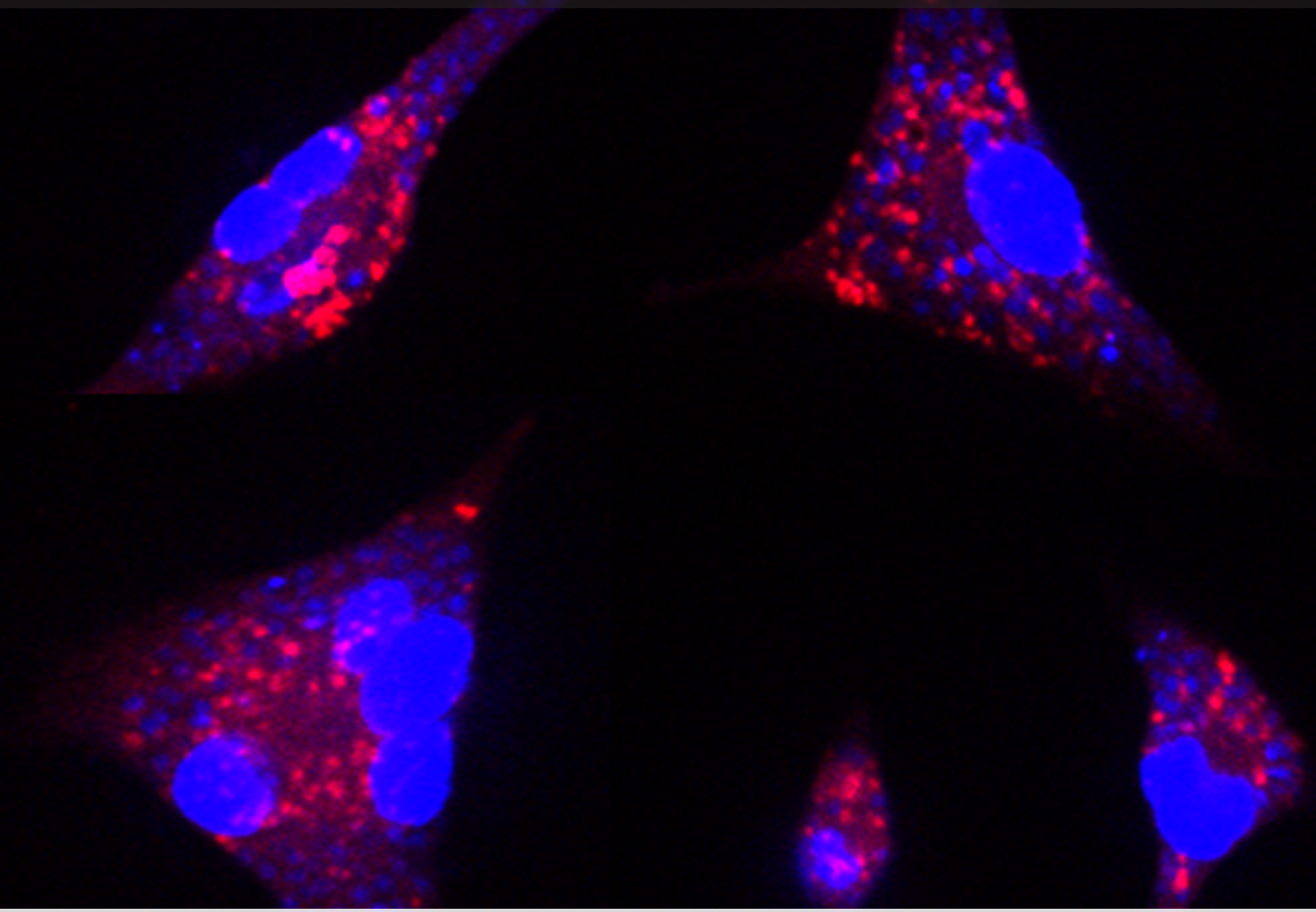
CONTROL OF VISCERAL LEISHMANIASIS BY IMMUNOTHERAPEUTIC AND PROPHYLACTIC STRATEGIES

EDITED BY: Nahid Ali, Hira L. Nakhasi, Jesus G. Valenzuela and Alexandre Barbosa Reis

PUBLISHED IN: Frontiers in Immunology and Frontiers in Public Health



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88919-552-7
DOI 10.3389/978-2-88919-552-7

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

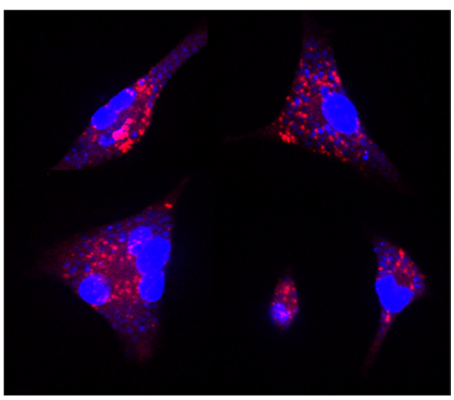
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

CONTROL OF VISCERAL LEISHMANIASIS BY IMMUNOTHERAPEUTIC AND PROPHYLACTIC STRATEGIES

Topic Editors:
Nahid Ali, CSIR-Indian Institute of Chemical Biology, India
Hira L. Nakhasi, US Food and Drug Administration, USA
Jesus G. Valenzuela, National Institute of Allergy and Infectious Diseases, USA
Alexandre Barbosa Reis, Federal University of Ouro Preto, Brazil



Murine peritoneal macrophages infected with promastigotes of Leishmania donovani. The macrophage F-actin cytoskeleton stained in red with Alexa-488 phalloidin. Host and parasite nuclei counterstained with DAPI (blue).

Visceral leishmaniasis (VL) or kala-azar is the most dreadful of all forms of leishmaniasis caused by Leishmania donovani in Old World and Leishmania chagasi and/or Leishmania infantum in New World affecting millions of people worldwide. In active VL, macrophages host the replicating amastigotes in phagolysosomal compartments leading to splenomegaly, hepatomegaly, hyperglobulinemia, anemia, weight-loss, incessant fever and ultimately death if not treated. Treatments available against the disease are limited by increased incidence of resistance, serious side-effects, high cost and long course of treatment. Immuno-chemotherapy is an alternative to overcome the limitations of the drugs against VL. Combination of one or more of immunotherapeutic agents like BCG, Alum, IFN-γ, antigen-pulsed dendritic cells (DC), etc. with chemotherapeutic drugs have been tested raising hopes for a suitable immuno-chemotherapy against VL and Post Kala-azar Dermal Leishmaniasis (PKDL). Antagonists of IL-10, TGF-β, IL-13 have been effectively used with pentavalent antimonials in treatment of experimental VL. Some parasitic antigens and liposomal formulations have also been shown

to impart superior therapeutic effectiveness to antileishmanial drugs. For socio-economic reasons prophylaxis is always more desirable than therapy. Although no vaccine against any form of leishmaniasis in humans is available, patients successfully treated show considerable protection from reinfection highlighting the possibility of developing prophylactic measures against the disease. Subsequently a lot of interest has been focused recently towards developing vaccines against VL and many potential vaccine candidates like whole cell (attenuated or heat killed), crude fractions, purified subunits, DNAs, recombinant proteins, fusion proteins, and genetically modified live attenuated parasites etc. have been reported. These vaccine candidates are either activators of CD4+Th1 cells and/or CD8+ T cells or neutralizers of immuno-suppression. Cationic liposomal formulations, nanoparticle and virosome delivery systems, etc. have been used to increase potency and durability of various vaccine candidates. Immuno-modulators like TLR agonists have been shown to be promising adjuvants in enhancing efficacy and overcoming the challenge of human administrable vaccine formulations. Recently role of sand fly salivary gland proteins as immune-modulators also has been explored. Various strategies such as heterologous prime boosting, targeted antigen delivery, adjuvant mediated protection, have been undertaken. Likewise, precise role of regulatory T cells (Tregs) in VL disease progression needs to be investigated and exploited to develop both immuno-therapeutic and prophylactic methods. A breakthrough in immunotherapy and prophylactic strategy would help in eradication of the parasites from the pool of natural reservoirs namely VL and PKDL patients, asymptomatic carrier individuals and infected dogs ensuring success of global VL control programmes.

Table of Contents

06 ***Targeted immunology for prevention and cure of VL***

Nahid Ali, Hira L. Nakhasi, Jesus G. Valenzuela and Alexandre Barbosa Reis

08 ***Immunotherapy and immunochemotherapy in visceral leishmaniasis: promising treatments for this neglected disease***

Bruno Mendes Roatt, Rodrigo Dian de Oliveira Aguiar-Soares, Wendel Coura-Vital, Henrique Gama Ker, Nádia das Dores Moreira, Juliana Vitoriano-Souza, Rodolfo Cordeiro Giunchetti, Cláudia Martins Carneiro and Alexandre Barbosa Reis

20 ***Immunotherapy and targeted therapies in treatment of visceral leishmaniasis: current status and future prospects***

Om Prakash Singh and Shyam Sundar

29 ***Exploring the role of medicinal plant-based immunomodulators for effective therapy of leishmaniasis***

Garima Chouhan, Mohammad Islamuddin, Dinkar Sahal and Farhat Afrin

36 ***Leishmania donovani nucleoside hydrolase terminal domains in cross-protective immunotherapy against Leishmania amazonensis murine infection***

Dirlei Nico, Daniele Crespo Gomes, Iam Palatnik-de-Sousa, Alexandre Morrot, Marcos Palatnik and Clarisa Beatriz Palatnik-de-Sousa

48 ***Visceral leishmaniasis: advancements in vaccine development via classical and molecular approaches***

Sumit Joshi, Keerti Rawat, Narendra Kumar Yadav, Vikash Kumar, Mohammad Imran Siddiqi and Anuradha Dube

66 ***Leishmania spp. proteome data sets: a comprehensive resource for vaccine development to target visceral leishmaniasis***

Toni Aebischer

74 ***Cross-protective immunity to Leishmania amazonensis is mediated by CD4+ and CD8+ epitopes of Leishmania donovani nucleoside hydrolase terminal domains***

Dirlei Nico, Daniele Crespo Gomes, Marcus Vinícius Alves-Silva, Elisangela Oliveira Freitas, Alexandre Morrot, Diana Bahia, Marcos Palatnik, Mauricio M. Rodrigues and Clarisa B. Palatnik-de-Sousa

84 ***Experimental validation of multi-epitope peptides including promising MHC class I- and II-restricted epitopes of four known Leishmania infantum proteins***

Maria Agallou, Evita Athanasiou, Olga Koutsoni, Eleni Dotsika and Evdokia Karagouni

100 Antigen-pulsed CpG-ODN-activated dendritic cells induce host-protective immune response by regulating the T regulatory cell functioning in Leishmania donovani-infected mice: critical role of CXCL10

Saikat Majumder, Amrita Bhattacharjee, Bidisha Paul Chowdhury, Suchandra Bhattacharyya Majumdar and Subrata Majumdar

109 Vector saliva in vaccines for visceral leishmaniasis: a brief encounter of high consequence?

Shaden Kamhawi, Hamide Aslan and Jesus G. Valenzuela

115 Live vaccination tactics: possible approaches for controlling visceral leishmaniasis

Noushin Saljoughian, Tahareh Taheri and Sima Rafati

126 Genetically modified organisms and visceral leishmaniasis

Rudra Chhajer and Nahid Ali

136 Biomarkers of safety and immune protection for genetically modified live attenuated Leishmania vaccines against visceral leishmaniasis – discovery and implications

Sreenivas Gannavaram, Ranadhir Dey, Kumar Avishek, Angamuthu Selvapandiyan, Poonam Salotra and Hira L. Nakhasi

frontiers in
IMMUNOLOGY

EDITORIAL
published: 19 December 2014
doi: 10.3389/fimmu.2014.00660

Targeted immunology for prevention and cure of VL

***Nahid Ali*[1]*, *Hira L. Nakhasi*[2], *Jesus G. Valenzuela*[3] *and Alexandre Barbosa Reis*[4]**

[1] *Infectious Diseases and Immunology, Indian Institute of Chemical Biology, Kolkata, India*
[2] *US Food and Drug Administration, Silver Spring, MD, USA*
[3] *National Institute of Allergy and Infectious Diseases, Rockville, MD, USA*
[4] *Universidade Federal de Ouro Preto, Ouro Preto, Brazil*
**Correspondence: nali@iicb.res.in*

Edited and reviewed by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Keywords: visceral leishmaniasis, immunotherapy, vaccine, immuno-modulator, Th1 response

Leishmaniasis is a neglected tropical disease caused by a group of protozoan parasites of the genus *Leishmania*. Clinical presentation of leishmaniasis can range from cutaneous, mucocutaneous, or visceral forms depending on the parasite species. Visceral leishmaniasis (VL) caused by *L. donovani* and *L. infantum* is the severest and one of the deadliest parasitic diseases of the tropics second only to malaria (1). Nearly, 20,000–40,000 annual deaths are estimated due to this disease (2). Except for the Indian subcontinent and West Africa, VL is frequent in dogs, which serve as the major reservoir for zoonosis (3).

Transmitted by the bite of an infected sandfly, *Leishmania* endure in the phagolysomal compartment of macrophages by evasion and attenuation of the microbicidal functions of the host (4, 5). *Leishmania* has evolved as a successful parasite chiefly by its ability to modulate the immunological and cyto-chemical responses of the host following infection. The key strategy for successful pathogenesis is to subvert the nitric oxide burst in the host macrophage. This opportunistic parasite thus establishes a safe niche in the inactivated phagocyte and uncontrolled parasitization in liver, spleen, and bone marrow leads to symptomatic VL characterized by fever, weight loss, hepatosplenomegaly, and anemia (3).

Since the pathogenesis of the disease is based on subversion and modulation of both innate and adaptive arms of immunity, the disease is opportunistic to immuno-suppression (6). Hence, commencement of an appropriate immune response is a challenge for the control of VL infection. Indeed, therapeutic drugs like SSG and miltefosine are immuno-modulators that trigger Th1 responses essential for activation of oxidative burst in the macrophages (7, 8). However, major limitations of narrow therapeutic index and increasing incidence of resistance with currently used drugs for VL are encumbrances in effective disease management. Recent approaches like combination therapy, targeted delivery, and use of immune-adjunct are efforts to bring down the effective doses of these toxicity-associated drugs. Most promising are the prospects of various immune-targeted therapeutic approaches for treatment of VL (9). Various leishmanial antigens, cytokines, and antibodies that initiate protective Th1-biased cell-mediated immune responses used singly or as an adjunct to conventional chemotherapy are potent immuno-chemotherapeutic agents for the cure of VL (10, 11). Additionally, medicinal plants and their products have opened new dimensions in search of safer and cheaper anti-leishmanial immuno-modulators. These phytochemicals are not only promising as immuno-chemotherapeutic agents against VL but also have potential as immuno-adjuvants and adjuncts to chemotherapy for a number of other immuno-regulatory diseases (12).

