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Original article

Immunogenicity in dogs of three recombinant antigens (TSA, LeIF and LmSTI1) potential vaccine candidates for canine visceral leishmaniasis

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Abstract – Control of canine visceral leishmaniasis (VL) remains a difficult and serious problem mostly because there is no reliable and effective vaccine available to prevent this disease. A mixture of three recombinant leishmanial antigens (TSA, LeIF and LmSTI1) encoded by three genes highly conserved in the *Leishmania* genus have been shown to induce excellent protection against infection in both murine and simian models of cutaneous leishmaniasis. A human clinical trial with these antigens is currently underway. Because of the high degree of conservation, these antigens might be useful vaccine candidates for VL as well. In the present study, using the dog model of the visceral disease, we evaluated the immunogenicity of these three antigens formulated with two different adjuvants, MPL-SE® and AdjuPrime®. The results were compared with a whole parasite vaccine formulated with BCG as the adjuvant. In order to investigate if sensitization with the recombinant antigens would result in recognition of the corresponding native parasite antigens upon infection, the animals were exposed for four weeks after the termination of the immunization protocol with the recombinant antigens to a low number of *L. chagasi* promastigotes, an etiological agent of VL. Immune response was evaluated by quantitative ELISA in the animal sera before and after exposure

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to the viable parasites. Both antigen specific IgG1 and IgG2 antibody levels were measured. Immunization of dogs with the recombinant antigens formulated in either MPL-SE® or AdjuPrime® resulted in high antibody levels particularly to LmSTI1. In addition, this immunization although to low levels, resulted in the development of antibody response to the whole parasite lysate. Importantly, experimental exposure with low numbers of culture forms of *L. chagasi* promastigotes caused a clear boost in the immune response to both the recombinant antigens and the corresponding native molecules. The boost response was predominantly of the IgG2 isotype in animals primed with the recombinant antigens plus MPL-SE®. In contrast, animals primed with the recombinant antigens formulated in AdjuPrime® as well as animals vaccinated with crude antigen preparation responded with mixed IgG1/IgG2 isotypes. These results point to the possible use of this antigen cocktail formulated with the adjuvant MPL-SE® in efficacy field trials against canine VL.

visceral leishmaniasis / dogs / vaccine / *Leishmania chagasi*

1. INTRODUCTION

Leishmaniasis are a complex group of diseases caused by several intracellular protozoa of the genus *Leishmania* that infect macrophages of a variety of mammals including humans and dogs [13, 37]. Both, tegument (skin and external mucosa) and viscera (primarily liver, spleen, and bone marrow) are the target organs of different species of *Leishmania*. Visceral leishmaniasis (VL) or kala-azar is fatal without treatment. About 500 000 new human cases of VL are registered annually [19]. Moreover, VL has been shown to be a serious opportunistic disease associated to AIDS and other immunocompromised patients [19]. VL is caused by the so-called *Leishmania donovani* complex [24]. Domesticated and wild dogs are the main reservoir of the disease [16]. Because drug treatment of infected dogs is expensive and poorly effective and because there is no effective vaccine for yet ready use despite many trials [5, 12, 18], elimination of seropositive animals has been used in some countries [16] where visceral leishmaniasis is zoonotic as a means to control the human disease.

Several laboratories have been dedicating much effort to the development of a prophylactic anti-leishmania vaccine constituted of killed organisms. Even though some promising early results were achieved against human cutaneous leishmaniasis, these observations were not easily reproduced [1, 2, 23, 25–27, 29, 32, 36]. Vaccination of dogs with a killed preparation of leishmania organisms mixed with the human vaccine BCG (bacille Calmette – Guerin) as the adjuvant results in protection against experimental VL in kennel dogs [28]. However, field trials with this vaccine showed no protection whatsoever [14, 15, 33, 34].

