore, be taken into account as hallmarks of resistance and susceptibility to canine leishmaniasis. Increased levels of parameters like parasite load, IL-10 and TGF-β expression, *Leishmania*-specific cell immunodepression or *Leishmania*-specific-IgG, IgM, IgA and IgE serum antibodies are related to clinical disease progression. By contrast, increased levels of parameters such as PBMCs proliferation after leishmanial antigen stimulation, IFN-γ and TNF-α expression, CD4+, CD8+ and B cell subsets, or positive leishmanin skin test are related to resistance. The different balances established between the factors involved in the host immune response are reflected in the spectrum of clinical forms that can be observed in naturally infected dogs, ranging from symptomatic severe canine leishmaniasis to asymptomatic animals.
search and development of a vaccine against canine leishmaniasis. This experimental determination will permit attainment of significant differences between vaccinated and control animals; moreover, they will establish the grade of individual protection induced by the vaccine and if it is strong enough to keep the animal healthy, avoid parasite transmission and confirm their applicability in national control programmes for ZVL.

Inducing protection against canine leishmaniasis

An ideal vaccine against canine leishmaniasis should have immunological features that include the elicitation of a long-lasting cell-mediated immune response and the ability to elicit a response in all of the vaccinated population. Several studies have reported the potential of different vaccine candidates to trigger immunoprotective mechanisms against canine leishmaniasis. In a first step, vaccine candidates are evaluated on the basis of their capability to be recognized in in vitro assays by T lymphocytes during early periods of infection (antigenicity) and/or their ability to produce T-cell response after immunization in animal models (immunogenicity). Monitoring different parameters over time (Box 1) helps assess the immunogenicity of the vaccine candidate. In addition to tolerance and safety tests, the selected vaccine candidates are then tested in different animal challenge models to confirm their protective efficacy. There have already been important advances in this area, as illustrated by the availability of two second-generation vaccines that are commercially available in Brazil: Leish-mune (Fort Dodge Animal Health) and Leishtec (Hertape Calier Saúde Animal SA). Nevertheless, more trials are in progress to characterize the protective immunity of other candidates for use in a canine vaccine against L. infantum infection, including live or dead Leishmania parasites, purified Leishmania antigens, live recombinant bacteria expressing Leishmania antigens and antigen-encoding DNA plasmid [29].

The specific immunological mechanisms elicited by these vaccine candidates are yet unelucidated, but diverse key aspects relating to the induction of protection have been identified. An important feature for the design and implementation of anti-parasite vaccines is the markedly different response of canine and murine hosts to leishmanial antigens due to the genetic variation between the two. For instance, whereas a partially purified 67 to 94 kDa promastigote fraction (LiF2) from L. infantum was shown to confer resistance in infected mice [30], the rate of disease in dogs exposed to natural infection was notably higher in vaccinated compared with control animals [31]. Elsewhere, the recombinant L. braziliensis eukaryotic ribosomal eIF4A (LeIF) and L. major stress-inducible 1 (LmSTI1) proteins have been shown to elicit Thelper cell type 1 (Th1) responses in experimentally infected cutaneous leishmaniasis mouse models [32,33]. Furthermore, LeIF and LmSTI1 as part of the multi-subunit recombinant vaccine, MML, together with monophosphoryl lipid A in a stable emulsion (MPL-SE) as adjuvant, were demonstrated to elicit at least partial protection against L. major infection in the BALB/c mouse model [33–35]. Additionally, the MML vaccine was also protective against L. infantum infection in mice and hamsters [36]. Yet, the multi-subunit MML vaccine with MPL-SE or AdjuPrime as adjuvant was ineffectual against leishmaniasis infection and disease progression in dogs [37,38]. By contrast, antigens that have confirmed protective capability against canine leishmaniasis such as fucose-mannose ligand (FML), A2 or excreted-secreted antigens were primarily tested with success in the murine model [39–41]. Although the murine model represents a necessary step to obtain experimental evidence of the candidate protective capability, the efficacy of a vaccine candidate against canine leishmaniasis requires assessment in dogs.

