

## REVIEW

# Vaccines to prevent leishmaniasis

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Leishmaniasis is a parasitic disease that encompasses a range of clinical manifestations affecting people in tropical and subtropical regions of the world. Epidemiological and experimental data indicate that protection from disease can be achieved in most people. In addition, we know how the host immune system must respond to infection in order to control parasite growth. However, there is still no vaccine for use in humans. Here, we review our understanding of host immunity following *Leishmania* infection and also discuss recent advances in the development of vaccines to prevent leishmaniasis, highlighting a new promising approach that targets the parasite hemoglobin receptor.

*Clinical & Translational Immunology* (2014) 3, e13; doi:10.1038/cti.2014.4; published online 14 March 2014

**Keywords:** immunity; leishmania; parasites; vaccines

Leishmaniasis is a vector-borne disease caused by obligate, protozoan parasites of the genus *Leishmania*. These parasites are transmitted by 30 different species of *Phlebotomine* sand flies as extracellular, flagellated promastigotes and replicate as intracellular, aflagellate amastigotes in mononuclear phagocytes in mammalian hosts.<sup>1,2</sup> Leishmaniasis ranges from self healing, asymptomatic infection to localized skin lesions, and can develop into a life-threatening progressive visceral form of disease. Leishmaniasis is one of the world's most neglected diseases, affecting mainly very poor people in developing countries. It is prevalent throughout the tropical and subtropical regions of Africa, Asia, the Mediterranean, Southern Europe (old world) and South and Central America (new world). The disease is endemic in 88 countries, of which 72 are developing countries. Approximately 350 million people are at risk of contracting leishmaniasis and 1.5–2 million new cases occur annually.<sup>3</sup> The transmission of *Leishmania* parasites is anthroponotic (human to vector to human) in the Indian subcontinent and Asia, while in Africa, Europe and the Americas, it is zoonotic (animal to vector to human), where dogs and rodents act as reservoir (Figure 1).<sup>4–6</sup>

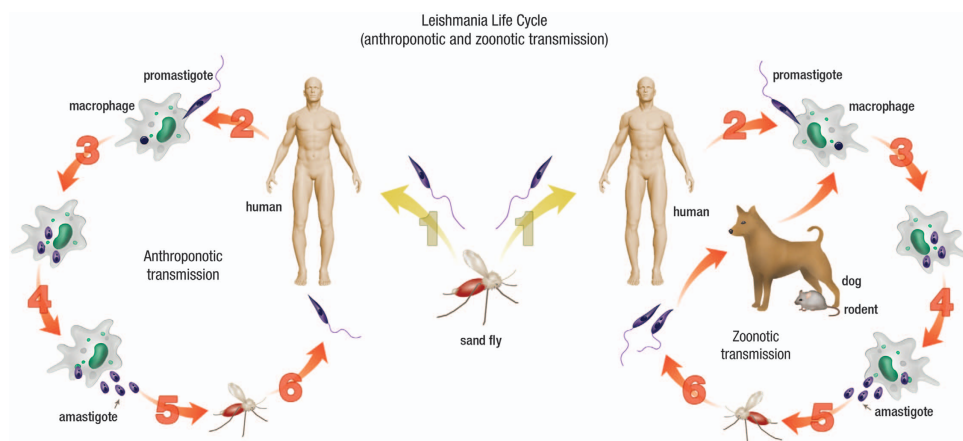
Visceral leishmaniasis (VL), commonly known as kala-azar, is caused by *L. donovani* and *L. infantum* in the Old World and *L. chagasi* in the New World. These parasites preferentially infect macrophages throughout the viscera, and parasites are usually found in the spleen, liver and bone marrow. Clinical features typically include long-term, low-grade fever, hepatosplenomegaly, weight loss, pancytopenia and polyclonal (IgG and IgM) hypergammaglobulinemia.<sup>7</sup> Untreated VL will in most cases ultimately lead to death. Approximately 500 000 new cases of VL occur annually.<sup>7</sup> Most (90%) VL cases occur in five countries, namely; India, Nepal, Bangladesh, Sudan and Brazil.<sup>3</sup> India is one of the most important foci in the world for VL.<sup>3,8</sup> The state of Bihar and neighboring areas of eastern Uttar Pradesh and West Bengal remain particularly badly

affected by VL.<sup>9</sup> The annual incidence of VL in India is approximately 100 000 cases, and the state of Bihar accounts for more than 90% of these.<sup>9</sup> The incidence of VL-human immunodeficiency virus/HIV coinfection is now also a serious concern in these endemic regions.<sup>10</sup>