Over the past years, several *Leishmania* recombinant antigens have been identified and demonstrated to have promising vaccine potential to cutaneous leishmaniasis [39, 44–46]. Three highly conserved antigens among the *Leishmania* genus, TSA, LmSTI1 and LeIF, have been shown to induce excellent protection in both the murine and non-human primate models of the human disease [8, 41]. Moreover, vaccination with plasmid DNA encoding these antigens conferred protection against *L. major* infection in BALB/c [9, 30, 31]. A single recombinant polyprotein comprising the sequences of all three recombinant proteins has been produced and tested in BALB/c mice against *L. major* infection formulated with MPL-SE® (Monophosphoryl Lipid A plus squalene) and the results showed excellent protective immunity against the challenge with virulent parasites [41]. This polyprotein is currently being tested in phase I/II human clinical trials against cutaneous leishmaniasis.

Because these three antigens are highly conserved in the *Leishmania* genus it is likely that they may be useful as an anti-VL vaccine as well. In the present studies, we began pre-clinical studies to address this possibility. Prime/boost experiments were

performed in dogs primed with a mixture of TSA, LmSTI1 and LeIF formulated with clinically approved adjuvants (MPL-SE® and AdjuPrime®) followed by boost with intra-venous inoculation of low numbers of viable *Leishmania chagasi* promastigotes (to mimic natural exposure to infection), the etiological agent of visceral leishmaniasis in humans and dogs in the New World.

2. MATERIALS AND METHODS

2.1. Animals and immunizations

Seven groups of dogs were used for the experiments in this study. Thirty-five animals, purpose-bred, parasite naïve, identified by ear tattoo, were selected from a colony of beagles, bed and maintained under conditions designed to exclude any possible contaminating *Leishmania* infections. The dogs were between 8 and 12 months old, well-fed animals under constant supervision by a veterinarian and had all received their routine vaccinations against parvovirosis, distemper, adenovirosis-2, hepatitis, parainfluenza and leptospirosis (Recombitek®, Merial Inc., USA). All animals also received a single intranasal dose against adenovirosis-2, parainfluenza and *Bordetella bronchiseptica* (Bronch Shield III®, Fort Dodge, USA). The dogs were also treated with anti-helminthic drugs (Endal Plus®, Schering Plough, Brazil) and with anti-ectoparasites (Frontline®, Merial Inc., USA), and were quarantined for approximately four weeks before beginning the vaccine trial. One group received a mixture of TSA, LmSTI1 and LeIF (10 μg each) with AdjuPrime® (1 mg, Pierce Chemical, USA) as the adjuvant; a second group was vaccinated with TSA, LmSTI1 and LeIF (10 μg each) and monophosphoryl lipid A (MPL) plus squalene (MPL-SE®) (50 μg, Corixa Co., USA) [41] as an adjuvant; a third group received a whole parasite vaccine (mix of *Leishmania amazonensis* – IFLA/BR/1967/PH8 – and *Leishmania braziliensis* – MCAN/BR/1972/C348 – crude extracts) and non-live lyophilized BCG (FAP, Rio de Janeiro, Brazil) as the adjuvant. The other four groups received AdjuPrime® (1 mg), MPL-SE® (50 μg), BCG (three decreasing doses –400 μg of BCG in the first dose, 300 μg in the second and 200 μg in the last dose) alone or 1 mL of sterile PBS (Phosphate Buffer Saline, pH = 7.2) as a placebo. All vaccines were administered subcutaneously. All animals received three doses of their respective vaccines at intervals of four weeks. The dogs were experimentally boosted intravenously with a low inoculum of 10^6 culture promastigotes of *Leishmania chagasi* (MHOM/BR/1972/BH46) four months after the third dose of the vaccine. The animals were followed up for 15 months and were sacrificed 9 months post-exposure with viable *L. chagasi*. The promastigotes used were obtained from 14 day cultures in NNN/LIT medium of macerated spleens from infected hamsters. The sacrifice was carried out with an overdose of barbiturates (Thionembutal®, Abbot, São Paulo, Brazil). All animals included in this investigation were treated following the guidelines for animal experimentation of the USA National Institute of Health in order to keep animal suffering to a minimum. This work was approved by the Ethical Committee of Animal Research of the Federal University of Minas Gerais, Belo Horizonte, Brazil (Protocol No. 008/02).

