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Review Article

Immunology of canine leishmaniasis

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SUMMARY

The role of dogs as the main reservoir of visceral leishmaniasis has led to an increased interest in the immune responses and in Leishmania *antigens implicated in protective cellular immunity in canine visceral leishmaniasis. The primary goal is to control the prevalence of human disease. Immune responses in canine visceral leishmaniasis are reviewed. Cellular immune responses toward a Th1 subset mediated by IFN-γ and TNF-α predominate in asymptomatic dogs exhibiting apparent resistance to visceral leishmaniasis. On the other hand, while the role of Th2 cytokines, such as IL-4 and IL-10, in symptomatic animals is still controversial, there is increasing evidence for a correlation of these cytokines with progressive disease. $CD8^+$ cytotoxic T cells seem also likely to be involved in resistance to visceral leishmaniasis. Several* Leishmania *antigens implicated in protective immune responses are described and some pivotal points for development of an effective vaccine against canine visceral leishmaniasis are discussed.*

Keywords *canine leishmaniasis, immune response,* Leishmania (L.) chagasi*,* Leishmania (L.) infantum*, vaccine*

GENERAL INTRODUCTION

Leishmaniases comprise a group of diseases caused by protozoan parasites of the *Leishmania* genus that include cutaneous, mucocutaneous and visceral leishmaniasis. *Leishmania* spp. are digenetic parasites that develop as promastigotes in the gut of the blood-sucking phlebotomine sandflies and as intracellular amastigotes in macrophages of the vertebrate hosts. Leishmaniases are mainly zoonotic diseases and a wide range of mammals act as reservoirs of *Leishmania* species, particularly rodents, edentates and marsupials in cutaneous leishmaniasis. Wild canines and domestic dogs are the main reservoirs of zoonotic visceral leishmaniasis (VL), a severe disease caused by *Leishmania (L.) infantum* in the Mediterranean area, Middle-East and Asian countries (1) and *Leishmania (L.) chagasi* in Latin America. *L. (L.) infantum* and *L. (L.) chagasi* are now regarded as a single species due to their genotypic relationships within the *L. (L.) donovani* complex (2). VL affects 500 000 people worldwide and there has been a dramatic increase in the number of reported human leishmaniasis cases (3). This increase is mainly due to urban migration from rural areas, which contributes to the spreading of VL in towns and suburbs of large cities, as has occurred in Brazil (4), and to failure of vector and reservoir control. Canine leishmaniasis control is based on the treatment or sacrifice of infected animals. Treatment of canine leishmaniasis shows low efficacy with drugs successfully used for human VL therapy, whereas the sacrifice of infected dogs is often not accepted for ethical and social reasons. Furthermore, elimination of infected dogs has shown contradictory results in Brazil (5). All of these features point to immunoprophylaxy as a promising alternative for prevention of canine VL. For this reason a considerable effort has been dedicated to studies on immune responses in canine VL, and several *Leishmania* antigens implicated in these responses have been characterized.

The present review focuses on immunological aspects of canine VL, emphasizing *Leishmania* antigens able to elicit

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protective immune responses with a potential for vaccine development.