Besides host responses, it is essential to point out the specificity of vaccines to different Leishmania species. In general, members of the genus Leishmania share specific biological and molecular characteristics and infection with a given species can confer cross-protection against other species of Leishmania. This has promoted the use of killed vaccines with L. braziliensis or L. major to induce heterologous protection against canine leishmaniasis [42,43]. However, the variability of immune responses elicited by different Leishmania species complicates the development of a pan-Leishmania second generation vaccine. In fact, some antigens demonstrated to protect against a species causing cutaneous leishmaniasis have proved ineffective against other species causing VL. As an example, the Leishmanida homolog of the receptor for activated C kinase (LACK) protected against L. major infection [44] but failed as a vaccine strategy against L. donovani [45]. Nevertheless, antigens highly conserved in the Leishmania genus retain the capability to cross-protect against other species and might be useful as vaccines against canine leishmaniasis [46].

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**Box 1. Methodologies to evaluate immunogenicity in vaccines for canine leishmaniasis**

The methodologies and techniques commonly used to evaluate the immunogenicity (humoral and cellular immune response after immunization with the vaccines for canine leishmaniasis) in different (ex vivo, in vitro and in vivo) contexts.

<table>
<thead>
<tr>
<th>CONTEXT</th>
<th>HUMORAL IMMUNITY</th>
<th>CELLULAR IMMUNITY</th>
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<tbody>
<tr>
<td><strong>Ex vivo and in vivo</strong></td>
<td>ELISA: Immunoglobulin Isotypes (IgG, IgG1 and IgG2)</td>
<td>Leishmanin skin test: To evaluate the specific intradermal reaction after leishmanin inoculation</td>
</tr>
<tr>
<td></td>
<td>Flow Cytometry: To evaluate levels of Leishmania-specific serum antibodies</td>
<td>Flow Cytometry: To quantify T- (CD5+, CD4+, CD8+), and B- (CD21+) cell subsets, monocytes (CD14+) and MHCI, MHCI, CD32 molecules expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ELISA and Real Time PCR: Cytokines (IFN-γ, IL-12, IL-10, IL-4 etc)</td>
</tr>
<tr>
<td><strong>In vitro</strong></td>
<td>Lymphocyte Proliferation Test: To evaluate Leishmania specific T-cell proliferation</td>
<td>Cytotoxicity Assay: To evaluate leishmanicidal activity in macrophages</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitric Oxide production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ELISPOT: To evaluate number of cells expressing specific cytokines</td>
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</tbody>
</table>
Another important feature to consider is the nature of the antigen. Significant differences in protection have been described based on the nature of the leishmanial antigen tested. For example, the LACK antigen induced comparable protection against *L. major* infection in mice when used as both a DNA vaccine or as recombinant protein plus IL-12 [47]. By contrast, in the case of the NH36 antigen, the level of protection induced in mice by the DNA vaccine was higher than that elicited by the recombinant protein or the FML antigen plus saponin in response to infection by *L. chagasi*, *L. mexicana* and *L. amazonensis* [48,49].

The choice of adjuvant system is also crucial since the use of an appropriate adjuvant has been shown to increase the vaccine efficacy of leishmanial purified antigens by up to 82% in phase III dog trials [49]. Adjuvants can potentiate the immunogenicity of leishmanial antigens, stimulate an appropriate immune response and reduce the dose of vaccine needed. Adjuvants influence the isotype and avidity of antibodies and affect the properties of cell-mediated immunity. They should be tested for specific leishmanial antigens, such as the FML antigen that was shown to be more effective when administered in combination with saponin, rather than incomplete Freund's adjuvant, aluminum hydroxide, or BCG and IL-12, in a murine model of visceral leishmaniasis [50]. In vaccination against canine leishmaniasis, the adjuvant selected not only must induce an appropriate immune stimulation, it must also be safe in dogs; saponin induces a selective cellular immune response without side effects, whereas BCG potentiates the establishment of innate and adaptive immune responses [51]. Unfortunately, live BCG has also proved to produce large local reactions after subcutaneous application [43,52]. Muramyl dipeptide is another adjuvant which was shown to be harmless and to confer protection in experimentally and naturally infected dogs when co-administered with secreted antigens purified from culture supernatants of *L. infantum* promastigotes (LiESAp), which correlated with the early establishment of a strong, long-lasting and predominantly Th1-type response against LiESAp [53,54].