2.2. Immunological evaluation

Peripheral blood samples from the jugular vein of the animals were taken before the immunizations (Pre-bleed), after each dose of immunization and every month after exposure to viable *L. chagasi*. Parasite specific antibodies were determined by conventional enzyme-linked immunosorbent assay (ELISA) and by indirect fluorescent assay (IFA). For ELISA the recombinant antigens TSA, LeIF, LmSTI1, rK26 [3] and rK39 [6] and a soluble lysate of *L. chagasi* (SLcA) were used. The antigens were coated onto 96-well microplates (MaxiSorp™, Nalge Nunc Intl., USA) at a concentration of 0.5 μg/well for recombinant

antigens and 10 μg/well for SLcA. The sera were added at a dilution of 1:80 followed by washes and addition of peroxidase conjugated goat anti-dog IgG1 or sheep anti-dog IgG and IgG2 (Bethyl Laboratories Inc., Montgomery, TX, USA). The wells were then washed and substrate and chromogen (O-Phenylenediamine, Sigma-Aldrich Co., USA) were added and the absorbance was read on an automatic ELISA microplate reader (Multiskan® MCC 340, Labsystems, Helsinki, Finland) at 492 nm. The conjugate anti-IgG1 was used at a dilution of 1:1 000 and the conjugates anti-IgG and IgG2 were used at 1:8 000 and 1:16 000 dilutions, respectively. IFA was carried out as described [7] using promastigotes of *Leishmania amazonensis* (MHOM/BR/1960/BH6) maintained by weekly passages in LIT medium. Serial dilutions of the sera were examined until a visual end-point was reached. IFA titers of 1:40 or higher were considered positive as established by The Department of Health of Brazil.

3. RESULTS

3.1. Antibody response to recombinant antigens in vaccinated and control dogs

Serum samples were obtained at the days indicated and they were assayed by ELISA using the recombinant antigens used in the vaccines TSA, LeIF and LmSTI1, and to the soluble *L. chagasi* antigen (SLcA). The results revealed that only dogs immunized with the recombinant antigens formulated either with MPL-SE® or AdjuPrime® produced specific IgG against each of the individual components of the vaccines (Figs. 1A and 1B). No specific IgG against the TSA, LeIF and LmSTI1 was found in the sera from control animals immunized only with adjuvants (data not shown). The absorbance value at 492 nm to LeIF was found to be the lowest of the three components. Antibody titers, particularly to LmSTI1 and TSA, reached maximal values after the third immunization, remained at a plateau for approximately four weeks and then declined steadily for the next six weeks. Moreover, immunization with the recombinant antigens, regardless of the adjuvant used, resulted in the production of low levels of anti-parasite antibodies (Figs. 1C and 1D).

3.2. Booster response to recombinant and native antigens after exposure of dogs to viable *L. chagasi*

One important requirement of successful vaccines to infectious diseases is that exposure to the disease etiological agent boosts the host memory cells generated by the vaccination leading to an effective amplification of effector immune cells. For most vaccines composed of complex mixtures of the microbe's native antigens (e.g. viable attenuated vaccines and killed organisms) such recognition is in general achieved because of the very complexity of the vaccine components. However, the memory cells generated by immunization with highly purified recombinant antigens may not necessarily be re-stimulated in vivo after exposure to the infectious agent because the corresponding native antigens may not be readily available or produced by the microbe in the in vivo milieu.