THE IMMUNE RESPONSE IN CANINE VISCERAL LEISHMANIASIS

The outcome of VL in dogs is highly variable. Infected dogs may develop symptomatic infection resulting in death, while others remain asymptomatic, or develop one or more mild symptoms and are classified as oligosymptomatic. The clinical spectrum of progressive VL in dogs includes lymphoadenopathy, anaemia, diarrhoea, alopecia, dermatitis, onychogryphosis, weight loss, cachexia, locomotion problems, conjunctivitis and epistaxis (6). Symptomatic VL in dogs has been associated with immunological changes involving T cells. These changes include absence of delayed type hypersensitivity (DTH) to *Leishmania* antigens (7–9), decreased T cell numbers in peripheral blood (8,10,11), and absence of gamma interferon (IFN-γ) and interleukin-2 (IL-2) production by peripheral blood mononuclear cells (PBMC) *in vitro* (8,12–14). Furthermore, high anti-*Leishmania* antibody titres, which are not immunoprotective, are detected in symptomatic animals (8,11,15). Resistance to canine VL has been associated with activation of Th1 cells producing IFN-γ, IL-2 and TNF-α (8,12). The main effector mechanism involved in protective immune response of dogs infected with *L. (L.) infantum* is the activation of macrophages by IFN-γ and TNF-α to kill intracellular amastigotes via the L-arginine nitric oxide pathway, as has been observed following successful chemotherapy of *L. (L.) infantum*-infected dogs (16). NO production and anti-leishmanial activity were also detected in a canine macrophage cell line infected with *L. (L.) infantum* after incubation with IFN-γ, TNF-α and IL-2 (17), as well as in macrophages from dogs immunized with killed *L. (L.) infantum* promastigotes (18). The role of IL-12 in inducing and maintaining a Th1 type response has been scarcely studied in canine VL. The simultaneous expression of IL-12p40, besides IL-2 and IFN-γ mRNA transcripts, was observed for a short period of time in dogs experimentally infected with *L. (L.) infantum*, indicating that these cytokines are involved in the delay of the disease establishment of these animals (14). IL-12 augmented IFN-γ production by PBMC from dogs with experimental or natural symptomatic VL, making it a likely candidate for cytokine therapy in canine VL (19). IL-12 was also detected in lymph node cells from dogs protected against *L. (L.) infantum* after immunization with DNA and vaccinia recombinant vectors expressing LACK (20). Similar findings with IL-18 have also been reported (21,22).

The role of Th2 type cytokines in canine VL has not yet been defined. Whereas in human *L. (L.) chagasi* infection IL-10 production has been correlated with pathology (23), data on the involvement of this cytokine with active disease in canine VL are controversial. Evidence for Th1 and Th2 mixed responses has been reported in antigen-stimulated PBMC from asymptomatic dogs which displayed IL-2, IFN-γ and IL-10 mRNA transcripts. However, IL-2 and IFN-γ predominated in asymptomatic dogs and the development of symptomatic infections could not be related to IL-10 expression (14,21). IL-10 mRNA transcripts were detected in Con A-stimulated PBMC derived from dogs with clinical signs of VL (13). All these results are in agreement with the experiments in which PBMC obtained from symptomatic VL dogs were induced by a recombinant *L. (L.) chagasi* cysteine proteinase and high levels of IL-10 were detected by an ELISA assay. In contrast, low or undetectable concentrations of this cytokine were found in PBMC supernatants from oligosymptomatic and asymptomatic animals, respectively (24). IL-10 mRNA transcripts were also reported in PBMC from control infected dogs 12 months after challenge with *L. (L.) infantum*, in contrast to uninfected animals vaccinated with cysteine proteinases type I and II (CPB and CPA, respectively) from *L. (L.) infantum*, which showed increased expression of IFN-γ mRNA transcripts (25). Although IL-10 secreted by $CD25^{+}CD4^{+}$-regulatory T cells has been implicated in murine and human leishmaniasis, the involvement of these cells in canine visceral leishmaniasis has not been explored. Expression of IL-4 mRNA was not observed in freshly isolated PBMC from asymptomatic dogs, although this cytokine was detected in asymptomatic dogs stimulated by soluble leishmanial antigen (SLA) (21). In symptomatic dogs the expression of IL-4 mRNA was observed in mitogen-stimulated PBMC (13) and detectable IL-4 mRNA was isolated from bone marrow aspirates of dogs that had more severe symptoms (22). Measurement of IL-4 by ELISA assays in supernatants from canine PBMC stimulated with a *L. (L.) chagasi* recombinant cysteine proteinase showed significant levels of this cytokine in supernatants from symptomatic dogs but not in supernatants from asymptomatic and oligosymptomatic animals (24).