Since both cure and resilience to *Leishmania* infection depend on the immunological status of the host, the antigens that can trigger healing responses can also induce prophylactic immunity. Therefore, identification of immunogens that can induce Th1 responses is the critical aspect of vaccine search against VL (13).

Although a number of defined antigens have been reported to impart protective immunity against experimental VL, recent trend of reverse vaccinology is a promising aspect for identification of key immunogens for a successful vaccine (13, 14). This requires rational inputs and algorithm for identification of a promising antigen from the whole proteome data analysis *in silico* (15). One of the key inputs is to identify the epitopes for activation of both $CD4^+$Th1 and $CD8^+$ T cells. Indeed, several studies have attempted to generate epitope-based vaccines from potent antigens that selectively targets MHC I and MHC II. These multi-epitope-based synthetic vaccines were found to stimulate Th1 and $CD8^+$ T cell responses and can be potentially used for prophylaxis against VL (16, 17). Since antigen presenting cells determine the activation of specific lymphocyte subsets, targeting dendritic cells that are known for activation of Th1 and $CD8^+$ T cells can serve as an important vaccination strategy against VL. Indeed, various reports of antigen-pulsed DCs as vaccine against experimental VL have been promising (18).

The vector (sandfly) salivary proteins play a pivotal role in parasite pathogenesis. Indeed, the infective dose of *Leishmania* parasites during natural transmission is much lower as compared to saliva free infectious inoculums (19). This has been primarily attributed to the initial immune responses to salivary component triggered following sandfly bite, which enhances the infectivity of *Leishmania* in the host. Rationally, priming the host against a number of sandfly salivary proteins have been shown to induce altered host immunity to the parasite imparting protection against *Leishmania* infection (20, 21). Therefore, salivary proteins alone or in combination with parasite antigens can be promising vaccine components against VL (22).

However, most defined protein based vaccines are limited by their inability to generate profound long lasting immunity. This is in part due to lack of antigen persistence and multiplicity of

antigens required to generate long lasting memory without a suitable adjuvant (23). In fact, lifelong immunity gained through natural infection is the gold standard of protection for VL. Therefore, apart from triggering appropriate immune responses, the immune correlates of long lasting protective immunity have to be determined. This can be achieved in part by the partial mimic of natural infection, which ensures antigen/parasite persistence and multi-antigenicity required for robust long lasting immunity. Although DNA vaccines can ensure antigen persistence, it is limited by multiplicity of antigens required and the potential adverse effects associated.

For this very reason, several genetically modified live parasites have been found to be the most efficient vaccination strategy (23, 24). However, despite reported success as a vaccination strategy against experimental VL, none of the genetically modified organisms have been approved for clinical trials. The primary concern is the safety issue associated with live parasites. Possibility of revert pathogenesis makes the use of live parasites speculative for human administration. However, understanding the biomarkers of safety of the live vaccines in human cell can be highly valuable in the development of a successful vaccine against VL (25).

ACKNOWLEDGMENTS

We would like to thank all support staff at the Frontiers Editorial Office for their guidance and cooperation and most importantly we are thankful to the authors for their valuable contributions to this research topic. We are also indebted to the timely and expert review by all the reviewers.

REFERENCES

1. Stauch A, Duerr HP, Picado A, Ostyn B, Sundar S, Rijal S, et al. Model-based investigations of different vector-related intervention strategies to eliminate visceral leishmaniasis on the Indian subcontinent. *PLoS Negl Trop Dis* (2014) **8**(4):e2810. doi:10.1371/journal.pntd.0002810
2. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) **7**(5):e35671. doi:10.1371/journal.pone.0035671
3. Palatnik-de-Sousa CB. Vaccines for canine leishmaniasis. *Front Immunol* (2012) **3**:69. doi:10.3389/fimmu.2012.00069
4. Kaye P, Scott P. Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol* (2011) **9**(8):604–15. doi:10.1038/nrmicro2608
5. Cecilio P, Perez-Cabezas B, Santarem N, Maciel J, Rodrigues V, Cordeiro da Silva A. Deception and manipulation: the arms of *Leishmania*, a successful parasite. *Front Immunol* (2014) **5**:480. doi:10.3389/fimmu.2014.00480
6. Kumar R, Nylen S. Immunobiology of visceral leishmaniasis. *Front Immunol* (2012) **3**:251. doi:10.3389/fimmu.2012.00251
7. Fan K, Borden E, Yi T. Interferon-gamma is induced in human peripheral blood immune cells in vitro by sodium stibogluconate/interleukin-2 and mediates its antitumor activity in vivo. *J Interferon Cytokine Res* (2009) **29**(8):451–60. doi:10.1089/jir.2008.0061
8. Wadhone P, Maiti M, Agarwal R, Kamat V, Martin S, Saha B. Miltefosine promotes IFN-gamma-dominated anti-leishmanial immune response. *J Immunol* (2009) **182**(11):7146–54. doi:10.4049/jimmunol.0803859
9. Singh OP, Sundar S. Immunotherapy and targeted therapies in treatment of visceral leishmaniasis: current status and future prospects. *Front Immunol* (2014) **5**:296. doi:10.3389/fimmu.2014.00296
10. Nico D, Gomes DC, Palatnik-de-Sousa I, Morrot A, Palatnik M, Palatnik-de-Sousa CB. *Leishmania donovani* nucleoside hydrolase terminal domains in cross-protective immunotherapy against *Leishmania amazonensis* murine infection. *Front Immunol* (2014) **5**:273. doi:10.3389/fimmu.2014.00273
11. Roatt BM, Aguiar-Soares RD, Coura-Vital W, Ker HG, Moreira N, Vitoriano-Souza J, et al. Immunotherapy and immunochemotherapy in visceral *Leishmaniasis*: promising treatments for this neglected disease. *Front Immunol* (2014) **5**:272. doi:10.3389/fimmu.2014.00272
12. Chouhan G, Islamuddin M, Sahal D, Afrin F. Exploring the role of medicinal plant-based immunomodulators for effective therapy of leishmaniasis. *Front Immunol* (2014) **5**:193. doi:10.3389/fimmu.2014.00193
13. Joshi S, Rawat K, Yadav NK, Kumar V, Siddiqi MI, Dube A. Visceral *Leishmaniasis*: advancements in vaccine development via classical and molecular approaches. *Front Immunol* (2014) **5**:380. doi:10.3389/fimmu.2014.00380
14. Nico D, Gomes DC, Alves-Silva MV, Freitas EO, Morrot A, Bahia D, et al. Cross-protective immunity to *Leishmania amazonensis* is mediated by CD4+ and CD8+ epitopes of *Leishmania donovani* nucleoside hydrolase terminal domains. *Front Immunol* (2014) **5**:189. doi:10.3389/fimmu.2014.00189
15. Aebischer T. *Leishmania* spp. proteome data sets: a comprehensive resource for vaccine development to target visceral leishmaniasis. *Front Immunol* (2014) **5**:260. doi:10.3389/fimmu.2014.00260
16. Das S, Freier A, Boussoffara T, Oswald D, Losch FO, Selka M, et al. Modular multiantigen T cell epitope-enriched DNA vaccine against human leishmaniasis. *Sci Transl Med* (2014) **6**(234):234ra56. doi:10.1126/scitranslmed.3008222
17. Agallou M, Athanasiou E, Koutsoni O, Dotsika E, Karagouni E. Experimental validation of multi-epitope peptides including promising MHC class I- and II-restricted epitopes of four known *Leishmania infantum* proteins. *Front Immunol* (2014) **5**:268. doi:10.3389/fimmu.2014.00268
18. Majumder S, Bhattacharjee A, Paul Chowdhury B, Bhattacharyya Majumdar S, Majumdar S. Antigen-pulsed CpG-ODN-activated dendritic cells induce host-protective immune response by regulating the t regulatory cell functioning in *Leishmania donovani*-infected mice: critical role of CXCL10. *Front Immunol* (2014) **5**:261. doi:10.3389/fimmu.2014.00261
19. Titus RG, Ribeiro JM. Salivary gland lysates from the sand fly *Lutzomyia longipalpis* enhance *Leishmania* infectivity. *Science* (1988) **239**(4845):1306–8. doi:10.1126/science.3344436
20. Gomes R, Oliveira F, Teixeira C, Meneses C, Gilmore DC, Elnaiem DE, et al. Immunity to sand fly salivary protein LJM11 modulates host response to vector-transmitted *Leishmania* conferring ulcer-free protection. *J Invest Dermatol* (2012) **132**(12):2735–43. doi:10.1038/jid.2012.205
21. Gomes R, Teixeira C, Teixeira MJ, Oliveira F, Menezes MJ, Silva C, et al. Immunity to a salivary protein of a sand fly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. *Proc Natl Acad Sci U S A* (2008) **105**(22):7845–50. doi:10.1073/pnas.0712153105
22. Kamhawi S, Aslan H, Valenzuela JG. Vector saliva in vaccines for visceral leishmaniasis: a brief encounter of high consequence? *Front Public Health* (2014) **2**:99. doi:10.3389/fpubh.2014.00099
23. Saljoughian N, Taheri T, Rafati S. Live vaccination tactics: possible approaches for controlling visceral leishmaniasis. *Front Immunol* (2014) **5**:134. doi:10.3389/fimmu.2014.00134
24. Chhajer R, Ali N. Genetically modified organisms and visceral leishmaniasis. *Front Immunol* (2014) **5**:213. doi:10.3389/fimmu.2014.00213
25. Gannavaram S, Dey R, Avishek K, Selvapandiyan A, Salotra P, Nakhasi HL. Biomarkers of safety and immune protection for genetically modified live attenuated *Leishmania* vaccines against visceral leishmaniasis – discovery and implications. *Front Immunol* (2014) **5**:241. doi:10.3389/fimmu.2014.00241

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 November 2014; accepted: 08 December 2014; published online: 19 December 2014.
*Citation: Ali N, Nakhasi HL, Valenzuela JG and Reis AB (2014) Targeted immunology for prevention and cure of VL. Front. Immunol. **5**:660. doi: 10.3389/fimmu.2014.00660 This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 13 June 2014
doi: 10.3389/fimmu.2014.00272

Immunotherapy and immunochemotherapy in visceral leishmaniasis: promising treatments for this neglected disease

Bruno Mendes Roatt[1,2,3]*, Rodrigo Dian de Oliveira Aguiar-Soares[1], Wendel Coura-Vital[1,2], Henrique Gama Ker[1,2], Nádia das Dores Moreira[1], Juliana Vitoriano-Souza[1], Rodolfo Cordeiro Giunchetti[1,4], Cláudia Martins Carneiro[1,2] and Alexandre Barbosa Reis[1,2,3]*

[1] *Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, Brazil*
[2] *Laboratório de Pesquisas Clínicas, Ciências Farmacêuticas, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Brazil*
[3] *Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais, Belo Horizonte, Brazil*
[4] *Laboratório de Biologia das Interações Celulares, Departamento de Morfologia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil*

Edited by:
Nahid Ali, Indian Institute of Chemical Biology, India

Reviewed by:
Emilio Luis Malchiodi, University of Buenos Aires, Argentina
Paula Mello De Luca, Funfação Oswaldo Cruz, Brazil
Jesus G. Valenzuela, National Institute of Allergy and Infectious Diseases, USA

***Correspondence:**
Bruno Mendes Roatt and Alexandre Barbosa Reis, Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas, ICEB II, Campus Morro do Cruzeiro, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais CEP 35400-000, Brazil
e-mail: eroatt@nupeb.ufop.br; alexreis@nupeb.ufop.br

Leishmaniasis has several clinical forms: self-healing or chronic cutaneous leishmaniasis or post-kala-azar dermal leishmaniasis; mucosal leishmaniasis; visceral leishmaniasis (VL), which is fatal if left untreated. The epidemiology and clinical features of VL vary greatly due to the interaction of multiple factors including parasite strains, vectors, host genetics, and the environment. Human immunodeficiency virus infection augments the severity of VL increasing the risk of developing active disease by 100–2320 times. An effective vaccine for humans is not yet available. Resistance to chemotherapy is a growing problem in many regions, and the costs associated with drug identification and development, make commercial production for leishmaniasis, unattractive. The toxicity of currently drugs, their long treatment course, and limited efficacy are significant concerns. For cutaneous disease, many studies have shown promising results with immunotherapy/immunochemotherapy, aimed to modulate and activate the immune response to obtain a therapeutic cure. Nowadays, the focus of many groups centers on treating canine VL by using vaccines and immunomodulators with or without chemotherapy. In human disease, the use of cytokines like interferon-γ associated with pentavalent antimonials demonstrated promising results in patients that did not respond to conventional treatment. In mice, immunomodulation based on monoclonal antibodies to remove endogenous immunosuppressive cytokines (interleukin-10) or block their receptors, antigen-pulsed syngeneic dendritic cells, or biological products like Pam3Cys (TLR ligand) has already been shown as a prospective treatment of the disease. This review addresses VL treatment, particularly immunotherapy and/or immunochemotherapy as an alternative to conventional drug treatment in experimental models, canine VL, and human disease.

Keywords: visceral leishmaniasis, immunology, immunotherapy, immunochemotherapy, *Leishmania infantum*, *Leishmania donovani*

INTRODUCTION OF VISCERAL LEISHMANIASIS: EPIDEMIOLOGY OF A ZOONOTIC AND ANTHROPONOTIC NEGLECTED DISEASE

Visceral leishmaniasis (VL) is a severe chronic systemic disease caused by *Leishmania donovani* or *L. infantum*. Occasionally, *L. tropica* in the Middle East and *L. amazonensis* in South America can produce VL (1). *Leishmania* spp. are transmitted to human and animal hosts through the bite of female sand flies from the genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World (2). Depending on whether or not a reservoir host is present, there are two basic types of epidemiological cycles: zoonotic, generally caused by *L. infantum*, which occurs in the Mediterranean Basin, China, the Middle East, and South America, and anthroponotic, generally caused by *L. donovani*, which is prevalent in East Africa, Bangladesh, India, and Nepal (3). The dogs, independent of the clinical form of VL, are the main urban reservoirs of *L. infantum* and represent the major source of contagion for the vectors by virtue of the high prevalence of infection and intense cutaneous parasitism (4, 5).