Cutaneous leishmaniasis (CL) is caused by *L. tropica*, *L. aethiopica* and *L. major* in the Old world, and by *L. mexicana*, *L. guyanensis*, *L. amazonensis* and *L. braziliensis* in the New world. CL is the most common form of leishmaniasis worldwide, representing 50–75% of all new cases. It can be very difficult to treat, long-lasting and disfiguring. According to the World Health Organization (WHO), the number of CL cases is around 1–1.5 million annually, and 90% of CL cases occur in seven countries; Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria.<sup>3,6</sup> CL is characterized by the development of an ulcerative skin lesion, which contains numerous parasites. Although the clinical features of CL can vary because of different causative species, a classical lesion starts as a papule or nodule at the site of parasite inoculation and slowly expands.<sup>6</sup>

Mucocutaneous leishmaniasis (MCL) occurs in the New World and is mainly caused by *L. braziliensis* and *L. panamensis*. These species can metastasize to mucosal tissue in the mouth and upper respiratory tract by lymphatic or haematogenous dissemination, and 90% of all MCL cases occur in Bolivia, Brazil and Peru. MCL can present from several months to years after the development of a cutaneous lesion. Although the pathogenesis of VL and CL is relatively well understood, the pathogenesis of MCL is still unclear, although there has been some recent headway in this area. It is believed that host genetic factors are important for the development of disease.

Control measures for leishmaniasis are heavily dependent on chemotherapy. Currently employed drugs are associated with severe toxic side effects and increasing parasite drug resistance.<sup>11,12</sup> This has forced researchers to think about other control measures, and in particular, the development and implementation of an effective



**Figure 1** Life cycle and transmission of *Leishmania* parasites. The promastigote form of *Leishmania* parasites responsible for human disease (VL, CL and MCL) are injected into the skin as a female sand fly takes a blood meal (1), and are then taken up by host macrophages (2). Promastigotes convert to the non-flagellated, amastigote form inside macrophages (3) and then divide by binary fission (4). The amastigotes are released by the rupture of macrophages (5) and can then be taken up by a female sand fly during another blood meal. The amastigote form then converts in the promastigote form in the midgut of the sand fly and can then again be transmitted to another human (anthroponotic transmission) or to animals that act as reservoirs (zoonotic transmission) (6).

vaccine. People cured of *Leishmania* infections develop lifelong immunity. Therefore, prevention of leishmaniasis through prophylactic vaccination is feasible. Advances in our understanding of *Leishmania* infection pathogenesis and the generation of host-protective immunity, together with completed *Leishmania* genome sequences, has opened new avenues for vaccine research. However, major challenges remain, including the translation of ideas from animal models to clinical settings, and the transition of products from the laboratory to the field. This review will highlight recent advancements in the development of vaccines to prevent and/or treat leishmaniasis, and discuss future prospects.

### PROTECTIVE IMMUNE RESPONSES IN THE HOST

A good understanding of immunity generated against pathogens is important for developing an effective vaccine. Our current understanding of host immune responses generated against *Leishmania* parasites is mainly based on the studies in animal models. Studies in mice show that protective immunity to *Leishmania* infection requires the development of interleukin-12-dependent, parasite-specific Th1 responses, characterized by interferon- $\gamma$  and tumor necrosis factor production by CD4<sup>+</sup> T cells.<sup>13–15</sup> These inflammatory cytokines are required for the generation of reactive oxygen and nitrogen species by infected macrophages that enables killing of intracellular parasites. Recent advances have also been made in understanding immunoregulatory mechanisms that suppress parasite-specific CD4<sup>+</sup> T-cell responses in human VL patients. These include the discovery that interleukin-10 produced by CD4<sup>+</sup> T cells is a potent, autocrine inhibitor of interferon- $\gamma$  production and promotes parasite persistence in spleen tissue from VL patients.<sup>16</sup> Thus, interleukin-10 has been identified as a potential therapeutic target for use in combination with drug therapy or to improve therapeutic vaccine efficacy.

