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Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives

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Visceral leishmaniasis is a major health problem in Latina America, as well as the Mediterranean region of Europe and Asia. We aimed to develop a vaccine against visceral leishmaniasis targeting the intracellular amastigotes, which is the parasite stage that persists throughout infections with *Leishmania* parasites. With this in mind, we identified an amastigote specific antigen (A2) that contains an immunogenic epitope for CD4+ T helper (Th) cells and multiple repetitive units encoding CD8+ cytotoxic T lymphocyte (CTL) epitopes. Vaccine formulations containing the recombinant A2 associated with saponin, alum and IL-12 or expressed by attenuated adenovirus were shown to be protective in mice, dogs and nonhuman-primates. We are currently identifying novel amastigote specific immunogenic proteins that could be aggregated to A2 to further improve the level of vaccine-induced cell-mediated immunity and protection against visceral leishmaniasis.

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Introduction

Leishmaniasis may be best defined as a diverse group of syndromes [1•], caused by several *Leishmania* species [2]. Visceral leishmaniasis (VL), also known as kala-azar, is the most severe and fatal syndrome, if diagnosis and treatment is not promptly established [3]. *Leishmania donovani*, *L. infantum*, *L. chagasi* (synonymous *L. infantum*) and *L. amazonensis* are the major species responsible for VL [4,5]. These species' parasites are able to migrate to visceral organs causing symptoms such as fever, weight loss, splenomegaly, hepatomegaly and anaemia [3].

Leishmaniasis still threatens 350 million worldwide, being one of the most neglected tropical diseases due to the lack of efficient tools and clear criteria for control [6]. Zoonotic VL is an emerging disease in countries around the Mediterranean basin, in the Middle East, and in Latin America [7]. In these areas, besides sylvatic reservoirs, domestic dogs are the principal urban reservoir of *L. infantum* and a significant source of infection for vectors. Currently, only few drugs are available for chemotherapy, presenting high toxicity, variable efficacy, inconvenient treatment schedules and costs. Combined therapies are advocated based on the emergence of drug resistant parasites but these remain costly for the majority of patients [8].

In this context, vaccines for human and canine populations are strongly desirable. Here, we discuss the rational for designing an anti-amastigote against VL, highlighting each of the critical steps: (i) antigen selection; (ii) choice of immunologic adjuvants and live attenuated vectors; (iii) testing in different animal models; (iv) development into a commercial vaccine for canine visceral leishmaniasis; and (v) the efforts to improve the current vaccine towards broaden epitopes and human clinical trials.

Biology and life cycle of leishmania parasites

Leishmania parasites are transmitted to the vertebrate host by sand flies that inject the parasite infective metacyclic promastigote forms under the skin [9]. Promastigotes are engulfed by phagocytic cells either resident or recruited to the wound site. Productive infections are established in macrophages, where promastigotes differentiate to amastigotes and replicate inside the parasitophorous vacuole [10•,11]. Importantly, except for the first hours of

infection, the amastigote is the only parasite stage persisting in the vertebrate host, and thus, need to be kept in check by the host immune response, in order to prevent disease. After macrophage rupture, free amastigotes reach the interstitial fluid or blood flow and are ingested by the female sand fly, during its blood meal. In the sand fly, amastigotes undergo a developmental differentiation to infective and flagellated metacyclic promastigotes. This final form reaches the fly saliva, which is introduced into the host skin at the next blood meal, closing the parasite cycle in nature [9,11].

Immunological correlates with the clinical outcome of visceral leishmaniasis

T cell-mediated (type 1) immune responses are necessary to control *Leishmania* multiplication and dissemination, both in humans and dogs [1•,12•]. Individuals with VL display an impaired ability to produce IFN-γ, allowing parasite multiplication and progressive disease [1•]. While IL-12, IFN-γ and TNF-α, are regarded as crucial for parasite control and resolution of disease, a growing body of evidences indicate an important role of IL-10 and TGF-β in down modulating type 1 responses, resulting in parasite persistence in the host and development of severe forms of both human and canine VL [12–17].