To more precisely evaluate if challenge with viable leishmania organisms would boost the immune response of dogs primed with the recombinant antigens it was important to determine in advance the kinetics of sensitization caused by the exposure of the animals with a low number of viable promastigote parasites. The dogs were inoculated intravenously with 10^6 culture promastigote of *L. chagasi* and their immune response to parasite specific antigens was monitored over a period of 9 months. Whole parasite lysate antigen as well as K26 and K39 antigens, which are accepted markers of parasite replication in vivo, were used to kinetically follow sensitization. The sera of dogs were obtained monthly after parasite exposure and the antibody response to these antigens was measured by ELISA.

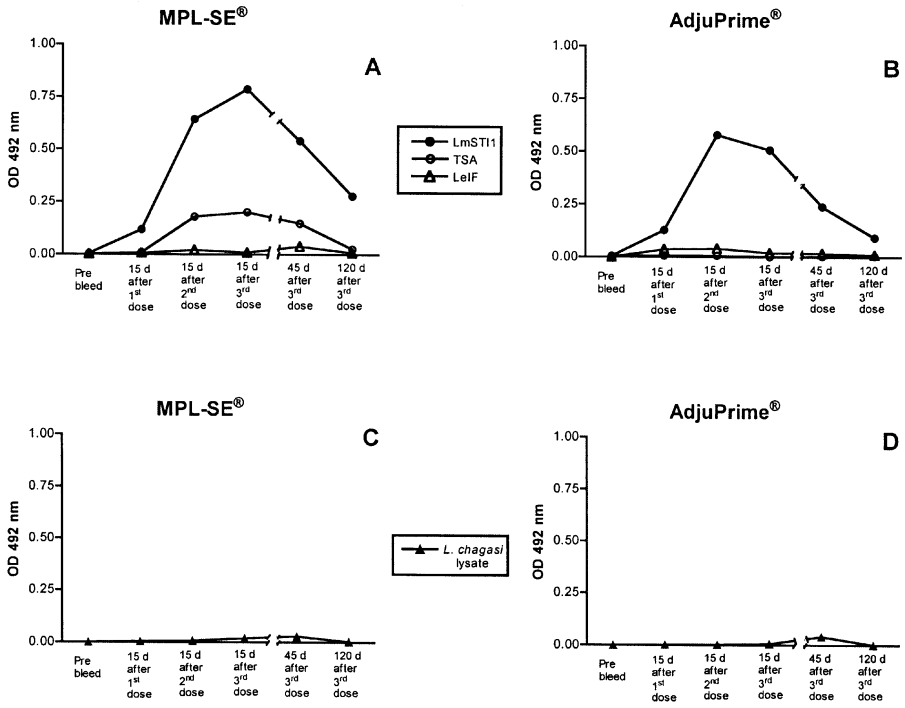

Figure 1. Immune response to recombinant antigens (LmSTI1, TSA and LeIF) and *L. chagasi* lysate after immunization with recombinant antigens formulated with MPL-SE® (**A** and **C**) or AdjuPrime® (**B** and **D**). The results are expressed as the mean average of absorbance in sera from each group ($n = 5$). The y-axis represents the ELISA absorbance values at 492 nm of the sera samples diluted 1:80.

The results are expressed in Figure 2 and indicate that this exposure of dogs to viable *L. chagasi* parasites requires at least three to four months in order to generate a detectable antibody response to crude parasite lysate as well as to the antigens K26 and K39.

Therefore, in order to ascertain that vaccination with the recombinant antigens LmSTI1, TSA, and LeIF resulted in memory cells that would be boosted in vivo by exposure of the animals to viable organisms, dogs previously sensitized with the recombinant antigens were inoculated intravenously with 10^6 culture *L. chagasi* promastigotes and the immune responses to both recombinant antigens and parasite lysate was kinetically evaluated by ELISA. The priming of the dogs with the recombinant antigens formulated with MPL-SE® generates a strong immunological memory to both recombinant antigens (Fig. 3) and parasite lysate (Fig. 4) that is clearly boosted by exposure to viable parasites. These results corroborate the findings from the IFA technique which shows that the dogs immunized with recombinant antigens formulated with MPL-SE® rather than with AdjuPrime® demonstrated higher levels of parasite specific antibodies (Tab. I). Specific low titers for IFA were found positive in control groups at 270 days after boost (data not shown). High levels of specific anti-recombinant