The generation of immunological memory is a requirement of effective vaccination. Studies on the generation of effector and central memory CD4<sup>+</sup> T cells indicate that central memory T cells mediate long-term immunity to *L. major* infection, even in the absence of persistent parasites.<sup>17</sup> Thus, defining the requirements and understanding the conditions for central memory CD4<sup>+</sup> T-cell formation and maintenance will be helpful in vaccine design.

Our knowledge that the majority of individuals infected with *Leishmania* parasites control parasite growth without causing serious disease,<sup>18,19</sup> combined with our understanding about the types of immune responses required for killing parasites and those that suppress this immunity, means that developing vaccines against leishmaniasis is a realistic goal.

### WHY DO WE NEED A VACCINE TO PREVENT AND/OR TREAT LEISHMANIASIS?

Treatment of leishmaniasis is dependent on chemotherapy. The most commonly used drugs are pentavalent antimonials, oral miltefosine, amphotericin B, liposomal amphotericin B and paramomycin. A major problem is that these drugs are associated with problems of cost, toxicity, length and duration of treatment, route of injection (for example, intravenous infusion) and the development of parasite drug resistance.<sup>20</sup> Pentavalent antimonials were the first line of treatment for many years, but increasing parasite resistance in endemic regions has limited their use. In the state of Bihar in India, almost 60% of cases are refractory to treatment with this drug.<sup>21</sup> Consequently, amphotericin B is now used as the main drug to treat VL patients. However, this drug is also associated with toxicity and there are reports of drug-resistant parasites.<sup>22</sup> Miltefosine was developed as an oral drug and showed an early promise; however, there are now increasing incidences of relapse in patients treated with this drug.<sup>23–25</sup> Recently, a single dose of ambisome (lipid formulation of amphotericin B) was shown to be effective in treating VL patients, with a lower incidence of toxicity, compared with conventional treatment, in a multicentre clinical trial.<sup>26</sup> Nevertheless, there are concerns that this type of drug-treatment protocol may promote the development of drug-resistant parasites. Therefore, combination drug therapy is being actively developed for use in endemic regions.<sup>27,28</sup> However, studies in a mouse model suggest that the *L. donovani* can develop resistance to drugs, even when they are used in combination.<sup>29</sup> Therefore, despite advances in chemotherapeutic options, it is unlikely that chemotherapy alone will enable disease elimination, and hence there is an urgent need for an effective vaccine if long-term goals to controlling and eliminating this disease are to be achieved.

## PAST AND PRESENT VACCINE CANDIDATES

Despite different *Leishmania* species causing a range of clinical manifestations, genomic analysis indicates a large degree of sequence homology between species, suggesting it may be possible to generate broadly effective vaccines against different clinical diseases. An effective vaccine against leishmaniasis has existed in the past. This involved inoculation with live, virulent parasites, in a process called leishmanization. It was practiced successfully in the former Soviet Union, Middle East and Israel.<sup>30,31</sup> However, it was abandoned in most countries because of logistical problems and safety concerns, due to some individuals developing non-healing lesions and immune suppression.<sup>32</sup>

Whole-killed (autoclaved) *Leishmania* promastigotes were also tested as vaccines against CL and VL. Testing of killed parasite vaccines took place in Brazil in the early 1940s, and was then tested either alone or in combination with adjuvant in phase-I, II and III trials.<sup>33,34</sup> Clinical trials with autoclaved *Leishmania*, adjuvanted with BCG, showed that this approach could reduce the incidence of CL by 18–78%.<sup>35,36</sup> Similar trials were conducted in Iran, Sudan and Ecuador with variable safety and efficacy.<sup>37–41</sup> Unfortunately, the autoclaved parasites showed decreasing potency with time, although studies with thimerosal preserved and non-autoclaved preparations have shown reduced effects of storage.<sup>42</sup> However, concerns remain regarding the feasibility of developing killed, whole-parasite vaccines, including the variation in results obtained from different field and clinical trial sites in the past, and potential difficulties in producing such a product to good clinical manufacturing standards.