When injected into the skin, *L. donovani* promastigotes first interact with resident cells, including dermal macrophages and dendritic cells (DCs) [10•]. The DCs will then secrete cytokines such as IL-12 or type I IFN that promote the polarization of naive T cells into Th1 effector lymphocytes [18,19]. Natural killer (NK) cells also participate in the innate immune response and control of the parasites, as an early source of IFN-γ and contribute to the development of Th1 cells that produce high levels of IFN-γ and TNF-α [20]. Whereas IFN-γ and TNF-α activate macrophages to generate toxic molecules, radical nitrogen intermediates (RNI) or radical oxygen intermediates (ROI) that destroy *Leishmania* parasites inside macrophages [21]. On the contrary, IL-10 and TGF-β suppress IL-12 production by macrophages and DCs, modulate the development of an adequate Th1 response [15,22,23], preventing resolution of infection.

$CD8^+$ Cytolytic T lymphocytes (CTL) are also clearly essential for the control of primary infections in mouse model of VL. Depletion of this cell subset abrogates the capacity of the host to eliminate amastigotes from the phagocytic cells [24]. Furthermore, CD8+ cells are the predominant population in liver granuloma in the late stages of the infection during resolution of infection [25]. Besides effective clearance of parasites [26], CD8+ cells also correlate with protection following vaccination and cure upon immunotherapy [27–29,30••]. In dogs, increased levels of CD8+ lymphocytes appear to be the major phenotypic feature of asymptomatic disease [31]. These cells have also been associated with cure in VL patients [26].

On the contrary, high titers of parasite-specific IgG positively correlate with symptomatic VL (12,13,17). Indeed, mice lacking B lymphocytes or the Fcγ receptor expressed by macrophages are resistant to infections with *Leishmania* [32,33]. It has been proposed that opsonization will favor parasite uptake, and therefore replication within macrophages. Furthermore, the importance of antibody-opsonized parasites in promoting VL may also be related to the induction IL-10 by macrophages [34]. B cells have also shown to produce IL-10 during VL [35]. Thus, the role of B-cells and IL-10 interfering with parasite/macrophage interaction and augmenting VL severity is an important issue and require further investigation.

Protective immunity and the development of vaccines for visceral leishmaniasis

Based on the immunological correlates of disease severity, as well as experimental infections of mice, an ideal protective immune response induced by vaccination against VL should aim to: (i) induce the production of IL-12 by professional antigen presenting cells (APCs) (i.e. DCs and macrophages); (ii) minimize the production of IL-10 by either T lymphocytes and APCs; (iii) induce a strong and long lasting response mediated by parasite-specific $CD4^+$Th1 lymphocytes that produce high levels of IL-2, IFN-γ and TNF-α; (iv) induce a strong and long lasting response mediated by parasite-specific $CD8^+$CTLs that produce high levels of IFN-γ and TNF-α; and (v) avoid the induction of high levels of parasite-specific antibodies that may favor development of disease. To achieve these aims one should carefully select the antigen that has both $CD4^+$ T and $CD8^+$ T cell epitopes, the immunological adjuvant or live attenuated vector, the site of immunization as well as the dose and schedule of immunization.

During the past decades, many *Leishmania* antigens have been identified and tested for vaccine development. A comprehensive compilation of candidate antigens, vaccine formulations and trials may be found in other publications [36–41,42••]. However, even with the most appropriate antigen, another critical issue is how antigens are presented to the immune system. It is generally agreed that adjuvants used in leishmaniasis vaccine formulations should induce type 1 immune responses such as IL-12, saponin, BCG, Monophosphoril Lipid A (MPL) [43,44], CpG and recombinant virus [45–48]. Induction of IL-12 is critical for vaccine efficiency [49] and many of these adjuvants activate the innate immune response via the Toll-like receptors (TLR), modeling the acquired immune responses [50,51]. Heterologous prime-boost immunization protocols involving recombinant virus, DNA or recombinant protein, have been also tested with

various antigens to increase vaccine potency [52–55]. Recently, however, long lasting protective immunity has been associated to induction of antigen specific poly-functional T-cells, which express simultaneously IFN-γ, TNF-α and IL-2 [47]. However, little is known about an immunization protocol that induces long-term memory T lymphocytes that mediate protective immunity.