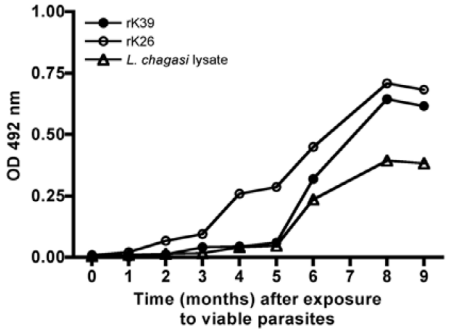

Figure 2. Kinetics of sensitization by low parasite inoculum. The results are expressed as the mean average of absorbance in sera from 40 beagles experimentally inoculated intravenously with 10^6 culture promastigotes of *L. chagasi*. The y-axis represents the ELISA absorbance values at 492 nm. All sera were used in a 1:80 dilution. No sign of infection or symptoms of disease were observed until the end of the study.

antigen antibodies were detected approximately three months previously to the appearance of the antibody to the parasite antigenic markers K26 and K29 and *L. chagasi* lysate in dogs not primed with the recombinant antigens (Fig. 2). In contrast, the boosting effect observed in dogs either primed with the recombinant antigens formulated with AdjuPrime® or previously vaccinated with the whole parasite vaccine was much less evident.

3.3. The phenotype of the humoral immune response to native parasite antigens generated in dogs immunized with recombinant antigens formulated in MPL-SE® and AdjuPrime®

Similarly to resistance to CL, several evidences suggest that resistance to VL is also correlated with the emergence of parasite specific Th1 response and that the Th2 response is non-protective. Therefore it became important to evaluate the anti-parasite phenotype (Th1 × Th2) response

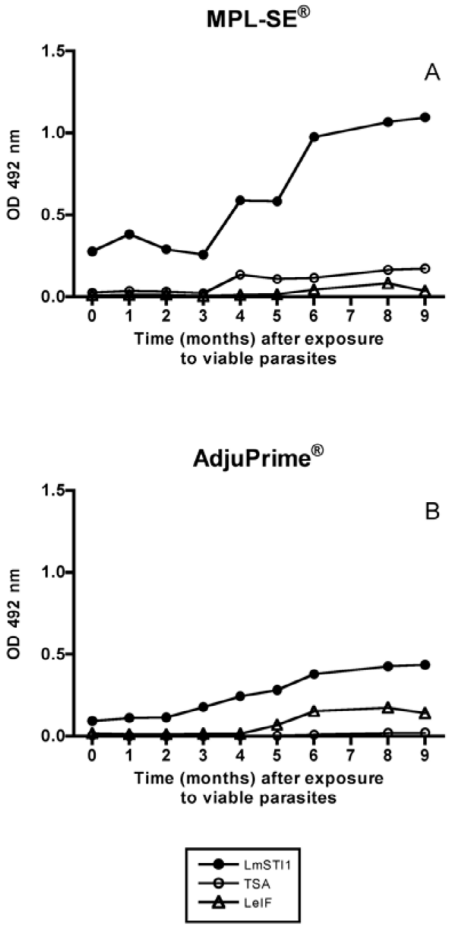

Figure 3. Boost effect in the specific antibody responses against the recombinant antigens caused by exposure to viable parasites (total IgG). The boost effect is represented by the anti-IgG absorbance values at 492 nm for LmSTI1, TSA and LeIF in dogs immunized with those recombinant antigens formulated with MPL-SE® (**A**) or AdjuPrime® (**B**) and experimentally inoculated with 10^6 culture promastigotes of *L. chagasi*, one month after the immunizations. All sera were used at a 1:80 dilution.