Various attenuated parasites have also been tested in animal models. These parasites are generally taken up by host cells in a similar way to virulent parasites, and persist for some time without replicating. This allows the host to mount robust immune responses against parasite antigens. Radio-attenuated and biochemically altered parasites have proven to confer good protection in mice and hamsters without adjuvant,<sup>43</sup> although concerns regarding conversion back to virulence make the latter option questionable for human use. However, targeted elimination of virulence genes may overcome this problem and could produce attractive vaccine candidates against leishmaniasis. Genetically modified *Leishmania* parasites lacking essential genes like dihydrofolate reductase, bipterin reductase or cysteine proteases have been shown to stimulate protection against challenge with virulent parasite strains.<sup>44–46</sup> The use of drug-sensitive *Leishmania* mutants<sup>47</sup> alone or with adjuvant has been proposed as a mechanism to induce anti-leishmanial immunity, as has the use of non-pathogenic *Leishmania* species like *L. tarantolae*, which can stimulate protection against virulent *L. donovani* strains.<sup>48</sup> However, the main problem with using killed or attenuated parasites are the concerns relating to safety and feasibility for large-scale use in the field.

Other approaches include using immunogenic surface antigens of *Leishmania* parasites as vaccine candidates. Several of these have been tested in mouse models and canine VL with data suggesting that protection against leishmaniasis can be achieved with defined candidate proteins. A saponin formulation of fucose mannose ligand that is expressed throughout the life cycle of parasite, was found to be safe, protective and immunogenic in an experimental mouse and hamster models.<sup>49,50</sup> This formulation has now become the Leishmune veterinary vaccine, licensed after a series of canine VL field studies.<sup>51,52</sup> Lipid formulations of soluble leishmania antigen from *L. donovani* were also tested as vaccine candidates in a hamster model of *L. donovani* infection, and this conferred protection with increased delayed type hypersensitive reactions in response to parasite antigen, enhanced parasite-specific antibody responses and improved parasite-

specific T-cell responses.<sup>53,54</sup> Liposomal soluble leishmania antigen (from *L. major*) incorporated with phosphorothioate CpG ODN (PS CpG) or phosphodiester CpG ODN (PO CpG) has also been tested in a mouse model of CL, and generated significant levels of protection.<sup>55</sup> The excretory/secretory proteins isolated from culture supernatants of *L. infantum* and adjuvanted with muramyl dipeptide were tested in dogs experimentally infected with *L. infantum*.<sup>56</sup> This vaccine, termed LiESAp-MDP, induced significant, long-lasting protection against canine VL in a field trial in an endemic area of France with naturally infected dogs.<sup>57</sup> However, a major hurdle with these fractionated vaccines for human applications is their production to good clinical manufacturing standards, as well as gene variation and polymorphisms in field isolates.

Recombinant proteins, either alone or combined with adjuvant or with bacteria/recombinant virus as a delivery vehicles,<sup>58,59</sup> have also been tested as vaccines in preclinical studies. There have been significant efforts in recent time to identify recombinant antigens that can protect against *Leishmania* infection in experimental models. Some of these antigens include kinetoplastid membrane protein-11,<sup>60,61</sup> sterol 24-c-methyltransferase,<sup>62</sup> amastigote specific protein A2,<sup>63</sup> cysteine proteinase B,<sup>64</sup> *L. braziliensis* elongation and initiation factor,<sup>65</sup> K26/HASPB,<sup>66</sup> *Leishmania*-activated C kinase,<sup>67</sup> promastigote surface antigen 2,<sup>68</sup> nucleoside hydrolase<sup>69</sup> and surface expressed glycoprotein gp63.<sup>70</sup> Although most of these recombinant antigens have been tested in animal models for their immunogenicity and protective efficacy, only a few have progressed to clinical trials in non-human primates, dogs or in preclinical human studies.<sup>71,72</sup> A multisubunit recombinant *Leishmania* vaccine, Leish-111E, containing a *L. major* homolog of eukaryotic thiole-specific antioxidant, *L. major* stress inducible protein-1 and *L. braziliensis* elongation and initiation factor, in formulation with MPL-SE, has been shown to provide protection in mouse models of CL and VL,<sup>73,74</sup> but failed to prevent canine VL caused by natural *L. infantum* infection.<sup>75</sup> Nevertheless, Leish-111E/MPL-SE is the first defined vaccine candidate to progress to human phase-I and phase-II clinical trials in healthy volunteers in South America, CL and ML patients in Brazil and Peru and patients cured of VL in India.<sup>76–79</sup> As with all subunit vaccines, potential problems include variations in immunogenicity, based on human lymphocyte antigen expression in individuals, gene variation and polymorphisms in parasites, as well as the potential to drive selective pressure of parasites away from the molecules used in vaccines.