The rational for an amastigote-specific visceral leishmaniasis vaccine

Amastigote antigens have been far least tested as vaccine candidates against VL. The easiness that promastigotes can be cultured *in vitro*, as opposed to the fact that, for many years, axenic amastigotes could not be cultivated and were obtained only from host tissues, has hampered the identification of stage specific antigens [56]. Thus, most studies on *Leishmania* vaccines have focused on promastigote antigens. However, the amastigotes seem to be the more appropriate target for the immune responses elicited by a vaccine, since after few hours of initial infection and during the active disease, only this parasite stage is present in the host tissues. In addition, in oppose to promastigotes, the amastigotes reside inside host cells, and are targets for $CD8^+$ CTLs, important elements for protective immunity against different species of *Leishmania* sp.

A main argument for an anti-promastigote vaccine is that sterile protection could be achieved by induction of an efficient immune response that eliminates the promastigotes soon after parasite transmission by the sand fly. However, protective immunity induced by promastigote antigens through vaccination is disrupted when challenge is performed through natural sand fly bite [57]. It is proposed that components of sand fly's saliva trigger the release of immunomodulators, for example, IL-10, at the site of parasite transmission, preventing the effective action of parasite specific T cell mediated immunity [11]. Since, these pharmacological active substances from the sand fly act locally and stay only for short periods in the host [58]; they are not likely to affected systemic anti-amastigote immune responses that will act at the later stages of infection. Importantly, when infection with *Leishmania* parasites is subclinical (asymptomatic) and the host become immunocompromised, as in HIV patients, the infection develops in severe disease [3]. In addition, solid protective immunity is achieved by vaccination with live attenuated *Leishmania*, but not with heat killed organisms or parasite lysates [59–61]. Together, these findings indicate the necessity of amastigote persistence in the host for maintenance of protective immunity. Thus, we conclude that a vaccine that elicits immune responses against intracellular amastigotes will have various advantages for both prophylactic and therapeutic vaccines: (i) it will act soon after promastigotes invade macrophages and transform in amastigotes, as well as later at stages of infection; (ii) it will allow the development of an CTL immune response that target intracellular parasites; (iii) it remains efficacious at later stages of infection, despite the initial immunomodulatory effects of sand fly saliva; and (iv) act on parasites that evade initial immune responses and manage to gain the inner compartments of host cells.