IgG1 and IgG2 subclasses have been used as a more adequate indicator of canine VL status than total IgG (26). A direct correlation between induction of high levels of IgG1 anti-*Leishmania* antibodies and the appearance of clinical symptoms was demonstrated in *L. (L.) infantum*-infected dogs, whereas IgG2 antibodies were associated with asymptomatic infection (27). However, these results were not corroborated by other studies in which dogs with positive DTH skin reactions exhibited a polymorphic humoral immune response ranging from seronegative to positive titre levels of IgG1 or IgG2 (9,28). High levels of

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IgG2 antibody were found in symptomatic dogs (29) and up-regulation of all IgG subclasses was reported in naturally infected Brazilian dogs, although IgG2 was up-regulated to a lesser extent (30). More recent data also showed a high expression of IgE, besides IgG1, in symptomatic dogs from different endemic areas, opening perspectives to their potential use as markers of the active disease (31,32).

Few studies have demonstrated the involvement of $CD8^+$ lymphocytes in resistance to canine VL. These lymphocytes were detected in asymptomatic dogs experimentally infected with *L. (L.) infantum* but not in symptomatic animals, suggesting that direct lysis of *L. (L.) infantum*-infected macrophages by cytotoxic T lymphocytes represents an additional effector mechanism in resistance to VL (12). In dogs naturally infected with *L. (L.) infantum*, a reduction in both $CD4^+$ and $CD8^+$ populations was observed and restoration of these cells occurred after drug treatment (28). Although not directly demonstrated, the involvement of $CD8^+$ in canine VL was suggested by two separate studies in which dogs were immunized either with DNA encoding DNA-LACK or DNA encoding cysteine proteinases CPA and CPB from *L. (L.) infantum* (20,25). In both studies, besides DNA, animals received a booster with recombinant vaccinia virus (rVV) expressing LACK or with recombinant CPA and CPB. Indeed, previous results have shown that $CD8^+$ T cells activated after DNA immunization could be restimulated with a recombinant viral or protein boost (33).

Figure 1 illustrates the main cellular mechanisms involved in resistance or progression of canine VL.

LEISHMANIA ANTIGENS IMPLICATED IN CELLULAR IMMUNE RESPONSES IN CANINE VISCERAL LEISHMANIASIS

Immunological studies have shown that Th1-type responses predominate in resistance to canine VL. Triggering a Th1 response in canine VL seems to depend on many factors, including host genetics, cytokine environment and nature of *Leishmania* antigens. Host genetic involvement in canine VL is supported by the recent demonstration that susceptibility to the disease is associated with polymorphisms of the canine *NRAMP1* gene (34) and MHC class II alleles (35). Implication of host genetic factors in canine VL is also strongly suggested by the Ibizian hound, a canine breed living in an endemic focus of canine VL, which rarely develops clinical VL. This resistance was correlated with a significant cellular immune response against *L. (L.) infantum* infection (9). The cytokine milieu plays a central role in the development of T cells into Th1 or Th2 subsets and IL-12 is required for induction, magnitude and sustaining of Th1 responses (36). In canine VL, IL-12 has been related to the delay of the disease and induction of IFN-γ secretion in dogs with symptomatic VL (14,19). Therefore, a critical question for screening and development of anti-leishmanial vaccines in canine VL is to define *Leishmania* antigens and adjuvant systems that elicit a favourable and sustained cytokine environment *in vivo*. This section summarizes the