In summary, all of the aspects related to vaccine development have to be evaluated in dog trials in order to obtain evidence of their protective capability. In the following section we will examine the immunological parameters used in dog trials.

**Hallmarks of immunity in vaccine trials for canine leishmaniasis**

Besides tissue parasitism, evaluation of the associated immunological parameters is an essential requisite for understanding the immunogenicity and efficacy elicited by candidate vaccines. Immunogenicity studies have been performed in kennel trials before [42,51,55–59] and after experimental challenge with *L. chagasi* or *L. infantum* promastigotes [38,46,53,60–68]. These studies allowed the pre-selection of a range of immunological features to be applied as biomarkers to determine the immunogenicity of vaccines against canine leishmaniasis, and to establish different approaches to evaluate the efficacy of such vaccines (Box 2). A summary of important biomarkers disclosed by these investigations of vaccine immunogenicity before and after challenge are summarized in Tables 1 and 2.

**Cellular markers**

In vitro PBMC proliferation was the first biomarker used to investigate cellular immune responses in vaccinated dogs [38,42,55–60,63,65–66,68]. This approach offers an important tool in determining the immunological responsiveness to both vaccine and parasite antigens. There is a general consensus that the presence of a positive lymphoproliferative response in vitro might represent a strong immunogenicity biomarker that can be associated with the vaccine efficacy.

In addition, an assessment of the delayed type of hypersensitivity (DTH) response, as determined by leishmanin skin test, has been also been applied to investigate cellular immune responses [61,64–66]. Given its simplicity and its use as a preliminary exploration of the in vivo cell-mediated immunity status, several field trials have indiscriminately employed a positive DTH to identify potential *Leishmania*-exposed dogs (for selection in population studies) or to assess the overall cellular immune response after vaccination or after natural exposure to *Leishmania* parasites [43,69–71]. It is important to note that with the lack of a DTH antigen standardization test in different studies as well as the apparent in vivo complexity of canine immune responses, the DTH test might be inappropriate for use in dogs. According to Reis et al., further investigations are necessary to define the role of the DTH test in natural and experimental *Leishmania* infections and its applications in vaccine trials to canine leishmaniasis [20].

To better characterize the immunogenicity of anti-canine leishmaniasis vaccines, the cellular response profiles elicited are now being investigated by immunophenotyping of circulating leukocytes (ex vivo assays) or antigen-specific immunophenotypic patterns of PBMCs (in vitro assays) through flow cytometry [42,51,57–59,68,71]. The increased frequency of T-cells (CD5+, CD3+, CD4+ and
**Review**