Canine visceral leishmaniasis (CVL) is present in approximately 50 countries, mainly in South America, the Mediterranean region, and Africa (6, 7). Several reports have revealed the emergence of canine infection in new locations, such as the United States and Canada (8, 9), and a northward spread in Europe, as found in Italy (10, 11). The seroprevalence of CVL ranges between 2 and 25% in endemic areas of Europe (2) and 5.9 and 29.8% in Brazil (12). In recent years, with the development of molecular techniques, infection rates have been shown to be underestimated. Studies in Europe have demonstrated an elevated prevalence of CVL (60–80%) by polymerase chain reaction (PCR) compared

with serology (25%) (13). During a cross-sectional study in an urban area of Brazil, we observed that approximately a quarter of seronegative dogs were infected by *L. infantum* according to PCR (14), and they had approximately twice the risk of seroconversion as those that were PCR negative (15). Finally, a high incidence of infection was demonstrated by PCR in endemic areas (16).

Official global estimates indicate that there are more than 58,000 cases of human VL (HVL) per year. However, the number may actually be as high as 0.2–0.4 million, and more than 90% of cases occur in five countries: India, Bangladesh, Sudan, Brazil, and Ethiopia (17). The incidence of VL is relatively low in southern Europe (2), but the disease has recently spread further northward as shown by reports of cases in northern Italy (18) and Germany (19). Additionally, the epidemiology of the disease has been influenced by the expansion of human immunodeficiency virus (HIV). Of the 70 countries that are endemic for VL, 35 have reported cases of *Leishmania*–HIV co-infection (20). One of the critical complications associated with co-infection is that HIV reduces the likelihood of a therapeutic response to treatment against *L. infantum*, and it also greatly increases the probability of a relapse (21).

Visceral leishmaniasis is clinically characterized by prolonged fever, weakness, anorexia, weight loss, hepatomegaly, splenomegaly, hypergammaglobulinemia, and pancytopenia. Without treatment, the disease may progress over time to severe cachexia, multisystem disease, bleeding, secondary infections, and death (22, 23). The case-fatality rates range from 1.5% in Bangladesh to 2.4% in India and 6.2% in Nepal (17). However, studies conducted by Ahluwalia et al. (24) in Bangladesh and by Barnett et al. (25) in India suggest that the rates are probably underestimated. In Brazil, data from the Ministry of Health were used to estimate 6.5% mortality from 2001 to 2011 (26). VL results in death mainly in untreated patients. The majority of leishmaniasis deaths go unrecognized, and even with treatment access, case-fatality rates can be as high as 10–20% (17). These findings underscore the need for further studies on the development of immunotherapeutic and prophylactic strategies for VL and *Leishmania*–HIV co-infection.

In this review, we discuss the recent advances in immunotherapy and immunochemotherapy in the treatment of VL, focusing on both canine and human disease and experimental models (murine). We also discuss some aspects of the epidemiology and immunology of VL, the most recent strategies and guidelines for chemotherapy, and new advances in modulating the host immune response (collectively called immunotherapy) with or without conventional chemotherapy.

IMMUNOBIOLOGY OF VISCERAL LEISHMANIASIS: CELLS AND IMMUNE MEDIATORS RELATED TO RESISTANCE AND SUSCEPTIBILITY

In visceral disease, the immunology and immunopathology in humans, dogs, and experimental rodent models has been extensively studied, with many points characterized and others still to be elucidated (27–29). A general consensus is that despite the peculiarities of each model, the outcome of the disease is critically influenced by the host immune response.

Several studies have demonstrated that susceptibility to HVL is related to a high titer of circulating antibodies and a depression of type-1 T cell-mediated immunity, mainly with decreased production of interferon (IFN)-γ and interleukin (IL)-12, including a marked up-regulation of IL-4 and IL-10 cytokines (30–32). In CVL, the protective response has also been associated with activation of Th1 cells producing IFN-γ, IL-2, and tumor necrosis factor (TNF)-α (33, 34). Similar to HVL, active CVL is characterized by polyclonal B-cell activation, specific immunosuppression, and the appearance of clinical symptoms depending on the parasite density in different visceral organs (35, 36). An interplay of Th1 and Th2 cytokines appears to exist during *Leishmania* infection, and this suggests important roles for different cytokines in disease protection and pathogenesis (37).

The innate immune response contributes to VL resistance, acting to control parasite growth during the early stages of infection. Furthermore, it directs cell recruitment and helps develop the cytokine microenvironment in which parasite-specific T cells are primed (38, 39).

The control of VL infection depends on a successful cell-mediated immune response (40), in which IFN-γ, produced mainly by $CD4^+$ T cells and natural killer (NK) cells stimulated by IL-12, leads to stimulation of microbicide action mediated by nitric oxide (41, 42). TNF-α exerts cytotoxic effects on invading parasites via its receptor, TNFR (43). There have been reports of the involvement of different Th17 cytokines in HVL, including IL-17, IL-22 (44), and IL-21 (45), which are important in the migration, recruitment, and activation of neutrophils. Recent work of Gautam et al. (46) evaluating patients with VL showed that individuals with active disease exhibit predominantly anergic splenic CD8 cells and CD8 peripheral blood mononuclear cells (PBMCs) with a mixture of anergic cells and cytotoxic T lymphocytes (CTLs). Following a cure after treatment, CD8 T cells contribute to *Leishmania*-induced IFN-γ production. The authors suggested that CD8 T cells are driven to anergy/exhaustion in HVL, which affects their ability to launch a protective immune response (46).

The expression of the various chemokine genes is observed in *Leishmania* infection (47, 48). Chemokines have been shown to play a crucial role in determining the outcome of leishmaniasis by coordinating the leukocyte recruitment involved in innate and adaptive immune responses (49, 50). Patients with VL show elevated concentrations of CXCL9 and CXCL10 in their serum during active infection, and it has been suggested that these chemokines play an important role along with IFN-γ in the disease (51). Dogs naturally or experimentally infected with *L. infantum* have CXCL10 mRNA overexpressed in the spleen, leading to a substantial type-1 immune response (52). A detailed analysis of chemokine expression in skin samples from dogs naturally infected with *L. infantum* demonstrated enhanced parasite density and a positive correlation with CCL2, CCL4, CCL5, CCL21, and CXCL8 (49). It is noteworthy that some chemokines such as CCL2 can activate macrophages to participate in reducing the parasite load (53).

The monocytes/macrophages, the main targets of *Leishmania*, represent one of the first steps of the innate immune response to kill intracellular parasite (54). The survival of the parasite relies

on evasion mechanisms such as the modulation of leishmanicidal activity of macrophages by production of tumor growth factor (TGF)-β with deactivation, inhibition of the action of IFN-γ, reduced expression of MHC class II molecules, and suppression of nitric oxide production (55). IL-10 is another cytokine produced by macrophages that contributes to the survival of *Leishmania* in these cells, and it has emerged as the most potent factor for VL pathogenesis. It inhibits synthesis of cytokines produced by macrophages, such as IL-1β, IL-6, IL-8, and TNF-α (56) and reduces the antigen-presenting function of these cells by decreasing the expression of MHC class II molecules (57). The association of IL-10 and VL in humans with active disease is well-established (32). Others cytokines, such as IL-27 and IL-21, have also emerged as being implicated in disease progression through the regulation of IL-10 (45). Other cells, such as NK cells, are important components of the immune response to combat infection. They connect the innate response to the development of efficient adaptive cellular immunity, mainly through TNF-α and IFN-γ production (58).

Successful immunity against *Leishmania* involves a complex immunological response of several mechanisms and factors, including the migration of appropriate cell populations to the infected sites, cytokine microenvironment, chemokines, and others. The elucidation and a better understanding of the immune response against *Leishmania* infection are relevant to establish a rational approach for immunomodulatory therapy and vaccine development.

CONVENTIONAL VL THERAPY

The drug policy in endemic countries and therapeutic decisions should be based on the individual benefit–risk ratio of drugs, the health service setting, and the availability of anti-leishmanial medicines in the context of public health concerns and the difference of the VL epidemiological aspects (anthroponotic and zoonotic) (59). For example, 70% of the anthropozoonotic VL burden occurs in the Indian subcontinent (17), and a critical challenge is related to widespread resistance to pentavalent antimony; resistance rates approach 60% in Bihar, India (60). In Europe, Asia, Africa, and the Americas, where zoonotic cases are observed, the risk of human disease is well-known to be associated with canine infection rates (61). Another serious problem that mainly occurs with zoonotic VL is that canine treatment does not effectively lead to a parasitological cure since these animals are constant sources of infection for sand flies (36).

Nevertheless, a few drugs are available. In most cases, the first-line treatment is pentavalent antimonials, and amphotericin B or pentamidine are commonly employed as second-line medicines. In recent years, other medicines have been extensively studied and became invaluable, such as liposomal amphotericin B (62), miltefosine (63, 64), and paromomycin (65). In line with this, current World Health Organization (WHO) treatment advice varies by global region, which is partially explained by differences in parasite susceptibility (59, 66, 67) (**Table 1**). Even so, the number of VL cases is increasing worldwide, and the enduring problems with current chemotherapy tools are still a critical issue. Furthermore, in many developing countries the cost of treatment is the greatest challenge faced by health authorities (**Table 2**). In the following section, we briefly review conventional chemotherapy, stressing essential issues in HVL and studies using different drugs and strategies for canine disease.

PENTAVALENT ANTIMONIALS

It is generally accepted that pentavalent antimonials (Sb^V) are the pro-drug, and that they must convert to trivalent antimonials (Sb^{III}) to have anti-leishmanial activity. The issues with the use of this drug are commonly attributed to serious side effects such as cardiotoxicity (68), pancreatitis (69), and nephrotoxicity (70). The doses and treatment durations of Sb^V have undergone constant changes over the years. The use of Sb^V in canine therapy does not lead to clinical and parasitological cure (71), and disease relapses are common (72). Moreover, prolonged or repeated use of this drug can induce resistance in *Leishmania* clones (73). Currently, an important strategy for therapy in dogs is the use of liposome-encapsulated Sb^V, which promotes improved clinical status and reduced parasite load in infected animals (74).

AMPHOTERICIN B DEOXYCHOLATE AND LIPOSOMAL AMPHOTERICIN B

The anti-*Leishmania* activity by amphotericin B is due to its complexation with 24-substituted sterols such as ergosterol and episterol, which are predominant in the plasma membranes of parasites. Amphotericin B deoxycholate is generally used for cases that are unresponsive to Sb^V, and it is a first-line drug in India. Unresponsiveness and relapses occur rarely and mostly in relation to HIV co-infection (75). The major limitation to using this drug is the necessity for prolonged hospitalization and close monitoring due to its high nephrotoxicity (76). The liposomal formulation improves the safety profile of amphotericin B and increases the anti-leishmanial activity, with selectivity to macrophage reticular–endothelial system (77). There are three formulations, liposomal amphotericin B, amphotericin B lipid complex, and amphotericin B cholesterol dispersion; all of which ensure a decrease in nephrotoxicity. Currently, liposomal amphotericin B is the first treatment choice for HVL in several endemic countries in Europe as well as in the United States. Following other countries, the Ministry of Health in Brazil, expanded the use of this medicine in the last years. In dogs, therapy with amphotericin B deoxycholate reduces serum antibody levels and parasite loads and increases the lymphoproliferative response, but the effects are transitory (78). In addition, renal failure is a common outcome (79), and the drug is not recommended for canine therapy. Treatment with liposomal amphotericin B resulted in recovery in dogs, but despite the initial effectiveness, relapses can occur (78, 80).

MILTEFOSINE

Miltefosine, which was initially developed as an anticancer drug, is the first effective oral drug for VL, and it represents a great breakthrough (81, 82). The main anti-leishmanial activity is due to modulation of cell surface receptors, inositol metabolism, phospholipase activation, and protein kinase C in addition to mitogenic pathways resulting in apoptosis (83). The main side effects of the drug include gastrointestinal disturbances, but the symptoms are transient or reversible; however, teratogenicity is a major problem (84). Careful use of this drug should be mandatory, since resistance

Table 1 | Recommendations of the World Health Organization for the treatment of visceral leishmaniasis per geographic region ranked by preference [World Health Organization (59)].

ANTHROPONOTIC VISCERAL LEISHMANIASIS CAUSED BY *L. donovani* IN THE INDIAN SUBCONTINENT
Liposomal amphotericin B: 3–5 mg/kg daily over 3–5 days to a total dose of 15 mg/kg by infusion or 10 mg/kg as a single dose
Combination therapy (co-administered following the sequence): (i) liposomal amphotericin B (5 mg/kg by infusion, single dose) + miltefosine (daily for 7 days, dosage as below), (ii) liposomal amphotericin B (5 mg/kg by infusion, single dose) + paromomycin (daily for 10 days, dosage as below), (iii), miltefosine + paromomycin both for 10 days (dosages as below)
Amphotericin B deoxycholate: 0.75–1.0 mg/kg daily or on alternate days for 15–20 doses by infusion
Miltefosine: children aged 2–11 years, 2.5 mg/kg daily; 12 years and older <25 kg body weight, 50 mg/day; 25–50 kg, 100 mg/day; >50 kg, 150 mg/day; orally for 28 days
Paromomycin: 15 mg (11 mg base)/kg/day by intramuscular route for 21 days
Pentavalent antimonials: 20 mg Sb^V/kg/day intramuscularly or by infusion for 30 days (areas where they are effective: Bangladesh, Nepal, and the Indian states of Jharkhand, West Bengal, and Uttar Pradesh)
VISCERAL LEISHMANIASIS CAUSED BY *L. donovani* IN EAST AFRICA
Combination therapy: pentavalent antimonials (20 mg Sb^V/kg/day intramuscularly or by infusion) + paromomycin [15 mg (11 mg base)/kg/day by intramuscular route] for 17 days
Pentavalent antimonials: same treatment scheme as above
Liposomal amphotericin B: 3–5 mg/kg daily given over 6–10 days for a total dose of 30 mg/kg by infusion
Amphotericin B deoxycholate: same treatment scheme as above
Miltefosine: same treatment scheme as above
VISCERAL LEISHMANIASIS CAUSED BY *L. infantum*
Liposomal amphotericin B: 3–5 mg/kg daily over 3–6 days for a total dose of 18–21 mg/kg by infusion
Pentavalent antimonials: 20 mg Sb^V/kg/day intramuscularly or by infusion for 28 days
Amphotericin B deoxycholate: 0.75–1.0 mg/kg daily or on alternate days for 10–20 doses by infusion (total dose: 2–3 g)

can be easily induced in *in vitro* experiments (85). Miltefosine has recently emerged as a potential tool for CVL treatment, and its use has been evaluated in monotherapy and in combination with other drugs (86, 87). There are no nephrotoxic effects reported, and vomiting is the most common side effect in dogs (88).