Amastigote antigen 2 (A2), the selected antigen for VL vaccine

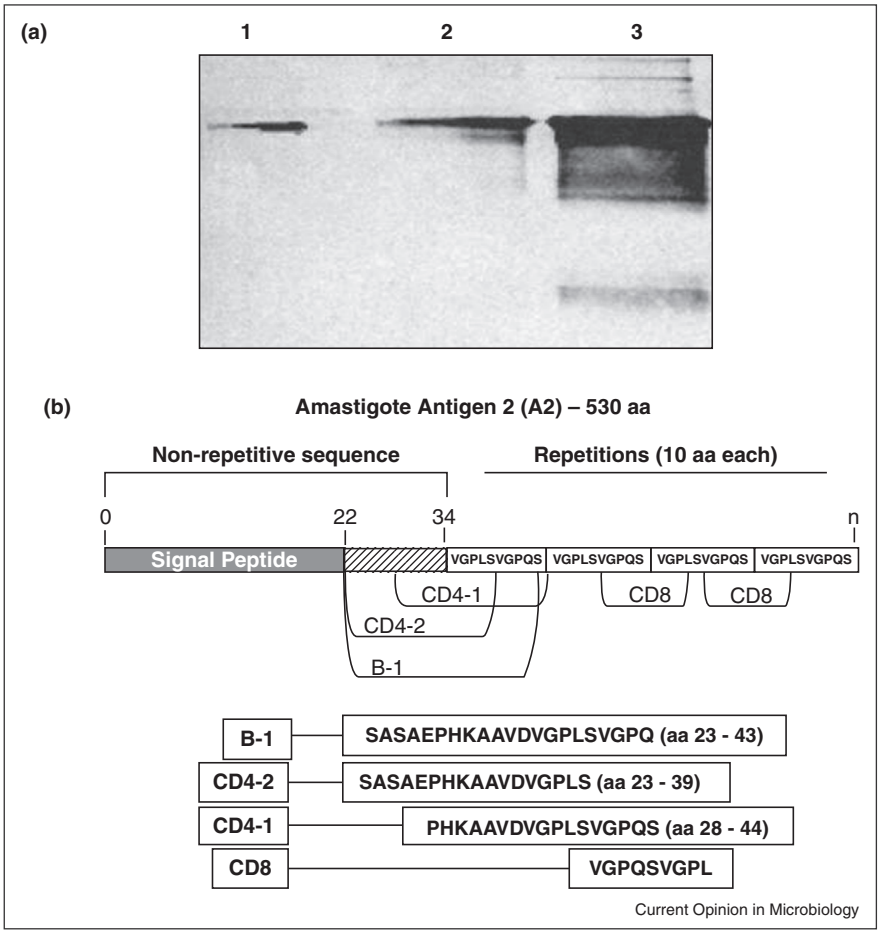
Among the few amastigote antigens tested so far as vaccine against VL, the A2 antigen has emerged as the most promising candidate. Table 1 summarizes the evidence for biological aspects, immunogenicity and protective responses induced by vaccination with A2, which is encoded by a multigene family that are abundantly expressed by amastigotes of leishmania species that cause VL [62–64]. The multigene family encode a set of

Table 1

Biological properties and evidence supporting A2 as a candidate antigen for development of vaccines against visceral leishmaniasis

Properties	Biological evidence	Reference
A2 antigen identification and characterization of biological role	Amastigote specific, species distribution, virulence factor, visceralization of parasite, stress protein	[62–64,65••,66–68]
Immunogenicity	*In silico* epitope prediction; Recognized by antibodies and lymphocytes of mice, dogs and patients with VL	[30••,64,70,72]
Protective responses in pre-clinical tests of vaccine formulations	Significant reduction of symptoms and parasite burdens upon vaccination either with recombinant protein, plasmid DNA, transfected non-pathogenic *L. tarentolae or Lactococcus lactis or* adenovirus expressing A2	[30••,73–78]
Protective immune responses against VL in Rhesus monkeys	Prime boost vaccination protocols consisting adenovirus expressing A2 and the recombinant protein A2 resulted in complete resolution of hepatic granuloma and elimination of parasites in liver	Unpublished results
Protective responses in dogs	Induction of type I protective immune responses in phase II trial in beagle dogs of a recombinant protein formulation	[79•]
Protective responses in dogs induced by a recombinant protein commercial brand (Leish-Tec®)	A randomized placebo controlled phase III trial in an endemic area for VL resulted in 71% of protection against infection based on parasitological tests and 83% among A2 responsive animals	Unpublished results