**Table 1. Biomarkers of immunogenicity in vaccine kennel trial before challenge with *L. chagasi* or *L. infantum***

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Immunogenicity biomarkers</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALM+BCG*</td>
<td><strong>in vitro</strong>: CD4+ cell proliferation</td>
<td>[55]</td>
</tr>
<tr>
<td>ALM+Saponin*</td>
<td><strong>ex vivo</strong>: anti-L. infantum antibodies</td>
<td></td>
</tr>
<tr>
<td>LiESAp**</td>
<td><strong>in vitro</strong>: IFN-γ; NO levels; apoptotic amastigotes, leishmanial effect</td>
<td>[56]</td>
</tr>
<tr>
<td>Leishvaccine**</td>
<td><strong>ex vivo</strong>: neutrophils (CD11b+MHC-I, CD11b+MHC-II, CD32, CD18), eosinophils (CD11b, T/B ratio), lymphocytes (CD4 CD18, CD6 CD18, CD32, CD4MHCII, CD8MHCII)</td>
<td>[51]</td>
</tr>
<tr>
<td>Leishmune**</td>
<td><strong>ex vivo</strong>: Neutrophils (CD11b+MHC-I, CD11b+MHC-II, CD32, CD18), eosinophils (CD11b, T/B ratio), lymphocytes (CD4 CD18, CD6 CD18, CD32, CD4CD4MHCI, CD8MHCI)</td>
<td></td>
</tr>
<tr>
<td>Leishvaccine**</td>
<td><strong>ex vivo</strong>: anti-L. chagasi IgG1 and IgG2 (IgG1-IgG2); <strong>in vitro</strong>: IFN-γ T-cell, IFN-γ CD4, IL-4 CD4, IL-4 CD8</td>
<td>[57]</td>
</tr>
<tr>
<td>Leishmune**</td>
<td><strong>ex vivo</strong>: anti-L. chagasi IgG1 and IgG2 (IgG1-IgG2); <strong>in vitro</strong>: IFN-γ T-cell, IFN-γ CD4, NO/monocyte index</td>
<td></td>
</tr>
<tr>
<td>LBSpa*</td>
<td><strong>ex vivo</strong>: anti-L. chagasi IgG1 and IgG2 (IgG1-IgG2); <strong>in vitro</strong>: CD4, CD8, NO index</td>
<td>[42]</td>
</tr>
<tr>
<td>LBSpaSal*</td>
<td><strong>ex vivo</strong>: anti-saliva IgG, IgG1 and IgG2, anti-L. chagasi IgG, IgG1 and IgG2, serum NO levels, lymphocytes (CD5, CD4, CD8, CD21, CD80); monocytosis (CD14); <strong>in vitro</strong>: cell proliferation, CD4, CD8, CD21, NO index</td>
<td>[58]</td>
</tr>
<tr>
<td>WPV*</td>
<td>anti-L. amazonensis and L. chagasi; anti-L. chagasi total IgG, IgG1 and IgG2; Thy-1 T-cell, CD4 T-cell, CD8 T-cell, CD21 B-cell, CD45RA Lymph; cell proliferation (L. chagasi stimulus)</td>
<td>[59]</td>
</tr>
</tbody>
</table>

**Abbreviations:** ALM autoclaved *L. major* promastigotes; Leishvaccine, *L. amazonensis* (IFLA/BR/1967/PBH) plus BCG; LBSpa *L. braziliensis* promastigote protein plus saponin; LBSpaSal *L. braziliensis* promastigote protein plus saponin and *Lusitania longipalpis* saliva; Leishmune, FML antigen (fucose mannose ligand) from *L. donovani* plus saponin; LiESAp vaccine purified naturally excreted-secreted antigens of *L. infantum* (MHOM/MA/67/ITMAP-263 clone 2) promastigotes plus muramyl dipeptide (MDP); NO nitric oxide evaluated as nitrite/nitrate; WPV whole parasite vaccine (mix of *L. amazonensis* (IFLA/BR/1967/PBH) and *L. braziliensis* (MCAN/BR/1972/C348 crude extracts) plus BCG.

* Killed Leishmania
* Purified Leishmania fractions or excreted-secreted antigens of Leishmania
* = increase in the biomarker levels when compared to controls groups
* = decrease in the biomarker levels when compared to controls groups

CD8+ and CD21+ B-cells, in addition to Leishmania-specific CD8+ T-cells and CD21+ B-cells are now seen as markers of protective immune responses in cases where anti-canine leishmaniasis vaccination has proved effective [42,51,71].

Another cellular marker for the assessment of anti-leishmanial activity is the determination of the macrophage killing ability. It has been shown that the Leishmania killing capacities of macrophages co-cultured with autologous lymphocytes derived from LiESAp-vaccinated dogs is significantly higher than that found in macrophages from placebo injected dogs [54,56].