induced by vaccination of the dogs with the recombinant antigens formulated with MPL-SE® and AdjuPrime®. Serum samples from vaccinated animals collected before and after exposure to viable *L. chagasi* were

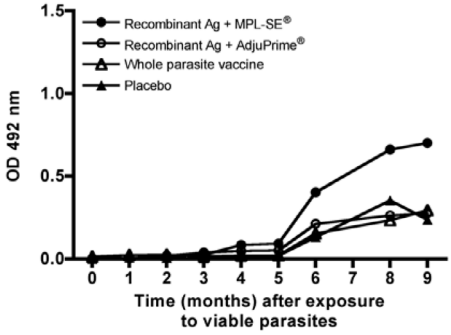

Figure 4. The boost effect caused by infection in animals immunized with recombinant antigens formulated either with MPL-SE® or AdjuPrime® as well as animals immunized with a whole parasite vaccine formulated with BCG. The boost effect is represented by anti-IgG absorbance values for soluble *L. chagasi* antigen at 492 nm in dogs immunized with recombinant antigens (LmSTI1/TSA/LeIF) formulated with MPL-SE® or AdjuPrime®, a whole parasite vaccine (mix of *L. amazonensis* and *L. braziliensis*) or PBS pH = 7.2 (Placebo). The animals from all groups ($n = 5$, each) were inoculated with 10^6 culture promastigotes of *L. chagasi*. The sera were used at a 1:80 dilution.

used in an ELISA format specially designed to detect both IgG1 and IgG2 anti-parasite antibodies. In the murine model, IgG1 and IgG2 isotypes of immunoglobulins are important surrogates of Th2 and Th1 phenotypes of immune responses respectively and the ratio of IgG2/IgG1 during the immune response to a particular antigen has been used as a faithful readout of the phenotype (Th1/Th2) response that is generated to that antigen. Figure 5 shows the results and clearly indicates that the ratio IgG2/IgG1 anti-parasite antibodies was approximately 40 times that in dogs immunized with the recombinant antigens formulated in MPL-SE®. In contrast, vaccination of dogs either with the recombinant antigens formulated in AdjuPrime® or with whole parasite vaccine generated low ratios (≤ 1–6). The dogs from the placebo, AdjuPrime® alone and MPL-SE® alone exposed to viable *L. chagasi* promastigote groups produced undetectable levels of both IgG1 and IgG2 anti-parasite antibodies.

4. DISCUSSION

Extensive vaccination trials in Brazil and Ecuador against cutaneous leishmaniasis in humans have demonstrated that a cocktail of five killed *Leishmania* stocks or a single strain of *L. amazonensis* induces protection from natural infection [1, 13, 23, 32]. However, to date, the protection efficacy of these or other vaccines in dogs

Table I. Antibody titers obtained by IFA[a].

Groups	Animal	0 days after boost	90 days after boost	180 days after boost	270 days after boost
MPL-SE® +	6	< 1:40	< 1:40	**1:160**	**1:80**
recombinant	9	< 1:40	< 1:40	**1:2560**	**1:10240**
antigens	15	< 1:40	< 1:40	**1:80**	**1:160**
	19	< 1:40	**1:40**	**1:10240**	**1:20480**
	24	< 1:40	< 1:40	< 1:40	< 1:40
AdjuPrime® +	4	< 1:40	**1:80**	**1:160**	**1:160**
recombinant	33	< 1:40	< 1:40	< 1:40	**1:40**
antigens	36	< 1:40	< 1:40	< 1:40	**1:40**
	38	< 1:40	< 1:40	< 1:40	**1:40**
	41	< 1:40	**1:80**	**1:640**	**1:1280**

[a] Positive titers (≥ 1:40) are presented in bold.