Figure 1

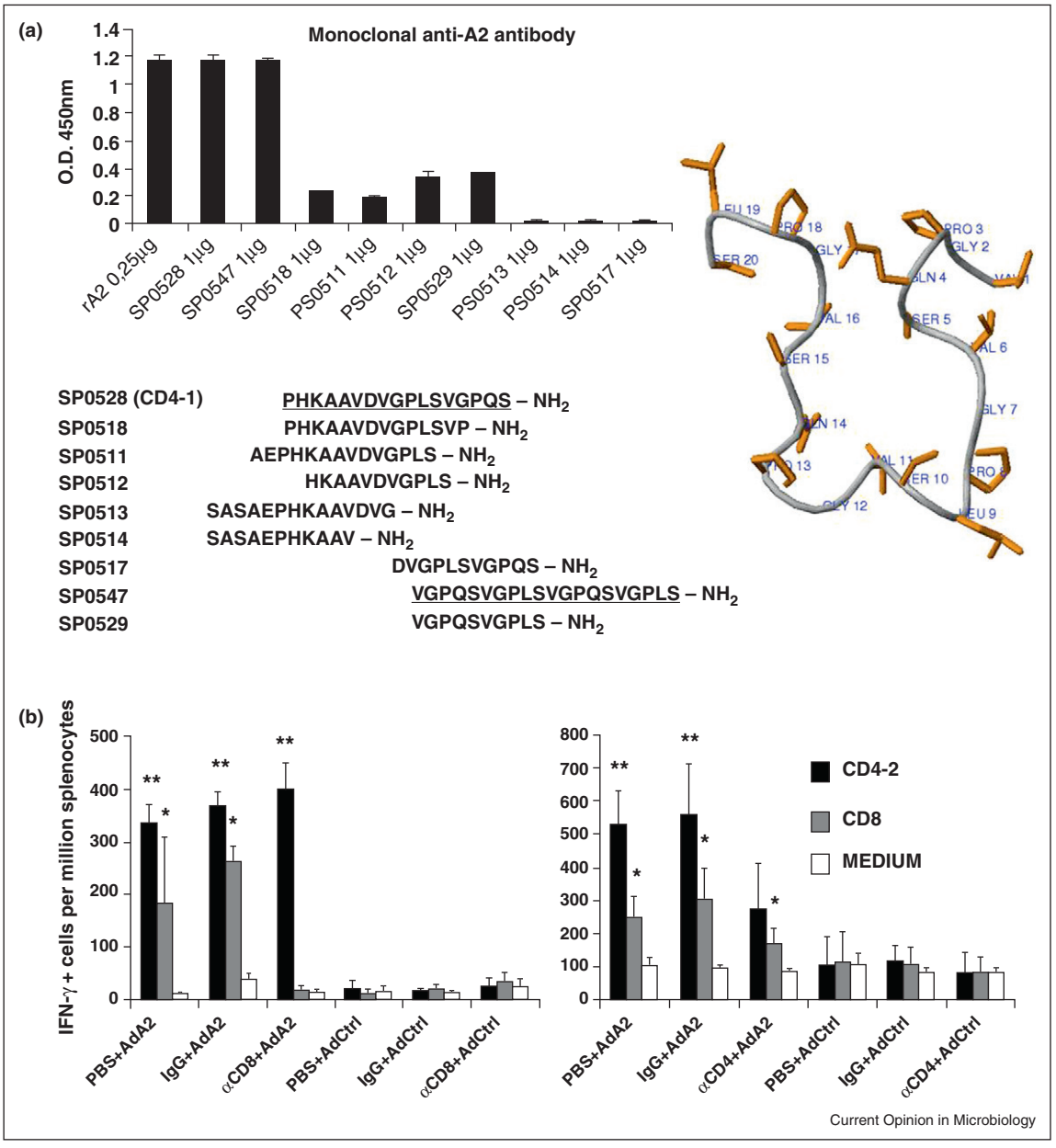


(A) Immunoblot analysis of amastigote extracts showing that besides *L. donovani*, *L. amazonensis* and *L. chagasi* amastigotes also express A2 proteins, as previously published [70]. Total protein lysates of *L. amazonensis* (lane 2) and *L. chagasi* (lane 3) amastigotes cultured at 37 °C and acid pH were submitted to 12% SDS-PAGE and transferred to nitrocellulose membranes. The C9 monoclonal antibody was used to detect the presence of A2 proteins. (License to use this figure – Elsevier license number: 2854920715893.)
(B) Epitope (CD4, CD8 and B cells) virtual mapping of A2, as previously described [30]. (License to use this figure – Elsevier license number: 2854910274786.)

proteins ranging from 45 to 110 kDa, primarily composed of repetitive units of 10 aminoacids. A2 proteins are closely associated with the parasite nuclei during infection with *L. donovani* and partly colocalize with HSP83, a cytosol protein. Moreover, they colocalize very similarly with BiP, a endoplasmic reticulum chaperone protein, suggesting an endoplasmic reticulum localization for A2 [65••]. Furthermore, the A2 is an important virulence factor [66], which is critical in the visceralization process of *L. donovani* [67–69].