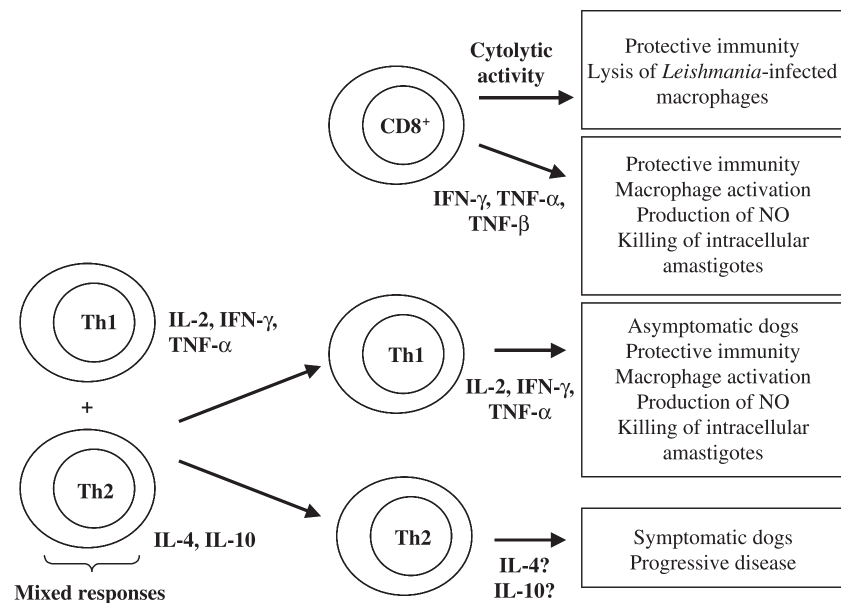

Figure 1 Schematic representation of immune regulation by Th cell types and cytotoxic T cells in canine VL. An early Th1 and Th2 mixed response may either drive toward a Th1 response, which leads to a protective immunity involving the mechanisms shown in the third box from the top or, alternatively, to a Th2 response resulting in disease exacerbation. Cytokines such as IL-12 may trigger Th1 responses and $CD8^+$ T cells exhibit cytolytic activity and may secrete macrophage-activating cytokines.

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Leishmania antigens implicated with cellular immune responses in canine VL with emphasis on vaccine candidates against VL.

Killed *Leishmania*

An autoclaved *L. (L.) major* promastigotes vaccine was used with either BCG or saponin in canine leishmaniasis. Humoral and cellular responses were evaluated in the vaccinated animals. Only dogs that received vaccine with BCG as adjuvant displayed specific and prolonged lymphocyte proliferation and low antibody titres to *L. (L.) infantum* antigen (37). However, the effect of the vaccine on canine protection against *L. (L.) infantum* infection was not evaluated. A vaccine composed of sonicated promastigotes of *L. (V.) braziliensis* treated with merthiolate and BCG was used in phases I and II clinical trials in *L. (L.) chagasi* infection in Brazilian dogs (38). In 90% of the dogs the vaccine induced significant lymphoproliferative responses to *Leishmania* antigens. The immunized dogs were also intravenously challenged with *L. (L.) chagasi* and absence of parasites was demonstrated by necropsy 26 months later. In contrast, all unvaccinated control dogs were unresponsive to *Leishmania* antigens and developed infection after challenge. These encouraging results led to a large phase III clinical trial of the vaccine which, however, did not protect the dogs against VL (39). These discrepant results between experimental and field studies may depend on the small numbers of vaccinated animals used in the experiments compared with the larger numbers involved in field trials. In addition, the difference in parasite infectivity between promastigotes present in axenic cultures and proboscide vector may have played a relevant role in dog infection. Vaccination with three doses of autoclaved *L. (L.) infantum* or *L. (L.) major* plus BCG in dogs before intraperitoneal challenge with *L. (L.) infantum* promastigotes was carried out in Iran (40). All control dogs were negative for the leishmanin skin test (LST) and showed parasite infection 6 months after challenge, as demonstrated by necropsy. In contrast, all vaccinated dogs converted to positive LST and only one of eight was infected. Extension of these tests was carried out in a natural *L. (L.) infantum* infection in a population of 45 vaccinated and 75 unvaccinated dogs living in the same endemic village. The follow-up evaluation was limited to measurement of specific anti-leishmanial antibodies. Seroconversion rates were significantly lower in the vaccinated animals compared to unvaccinated controls; however, the effect of this vaccine on *L. (L.) infantum* canine infection was not evaluated (41). More recently, a single dose of autoclaved *L. (L.) major* adsorbed onto aluminium hydroxide and mixed with BCG was used to vaccinate dogs in a field trial in Iran. The vaccine appeared to be safe and decreased the incidence of VL with an efficacy rate of 69·3%, leading to its further use in multiple doses for evaluation of protection and transmission of VL in vaccinated dogs in Iran (42).