PAROMOMYCIN

Paromomycin presents variable efficacy in distinct parts of the world (89). The drug's low-cost, relatively short duration of administration, and good safety profile strengthens its usefulness as a first-line drug (90). The drug has activity against *Leishmania* by altering plasma membrane fluidity, interfering in ribosomal function, and disrupting mitochondrial membrane potential (91). The most common side effects associated with paromomycin are ototoxicity and impaired liver function (92). Although it is the least expensive drug for VL, current demand for paromomycin is low, and production is irregular. In canine studies, the drug was associated with a decrease in anti-*Leishmania* IgG antibody titers (93). Following clinical recovery, relapse, and parasitologic cure in symptomatic CVL treated with paromomycin, only clinical improvement was verified (94). However, the search for an optimum dosage for the safe use in the treatment of CVL is necessary.

Table 2 | Cost of visceral leishmaniasis treatment (patient weighing 35 kg)*.

Medicine (compound)	Treatment regimen in days	Drug cost in US$
L-Amb 10 mg/kg	1	125
L-Amb 20 mg/kg	2–4	250
Amphotericin B deoxycholate 1 mg/kg (alternating days)	30	20
MF 100 mg/kg	28	65–150
PM 15 mg/kg/day	21	15
SSG 20 mg/kg/day	30	55
MA 20 mg/kg/day	30	59
L-Amb 5 mg/kg + MF 100 mg/kg	8	88–109
L-Amb 5 mg/kg + PM 15 mg/kg/day	11	78
MF 100 mg/kg + PM 15 mg/kg/day	10	30–60
SSG 20 mg + PM 15 mg/kg/day	17	43

**Calculations for SSG and MF based on exchange rate of €1 = US$ 1.40 (December 2013). Price range of MF depends on order volume. Price is based on generic SSG, World Health Organization (59).*

L-Amb, liposomal amphotericin B; MF, miltefosine; PM, paromomycin; SSG, sodium stibogluconate; MA, meglumine antimoniate.

COMBINED DRUG THERAPY

In general, the treatment of VL is clinically challenging, and the drugs have several drawbacks. Over the past few years, the WHO consensus has evolved toward the use of combination regimens, particularly in highly endemic regions. Combining drugs from various chemical classes has the following objectives: (i) shortening the duration of treatment, reducing total parenteral drug doses with fewer toxic effects, and improving adherence to the regimen; (ii) lowering the cost of the treatment (less burden on the health system), thus providing a more cost-effective option, and (iii) helping to delay the emergence of resistance. These strategies could increase the therapeutic lifespan of the respective drugs, as has been demonstrated with drugs for diseases like malaria, tuberculosis, and HIV. These strategies might also encourage a cure, especially in complicated cases like *Leishmania*–HIV co-infection, for which treatment outcomes with monotherapy have been consistently poor (1, 59, 66).

Recently, reports of treatment failure with Sb^V from the Indian subcontinent have increasingly raised the issue of acquired drug resistance (67). This concern also extends to miltefosine, which is worrisome given the drug's long half-life (84). More recently (95) reported unresponsiveness to liposomal amphotericin B in Sudanese patients, who experienced cured disease only with combination treatment. Specifically, a 17-day combination of antimonials with paromomycin presented 93% efficacy in East Africa. Combination regimens including liposomal amphotericin B (single dose), paromomycin, and/or miltefosine were also found to be extremely effective (98–99%) and safe, and are now included in WHO guidelines for the Indian subcontinent (see **Table 1**) (1, 59).

Substantial progress has been made in the chemotherapeutic approaches in recent years, but the current conventional drugs for VL are far from ideal (96). Combined therapy enhancement should be on-going, but exploratory studies that encompass highly efficient regimens in single dose treatments are urgently needed (97). The most effective strategies for protecting against resistance are uncertain, but overall monitoring of access to anti-leishmanial drugs should definitely be strengthened. In this context, canine treatment is still controversial, and strict action should be taken particularly for zoonotic VL. Worryingly, in Europe, dogs with active VL are routinely treated with first-line drugs for HVL, and this practice could generate parasites that are resistant to conventional therapies (98). Considering the success of combined therapy, the control and the effectiveness of current conventional medicines must be protected until new options arise.

PROMISING STRATEGIES FOR VL TREATMENT: IMMUNOTHERAPY AND IMMUNOCHEMOTHERAPY

The immunotherapy, involves the use of biological substances or molecules to modulate the immune responses for the purpose of achieving a prophylactic and/or therapeutic success. Currently, immunotherapy is a strategy applied against various diseases such as cancer, allergies, and some viruses (hepatitis). It is based on the idea that our organism's defense systems are capable of protecting us against a variety of diseases (in most circumstances). Normally, it is known that disease occurs when there is either a failure, suboptimal, or excessive immune response and this could be remedied by appropriate immune modulation or interventions using immunomodulatory agents or biological response modifiers. Thus, immunotherapeutic agents can exert their effect by directly or indirectly augmenting the host natural defenses, restoring the impaired effectors functions or decreasing host excessive response (99–101). Moreover, the combination of immunotherapy with chemotherapeutic drugs (immunochemotherapy), especially when applied against infectious diseases, results in an increased synergic action with activation of the immune system and direct action of drugs against the infectious agent. Therefore, immunotherapy and immunochemotherapy have been used to accelerate the specific immunity in responsive and non-responsive patients (102, 103). The underlying idea is to selectively induce Th1 responses that are fundamental for resistance in VL. Protective immunity usually follows recovery from leishmaniasis in immunocompetent patients, but the behavior of disease in these individuals suggests that their immune responses are not sterile. VL has emerged as an important opportunistic infection associated with HIV, with the risk of developing active/severe disease increasing 100–2320 times the average (20). Depending on the stage of infection and the clinical condition, the use of conventional chemotherapy can be inefficient. In such cases, combination therapy with immunomodulators that potentiate the cellular immune response can lead to more satisfactory results.

Immunotherapy with or without chemotherapy has been used for the treatment of cutaneous leishmaniasis (CL) in the last two decades. Convit et al. (104), using three injections of a vaccine composed of a lysate of *L. mexicana amazonensis* with BCG as an adjuvant, demonstrated a 94% of cure rates in CL patients in Venezuela. These authors also showed that 5341 patients from four different regions of Venezuela, who had different forms of CL (mucosal and chronic CL) and received the vaccine treatment between 1990 and 1999, demonstrated a high cure rate (91.2–98.7%) (105). In Brazil, Mayrink et al. (106) evaluated an immunotherapy protocol using a mixture of five strains of *Leishmania* vaccine and observed a 76% cure rate in patients with CL. Moreover, years later, Mayrink et al. (107) used repeated daily doses of killed *L. amazonensis* in a human clinical trial comprising 542 patients and observed that 98.1% of the individuals treated with immunotherapy ($n = 53$; *L. amazonensis* vaccine + BCG) showed a clinical cure. A similar cure rate was found in patients treated with conventional chemotherapy and an immunochemotherapy scheme (100%). The immunochemotherapy protocol was also associated with a reduction in the total volume of the drug used (17.9%) and a shorter treatment time (94.6 days for chemotherapy alone to 64.7 days for immunochemotherapy) (107). In the Sudan, a trial involving patients with persistent post-kala-azar dermal leishmaniasis and using a vaccine composed of a mixture of killed *L. major* adsorbed on alum + BCG, given four times at weekly intervals, showed that the cure rate with immunochemotherapy was significantly higher than with chemotherapy alone (final cure rates: 87 and 53%, respectively) (108).

As we observed, therapeutic vaccines in CL can be rapidly evaluated at lower cost, appear to be safe, and are not associated with the adverse effects of conventional treatment, encouraging the use of this strategy for treatment of VL. Furthermore, using immunomodulators to enhance host immunity combined with conventional chemotherapy may have several advantages

Table 3 | Immunotherapy and immunochemotherapy strategies against VL for humans and dogs.

Immunotherapeutic agent	Chemotherapy agent	Visceral disease	Improvements	Treatment efficacy	Reference
IFN-γ	Sb^V	Human	Accelerated parasitological control, enhanced the clinical efficacy of conventional Sb^V treatment, 83.2% cure rate	Marked	(109–111)
IFN-γ for 15 or 30 days (10^7 U/mg/day)	Sb^V (20 mg/kg/day) at 30 days	Human	No difference was observed in patients treated with Sb^V alone	Moderate	(112)
IFN-γ	Sb^V (20 mg/kg/day) at 30 days	Human	All patients responded clinically to treatment, more quickly splenic culture-negative	Moderate	(113)
Antigenic preparation of *L. infantum* (soluble antigen)	100 mg/kg SC of *N*-methyl-d-glucamine antimoniate	Canine	Increase in the T lymphocytes, especially $CD4/TcR\alpha\beta^+$ and $CD4/CD45RA^+$ cells in PBMC; reduction of infection to *Phlebotomus perniciosus*	Low	(114)
Enriched-Leishmune® vaccine (plus 0.5 mg of saponin)	n.d.	Canine	Higher levels of anti-FML IgG (IgG2), positive delayed type hypersensitivity reaction, lower clinical scores	Moderate	(115, 116)
Enriched-Leishmune® vaccine (plus 0.5 mg of saponin)	Allopurinol (10 mg/kg) and amphotericin B (0.5 mg/kg)	Canine	Positive DTH reaction, reduction of symptomatic cases and low numbers of animals with parasites in lymph nodes and deaths	Marked	(117)
Vaccine composed by 20 μg of rLeish-110f® + 25 μg of MPL-SE®	100 mg/kg/day IM of Glucantime®	Canine	Improvement in the clinical parameters (hematological, biochemical, cellular); reduction in parasitological positive animals (bone marrow smears or culture); reduced number of deaths; 33% xenodiagnosis negative of by PCR	Marked	(118)
Vaccine composed by 20 μg of Leish-111f® plus 20 μg of MPL-SE®	20 mg/kg/day IV of Glucantime®	Canine	Cure rates 50%; 92% clinical improvement	Moderate	(119)
Immunomodulator P-MAPA (2.0 mg/kg) intramuscularly	n.d.	Canine	Increase $CD8^+$ T cells, IL-2, and IFN-γ; decrease in IL-10 and improvement in clinical signs and reduction in parasite load in skin	Marked	(120)

SC, subcutaneous; IM, intramuscular; IV, intravenous; n.d., not done.

as a means to improve current therapeutic regimens in this neglected disease (109). On this topic, we discuss advances in immunotherapy and immunochemotherapy for VL by focusing mainly on approaches used in humans and dogs (**Table 3**) and recent advances in murine models.

APPROACHES USED IN HUMANS

Increasing reports of treatment failure (Sb^V, miltefosine, and liposomal amphotericin B) and complicated cases (*Leishmania*–HIV co-infection) in HVL increase the urgency of using combination therapies and developing new treatment strategies for the disease (67, 95). In fact, the added effects produced by immunotherapy and/or immunochemotherapy could be potentially useful against HVL; however, these approaches are still very rarely used.

In this context, IFN-γ is well-recognized as a cytokine capable of inducing macrophages to kill intracellular *Leishmania* (110). It is clinically well-tolerated (111), and repeat treatment with IFN-γ plus Sb^V has been shown to be effective in patients with Sb^V-refractive disease, yielding a >80% cure rate in VL (112, 113, 121). Studies in untreated patients with VL demonstrated that the addition of IFN-γ as immunotherapy accelerated parasitological control (122, 123) and enhanced the clinical efficacy of conventional Sb^V therapy (123). However, another human trial in India showed no differences among patients treated with Sb^V alone (30 days, 20 mg/kg/day), Sb^V plus IFN-γ (30 days, 10^7 U/mg/day), or Sb^V plus IFN-γ for 15 days (114). Six months after treatment, a low percentage of individuals were cured (36, 49, 42%, respectively), but the immunochemotherapy protocol was the most efficient.

A similar study was conducted in Kenyan patients with VL treated for 30 days with either conventional therapy with Sb^V or immunochemotherapy (daily Sb^V plus IFN-γ) (122). All patients responded clinically to treatment, and microscopic splenic aspirate scores rapidly decreased in both groups. Interestingly, the patients treated with immunochemotherapy had a negative spleen culture more quickly, which may demonstrate the potential of this protocol to accelerate early parasitological control (122). These results suggest the beneficial effects of using IFN-γ in the treatment of HVL. The combination of this immunotherapy or

another (therapeutic vaccines, immunomodulators) with other drugs (miltefosine, liposomal amphotericin B) could provide more satisfactory results with better cure rates mainly in VL patients unresponsive to Sb^V.

PROGRESS FOR VL TREATMENT IN DOGS

The drugs generally used to treat CVL are highly toxic, expensive, and ineffective. They promote clinical remission without parasite reduction or sterilization, and once the treatment is withdrawn, relapses of the disease are always observed (115). Moreover, the WHO does not recommend the use of human chemotherapy in dogs due to concerns about selecting for drug-resistant parasites, which might then be untreatable in subsequent HVL infection. Also, primary resistance to these drugs is considerable, and treated dogs still have parasites in different organs even if they are asymptomatic (116).

Along with vaccine development, new drugs and new treatment strategies (immunotherapy and immunochemotherapy) are the most important alternatives for CVL control. Guarga et al. (117) evaluated the efficacy of a novel immunochemotherapy protocol in dogs naturally infected with *L. infantum*. The protocol consisted of 21 consecutive subcutaneous injections of *N*-methyl-D-glucamine antimoniate (100 mg/kg) and three applications of an antigenic preparation of *L. infantum* (soluble antigen). The animals showed an increase in the proportion of T lymphocytes, especially of CD4/TcR$\alpha\beta^+$ and CD4/CD45RA$^+$ cells in PBMCs, and reduction in the infection from *Phlebotomus perniciosus* after immunochemotherapy (117).