In silico analyses revealed two $CD4^+$ T cell epitopes (SASAEPHKAAVDVGPLS/PHKAAVDVGPLSVGPQS) in the N terminal portion of 34 aminoacids that correspond to the non-repetitive segment of A2 [30]. It was also determined that the repetitive unit composed by the VGPQSVGPL motif has high affinity for different MHC I class haplotypes, and TAP, being a potential epitope for $CD8^+$ CTLs (Figure 1B). The main B cell epitope of A2 was mapped and shown to be the secondary structure of a peptide containing two repetitive units of A2 (Figure 2A). The two $CD4^+$ T cell epitopes induced production of IFN-γ in recall response of splenocytes from infected or A2 vaccinated BALB/c mice (Figure 2B). The $CD8^+$ T cell epitopes (repetitive unit) induced, besides production of INF-γ (Figure 2B), cytotoxic activity *in vivo* in mice that were vaccinated with adenovirus expressing A2 [30]. Importantly, the levels of anti-A2 antibodies are higher in asymptomatic animals as compared with the symptomatic ones [70,71], thus indicating a potential role in protective immunity elicited in infection with *L. infantum*. The A2 antigen also contains B

Figure 2



(A) B cell epitope mapping of A2. Shorter peptide sequences based on reactive peptides CD4-1 and B-1 (Figure 1B) and peptides composed of A2 repetitions were tested in ELISA against anti-A2 monoclonal antibody in order to determine the minimal B epitope, as previously described [30]. (License to use this figure – Elsevier license number: 2854910274786.)
(B) T cell epitope mapping of A2, as previously described [30]. ELISPOT assay performed with spleen cells of BALB/c mice submitted to depletion of CD8+ or CD4+ T cells followed by immunization with one dose of adenovirus. Spleen cells were stimulated with the peptides CD4-2 and CD8, described in Figure 1B. (License to use this figure – Elsevier license number: 2854910274786.)

and T cell epitopes recognized by human cells, which is an important requirement for induction of protection against leishmaniasis [64,70,72].

During infection, since A2 contains both CD4 and CD8 epitopes, infected cells present these epitopes on the context of MHC II and MHC I molecules, resulting in activation of $CD4^+$ T as well as $CD8^+$ T lymphocytes, respectively. In the case of an A2 vaccinated host, the CD4 and CD8 A2 epitopes that were presented to immune system during vaccination will induce high production of IFN-gamma from both $CD4^+$ T and $CD8^+$ T lymphocytes, resulting in activation of macrophages that will kill infective parasites. In addition, the A2 CD8

epitope induces *in vivo* CD8 cytotoxic cells that will recognize and also kill infected macrophages.