Purified *Leishmania* fractions

Purified *Leishmania* fractions have been tested in vaccination studies in canine VL with different degrees of protection. A semi-purified lyophilized preparation of *L. (L.) infantum* promastigotes, with an apparent molecular weight ranging from 67 to 94 kDa (LiF2), induced humoral responses in vaccinated dogs. These antibodies neutralized the multiplication of *L. (L.) infantum in vitro*, but they were unable to totally block the infection by *L. (L.) major* promastigotes *in vivo* (43). In a second trial, domestic dogs living in a holoendemic area in southern France were immunized with LiF2 and thereafter subjected for 2 years to field *L. (L.) infantum* exposure. Surprisingly, in the first year of follow-up, the rate of both infection and clinical disease was significantly higher in the vaccinated dogs compared to non-vaccinated controls (44). These results strengthened the concept that resistance in VL is mainly linked to cell-mediated immunity.

The fucose mannose ligant (FML), a glycoproteic complex purified from promastigotes of *L. (L.) donovani*, was also tested for vaccination against canine VL. Phase III efficacy trials in a Brazilian endemic area for human and canine VL, using either FML or FML-QuilA, induced 92% and 95% protection, respectively, in naturally exposed dogs after a follow-up of 2 years (45,46). A comparison of the anti-FML antibody subtypes in sera of FML-vaccinated dogs from a non-endemic area with those of naturally infected dogs in an endemic area showed that IgG1 was associated with natural infection, whereas IgG2 was associated with the highly protective FML vaccination (47). The FML vaccine was also effective in immunotherapy against VL of asymptomatic infected dogs (48). More recently, the ability of the vaccine to block the transmission of canine VL was tested in a Brazilian epidemic area of VL. Absence of disease symptoms and of *Leishmania* parasites in blood, skin and lymph nodes was observed in FML-vaccinated dogs. In contrast, untreated controls showed clear evidence of *Leishmania* infection by lymph node and blood PCR, in addition to VL symptoms (49). Overall these results support the protective effect of FML against *L. (L.) chagasi* infection. However, a higher number of dogs would have to be investigated in order to evaluate the efficacy of FML-canine vaccination on human VL transmission in endemic areas.

Secreted antigens purified from culture supernatants of *L. (L.) infantum* promastigotes, *Li*ESAp, were used for

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vaccination of healthy Beagle dogs with muramyl dipeptide (MDP) as the adjuvant; a protective efficacy of 100% was reported after experimental infection. It was also shown that *Li*ESAp-induced protection correlated with an early establishment of a strong and long-lasting predominantly Th1-type response specifically directed against *Li*ESAp (50). These promising results open perspectives to test the ability of the *Li*ESAp to protect dogs in natural conditions of *L. (L.) infantum* infection. Flagellar and microsomal fractions previously used to elicit cell-mediated immunity and acquired resistance to infection with *Leishmania (L.) amazonensis* in mice (51) were shown to induce significant lymphoproliferative responses in dogs naturally infected with *L. (L.) chagasi*. Vaccination tests with these fractions isolated from *L. (L.) chagasi* have been initiated in some Brazilian regions endemic for VL (personal communication).

Recombinant *Leishmania* antigens

Defined recombinant *Leishmania* antigens have also been tested for their potential protection in canine VL. A chimeric multi-component antigenic protein, named Q, formed by the genetic fusion of five fragments from the acidic ribosomal proteins Lip2a, Lip2b, P0 and the histone H2A, previously used for serodiagnosis of canine VL (52), was tested in dogs experimentally infected with *L. (L.) infantum*. The animals received the chimeric protein mixed with live BCG and the data showed 90% protection against experimental infection in the vaccinated dogs (53). A mixture of three recombinant leishmanial antigens (TSA, LeIF and LmSTI1) formulated with two adjuvants, MPL-SE® and AdjuPrime®, was used for dog immunization, followed by infection with *L. (L.) chagasi* promastigotes. Levels of antigen-specific IgG1 and IgG2 antibodies were measured in vaccinated dogs and results showed a predominance of the IgG2 isotype in animals primed with the recombinant antigens plus MPL-SE®. In contrast, animals immunized with the recombinant antigens formulated with AdjuPrime® displayed mixed IgG1/IgG2 isotypes. The protective effect of the two vaccine formulations was not evaluated in immunized dogs (54). More recently, another chimeric antigen, MML, generated from three recombinant *Leishmania* antigens and able to confer protective immunity in a murine model, was used in Italy in a Phase III trial to evaluate the protection of dogs against infection by *L. (L.) infantum*. It was shown that vaccination with MML did not prevent infection and VL progression in dogs exposed to natural transmission in an endemic area. These data strengthen the differences between experimental and field trials in vaccination studies (55).