Different studies are being done to evaluate the potential of fucose–manose-ligand (FML) antigen plus saponin as an immunotherapy. Borja-Cabrera et al. (118) used three vaccine doses (1.5 mg FML +1 mg saponin) in asymptomatic dogs and observed them for 22 months after immunotherapy was complete. No deaths due to disease were recorded, and 90% of the dogs remained asymptomatic, healthy, and parasite free. In contrast, 37% of kala-azar deaths were recorded in non-treated animals (118). Another vaccine formulation (enriched-Leishmune® vaccine plus 0.5 mg of saponin) was evaluated by Santos et al. (119) in dogs experimentally infected with *L. infantum*. The enriched-Leishmune was injected when dogs were seropositive and symptomatic. After immunotherapy, the dogs showed higher levels of anti-FML IgG (higher IgG2 and lower IgG1), positive delayed type hypersensitivity reactions, lower clinical scores, and normal CD4$^+$ counts (119). The association of enriched-Leishmune vaccine with chemotherapy (allopurinol or amphotericin B/allopurinol) demonstrated synergistic efficacy in naturally infected animals. For both immunotherapy and immunochemotherapy, dogs showed an intradermal response to *Leishmania* antigen, reduction of symptomatic cases, a lower proportion of animals presenting with parasites in lymph nodes, and fewer deaths (120).

Miret et al. (124) evaluated immunochemotherapy using Leish-110f® + MPL-SE® vaccine in combination with Glucantime® and showed in symptomatic dogs improved clinical parameters (hematological, biochemical, and immunological), reduced parasite-positive animals, and reduced number of deaths compared to control groups (adjuvant alone or placebo). Trigo et al. (125) performed two separate trials to evaluate the recombinant polyprotein vaccine antigen Leish-111f®, formulated with MPL-SE® for therapeutic purposes against CVL. In both trials, a therapeutic efficacy of the vaccine in preventing mild cases of disease was demonstrated, and weekly injections (three doses) promoted clinical cure for many dogs with VL.

Using an immunomodulator, Santiago et al. (126) tested the immunotherapeutic effect for CVL of a protein aggregate of magnesium–ammonium phospholinoleate–palmitoleate anhydride (P-MAPA) obtained by fermenting the fungus *Aspergillus oryzae*. P-MAPA showed immunomodulatory activity, with greater stimulation of cellular immunity and no toxic effects in mice and dogs (127). To investigate the immunotherapeutic potential of P-MAPA, symptomatic dogs were submitted to a protocol of 15 doses of the immunomodulator (2.0 mg/kg) intramuscularly. An increase in CD8$^+$ T cells in peripheral blood, a decrease in IL-10 levels, and an increase in IL-2 and IFN-γ, improved clinical signs, and reduced skin parasitism were obtained after immunotherapy (126).

Some CVL vaccines candidates have been developed by our research group, called LBSap and LBSapSal, demonstrating important results of immunogenicity and efficacy in phase I and II trials (128, 129). Currently, we are investigating the potential immunotherapeutic of these and other vaccines in the treatment of CVL. Given these results, we believe that we could use immunotherapy/immunochemotherapy to treat dogs in endemic areas to eliminate their reservoir condition mainly by decreasing the skin parasite load, which would block the zoonotic transmission cycle.

RECENTS ADVANCES IN MURINE MODELS

With the current status of *Leishmania* treatment, use of a low-dose drug or a short course of an effective drug in combination with an immunomodulator is an approach for effective treatment of disease (130). Thus, murine models of leishmaniasis are being extensively used to obtain preliminary information on the anti-*Leishmania* potential of different compounds (67). Many researchers have worked on the development and discovery of new agents against the parasite, and several studies have shown that the use of immunotherapy would be an important tool in control of VL.

Because Sb^V-based anti-leishmanial chemotherapy depends in part on the Th1 response, which can be induced by dendritic cell (DC)-based treatment (131). DC-based immunotherapy combined with Sb^V chemotherapy was very effective against murine VL (132). While three weekly injections of *L. donovani*-soluble, antigen-pulsed syngeneic bone marrow-derived DCs into mice infected with *L. donovani* only reduced the number of spleen and liver amastigotes, when combined with sodium stibogluconate, the treatment resulted in a complete eradication of the parasites from both organs (132).

A fusion protein that stimulates T cells through OX40, as well as a monoclonal antibody (mAb) agonist against CD40, enhanced host immunity, and supported low-dose Sb^V in a murine VL model (133, 134). The treatment enhanced both the rate of granuloma maturation and CD4$^+$ T cell proliferation and promoted greater reduction in the parasite burden, without causing excess

tissue damage. Moreover, the blockade of cytotoxic T lymphocyte-associated (CTLA)-4, a negative regulator of T cell co-stimulation using mAb, has a beneficial effect in experimental VL, inducing the destruction of 60% of the parasites within liver macrophages, stimulating IFN-γ secretion, and enhancing mononuclear cell recruitment with significant synergy with Sb^V (134).

In VL, cytokine-mediated immunosuppression is dominated by IL-10 and TGF-β (135). Hence IL-10-deficient mice are highly resistant to VL (27, 135). This cytokine also impairs responsiveness to Sb^V. In experimental models of VL, treatment with mAb against the IL-10 receptor allowed a 10-fold reduction in the effective dose of Sb^V compared with the drug alone, as well as considerable shortening of the time needed for effective therapy (135, 136). Inhibition of TGF-β has been shown to decrease parasite burdens in experimental VL; however, TGF-β blockade has no apparent effect on Sb^V activity (136).

Using lower doses of miltefosine in combination with Pam3Cys (an immunomodulator synthetic bacterial lipopeptide (BLP) and TLR-2/1 ligand) in a BALB/c mouse model of VL, Shakya et al. (137) demonstrated significantly enhanced parasitic inhibition and Th1 cytokine production and an increased phagocytosis index. Another study, conducted by Karmakar et al. (138), demonstrated the interactions between a TLR ligand and invariant natural killer T (iNKT) cell activation as immunotherapy in VL. The authors evaluated the anti-*Leishmania* immune responses and the protective efficacy of the b-(1–4)-galactose terminal NKT cell ligand glycosphingophospholipid (GSPL) antigen of *L. donovani* parasites. Their findings suggested that TLR4 can function as an upstream sensor for GSPL and promote intracellular inflammatory signaling necessary for parasite killing. Furthermore, the treatment with GSPL induced a strong, effective T cell response, with control of acute parasite burden leading to undetectable parasite persistence (138).

The remarkable improvement in clinical signs and decrease in parasite burden in the immunotherapy or immunochemotherapy protocols described mostly arise from the restoration and activation of an effective immune response. In this context, the search for new therapeutic vaccines or substances with strong immunomodulatory effects as adjuvants (immunotherapy) may lead to the next generation of drugs, and associations with conventional chemotherapy (immunochemotherapy) will form the treatment strategy to cure visceral disease or reverse severe clinical forms of HVL.

CONCLUDING REMARKS

Most traditional and low-cost treatment options for VL are toxic and have many side effects, and the use of more effective drugs is limited mainly by the high cost. Successful immunotherapy using killed parasite vaccines or immunomodulators has been extensively reported in leishmaniasis. Another approach is immunochemotherapy, in which a low-dose or short course of chemotherapy associated with a vaccine or immunomodulator quickly induces an effective immune response. In VL, many efforts in the development and application of immunotherapy or immunochemotherapy have been made in the last decade, mainly due to the emergence of drug resistance and the increase in HIV co-infection. Many researchers have treated CVL using vaccines and immunomodulators with or without chemotherapy. In humans, the use of cytokines like IFN-γ associated with Sb^V has demonstrated promising results in patients that are unresponsive to conventional treatment. In murine models, immunomodulation based on mAbs to remove endogenous immunosuppressive cytokines or block their receptors, antigen-pulsed syngeneic DCs, and biological products like Pam3Cys (TLR ligand) has demonstrated future prospects for the treatment of VL. Efforts need to be directed to standardization and additional carefully controlled studies in animals and humans to understand the immunologic basis of these new vaccines and other immunomodulators in conjunction with chemotherapeutic agents for treatment of this important neglected disease.

ACKNOWLEDGMENTS

The authors are grateful for the Federal University of Ouro Preto (UFOP). This study was supported by the following grants: FAPEMIG grant: CNPq/BR/grant: 554757/2010-9; 476951/2013-5; GENOPROT 560943/2010-5; PPSUS/MS/CNPq/FAPEMIG/SES-MG/grant CBB-APQ-00356-10; APQ-01358-12. Alexandre Barbosa Reis, Rodolfo Cordeiro Giunchetti, and Cláudia Martins Carneiro are grateful for CNPq fellowships, Wendel Coura-Vital and Nádia das Dores Moreira are grateful to the PNPD/CAPES fellowships and Bruno Mendes Roatt is grateful to the CNPq/DTI-A fellowship.

REFERENCES

1. van Griensven J, Diro E. Visceral leishmaniasis. *Infect Dis Clin North Am* (2012) **26**(2):309–22. doi:10.1016/j.idc.2012.03.005
2. Dujardin JC, Campino L, Canavate C, Dedet JP, Gradoni L, Soteriadou K, et al. Spread of vector-borne diseases and neglect of leishmaniasis, Europe. *Emerg Infect Dis* (2008) **14**(7):1013–8. doi:10.3201/eid1407.071589
3. Desjeux P. Leishmaniasis: current situation and new perspectives. *Comp Immunol Microbiol Infect Dis* (2004) **27**(5):305–18. doi:10.1016/j.cimid.2004.03.004
4. Laurenti MD, Rossi CN, Matta VL, Tomokane TY, Corbett CE, Secundino NF, et al. Asymptomatic dogs are highly competent to transmit *Leishmania (Leishmania) infantum chagasi* to the natural vector. *Vet Parasitol* (2013) **196**(3–4):296–300. doi:10.1016/j.vetpar.2013.03.017
5. Molina R, Amela C, Nieto J, San-Andres M, Gonzalez F, Castillo JA, et al. Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*. *Trans R Soc Trop Med Hyg* (1994) **88**(4):491–3. doi:10.1016/0035-9203(94)90446-4
6. Solano-Gallego L, Koutinas A, Miro G, Cardoso L, Pennisi MG, Ferrer L, et al. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Vet Parasitol* (2009) **165**(1–2):1–18. doi:10.1016/j.vetpar.2009.05.022
7. Dantas-Torres F, Solano-Gallego L, Baneth G, Ribeiro VM, de Paiva-Cavalcanti M, Otranto D. Canine leishmaniosis in the Old and New Worlds: unveiled similarities and differences. *Trends Parasitol* (2012) **28**(12):531–8. doi:10.1016/j.pt.2012.08.007
8. Freeman KS, Miller MD, Breitschwerdt EB, Lappin MR. Leishmaniasis in a dog native to Colorado. *J Am Vet Med Assoc* (2010) **237**(11):1288–91. doi:10.2460/javma.237.11.1288
9. Duprey ZH, Steurer FJ, Rooney JA, Kirchhoff LV, Jackson JE, Rowton ED, et al. Canine visceral leishmaniasis, United States and Canada, 2000-2003. *Emerg Infect Dis* (2006) **12**(3):440–6. doi:10.3201/eid1203.050811
10. Baldelli R, Piva S, Salvatore D, Parigi M, Melloni O, Tamba M, et al. Canine leishmaniasis surveillance in a northern Italy kennel. *Vet Parasitol* (2011) **179**(1–3):57–61. doi:10.1016/j.vetpar.2011.01.052
11. Maroli M, Rossi L, Baldelli R, Capelli G, Ferroglio E, Genchi C, et al. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. *Trop Med Int Health* (2008) **13**(2):256–64. doi:10.1111/j.1365-3156.2007.01998.x