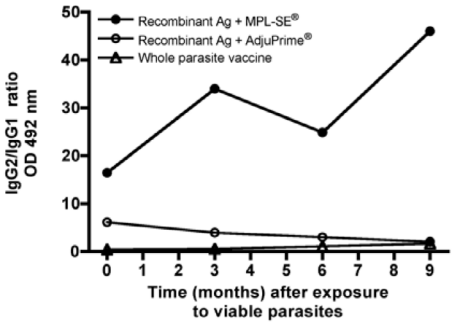

Figure 5. IgG2/IgG1 ratio as indicative of Th1/Th2 immune response after vaccination with recombinant antigens formulated with MPL-SE® or AdjuPrime® as well as vaccination with a whole parasite vaccine formulated with BCG. The results are expressed as the ratio of anti-dog IgG1 and anti-dog IgG2 horseradish-peroxidase conjugated absorbance values at 492 nm of each dog serum (1:80) from animals immunized with recombinant antigens (LmSTI1/TSA/LeIF) formulated with MPL-SE® or AdjuPrime® or immunized with a whole parasite vaccine (mix of *L. amazonensis* and *L. braziliensis*). All animals were experimentally infected with a low inoculum (10^6 promastigotes) of viable parasites of *L. chagasi*. Goat anti-IgG1 and anti-IgG2 heavy chain specific antibodies were used at dilutions 1:1 000 and 1:16 000, respectively.

against visceral leishmaniasis is highly controversial [15, 28, 33, 34]. Therefore several efforts have been dedicated to the development of such vaccines.

Here, we began the evaluation of a mixture of the recombinant antigens TSA, LmSTI1 and LeIF as candidate vaccine for VL. These antigens have been successfully shown to induce an excellent protection against cutaneous leishmaniasis in the murine and non-human primate models [9, 30, 41] and currently are been tested in Phase I/II human vaccine clinical trials. Because these antigens are highly conserved among the *Leishmania* species and are expressed in both the amastigote and the promastigote forms of the parasites [39, 40, 46], they could be useful as a component of a pan-*Leishmania* vaccine [20]. Despite the possibility that a single antigen could by itself induce good protection, we chose to test a mixture of these three proteins because a cocktail of several antigens is conceivably a better vaccine for both prophylactic and therapeutic applications because a vaccine containing a broader range of protective epitopes is unlikely to suffer from MHC related unresponsiveness in a heterogeneous population. Moreover, the use of several antigens can potentially decrease the effects of the selective pressure on the parasite to modify multiple genes [41].

The primary aim of the current studies was the evaluation of the immunogenicity in dogs of the recombinant antigens formulated in two commercially available adjuvants. These studies were primarily concentrated in determining two key immunological aspects of an anti-leishmania vaccine: first, to investigate if the recombinant antigens could prime an immune response that was boosted by viable *L. chagasi*. Second, to evaluate the phenotype (Th1 × Th2) of the immune response that is generated by the two vaccine formulations. These two aspects of the immune response are critical because, as for any effective anti-microbial vaccine, the immune response needs to be boosted by infection or exposure to the infectious agent. In view of the fact that the current studies are testing recombinant antigens, this crucial pre-requisite in vaccine development was imperative to be investigated because an immune response to recombinant antigens not necessarily recognizes the corresponding native antigens. Moreover, since immunity to VL is primarily mediated by Th1 cells [17, 35], the evaluation of the Th1/Th2 paradigm induced by a vaccine candidate is also a critical element to be investigated. The current studies were not aimed at investigating any protective effect of the two vaccine formulations because protection studies against VL in kenneled dogs have been a controversial issue mostly because the results obtained from this kind of experiment are not necessarily translated into the outcome of vaccine trials with unconfined dogs [15, 28].