The antigenicity of *Leishmania* cysteine proteinases for humoral and cellular responses has been demonstrated in humans and dogs (56–58). In canine VL, the humoral and cellular immune responses of naturally infected dogs were evaluated against two recombinant cysteine proteinases from *L. (L.) infantum*, rCPA and rCPB. The data showed a low level of antibodies reactive to rCPA in both symptomatic and asymptomatic dogs. However, high levels of antibody reactive to rCPB (mostly of the IgG2 subclass) were found in asymptomatic dogs compared to symptomatic animals. The PBMC reactivity was low in the presence of rCPA and rCPB, although always higher for PBMC recovered from asymptomatic dogs (58). A recombinant cysteine proteinase from *L. (L.) chagasi* was shown to constitute a suitable immunological marker for several stages of VL in humans and dogs (24). The ability of this recombinant antigen to elicit a Th1 response in asymptomatic dogs suggested its use in new schedules of vaccination against canine VL which are currently in progress in Brazilian zoonotic areas of VL transmission (59).

More recently, interest in genetic immunization in canine VL was aroused because the plasmids used are stable, inexpensive and easily produced with high efficiency. Furthermore, immunization with DNA induces $CD4^+$ helper and $CD8^+$ cytotoxic T-cell responses. Genetic vaccination using a combination of DNA encoding the cysteine proteinases CPA and CPB from *L. (L.) infantum* followed by a booster with rCPA and rCPB was carried out in canine experimental VL in Iran. Vaccinated animals were followed for 12 months and did not exhibit parasites in their bone marrow; their peripheral lymphocytes had high proliferative responses to rCPA and rCPB and elevated IFN-γ mRNA transcripts were detected in PBMC. In contrast, unvaccinated controls presented parasites in their bone marrow, displayed low proliferation responses to *L. (L.) infantum* antigens and increased level of IL-10 mRNA in PBMC. Furthermore, the levels of total IgG and IgG2, but not of IgG1, to rCPA and rCPB, were significantly higher in the vaccinated group than in controls; the DTH responses were strong in vaccinated dogs and absent in controls (25). In another example of genetic immunization in canine VL, prime-boost vaccination was tested by use of a plasmid carrying the gene for the LACK antigen from *L. (L.) infantum*, DNA-LACK, followed by a booster with a recombinant vaccinia virus (rVV) containing the same gene (rVV-LACK) and a challenge with *L. (L.) infantum*. After 17 months of infection, 60% of animals vaccinated by prime-boost with DNA-LACK/rVV-LACK remained free of infection and did not present disease symptoms, whereas dogs immunized with two doses of the DNA-LACK plasmid were not protected from infection. The unvaccinated controls were all clinically affected, showing high levels of parasite load in tissues and high titres of specific anti-*Leishmania* antibodies. Cytokine production, evaluated by mRNA determination in PBMC from immunized dogs, showed immune responses characterized

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Table 1 Summary of vaccination studies in dogs