12. Malaquias LC, do Carmo Romualdo R, do Anjos JB Jr, Giunchetti RC, Correa-Oliveira R, Reis AB. Serological screening confirms the re-emergence of canine leishmaniosis in urban and rural areas in Governador Valadares, Vale do Rio Doce, Minas Gerais, Brazil. *Parasitol Res* (2007) **100**(2):233–9. doi:10.1007/s00436-006-0259-z
13. Solano-Gallego L, Morell P, Arboix M, Alberola J, Ferrer L. Prevalence of *Leishmania infantum* infection in dogs living in an area of canine leishmaniasis endemicity using PCR on several tissues and serology. *J Clin Microbiol* (2001) **39**(2):560–3. doi:10.1128/JCM.39.2.560-563.2001
14. Coura-Vital W, Marques MJ, Veloso VM, Roatt BM, Aguiar-Soares RD, Reis LE, et al. Prevalence and factors associated with *Leishmania infantum* infection of dogs from an urban area of Brazil as identified by molecular methods. *PLoS Negl Trop Dis* (2011) **5**(8):e1291. doi:10.1371/journal.pntd.0001291
15. Coura-Vital W, Reis AB, Fausto MA, Leal GG, Marques MJ, Veloso VM, et al. Risk factors for seroconversion by *Leishmania infantum* in a Cohort of dogs from an endemic area of Brazil. *PLoS One* (2013) **8**(8):e71833. doi:10.1371/journal.pone.0071833
16. Coura-Vital W, Reis AB, Reis LE, Braga SL, Roatt BM, Aguiar-Soares RD, et al. Canine visceral leishmaniasis: incidence and risk factors for infection in a cohort study in Brazil. *Vet Parasitol* (2013) **197**(3–4):411–7. doi:10.1016/j.vetpar.2013.07.031
17. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) **7**(5):e35671. doi:10.1371/journal.pone.0035671
18. Gramiccia M, Scalone A, Di Muccio T, Orsini S, Fiorentino E, Gradoni L. The burden of visceral leishmaniasis in Italy from 1982 to 2012: a retrospective analysis of the multi-annual epidemic that occurred from 1989 to 2009. *Euro Surveill* (2013) **18**(29):20535.
19. Bogdan C, Schonian G, Banuls AL, Hide M, Pratlong F, Lorenz E, et al. Visceral leishmaniasis in a German child who had never entered a known endemic area: case report and review of the literature. *Clin Infect Dis* (2001) **32**(2):302–6. doi:10.1086/318476
20. Alvar J, Aparicio P, Aseffa A, Den Boer M, Canavate C, Dedet JP, et al. The relationship between leishmaniasis and AIDS: the second 10 years. *Clin Microbiol Rev* (2008) **21**(2):334–59. doi:10.1128/CMR.00061-07
21. Pintado V, Martin-Rabadan P, Rivera ML, Moreno S, Bouza E. Visceral leishmaniasis in human immunodeficiency virus (HIV)-infected and non-HIV-infected patients. A comparative study. *Medicine (Baltimore)* (2001) **80**(1):54–73. doi:10.1097/00005792-200101000-00006
22. Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. *Lancet* (2005) **366**(9496):1561–77. doi:10.1016/S0140-6736(05)67629-5
23. Costa DL, Rocha RL, Carvalho RM, Lima-Neto AS, Harhay MO, Costa CH, et al. Serum cytokines associated with severity and complications of kala-azar. *Pathog Glob Health* (2013) **107**(2):78–87. doi:10.1179/2047773213Y.0000000078
24. Ahluwalia IB, Bern C, Costa C, Akter T, Chowdhury R, Ali M, et al. Visceral leishmaniasis: consequences of a neglected disease in a Bangladeshi community. *Am J Trop Med Hyg* (2003) **69**(6):624–8.
25. Barnett PG, Singh SP, Bern C, Hightower AW, Sundar S. Virgin soil: the spread of visceral leishmaniasis into Uttar Pradesh, India. *Am J Trop Med Hyg* (2005) **73**(4):720–5.
26. Ministério da saúde. *Sistema de Informação de Agravos de Notificação – Sinan Net*. (2013) [cited 2013 December 2]. Available from: http://dtr2004.saude.gov.br/sinanweb/tabnet/tabnet?sinannet/leishvi/bases/leishvbrnet.def
27. Kaye PM, Svensson M, Ato M, Maroof A, Polley R, Stager S, et al. The immunopathology of experimental visceral leishmaniasis. *Immunol Rev* (2004) **201**:239–53. doi:10.1111/j.0105-2896.2004.00188.x
28. Stager S, Joshi T, Bankoti R. Immune evasive mechanisms contributing to persistent *Leishmania donovani* infection. *Immunol Res* (2010) **47**(1–3):14–24. doi:10.1007/s12026-009-8135-4
29. Nylen S, Gautam S. Immunological perspectives of leishmaniasis. *J Glob Infect Dis* (2010) **2**(2):135–46. doi:10.4103/0974-777X.62876
30. Carvalho EM, Bacellar O, Barral A, Badaro R, Johnson WD Jr. Antigen-specific immunosuppression in visceral leishmaniasis is cell mediated. *J Clin Invest* (1989) **83**(3):860–4. doi:10.1172/JCI113969
31. Bacellar O, Brodskyn C, Guerreiro J, Barral-Netto M, Costa CH, Coffman RL, et al. Interleukin-12 restores interferon-gamma production and cytotoxic responses in visceral leishmaniasis. *J Infect Dis* (1996) **173**(6):1515–8. doi:10.1093/infdis/173.6.1515
32. Nylen S, Sacks D. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. *Trends Immunol* (2007) **28**(9):378–84. doi:10.1016/j.it.2007.07.004
33. Pinelli E, Killick-Kendrick R, Wagenaar J, Bernadina W, del Real G, Ruitenberg J. Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania infantum*. *Infect Immun* (1994) **62**(1):229–35.
34. Pinelli E, van der Kaaij SY, Broeren CP, Ruitenberg EJ, Rutten VP. Measurement of dog cytokines by reverse transcription-quantitative competitive polymerase chain reaction. *Immunogenetics* (1999) **49**(7–8):696–9. doi:10.1007/s002510050667
35. Reis AB, Martins-Filho OA, Teixeira-Carvalho A, Carvalho MG, Mayrink W, Franca-Silva JC, et al. Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis. *Res Vet Sci* (2006) **81**(1):68–75. doi:10.1016/j.rvsc.2005.09.011
36. Reis AB, Martins-Filho OA, Teixeira-Carvalho A, Giunchetti RC, Carneiro CM, Mayrink W, et al. Systemic and compartmentalized immune response in canine visceral leishmaniasis. *Vet Immunol Immunopathol* (2009) **128**(1–3):87–95. doi:10.1016/j.vetimm.2008.10.307
37. Bhattacharya P, Ali N. Involvement and interactions of different immune cells and their cytokines in human visceral leishmaniasis. *Rev Soc Bras Med Trop* (2013) **46**(2):128–34. doi:10.1590/0037-8682-0022-2012
38. Peruhype-Magalhaes V, Martins-Filho OA, Prata A, Silva Lde A, Rabello A, Teixeira-Carvalho A, et al. Immune response in human visceral leishmaniasis: analysis of the correlation between innate immunity cytokine profile and disease outcome. *Scand J Immunol* (2005) **62**(5):487–95. doi:10.1111/j.1365-3083.2005.01686.x
39. Manna PP, Chakrabarti G, Bandyopadhyay S. Innate immune defense in visceral leishmaniasis: cytokine mediated protective role by allogeneic effector cell. *Vaccine* (2010) **28**(3):803–10. doi:10.1016/j.vaccine.2009.10.053
40. Murray HW. Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis. *J Infect Dis* (1997) **175**(6):1477–9. doi:10.1086/516482
41. Bogdan C, Rollinghoff M, Diefenbach A. The role of nitric oxide in innate immunity. *Immunol Rev* (2000) **173**:17–26. doi:10.1034/j.1600-065X.2000.917307.x
42. Brandonisio O, Panaro MA, Fumarola I, Sisto M, Leogrande D, Acquafredda A, et al. Macrophage chemotactic protein-1 and macrophage inflammatory protein-1 alpha induce nitric oxide release and enhance parasite killing in *Leishmania infantum*-infected human macrophages. *Clin Exp Med* (2002) **2**(3):125–9. doi:10.1007/s102380200017
43. Medeiros IM, Reed S, Castelo A, Salomao R. Circulating levels of sTNFR and discrepancy between cytotoxicity and immunoreactivity of TNF-alpha in patients with visceral leishmaniasis. *Clin Microbiol Infect* (2000) **6**(1):34–7. doi:10.1046/j.1469-0691.2000.00011.x
44. Pitta MG, Romano A, Cabantous S, Henri S, Hammad A, Kouriba B, et al. IL-17 and IL-22 are associated with protection against human kala azar caused by *Leishmania donovani*. *J Clin Invest* (2009) **119**(8):2379–87. doi:10.1172/JCI38813
45. Ansari NA, Kumar R, Gautam S, Nylen S, Singh OP, Sundar S, et al. IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral leishmaniasis. *J Immunol* (2011) **186**(7):3977–85. doi:10.4049/jimmunol.1003588
46. Gautam S, Kumar R, Singh N, Singh AK, Rai M, Sacks D, et al. CD8 T cell exhaustion in human visceral leishmaniasis. *J Infect Dis* (2014) **209**(2):290–9. doi:10.1093/infdis/jit401
47. Racoosin EL, Beverley SM. *Leishmania major*: promastigotes induce expression of a subset of chemokine genes in murine macrophages. *Exp Parasitol* (1997) **85**(3):283–95. doi:10.1006/expr.1996.4139
48. Antoniazi S, Price HP, Kropf P, Freudenberg MA, Galanos C, Smith DF, et al. Chemokine gene expression in toll-like receptor-competent and -deficient mice infected with *Leishmania major*. *Infect Immun* (2004) **72**(9):5168–74. doi:10.1128/IAI.72.9.5168-5174.2004
49. Menezes-Souza D, Guerra-Sa R, Carneiro CM, Vitoriano-Souza J, Giunchetti RC, Teixeira-Carvalho A, et al. Higher expression of CCL2, CCL4, CCL5, CCL21, and CXCL8 chemokines in the skin associated with parasite density in canine visceral leishmaniasis. *PLoS Negl Trop Dis* (2012) **6**(4):e1566. doi:10.1371/journal.pntd.0001566

50. Stanley AC, Engwerda CR. Balancing immunity and pathology in visceral leishmaniasis. *Immunol Cell Biol* (2007) **85**(2):138–47. doi:10.1038/sj.icb7100011
51. Hailu A, van der Poll T, Berhe N, Kager PA. Elevated plasma levels of interferon (IFN)-gamma, IFN-gamma inducing cytokines, and IFN-gamma inducible CXC chemokines in visceral leishmaniasis. *Am J Trop Med Hyg* (2004) **71**(5):561–7.
52. Strauss-Ayali D, Baneth G, Jaffe CL. Splenic immune responses during canine visceral leishmaniasis. *Vet Res* (2007) **38**(4):547–64. doi:10.1051/vetres:2007015
53. Ritter U, Moll H. Monocyte chemotactic protein-1 stimulates the killing of *Leishmania major* by human monocytes, acts synergistically with IFN-gamma and is antagonized by IL-4. *Eur J Immunol* (2000) **30**(11):3111–20. doi:10.1002/1521-4141(200011)30:11<3111::AID-IMMU3111>3.0.CO;2-O
54. Trinchieri G. Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-gamma). *Curr Opin Immunol* (1997) **9**(1):17–23. doi:10.1016/S0952-7915(97)80154-9
55. Kemp M, Kurtzhals JA, Kharazmi A, Theander TG. Interferon-gamma and interleukin-4 in human *Leishmania donovani* infections. *Immunol Cell Biol* (1993) **71**(Pt 6):583–7. doi:10.1038/icb.1993.64
56. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* (1991) **174**(5):1209–20. doi:10.1084/jem.174.5.1209
57. de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* (1991) **174**(4):915–24. doi:10.1084/jem.174.4.915
58. Scharton-Kersten T, Afonso LC, Wysocka M, Trinchieri G, Scott P. IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. *J Immunol* (1995) **154**(10):5320–30.
59. World Health Organization. *Control of the Leishmaniasis: Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases*. Geneva: WHO (2010). p. 202. Available from: http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf
60. Singh N, Kumar M, Singh RK. Leishmaniasis: current status of available drugs and new potential drug targets. *Asian Pac J Trop Med* (2012) **5**(6):485–97. doi:10.1016/S1995-7645(12)60084-4
61. Fraga DB, Solca MS, Silva VM, Borja LS, Nascimento EG, Oliveira GG, et al. Temporal distribution of positive results of tests for detecting *Leishmania* infection in stray dogs of an endemic area of visceral leishmaniasis in the Brazilian tropics: a 13 years survey and association with human disease. *Vet Parasitol* (2012) **190**(3–4):591–4. doi:10.1016/j.vetpar.2012.06.025
62. Davidson RN, di Martino L, Gradoni L, Giacchino R, Gaeta GB, Pempinello R, et al. Short-course treatment of visceral leishmaniasis with liposomal amphotericin B (AmBisome). *Clin Infect Dis* (1996) **22**(6):938–43. doi:10.1093/clinids/22.6.938
63. Jha TK, Sundar S, Thakur CP, Bachmann P, Karbwang J, Fischer C, et al. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. *N Engl J Med* (1999) **341**(24):1795–800. doi:10.1056/NEJM199912093412403
64. Sundar S, Jha TK, Thakur CP, Engel J, Sindermann H, Fischer C, et al. Oral miltefosine for Indian visceral leishmaniasis. *N Engl J Med* (2002) **347**(22):1739–46. doi:10.1056/NEJMoa021556
65. Jha TK, Olliaro P, Thakur CP, Kanyok TP, Singhania BL, Singh IJ, et al. Randomised controlled trial of aminosidine (paromomycin) v sodium stibogluconate for treating visceral leishmaniasis in North Bihar, India. *BMJ* (1998) **316**(7139):1200–5. doi:10.1136/bmj.316.7139.1200
66. Alvar J, Croft S, Olliaro P. Chemotherapy in the treatment and control of leishmaniasis. *Adv Parasitol* (2006) **61**:223–74. doi:10.1016/S0065-308X(05)61006-8
67. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. *Clin Microbiol Rev* (2006) **19**(1):111–26. doi:10.1128/CMR.19.1.111-126.2006
68. Matoussi N, Ameur HB, Amor SB, Fitouri Z, Becher SB. Cardiotoxicity of n-methyl-glucamine antimoniate (Glucantime). A case report. *Med Mal Infect* (2007) **37**(Suppl 3):S257–9. doi:10.1016/j.medmal.2007.08.001
69. Gasser RA Jr, Magill AJ, Oster CN, Franke ED, Grogl M, Berman JD. Pancreatitis induced by pentavalent antimonial agents during treatment of leishmaniasis. *Clin Infect Dis* (1994) **18**(1):83–90. doi:10.1093/clinids/18.1.83
70. Zaghloul IY, Al-Jasser M. Effect of renal impairment on the pharmacokinetics of antimony in hamsters. *Ann Trop Med Parasitol* (2004) **98**(8):793–800. doi:10.1179/000349804X3171
71. Mancianti F, Gramiccia M, Gradoni L, Pieri S. Studies on canine leishmaniasis control. 1. Evolution of infection of different clinical forms of canine leishmaniasis following antimonial treatment. *Trans R Soc Trop Med Hyg* (1988) **82**(4):566–7. doi:10.1016/0035-9203(88)90510-X
72. Slappendel RJ, Teske E. The effect of intravenous or subcutaneous administration of meglumine antimoniate (Glucantime) in dogs with leishmaniasis. A randomized clinical trial. *Vet Q* (1997) **19**(1):10–3. doi:10.1080/01652176.1997.9694729
73. Carrio J, Portus M. In vitro susceptibility to pentavalent antimony in *Leishmania infantum* strains is not modified during in vitro or in vivo passages but is modified after host treatment with meglumine antimoniate. *BMC Pharmacol* (2002) **2**:11. doi:10.1186/1471-2210-2-11
74. da Silva SM, Amorim IF, Ribeiro RR, Azevedo EG, Demicheli C, Melo MN, et al. Efficacy of combined therapy with liposome-encapsulated meglumine antimoniate and allopurinol in treatment of canine visceral leishmaniasis. *Antimicrob Agents Chemother* (2012) **56**(6):2858–67. doi:10.1128/AAC.00208-12
75. Lachaud L, Bourgeois N, Plourde M, Leprohon P, Bastien P, Ouellette M. Parasite susceptibility to amphotericin B in failures of treatment for visceral leishmaniasis in patients coinfected with HIV type 1 and *Leishmania infantum*. *Clin Infect Dis* (2009) **48**(2):e16–22. doi:10.1086/595710
76. Sundar S, Chakravarty J, Rai VK, Agrawal N, Singh SP, Chauhan V, et al. Amphotericin B treatment for Indian visceral leishmaniasis: response to 15 daily versus alternate-day infusions. *Clin Infect Dis* (2007) **45**(5):556–61. doi:10.1086/520665
77. Sinha PK, Roddy P, Palma PP, Kociejowski A, Lima MA, Rabi Das VN, et al. Effectiveness and safety of liposomal amphotericin B for visceral leishmaniasis under routine program conditions in Bihar, India. *Am J Trop Med Hyg* (2010) **83**(2):357–64. doi:10.4269/ajtmh.2010.10-0156
78. Oliva G, Gradoni L, Ciaramella P, De Luna R, Cortese L, Orsini S, et al. Activity of liposomal amphotericin B (AmBisome) in dogs naturally infected with *Leishmania infantum*. *J Antimicrob Chemother* (1995) **36**(6):1013–9. doi:10.1093/jac/36.6.1013
79. Rigo RS, Carvalho CM, Honer MR, Andrade GB, Silva IS, Rigo L, et al. Renal histopathological findings in dogs with visceral leishmaniasis. *Rev Inst Med Trop Sao Paulo* (2013) **55**(2):113–6. doi:10.1590/S0036-46652013000200008
80. Cortadellas O. Initial and long-term efficacy of a lipid emulsion of amphotericin B desoxycholate in the management of canine leishmaniasis. *J Vet Intern Med* (2003) **17**(6):808–12. doi:10.1111/j.1939-1676.2003.tb02518.x
81. Croft SL, Coombs GH. Leishmaniasis – current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol* (2003) **19**(11):502–8. doi:10.1016/j.pt.2003.09.008
82. Sundar S, Jha TK, Thakur CP, Bhattacharya SK, Rai M. Oral miltefosine for the treatment of Indian visceral leishmaniasis. *Trans R Soc Trop Med Hyg* (2006) **100**(Suppl 1):S26–33. doi:10.1016/j.trstmh.2006.02.011
83. Verma NK, Dey CS. Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. *Antimicrob Agents Chemother* (2004) **48**(8):3010–5. doi:10.1128/AAC.48.8.3010-3015.2004
84. Sundar S, Olliaro PL. Miltefosine in the treatment of leishmaniasis: clinical evidence for informed clinical risk management. *Ther Clin Risk Manag* (2007) **3**(5):733–40.
85. Perez-Victoria FJ, Gamarro F, Ouellette M, Castanys S. Functional cloning of the miltefosine transporter. A novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. *J Biol Chem* (2003) **278**(50):49965–71. doi:10.1074/jbc.M308352200
86. Andrade HM, Toledo VP, Pinheiro MB, Guimaraes TM, Oliveira NC, Castro JA, et al. Evaluation of miltefosine for the treatment of dogs naturally infected with *L. infantum* (=*L. chagasi*) in Brazil. *Vet Parasitol* (2011) **181**(2–4):83–90. doi:10.1016/j.vetpar.2011.05.009
87. Farca AM, Miniscalco B, Badino P, Odore R, Monticelli P, Trisciuoglio A, et al. Canine leishmaniosis: in vitro efficacy of miltefosine and marbofloxacin alone or in combination with allopurinol against clinical strains of *Leishmania infantum*. *Parasitol Res* (2012) **110**(6):2509–13. doi:10.1007/s00436-011-2792-7
88. Woerly V, Maynard L, Sanquer A, Eun HM. Clinical efficacy and tolerance of miltefosine in the treatment of canine leishmaniosis. *Parasitol Res* (2009) **105**(2):463–9. doi:10.1007/s00436-009-1404-2