**Molecular markers**

More recently, analyses measuring cytokines (IFN-γ, IL-12, IL-4, IL-10) in supernatants by ELISA [53,56,67] or mRNA expression in *Leishmania*-stimulated PBMCs by real time qRT-PCR [63-65,68] have also been used to identify immunological patterns in vaccinated dogs before and after experimental challenge. These studies showed that IFN-γ is a high-quality biomarker of immunogenicity and protection against *Leishmania*.

To further evaluate the impact of dog immunization on the T-cell cytokine patterns that are elicited, the recent characterization of both IFN-γ and IL-4 production in PBMCs in dogs (as well as their major T-cell subsets CD4+ and CD8+) as a consequence of anti-canine leishmaniasis vaccination has provided an innovative approach for immunogenicity vaccine studies [57]. This study further emphasized the importance of T-cell-derived IFN-γ as a valuable hallmark of immunity. In addition to identifying the cellular source of IFN-γ, precise determination by ELSpot analysis of the frequency of IFN-γ-secreting cells after leishmanial antigen stimulation constitutes substantial progress in the quantitative evaluation of vaccines. This type of assay has shown its utility in determining the immunological status in *Leishmania*-infected humans and macaques [72-73], although it has been scarcely used to evaluate immune response in dogs.

Analyses of nitric oxide (NO) production in the serum or supernatant of *in vitro* PBMC cultures have also been applied as a biomarker for immunity against *Leishmania*. NO levels have been shown to increase in vaccinated dogs, indicating that it might be included as an important immunological feature when assessing the immunogenicity and protection induced by vaccines [42,56-57].

Although production of a humoral immune response does not appear to correlate directly with protection derived from vaccination, some studies have proposed the analysis of IgG, IgG1 and IgG2 isotypes as additional immune biomarkers. Considering that IgG1 and IgG2 responses are largely T-cell dependent, the evaluation of anti-Leishmania isotypes have been considered as important readouts when assessing the overall immunity [46]. IgG1 and IgG2, which are frequently used as markers in studies of canine leishmaniasis immunopathology or vaccines developed against canine leishmaniasis, probably cannot be associated with resistance or susceptibility, as these polyclonal antisera are not subclass specific [74]. By contrast, an increase of anti-Leishmania total IgG after vaccination is considered crucial for differentiating both immunized dogs and *Leishmania*-infected dogs in endemic areas during governmental control campaigns [75].

**Kennel and field trials**

Kennel trials that include experimental challenge represent a crucial prerequisite for selecting vaccine candidates to be forwarded for clinical and field trials. Nevertheless, these studies, in which the experimental inoculum is administered intravenously, have used variable challenge doses, ranging from 5×10^5 to 10^8 *L. chagasi* or *L. infantum* promastigotes [38,46,53,60-68]. Considering
**Table 2. Biomarkers of immunogenicity in vaccine kennel trial after challenge with *L. chagasi* or *L. infantum***