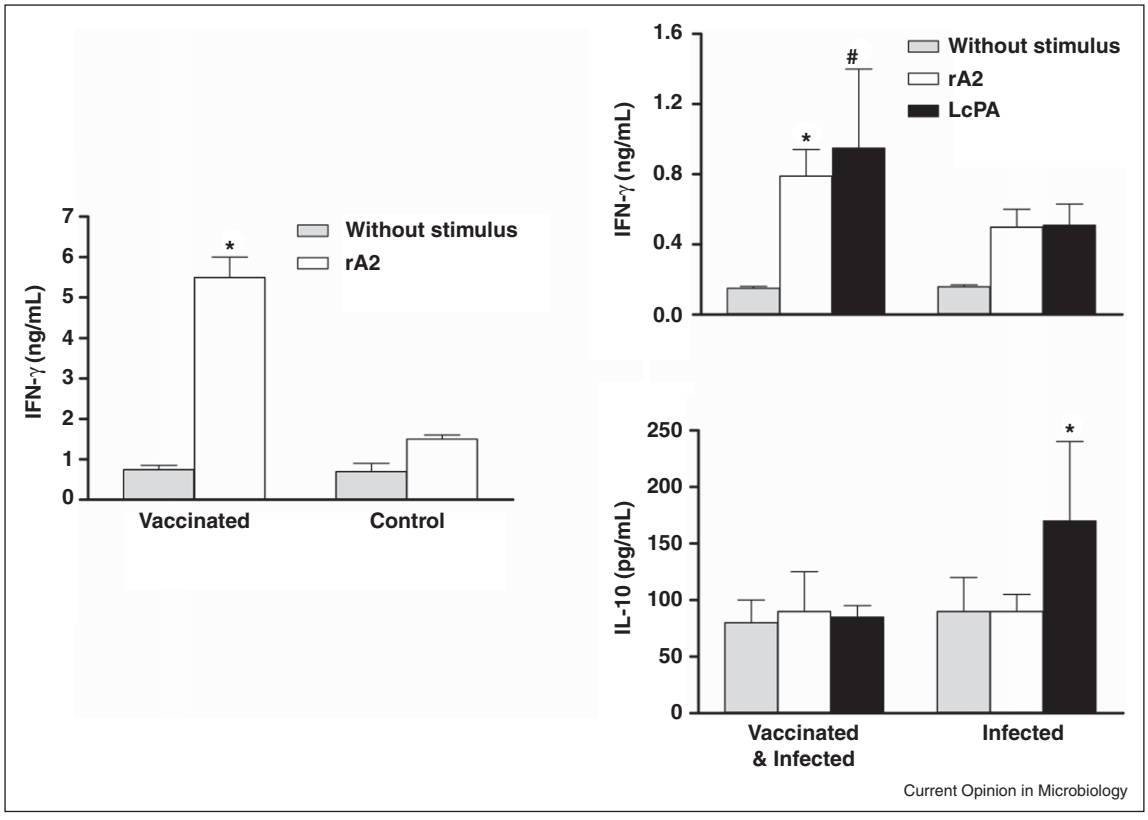
Together, the fact that A2 is (i) conserved among the different species that cause VL, (ii) expressed in high levels by intracellular amastigotes, (iii) encode potent immunogenic CD4+ T cell epitopes, and (iv) in particular expresses at least 30 CD8+ T cell epitopes, makes it a very attractive vaccine candidate for VL. The next major challenge is to define a vaccine formulation, and an immunization protocol that elicits an appropriate immune response that leads to protection against VL.

Formulations of A2 vaccine: aiming induction of a prominent T-cell mediated immunity

Several pre-clinical tests, which included administration of A2 antigens either as recombinant protein associated to different adjuvants [73,74], DNA [75,76], attenuated non-replicative viruses [30], non-pathogenic bacteria [77] or non-virulent parasites (*L. tarentolae*) [78], have provided evidence of the protective effect of vaccination in mice. In general, A2 vaccine induced protective immunity was associated to parasite specific IgG2a antibodies, as well as high levels of IFN-γ and low levels of IL-10 produced by T cells in recall response to parasite antigens. Protective immunity results in reduced lesion size and numbers of parasites in protected animals [73–78]. Given the impressive reductions in parasite burdens at the site of infection and in distant body sites, anti-A2 immune responses may act by promoting clearance of parasites instead of only preventing their dissemination.

A recombinant protein formulation consisting of saponin, as adjuvant and the recombinant protein A2 has also been tested in a phase II trial in dogs [79•]. Vaccinated dogs presented high levels of anti-A2 IgG and IgG2, but not IgG1 antibodies, and high IFN-γ and low IL-10 levels in response to both A2 and total antigenic extracts of parasites. After challenge, 5 out of 7 control animals presented severe symptoms of VL as early as three months, such as bloody diarrhea and intense weight losses. By contrast, 5 out of 7 vaccinated animals remained asymptomatic throughout the tests. The 2 symptomatic animals presented low grade of symptoms, which appeared only one year after the

Figure 3



Immune responses induced by a vaccine formulation containing the recombinant protein A2 and saponin in dogs (phase II trial), as previously described [79•]. Cytokine levels detected in culture supernatants of canine PBMC. The left panel shows IFN-γ levels detected after immunization and before infection in PBMC culture supernatants stimulated with rA2. The right upper panel shows levels of IFN-γ detected in PBMC culture supernatants produced in response to rA2 or LcPA, seven months after infection. The right lower panel shows levels of IL-10 in response to rA2 or LcPA seven months after infection. The asterisks and # indicate that differences are statistically significant ($p < 0.05$). (License to use these figures – Elsevier license number: 2854901184204.)

infection (Figure 3). Importantly, after vaccination, the dogs remained negative in serological tests using promastigote antigens, indicating that vaccination with A2 allows the serology distinction between immunized and infected dogs.