89. Hailu A, Musa A, Wasunna M, Balasegaram M, Yifru S, Mengistu G, et al. Geographical variation in the response of visceral leishmaniasis to paromomycin in East Africa: a multicentre, open-label, randomized trial. *PLoS Negl Trop Dis* (2010) **4**(10):e709. doi:10.1371/journal.pntd.0000709
90. Sundar S, Chakravarty J. Paromomycin in the treatment of leishmaniasis. *Expert Opin Investig Drugs* (2008) **17**(5):787–94. doi:10.1517/13543784.17.5.787
91. Fernandez MM, Malchiodi EL, Algranati ID. Differential effects of paromomycin on ribosomes of *Leishmania mexicana* and mammalian cells. *Antimicrob Agents Chemother* (2011) **55**(1):86–93. doi:10.1128/AAC.00506-10
92. Sundar S, Jha TK, Thakur CP, Sinha PK, Bhattacharya SK. Injectable paromomycin for visceral leishmaniasis in India. *N Engl J Med* (2007) **356**(25):2571–81. doi:10.1056/NEJMoa066536
93. Poli A, Sozzi S, Guidi G, Bandinelli P, Mancianti F. Comparison of aminosidine (paromomycin) and sodium stibogluconate for treatment of canine leishmaniasis. *Vet Parasitol* (1997) **71**(4):263–71. doi:10.1016/S0304-4017(97)00014-9
94. Vexenat JA, Olliaro PL, Fonseca de Castro JA, Cavalcante R, Furtado Campos JH, Tavares JP, et al. Clinical recovery and limited cure in canine visceral leishmaniasis treated with aminosidine (paromomycin). *Am J Trop Med Hyg* (1998) **58**(4):448–53.
95. Mueller M, Ritmeijer K, Balasegaram M, Koummuki Y, Santana MR, Davidson R. Unresponsiveness to AmBisome in some Sudanese patients with kala-azar. *Trans R Soc Trop Med Hyg* (2007) **101**(1):19–24. doi:10.1016/j.trstmh.2006.02.005
96. Freitas-Junior LH, Chatelain E, Kim HA, Siqueira-Neto JL. Visceral leishmaniasis treatment: what do we have, what do we need and how to deliver it? *Int J Parasitol Drugs Drug Resist* (2012) **2**:11–9. doi:10.1016/j.ijpddr.2012.01.003
97. Sundar S, Chakravarty J. Leishmaniasis: an update of current pharmacotherapy. *Expert Opin Pharmacother* (2013) **14**(1):53–63. doi:10.1517/14656566.2013.755515
98. Oliva G, Roura X, Crotti A, Maroli M, Castagnaro M, Gradoni L, et al. Guidelines for treatment of leishmaniasis in dogs. *J Am Vet Med Assoc* (2010) **236**(11):1192–8. doi:10.2460/javma.236.11.1192
99. Okwor I, Uzonna JE. Immunotherapy as a strategy for treatment of leishmaniasis: a review of the literature. *Immunotherapy* (2009) **1**(5):765–76. doi:10.2217/imt.09.40
100. Oldham RK. Biologicals: new horizons in pharmaceutical development. *J Biol Response Mod* (1983) **2**(3):199–206.
101. Oldham RK, Smalley RV. Immunotherapy: the old and the new. *J Biol Response Mod* (1983) **2**(1):1–37.
102. El-On J. Current status and perspectives of the immunotherapy of leishmaniasis. *Isr Med Assoc J* (2009) **11**(10):623–8.
103. Genaro O, de Toledo VP, da Costa CA, Hermeto MV, Afonso LC, Mayrink W. Vaccine for prophylaxis and immunotherapy, Brazil. *Clin Dermatol* (1996) **14**(5):503–12. doi:10.1016/0738-081X(96)00040-5
104. Convit J, Castellanos PL, Rondon A, Pinardi ME, Ulrich M, Castes M, et al. Immunotherapy versus chemotherapy in localised cutaneous leishmaniasis. *Lancet* (1987) **1**(8530):401–5. doi:10.1016/S0140-6736(87)90116-4
105. Convit J, Ulrich M, Zerpa O, Borges R, Aranzazu N, Valera M, et al. Immunotherapy of American cutaneous leishmaniasis in Venezuela during the period 1990-99. *Trans R Soc Trop Med Hyg* (2003) **97**(4):469–72. doi:10.1016/S0035-9203(03)90093-9
106. Mayrink W, Magalhaes PA, Michalick MS, da Costa CA, Lima Ade O, Melo MN, et al. Immunotherapy as a treatment of American cutaneous leishmaniasis: preliminary studies in Brazil. *Parassitologia* (1992) **34**(1–3):159–65.
107. Mayrink W, Botelho AC, Magalhaes PA, Batista SM, Lima Ade O, Genaro O, et al. Immunotherapy, immunochemotherapy and chemotherapy for American cutaneous leishmaniasis treatment. *Rev Soc Bras Med Trop* (2006) **39**(1):14–21. doi:10.1590/S0037-86822006000100003
108. Musa AM, Khalil EA, Mahgoub FA, Elgawi SH, Modabber F, Elkadaru AE, et al. Immunochemotherapy of persistent post-kala-azar dermal leishmaniasis: a novel approach to treatment. *Trans R Soc Trop Med Hyg* (2008) **102**(1):58–63. doi:10.1016/j.trstmh.2007.08.006
109. Dalton JE, Kaye PM. Immunomodulators: use in combined therapy against leishmaniasis. *Expert Rev Anti Infect Ther* (2010) **8**(7):739–42. doi:10.1586/eri.10.64
110. Murray HW, Rubin BY, Rothermel CD. Killing of intracellular *Leishmania donovani* by lymphokine-stimulated human mononuclear phagocytes. Evidence that interferon-gamma is the activating lymphokine. *J Clin Invest* (1983) **72**(4):1506–10. doi:10.1172/JCI111107
111. Murray HW. Interferon-gamma and host antimicrobial defense: current and future clinical applications. *Am J Med* (1994) **97**(5):459–67. doi:10.1016/0002-9343(94)90326-3
112. Sundar S, Rosenkaimer F, Murray HW. Successful treatment of refractory visceral leishmaniasis in India using antimony plus interferon-gamma. *J Infect Dis* (1994) **170**(3):659–62. doi:10.1093/infdis/170.3.659
113. Badaro R. The use of recombinant gamma interferon associated with pentavalent antimony in therapy for visceral leishmaniasis. *Mem Inst Oswaldo Cruz* (1988) **83**(Suppl 1):376–7. doi:10.1590/S0074-02761988000500028
114. Sundar S, Singh VP, Sharma S, Makharia MK, Murray HW. Response to interferon-gamma plus pentavalent antimony in Indian visceral leishmaniasis. *J Infect Dis* (1997) **176**(4):1117–9. doi:10.1086/516526
115. Baneth G, Shaw SE. Chemotherapy of canine leishmaniosis. *Vet Parasitol* (2002) **106**(4):315–24. doi:10.1016/S0304-4017(02)00115-2
116. Riera C, Valladares JE, Gallego M, Aisa MJ, Castillejo S, Fisa R, et al. Serological and parasitological follow-up in dogs experimentally infected with *Leishmania infantum* and treated with meglumine antimoniate. *Vet Parasitol* (1999) **84**(1–2):33–47. doi:10.1016/S0304-4017(99)00084-9
117. Guarga JL, Moreno J, Lucientes J, Gracia MJ, Peribanez MA, Castillo JA. Evaluation of a specific immunochemotherapy for the treatment of canine visceral leishmaniasis. *Vet Immunol Immunopathol* (2002) **88**(1–2):13–20. doi:10.1016/S0165-2427(02)00128-9
118. Borja-Cabrera GP, Cruz Mendes A, Paraguai de Souza E, Hashimoto Okada LY, de A Trivellato FA, Kawasaki JK, et al. Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. *Vaccine* (2004) **22**(17–18):2234–43. doi:10.1016/j.vaccine.2003.11.039
119. Santos FN, Borja-Cabrera GP, Miyashiro LM, Grechi J, Reis AB, Moreira MA, et al. Immunotherapy against experimental canine visceral leishmaniasis with the saponin enriched-Leishmune vaccine. *Vaccine* (2007) **25**(33):6176–90. doi:10.1016/j.vaccine.2007.06.005
120. Borja-Cabrera GP, Santos FN, Santos FB, Trivellato FA, Kawasaki JK, Costa AC, et al. Immunotherapy with the saponin enriched-Leishmune vaccine versus immunochemotherapy in dogs with natural canine visceral leishmaniasis. *Vaccine* (2010) **28**(3):597–603. doi:10.1016/j.vaccine.2009.09.071
121. Badaro R, Falcoff E, Badaro FS, Carvalho EM, Pedral-Sampaio D, Barral A, et al. Treatment of visceral leishmaniasis with pentavalent antimony and interferon gamma. *N Engl J Med* (1990) **322**(1):16–21. doi:10.1056/NEJM199001043220104
122. Squires KE, Rosenkaimer F, Sherwood JA, Forni AL, Were JB, Murray HW. Immunochemotherapy for visceral leishmaniasis: a controlled pilot trial of antimony versus antimony plus interferon-gamma. *Am J Trop Med Hyg* (1993) **48**(5):666–9.
123. Sundar S, Murray HW. Effect of treatment with interferon-gamma alone in visceral leishmaniasis. *J Infect Dis* (1995) **172**(6):1627–9. doi:10.1093/infdis/172.6.1627
124. Miret J, Nascimento E, Sampaio W, Franca JC, Fujiwara RT, Vale A, et al. Evaluation of an immunochemotherapeutic protocol constituted of N-methyl meglumine antimoniate (Glucantime) and the recombinant Leish-110f + MPL-SE vaccine to treat canine visceral leishmaniasis. *Vaccine* (2008) **26**(12):1585–94. doi:10.1016/j.vaccine.2008.01.026
125. Trigo J, Abbehusen M, Netto EM, Nakatani M, Pedral-Sampaio G, de Jesus RS, et al. Treatment of canine visceral leishmaniasis by the vaccine Leish-111f+MPL-SE. *Vaccine* (2010) **28**(19):3333–40. doi:10.1016/j.vaccine.2010.02.089
126. Santiago ME, Neto LS, Alexandre EC, Munari DP, Andrade MM, Somenzari MA, et al. Improvement in clinical signs and cellular immunity of dogs with visceral leishmaniasis using the immunomodulator P-MAPA. *Acta Trop* (2013) **127**(3):174–80. doi:10.1016/j.actatropica.2013.04.005
127. Favaro WJ, Nunes OS, Seiva FR, Nunes IS, Woolhiser LK, Duran N, et al. Effects of P-MAPA immunomodulator on toll-like receptors and p53: potential therapeutic strategies for infectious diseases and cancer. *Infect Agent Cancer* (2012) **7**(1):14. doi:10.1186/1750-9378-7-14
128. Roatt BM, Aguiar-Soares RD, Vitoriano-Souza J, Coura-Vital W, Braga SL, Correa-Oliveira R, et al. Performance of LBSap vaccine after intradermal