Finally, DNA vaccines to prevent leishmaniasis are also undergoing development and testing. This approach is not new,<sup>80</sup> but has several advantages, such as low costs of production, stability of materials, sustained expression of relevant antigens and efficient generation of effector and memory immune responses.<sup>81</sup> In addition, more than one antigen can be produced by a single construct. The non-methylated CpG motif of bacterial DNA provides the further advantage of activating innate immune cells to produce interleukin-12, which can prime CD4<sup>+</sup> T cells to develop into Th1 cells.<sup>82</sup> A list of vaccine antigen candidates being tested in DNA vaccines for CL and VL is shown in Table 1.

Therefore, despite many years of effort in identifying immunogenic parasite antigens and advances in vaccine technologies, there does not yet appear to be a vaccine candidate capable of delivering the level of protection needed for a disease elimination program. However, a significant advance was recently described in a study by Guha *et al.*,<sup>83</sup> where they targeted the parasite hemoglobin receptor (HbR) using a DNA vaccine approach and tested it in an experimental model of VL. *Leishmania* parasites require heme for various metabolic activities; however, they lack an endogenous heme synthesis pathway, thus

making them dependent on the host. HbR is expressed on the cell surface of the parasite and is conserved among different species. This receptor is not only important for hemoglobin endocytosis,<sup>84</sup> but also has hexokinase activity, distinct from host hexokinase,<sup>85</sup> which is important for regulating glycolysis. These important properties of parasite HbR led Guha and colleagues to test this molecule as a DNA vaccine candidate.

The success of any vaccine depends on many factors, including the generation of effective antigen-specific antibody responses, the priming and maintenance of parasite-specific T-cell responses and the generation of T cells with appropriate effector functions. Guha *et al.* found that patients with active VL produced reactive antibodies against HbR,

**Table 1** *Leishmania* vaccine antigens being tested as candidate DNA vaccines

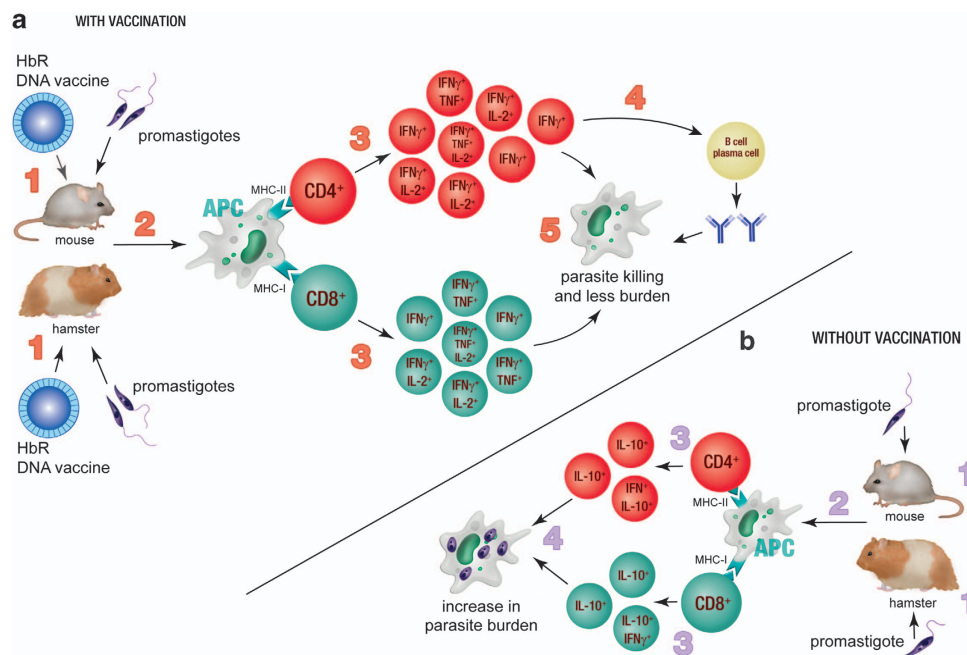
Candidate antigen	Models	Disease	Species	Reference
LACK	Dog, mice	VL, CL	<i>L. donovani</i> , <i>L. chagasi</i> , <i>L. major</i> , <i>L. infantum</i>	86–90
gp63	Mice, dogs	CL, VL	<i>L. major</i> , <i>L. infantum</i>	88,90
KMP11	Mice	CL, VL	<i>L. major</i> , <i>L. donovani</i>	91–93
CPB	Dogs, mice	VL, CL	<i>L. infantum</i> , <i>L. major</i>	94,95
ORFF	Mice	VL	<i>L. donovani</i>	96
NH 36	Mice	VL, CL	<i>L. chagasi</i> , <i>L. maxicana</i>	97,98
TRYP	Dogs	VL	<i>L. infantum</i>	90
PSA-2	Mice	CL	<i>L. major</i>	88