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Experimental challenge</th>
<th>Immunogenicity biomarkers/clinical signs</th>
<th>Tissue parasitism (positivity)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L. braziliensis</strong>&lt;br&gt; sound-disrupted promastigotes + BCG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3x10⁷&lt;br&gt; L. chagasi promastigotes iv/60dai</td>
<td>Before challenge:</td>
<td>BM: vaccinees (1/9) and control (9/10)</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cell proliferation in vaccinees (8/9) and unresponsiveness in controls (10/10); After challenge: IFAT positive in vaccinees (1/9) and controls (9/10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Q + BCG&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td>5x10⁷&lt;br&gt; L. infantum promastigotes iv/66dai</td>
<td>After challenge:</td>
<td>LN: vaccinees (5/10-150dpi, 3/10 - 450dpi, 1/10 - 634 dpi) and controls (10/10-150dpi, 9/10 - 450dpi, 9/10 - 634 dpi); spleen: negative in all vaccinees and controls (3/10)</td>
<td>[61]</td>
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<tr>
<td></td>
<td></td>
<td>anti-Leishmania IgG in all vaccinees (10/10) and controls (0/10); DTH positive in vaccinees (8/10) and controls (1/10)</td>
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<tr>
<td><strong>LESAp&lt;sup&gt;b&lt;/sup&gt;</strong></td>
<td>10⁸&lt;br&gt; L. infantum promastigotes iv/240dai</td>
<td>Before and after challenge:</td>
<td>BM: LESAp (1/3 - 60g, 0/3 - 100g, 0/3 - 200g) and controls (3/3)</td>
<td>[53]</td>
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<tr>
<td></td>
<td></td>
<td>IgG2; LESAp-specific PBMC proliferation; in vitro leishmanicidal effect; NO levels and IFN-γ in vaccinees</td>
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<tr>
<td></td>
<td></td>
<td>Absence of clinical signs in vaccinees</td>
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<td><strong>H1&lt;sup&gt;c&lt;/sup&gt;; HASPB1&lt;sup&gt;c&lt;/sup&gt;; H1&lt;sup&gt;d&lt;/sup&gt;; MML&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td>10⁸&lt;br&gt; L. infantum promastigotes iv/45dai</td>
<td>Before and challenge:</td>
<td>BM (culture, PCR): HASPB1 (3/8, 5/8); H1 (3/8, 3/8); HASPB1+H1 (5/8, 6/8); MML (3/7, 4/7) and controls (3/8, 2/8); LN (culture, PCR): HASPB1 (6/8, 5/8); H1 (4/8, 5/8); HASPB1+H1 (3/8, 5/8); MML (5/7, 5/7) and controls (5/8, 2/8)</td>
<td>[38]</td>
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<td>specific IgG in vaccinees</td>
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<td>After challenge:</td>
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<td>specific IgG in vaccinees and controls,</td>
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<td>leukocytes, lymphocytes, platelets in vaccinees</td>
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<td>Absent Leishmania-specific cell proliferation before and after challenge</td>
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<td>Presence of clinical signs in, HASPB1 (4/8) H1(3/8); HASPB1+H1 (4/8), MML (5/7) and in controls (6/8)</td>
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<td><strong>A2&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td>5x10⁷&lt;br&gt; L. chagasi promastigotes iv/28dai</td>
<td>Before and after challenge:</td>
<td>BM: vaccinees (4/7) and controls (7/7); peripheral blood PCR: vaccinees (2/7) and controls (5/7)</td>
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<td>anti-A2 IgG and IgG2 in vaccinees</td>
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<td>anti-L. chagasi IgG and IgG2 in infected vaccinees and controls; IFN-γ in infected vaccinees</td>
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<td>Presence of clinical signs in infected vaccinees (2/7) and controls (5/7)</td>
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Abbreviations: A2 purified recombinant protein of *L. donovani* plus saponin; BM bone marrow; dpi days post infection; DTH delayed type hypersensitivity; H1 L. infantum recombinant histone H1 plus MontanideTM ISA 720 (used only in first and second immunization); HASPB1 *L. donovani* hydrophilic acylated surface protein B1 plus Montanide<sup>™</sup> ISA 720 (used only in first and second immunization); IFAT: Anti-Leishmania immunofluorescent antibody test; IV: intravenously route; LESAp vaccine: purified naturally excreted-secreted antigens of *L. infantum* (MHOM/MA/67/ITMAP-263 clone 2) promastigotes plus muramyl dipeptide (MDP); LN popliteous lymph node; MML mixture of TSA LmST11 and LeIF Leishmania antigens plus MPL-SE; NO nitric oxide evaluated by nitrite/nitrate levels; PBMC peripheral blood mononuclear cells; Q + BCG 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 Calmette Guérin);