Based on this, a protein formulation (Leish-Tec®) was licensed in Brazil, the third prophylactic vaccine against canine VL, but the first recombinant one commercialized in world. Leish-Tec® was also immunogenic and safe for a heterogeneous dog population. Increased anti-A2 total IgG, IgG2 and IgG1 antibody titers were detected one month after vaccination, in a high percentage of the animals, increasing again after the boosting [manuscript in preparation].

A double-blinded randomized phase III trial was also performed to test the efficacy of Leish-Tec® in an endemic area of VL, located in the city of Porteirinha, Minas Gerais, Brazil. In this study, 1650 healthy dogs were included, following statistical analysis. Dogs were accompanied for follow up period of one year, when 96% of the Leish-Tec® vaccinated dogs remained not infected leading to a significant protective efficacy (71%), based on the recovery of parasites through culture of bone marrow aspirates. Among the animals that presented anti-A2 antibodies in response to Leish-Tec®, 82% of protection has been achieved [manuscript in preparation].

Finally, a pre-clinical trial in Rhesus monkeys was also conducted, aiming to test, through homologous or heterologous prime-boost protocols, the protective responses induced by adenovirus, plasmids expressing A2 or the recombinant A2. In comparison to the control groups, the vaccinated monkeys displayed a significant capacity to control parasite replication. The vaccination with recombinant protein and adenovirus expressing A2 provided the best results, leading to complete granuloma resolution and elimination of parasites in hepatic biopsies, as confirmed by real time PCR analysis. These results are quite promising from the perspective of developing vaccines against human VL, since Rhesus monkeys are one of the closest infection models to human VL [Grimaldi *et al.*, manuscript in preparation].

Efforts to further improve A2 based vaccines

While a large amount of encouraging results have been obtained, employing different vaccine formulations containing the A2, the heterogeneity of MHC molecules is still a major barrier to make a vaccine that protects a vast majority of the target canine and human populations. Thus, it is essential to identify other *L.* (*L.*) *infantum* antigens that could be aggregated to the A2 based vaccine. By employing immunoproteomics our group has identified new promising multi-epitope *Leishmaniaantigens*.

Differential expression of proteins between tissue amastigotes and promastigotes forms of *L. infantum* was analyzed by immunoproteomics [80]. Several proteins were identified by mass spectrometry (MS), including 25 expressed in promastigotes, 5 expressed in amastigotes and 10 proteins expressed in both *Leishmania* stages. Many of these proteins were hypothetical, as previously detected by genomic approaches. In a second study, we compared the reactivity of sera of asymptomatic and symptomatic dogs with proteins expressed by axenic promastigotes and amastigotes [81•]. Among the proteins identified in promastigotes and amastigotes extracts, we found various known and hypothetical proteins that are recognized by either sera of symptomatic or asymptomatic dogs. All these proteins identified by immunoproteomics were screened *in silico* for potential T cell epitopes. We also tested the ability of each peptide/protein to bind to 10 different HLA supertypes, MHC I potential B cell epitope prediction. Altogether, the data obtained disclosed many new antigens that are potential vaccine candidates, which are currently been tested in experimental murine model of VL.

Perspectives

Altogether, the results discussed above constitute a solid base for the development of a vaccine for human VL. Our proposal is to initiate a phase I clinical trial to evaluate safety, immunogenicity of the selected vaccine formulation containing A2. The adenovirus expressing A2 and recombinant A2 associated to adjuvants that are licensed to be used in humans are the natural candidates for the vaccine formulation. However, a continuous search for new adjuvants, live attenuated vaccine vectors, as well as additional immunogenic parasite proteins may further improve efficacy of A2 vaccine.

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