Abbreviations: CPB, cysteine proteinase B; CL, cutaneous leishmaniasis; gp63, glycoprotein 63; KMP11, kinetoplastid membrane protein-11; LACK, *Leishmania*-activated C kinase; NH36, nucleoside hydrolase 36; ORFF, open reading frame F; PSA-2, promastigote surface antigen 2; TRYP, trypanothione peroxidase; VL, visceral leishmaniasis.

and that these antibodies were able to inhibit parasite growth in a complement dependent manner *in vitro*. They also showed that HbR-DNA vaccination of mice stimulated the production of antigen-specific IgG2a antibodies and promoted the generation of antigen-specific T-cell responses that were able to produce multiple Th1-related cytokines simultaneously (that is, a polyfunctional T-cell response). Moreover, immunization with this DNA vaccine enabled sterile cure in hamsters and mice following challenge with virulent *L. donovani* (Figure 2). This is remarkable. These results were obtained in the absence of adjuvant, and thus highlight the potential of HbR as a DNA vaccine candidate for human use. However, further testing, including independent validation of efficacy, must be performed. In addition, and as mentioned earlier, DNA vaccines have shown great promise in animal models, but have not yet proven their utility in humans. There have been no clinical trials beyond phase-II to test DNA vaccines in humans. Thus, a major challenge for DNA vaccine candidates, such as parasite HbR, remains the demonstration of safety and efficacy in humans in both clinical trial and field settings.

## CONCLUDING REMARKS: PROBLEMS AND FUTURE DIRECTION

Vaccination is the most cost-effective way of controlling infectious diseases. The success of vaccine development depends upon understanding the immunobiology of pathogen/host interactions, selection of appropriate vaccine candidates and choosing the right adjuvant or delivery vehicle. In addition, the vaccine must be able to generate long-lasting immunity, the best immune correlates of protection must be identified so vaccine efficacy can be efficiently evaluated and it must be able to transition from preclinical testing to human trials. However, despite a better understanding of immune regulatory



**Figure 2** The effect of HbR-DNA vaccination. (a) Mice and hamsters were vaccinated with the HbR-DNA vaccine before infection with *L. donovani* promastigotes (1). The antigens were presented by antigen-presenting cells (APC) to CD4<sup>+</sup> and CD8<sup>+</sup> T cells in context of MHC-II and MHC-I molecules, respectively (2). This led to the enhanced proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (3) and generation of multifunctional CD4<sup>+</sup> and CD8<sup>+</sup> T cells. CD4<sup>+</sup> T cells enhanced antibody generation by B cells/plasma cells (4). The combined effect of increased multifunctional CD4<sup>+</sup> and CD8<sup>+</sup> T cells and antibody resulted in complete clearance of the parasite (5). (b) Non-vaccinated mice and hamsters were infected with *L. donovani* promastigotes (1). Antigens were presented by APC to CD4<sup>+</sup> and CD8<sup>+</sup> T cells, (2) which resulted in limited T-cell proliferation and the generation of interleukin-10 producing cells (3) leading to enhanced parasite growth (4).



pathways established following infection or vaccination, we still have a limited capacity to modulate these to clinical advantage with available adjuvants or drugs. Ideally, the vaccine should also be effective against all causative agents for a particular disease. This would allow significant saving in product development and testing, which will be an important consideration in future vaccine development programs. The development of a vaccine against leishmaniasis has been slow. However, increased knowledge gained in recent years in all of the above areas is paving the way for renewed efforts to make and test new vaccines aimed at preventing and/or treating leishmaniasis. If funding sources can be identified and commit to the long road of vaccine development, we are confident this is one parasitic disease that can ultimately be controlled.

## ACKNOWLEDGEMENTS

Research in the authors' laboratory is funded by the Australian NHMRC and the Australia-India Strategic Research Fund. We thank Madeleine Flynn for help in preparing both figures.

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