<sup>a</sup>Killed Leishmania
<sup>b</sup>Purified Leishmania fractions or excreted-secreted antigens of Leishmania
<sup>c</sup>Recombinant antigens
<sup>d</sup>| = increase in the biomarker levels when compared to controls groups
<sup>e</sup>| = decrease in the biomarker levels when compared to controls groups

**Figure 2. Biomarkers of immunogenicity and efficacy in vaccinated dogs.** Different surrogate markers have been identified in dog trial after immunization with effective and ineffective vaccines against canine leishmaniasis and challenge. Effective vaccination should be able to induce a specific response confirmed by increased levels of parameters like PBMCs proliferation after leishmanial antigen stimulation, leishmanicidal activity of macrophages, IFN-γ and TNF-α expression, cytotoxic T cells, NO production, Leishmania-specific IgG2 serum antibodies, or positive leishmanin skin test. Determination of these parameters will also allow attainment of a quantitative confirmation that the grade of protection induced by the vaccine candidate is strong and long-lasting enough to control the parasite in all the vaccinated animals. By contrast, increased levels after challenge of parameters like parasite burden, IL-10 and IL-4 expression, or Leishmania-specific serum antibodies are indicative that the vaccine candidate is ineffective and the immune conditions of the vaccinated animals are non-protected or partially protected, depending on the grade of immunization obtained.
that the systemic route has thus far been the only challenge route used in all these kennel trials, the varying amount of parasites might contribute to the different levels of protection observed. Hence, the future standardization of experimental challenges is both fundamental and necessary for comparative analyses. Whereas using a high intravenous inoculum of promastigotes might be useful as a rapid strategy for efficacy evaluation, it could also mask immunological events important to achieving protection by immunization [38].

In general, *Leishmania* vaccine protective efficacy has been assessed by several approaches, including the isolation of parasites in bone marrow culture, tissues imprints or smears (bone marrow, kidney, liver, lymph node, spleen) and PCR (blood, bone marrow, lymph node) [49,62,46,63,76]. The use of alternative approaches to determine the parasite load is relevant when evaluating distinct levels of vaccine efficacy. Besides conventional methodologies, the application of alternative parasitological investigatory protocols such as the Leishman donovan units (LDU) determined by counting the number of amastigotes forms amongst 1000 nucleated cells and multiplied by the organ weight [16–18,20], anti- *Leishmania* immunohistochemistry [16–18,20] and QRT-PCR [22,77] in different tissues (bone marrow, skin, spleen, liver, lymph node) should also be considered. The use of clinical status evaluation as a foremost tool to access vaccine efficacy should not be recommended, considering the subjectivity and unspecificity of this approach.

The last step to assess the efficacy of a vaccine against canine leishmaniasis is the phase III field trial where vaccinated and control dogs are exposed to natural infection by sand fly bites. In contrast to laboratory experimental challenge, natural infection depends on many variable factors related to the host, the vector and the parasite itself. Recent reports have described field clinical trials evaluating vaccines against canine leishmaniasis [43,54,69–71]. Immunogenicity biomarkers such as *in vivo* DTH, *ex vivo* immunophenotypic patterns (CD5+ T-cells, CD21+ B-cells, CD4+ and CD8+ T-cell subsets) and anti-*Leishmania* IgG profiles have been employed in these studies, and they also allow confirmation that natural infection is able to induce cell-mediated immunity in those animals from the placebo group. Similar to kennel trials, the markers of *Leishmania*-infection resistance in these field clinical trials have included a positive DTH, increased levels of circulating CD8+ T-cells and CD21+ B-cells, as well as *in vitro* IFN-γ and NO production. Future vaccine field trials should include analysis of immunogenicity biomarkers in order to assess not only the efficacy of the vaccine candidate but also to establish differences of nature and quantity between the naturally and the vaccine-induced immunity.