The choice of the adjuvants tested in the current study was based on reports of the literature indicating that they have been previously shown to work in anti-leishmania vaccine experiments and that they were commercially available and licensed to be used in humans and/or animals. Both AdjuPrime® and MPL-SE® fulfill these criteria [10, 21, 22, 42, 43]. Immunogenicity of the recombinant antigens was measured primarily by ELISA specifically designed to detect canine antibodies of IgG isotypes specific for the recombinant antigens. Unfortunately, a direct evaluation of the T cell response to investigate the pattern of cytokine production upon stimulation with antigens could not be performed because canine reagents necessary for these assays were not commercially available. However, because IgG1 and IgG2 responses are strictly T cell dependent we used them as readouts to evaluate the overall immunogenicity of the recombinant antigens in dogs. In addition, for humans and mice, IgG1 and IgG2 subtypes have been traditionally used as surrogates of the Th2 and Th1 phenotypes of immune responses respectively. For dogs, although the association between IgG subtypes and the immune response phenotype were not demonstrated yet, experimental evidences indicate that this association should occur [4, 11, 38].

After the immunizations, the anti-recombinant antigens antibody response was readily detected by both LmSTI1 and TSA being stronger in animals immunized with the antigens formulated in MPL-SE® than AdjuPrime®. In contrast the immune response to LeIF, even after three immunizations was only borderline detected in animals immunized with the antigen formulated in MPL-SE® and undetected in animals immunized with the antigens formulated in AdjuPrime®. It is noteworthy that the antibody response to LmSTI1 was much stronger than to the responses to TSA and LeIF, regardless of the adjuvant. These results were consistent with previous observations that indicate that LmSTI1 is a stronger immunogen than TSA and LeIF in both mice and monkeys using MPL-SE® or Alum plus IL-12 as adjuvants [8, 41]. More importantly though was the observation that the immune response generated after immunization of the dogs with the recombinant antigens (regardless of the adjuvants) although at low levels reacted with the parasite lysate and was clearly boosted by exposure of the dogs with viable *L. chagasi*. This recognition is crucial in vaccine development particularly when recombinant proteins constitute the immunizing antigen of a vaccine. It is not rare that the immune response to recombinant antigens, produced for example in *E. coli*, does not recognize the corresponding native molecule produced by the infectious agent. Different pos-translation modifications can lead to altered conformation of the recombinant molecule. This modification may result in the recognition of epitopes in the recombinant antigens that are different from the ones that are normally recognized in the original native molecule. In the present study no such restriction occurred because viable leishmanial organisms readily boosted the immune response to the recombinant antigens. Therefore these results fulfill this important requisite to validate a recombinant antigen as a vaccine candidate.

The second pre-requisite in vaccine development against leishmaniasis i.e. the induction of a predominantly Th1 response may also have been fulfilled with the adjuvant MPL-SE®. By using IgG2 and IgG1 subtypes of IgG response as surrogates of the Th1 and Th2 helper phenotype of immune responses respectively, it was evident that immunization with the recombinant antigens formulated with MPL-SE® resulted in an immune response preponderantly of the IgG2 sub-type (IgG2/IgG1 ratio ≥ 40). This would likely point to a dominant Th1 response to the recombinant antigens. Indeed, this bias towards inducing a Th1 response has also been observed in mice immunized with LmSTI1 mixed with MPL-SE® [41]. In contrast, immunization of the dogs with the

recombinant antigens formulated with Adju-Prime®, or vaccination with a whole parasite vaccine result in higher IgG1 production (IgG2/IgG1 of ≤ 1–6), which suggests a Th2 response or at best, points to a mixed Th1/Th2 response.

In conclusion, given the facts that the recombinant antigens TSA/LmSTI1/LeIF have already been shown to be highly protective against CL in two different animal models of the disease and that the current work demonstrates that immunization of dogs with these antigens formulated with the adjuvant MPL-SE® induces almost exclusively a Th1 response (IgG2/IgG1 ≥ 40), it is reasonable to assume that a combination of TSA/LmSTI1/LeIF with the adjuvant MPL-SE® constitutes a highly attractive vaccine formulation to be used in field trials against canine VL.

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