Antigen	Protection	Reference(s)
Killed *Leishmania*		
Heat-killed *L. (L.) major* promastigotes + BCG	Not evaluated	37
Merthiolate-killed *L. (V.) braziliensis*	Good in phases I and II	38
Merthiolate-killed *L. (V.) braziliensis*	No protection in phase III	39
Heat-killed *L. (L.) major* or *L. (L.) infantum* + BCG	Good in phases I and II	40
Heat-killed *L. (L.) major* or *L. (L.) infantum* + BCG	Not evaluated in phase III	41
Heat-killed *L. (L.) major* + BCG + alum	Good in phase III	42
Purified *Leishmania* fractions		
67–94 kDa *L. (L.) infantum* fraction LiF2	Partial protection	43
67–94 kDa *L. (L.) infantum* fraction LiF2	Disease exacerbation	44
L. (L.) donovani FML-ligand	Good in phase III and immunotherapy	45–49
L. (L.) infantum secreted antigens *Li*ESAp	Good in phases I and II	50
Defined recombinant *Leishmania* antigens		
Chimeric multi-component protein Q + BCG	Good in phases I and II	53
Multi-subunit recombinant leishmanial MML	No protection in phase III	54
rLeIF + rTSA + rLmSTI1 + adjuvant	Not evaluated	55
Antigen-encoding plasmid DNA		
LACK-plasmid	Good in phases I and II	20
Cysteine proteinases type I and II plasmid	Good in phases I and II	25

by an initial Th1/Th2 proliferation and a Th1 final outcome of response, whereas there was a predominance of Th2 responses in unvaccinated controls (20). The promising results observed by use of recombinant cysteine proteinases and LACK, as well as the DNA encoding these antigens, warrant field studies for evaluation of these antigens and their further use as vaccines against canine VL.

A summary of vaccination studies in dogs is given in Table 1.

CONCLUDING REMARKS

There is now a consensus among parasitologists that prevention of canine VL may reduce the prevalence of human VL in endemic areas. As outlined in this review, this approach led to a significant investment in research on the immune responses in dogs naturally and experimentally infected with *L. (L.) infantum/chagasi*. Elucidation of the mechanisms mediating immune responses in canine VL may improve the development of vaccines or strategies for immunotherapy. The characterization of the relevant *Leishmania* antigens implicated in protective responses led to several vaccine candidates that have been tested for their efficacy against VL. However, a critical evaluation of vaccine candidates in dogs points to some parameters that should be considered before a vaccine is routinely used. Most of the antigens that induced significant protection in dogs were tested in animals subjected to a single parasite challenge (38,40). However, in field assays either no significant difference was found between vaccinated and control dogs (39), or rate of infection was higher in the vaccinated animals compared to unvaccinated controls (44). Another point to be considered in testing the efficacy of a vaccine against canine VL is the choice of the method used to estimate the parasite load in the vaccinated animals. Since asymptomatic dogs were also shown to infect the vector (60), *Leishmania* infection in vaccinated animals has to be evaluated by xeno-diagnosis studies and PCR analysis for leishmanial DNA. Recent findings showed a high sensitivity in detection of *L. (L.) infantum* DNA by real-time PCR. These results indicated the usefulness of this method for quantification of *Leishmania* DNA (61,62) over the conventional PCR technique, which failed to detect *Leishmania* DNA in samples with very low or very high numbers of parasites (62).

The FML-vaccine tested in an endemic Brazilian area for both human and canine VL is the first licensed vaccine against canine VL and has shown encouraging results in terms of protection and long lasting immunity (45,46). However, the number of vaccinated dogs should be increased to analyse the impact of FML-canine vaccination on the human VL prevalence before its routine use for prophylaxis in endemic areas. The few genetic immunization schedules tested in canine VL showed good protection against experimental challenge, but have not yet been applied in field trials (20,25).

Two other important parameters have to be taken into account in the evaluation of vaccine candidates in dogs. The first is the need for long follow-up periods after dog

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exposure to field infection. This is necessary because parasite transmission in most endemic areas is seasonal, with higher prevalence in the warm months (63). The second is to consider the occurrence of *Ehrlichia* (54) and the better known helminth co-infections in endemic regions of VL transmission. *Ehrlichia* can down-regulate canine MHC class II receptors (64), whereas helminth infections preferentially elicit Th2 responses. Therefore, the impairment of protective cellular immune responses by these frequent co-infections in endemic areas should be considered in designing vaccines against canine VL.

In conclusion, development of a vaccine against canine VL is obviously a difficult task, and several points that have arisen here are of key relevance for the development of an effective vaccine against canine VL.

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