Concluding remarks

These studies confirm that determination of *Leishmania* specific IgG isotype levels, leishmanin skin test, T and B lymphocyte subsets levels, IFN-γ expression, proliferative response to leishmanial antigen, leishmanicidal activity in macrophages and parasite burden all represent valuable, reliable and measurable biomarkers when determining the immunogenicity and protection induced by vaccines against canine leishmaniasis. Currently there are different tools and approaches reviewed in this article (see Box 2) to obtain rational evidence concerning the efficacy of vaccine candidates in laboratory and field trials, and it is desirable, consequently, to use them in future studies to assess and quantify immune correlates of protection. The correspondence between different surrogate markers will confirm a statistically significant level of immunity despite the low number of animals used in dog trials and also a quantitative confirmation that the grade of protection induced by the vaccine candidate is strong and long-lasting enough to control *Leishmania* infection and disease progression (Figure 2). The advances obtained in this field will represent not only a significant step in the control of ZVL, but no doubt will be a decisive step in obtaining a vaccine for human VL.

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Appendix A. Supplementary data


References


Skeiky, Y.A. et al. (2002) Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. Vaccine 20, 3292–3303

Coler, R.N. et al. (2007) Leish-111f, a recombinant polypeptide vaccine that protects against visceral Leishmaniasis by elicitation of CD4+ T cells. Infect. Immun. 75, 4648–4654

Gradoni, L. et al. (2005) Failure of a multi-subunit recombinant leishmanial vaccine (MML) to protect dogs from Leishmania infantum infection and to prevent disease progression in infected animals. Vaccine 23, 5245–5251

Moreno, J. et al. (2007) Immunization with H1, HASPB1 and MML Leishmania proteins in a vaccine trial against experimental canine leishmaniasis. Vaccine 25, 5290–5300

Paraguai de Souza, E. et al. (2001) Vaccination of Balb/c mice against experimental visceral leishmaniasis with the GP36 glycoprotein antigen of Leishmania donovani. Vaccine 19, 3104–3115

Ghosh, A. et al. (2001) Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against Leishmania donovani infections. Vaccine 19, 59–68


Soussi, N. et al. (2002) Effect of intragastric and intraperitoneal immunisation with attenuated and wild-type LACK-expressing Listeria monocytogenes on control of murine Leishmania major infection. Vaccine 20, 2702–2712

Melby, P.C. et al. (2001) Leishmania donovani p36LACK DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. Infect. Immun. 69, 4719–4725


Aguilar-Be, I. et al. (2005) Cross-protective efficacy of a prophylactic Leishmania donovani DNA vaccine against visceral and cutaneous murine leishmaniasis. Infect. Immun. 73, 812–819


Araújo, M.S. et al. (2008) Despite Leishvaccine and Leishmune trigger distinct immune profiles, their ability to activate phagocytes and CD8+ T-cells support their high-quality immunogenic potential against canine visceral leishmaniasis. Vaccine 26, 2211–2224

Poot, J. et al. (2009) Vaccination of dogs with six different candidate leishmaniasis vaccines composed of a chimerical recombinant protein containing ribosomal and histone protein epitopes in combination with different adjuvants. Vaccine 27, 4439–4446


Araújo, M.S. et al. (2009) T-cell-derived cytokines, nitric oxide production by peripheral blood monocytes and seric anti-Leishmania (Leishmania) chagasi IgG subclass patterns following immunization against canine visceral leishmaniasis using Leishvaccine and Leishmune. Vaccine 27, 1008–1017


62 Ramiro, M.J. et al. (2003) Protection in dogs against visceral leishmaniasis caused by *Leishmania infantum* is achieved by immunization with a heterologous prime-boost regime using DNA and vaccinia recombinant vectors expressing LACK. *Vaccine* 21, 2474–2484


64 Poot, J. et al. (2006) Vaccination with a preparation based on recombinant cysteine peptidases and canine IL-12 does not protect dogs from infection with *Leishmania infantum*. *Vaccine* 24, 2460–2468


73 Nylen, S. et al. (2006) Surrogate markers of immunity to *Leishmania major* inleishmanin skin test negative individuals from an endemic area re-visited. *Vaccine* 24, 6944–6954