challenge with *L. infantum* and saliva of *Lu. longipalpis*: immunogenicity and parasitological evaluation. *PLoS One* (2012) **7**(11):e49780. doi:10.1371/journal.pone.0049780
129. Aguiar-Soares RD, Roatt BM, Ker HG, Moreira N, Mathias FA, Cardoso JM, et al. LBSapSal-vaccinated dogs exhibit increased circulating T-lymphocyte subsets (CD4(+) and CD8(+)) as well as a reduction of parasitism after challenge with *Leishmania infantum* plus salivary gland of *Lutzomyia longipalpis*. *Parasit Vectors* (2014) **7**:61. doi:10.1186/1756-3305-7-61
130. Musa AM, Noazin S, Khalil EA, Modabber F. Immunological stimulation for the treatment of leishmaniasis: a modality worthy of serious consideration. *Trans R Soc Trop Med Hyg* (2010) **104**(1):1–2. doi:10.1016/j.trstmh.2009.07.026
131. Moll H, Berberich C. Dendritic cell-based vaccination strategies: induction of protective immunity against leishmaniasis. *Immunobiology* (2001) **204**(5):659–66. doi:10.1078/0171-2985-00105
132. Ghosh M, Pal C, Ray M, Maitra S, Mandal L, Bandyopadhyay S. Dendritic cell-based immunotherapy combined with antimony-based chemotherapy cures established murine visceral leishmaniasis. *J Immunol* (2003) **170**(11):5625–9. doi:10.4049/jimmunol.170.11.5625
133. Murray HW, Lu CM, Brooks EB, Fichtl RE, DeVecchio JL, Heinzel FP. Modulation of T-cell costimulation as immunotherapy or immunochemotherapy in experimental visceral leishmaniasis. *Infect Immun* (2003) **71**(11):6453–62. doi:10.1128/IAI.71.11.6453-6462.2003
134. Zubairi S, Sanos SL, Hill S, Kaye PM. Immunotherapy with OX40L-Fc or anti-CTLA-4 enhances local tissue responses and killing of *Leishmania donovani*. *Eur J Immunol* (2004) **34**(5):1433–40. doi:10.1002/eji.200324021
135. Murray HW, Lu CM, Mauze S, Freeman S, Moreira AL, Kaplan G, et al. Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. *Infect Immun* (2002) **70**(11):6284–93. doi:10.1128/IAI.70.11.6284-6293.2002
136. Murray HW. Interleukin 10 receptor blockade – pentavalent antimony treatment in experimental visceral leishmaniasis. *Acta Trop* (2005) **93**(3):295–301. doi:10.1016/j.actatropica.2004.11.008
137. Shakya N, Sane SA, Vishwakarma P, Gupta S. Enhancement in therapeutic efficacy of miltefosine in combination with synthetic bacterial lipopeptide, Pam3Cys against experimental visceral leishmaniasis. *Exp Parasitol* (2012) **131**(3):377–82. doi:10.1016/j.exppara.2012.05.007
138. Karmakar S, Bhaumik SK, Paul J, De T. TLR4 and NKT cell synergy in immunotherapy against visceral leishmaniasis. *PLoS Pathog* (2012) **8**(4):e1002646. doi:10.1371/journal.ppat.1002646

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 March 2014; accepted: 27 May 2014; published online: 13 June 2014.
*Citation: Roatt BM, Aguiar-Soares RDdO, Coura-Vital W, Ker HG, Moreira NdD, Vitoriano-Souza J, Giunchetti RC, Carneiro CM and Reis AB (2014) Immunotherapy and immunochemotherapy in visceral leishmaniasis: promising treatments for this neglected disease. Front. Immunol. **5**:272. doi: 10.3389/fimmu.2014.00272*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 26 June 2014
doi: 10.3389/fimmu.2014.00296

Immunotherapy and targeted therapies in treatment of visceral leishmaniasis: current status and future prospects

Om Prakash Singh and Shyam Sundar*

Infectious Disease Research Laboratory, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Edited by:
Nahid Ali, Indian Institute of Chemical Biology, India

Reviewed by:
Scott D. Gray-Owen, University of Toronto, Canada
Lynn Soong, The University of Texas Medical Branch, USA

****Correspondence:***
Shyam Sundar, Infectious Disease Research Laboratory, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh 221005, India
e-mail: drshyamsundar@hotmail.com

Visceral leishmaniasis (VL) is a vector-borne chronic infectious disease caused by the protozoan parasite *Leishmania donovani or Leishmania infantum*. VL is a serious public health problem, causing high morbidity and mortality in the developing world with an estimated 0.2–0.4 million new cases each year. In the absence of a vaccine, chemotherapy remains the favored option for disease control, but is limited by a narrow therapeutic index, significant toxicities, and frequently acquired resistance. Improved understanding of VL pathogenesis offers the development and deployment of immune based treatment options either alone or in combination with chemotherapy. Modulations of host immune response include the inhibition of molecular pathways that are crucial for parasite growth and maintenance; and stimulation of host effectors immune responses that restore the impaired effector functions. In this review, we highlight the challenges in treatment of VL with a particular emphasis on immunotherapy and targeted therapies to improve clinical outcomes.

Keywords: immunotherapy, visceral leishmaniasis, treatment, resistance, IL-10, anti-IL-10 mAb

INTRODUCTION

Leishmaniasis , a spectrum of diseases caused by *Leishmania* species, affects ~12 million people around the world, mostly in developing countries. It is transmitted by sand flies (*Phlebotomus* species) as extracellular flagellated promastigotes and replicate as intracellular, aflagellated amastigotes in mononuclear phagocytes in mammalian host (1). Depending on the species, the disease symptoms may range from self-healing skin lesions to the fatal visceral form known as kala-azar or visceral leishmaniasis (VL). Kala-azar is the most severe form of the leishmaniasis and accounts for 200–400 thousands new cases and over 50,000 deaths annually (2). Anthroponotic transmission of VL is caused by *Leishmania donovani* and prevails in Indian subcontinent and East Africa; while zoonotic transmission of VL is caused by *L. infantum* (syn. *L. chagasi*) in the Mediterranean region, South America, and Southwest and Central Asia. The majority of all cases (90%) are found in India, Nepal, Bangladesh, Brazil, South Sudan, and Ethiopia (3), where transmission typically occurs from humans infected with kala-azar or post kala-azar dermal leishmaniasis (PKDL) (4, 5). More specifically, an estimated 80% of the global burden of VL occurs in South Asia (e.g. in 2007, 100,000–150,000 of the cases occurred in India alone). The situation is especially severe in Bihar State in eastern India, where some districts have faced the worst epidemic since the 1970s. Left untreated, VL is fatal, and the burden of disease expressed in disability-adjusted life years is estimated to be ~2.5 million. Furthermore, over 90% of all individuals with VL earn an income of <2 United States (US) dollars per day. Because VL is associated with resource-poor regions, access to care is another challenge in the overall management and treatment of VL (6, 7). The situation is further complicated by the emergence of resistant strains to currently available anti-leishmanial drugs and by the limited availability of inexpensive, non-toxic drugs (**Table 1**). Antimonial chemotherapy has been the mainstay for VL treatment for more than 50 years, and continues to be the recommended first line treatment in most parts of the world (8). Resistance to pentavalent antimonials (Sb^v) has first been reported in northern Bihar, where nearly 60% of individuals are now unresponsive to this drug (9). Pentamidine has been the second line drug used in Sb^v refractory patients. Unfortunately, its efficacy has also declined over the years, and now curing only ~70% patients. Resistance has also been reported with pentamidine and miltefosine (10, 11), and there is growing concern for resistance with paromomycin monotherapy (12). Increasing parasite drug resistance, longer treatment times, and associated toxicity to patients has resulted in the need to use more expensive drugs such as AmBisome® (liposomal amphotericin B) and miltefosine (8). A recent study demonstrated a single dose of liposomal amphotericin B is an effective VL treatment (13). However, concerns about emerging drug resistance with single drug therapy have led to testing liposomal amphotericin B in combination with oral miltefosine (14, 15). This strategy is still requiring administration of the drugs over an extended period and cost and toxic side effects are major issues. Hence, dose-sparing strategies that shorten treatment times are likely to be of major benefit to VL treatment programs. In addition, an intervention that can reduce the risk of developing PKDL is also highly desired. Importantly and relevant to this discussion, drug therapy works most effectively with help from the host immune system, and in particular, cell mediated immune (CMI) responses. Hence, immune modulation that stimulates immunity and work synergistically with drugs has enormous potential for drug-sparing strategies that would help in the treatment of a broad range of diseases.

Currently, there is no effective human vaccine available for any form of leishmaniasis. One of the major challenges in vaccine development has been a limited understanding of the precise immune mechanisms required for controlling parasite growth

Table 1 | Current VL treatments with anti-leishmanial drugs: their mode(s) of action on parasites, dosage, efficacy, advantages and limitations.

	Drugs	Mode(s) of action	Dosage	Efficacy (%)	Advantages	Limitations	Reference
1	Pentavalent antimonials: sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime)	Acts as pro-drug that is converted to active and more toxic trivalent form within the amastigote/macrophage; and this active trivalent SbIII form inhibits trypanothione reductase and exposes parasite to oxidative stress of the host	20 mg/kg/day (i.m.) for 20–30 days in India	80–90 (50% in Bihar, India)	Low cost and easily availability in endemic area	Pancreatitis, cardiac arrhythmias, acquired resistance in the Indian subcontinent	(8, 16, 17)
2	Amphotericin B (Fungizone)	Form complexes and bind to ergosterol in parasite membranes that create pores, which alter ion balance, increase membrane permeability resulting in cell death; also acts as an inhibitor of ergosterol biosynthesis	0.75–1.0 mg/kg for 15–20 infusions either daily on alternate days in India (i.v)	>95%	Effective in antimony resistant regions, primary resistance is unknown	High cost and need of prolonged hospitalization, rigor, and fever with renal complications, hypokalemia	(16, 18, 19)
3	Liposomal amphotericin B (AmBisome)	Targeted delivery of drug to the infected macrophage and mechanism of action is same as amphotericin	3.0 mg/kg/day for 5 days (total 15 mg) OR 10 mg/kg as a single dose, i.v	>96%	Highly effective, low toxicity, resistance is not documented	High cost	(13)
4	Paromomycin (aminoglycoside antibiotic), also known as aminosidine	Exact mechanism is not known. In bacteria, inhibits protein synthesis, but in *Leishmania*, it decreases the mitochondrial membrane potential of *L. donovani* promastigotes	11 mg/kg of base/day for 21 days (i.m.)	95%	Acts synergistically with antimonials, effective, well tolerated, and cheapest drug for VL	Reversible ototoxicity but no nephrotoxicity, lack of efficacy in East Africa	(20–22)
5	Miltefosine	Interacts with the cell membrane of *Leishmania* parasites by modulation of cell surface receptors, inositol metabolism, and phospholipase activation, Cell death being mediated by apoptosis	50 mg/day for adults <25 kg and 100 mg/day >50 kg adults (oral)	85–95%	First oral drug for VL. Currently first line of treatment in Indian subcontinent	Potentially teratogenic, vomiting, and diarrhea with occasional hepatic and renal toxicity	(15, 19)
6	Pentamidine	Accumulate in parasite mitochondria and inhibit mitochondrial topoisomerase II, binding to AT-rich sites in the minor groove of DNA followed by inhibition of transcription process	4 mg/kg/day for three times weekly for 15–20 dose (i.m or i.v)	70–80%	Low efficacy, toxic. May be used in combination with other drugs	Gastrointestinal side effects, cardiac, arrhythmias, hypotension, pancreatitis, and irreversible insulin-dependent diabetes mellitus	(23, 24)

(25, 26). In the present review, we highlight the current status and challenges in treatment of leishmaniasis with focus on immune based strategy for improving treatment regimens for VL.

IMMUNE REGULATION AND IMMUNOPATHOGENESIS

Mammals have evolved to recognize and control pathogens, including the recognition of infected cells. This is achieved by the coordinated actions of innate and adaptive immune mechanisms

[reviewed in Ref. (27)]. The innate immune response involves the recognition and early control of threats to the body as well as for the activation of adaptive immunity. Adaptive immune response involves B cells that produce specific antibodies; and T cells that recognize peptide antigens. T cell responses are mediated by $CD8^+$ T cells that recognize peptides derived from both inside and outside of cells and presented by major histocompatibility class (MHC) I molecules on the cell surface or $CD4^+$ T cells that recognize peptides from microbes or antigens engulfed by professional phagocytes and then presented on the context of MHC II molecules. The main targets of immunomodulatory strategies should be $CD4^+$ T cells because they play critical roles in coordinating immune responses by producing molecules critical for the production of high affinity antibodies by B cells, essential for activation of $CD8^+$ T cells to kill infected and transformed cells.

Based on the studies in the *L. major*/BALB/c mouse model, the immune dysregulation associated with non-healing and disseminating forms of leishmaniasis has been associated with a parasite-driven Th2 polarized response, in which interleukin (IL)-4 is especially dominant [reviewed in Ref. (28)]. Accumulating data in human VL, however, indicate that the cytokine responses are not highly polarized, and even during the acute phase of disease, elevated levels of interferon-γ (IFN-γ) mRNA have been found in lesional tissue, such as the spleen and bone marrow (29–31). Furthermore, in human VL, overproduction of IL-10 provides a much better correlate of susceptibility than IL-4. The vast array of cytokines, chemokines, and immune mechanisms involved in the host immune response to *Leishmania* clearly highlights the complexity of diseases (32, 33). Based on studies in mice, production of interleukin-12 (IL-12) by antigen-presenting cells (APCs) and IFN-γ by T cells appear to be required for the control of the parasites and development of acquired resistance (34, 35). IL-12 is regulatory cytokine for initiation and maintenance of the Th1 response and plays an important role in the induction of IFN-γ production by T and NK cells (36–40). Priming of susceptible BALB/c mice with exogenous rIL-12 during *Leishmania* infection also promotes protection and gives self-healing phenotype (41, 42). On the other hand, *Leishmania* parasites have been shown to inhibit IL-12 production, resulting in decreased leishmanicidal activity of macrophage (43). Maintenance of the proportion of $CD4^+$ and $CD8^+$ T cells required for cytokines secretion is the crucial step in generation of immunity against leishmaniasis. In active VL, both CD4 and CD8 cells are activated and play distinct but cooperative role in disease resolution. $CD4^+$ cells play a role in the control of primary infection, while $CD8^+$ cells are thought to be more important during secondary immune response (44).

Human VL is characterized by very high titers of *Leishmania*-specific antibodies, appearing soon after infection but before the development of cellular immunological abnormalities (45, 46). These anti-leishmanial antibodies persist up to 16 years after treatment, suggesting its possible involvement in immunity (47). There are reports that B cells and antibodies correlate with pathology, but role of these antibodies in disease resolution or protection is unknown. Studies have also shown that animals lacking B cells are resistant to *Leishmania* infections (48), but such evidence on human VL are still lacking. Importantly, in endemic area of Bihar (India), strong association were found between seropositivity and progression to clinical diseases in healthy individual (49), suggesting its role in disease pathogenesis. Therefore, in-depth studies are required before any conclusion can be drawn. More recently, we have reported high anti-leishmanial antibodies in Indian VL patients compared to Sudanese patients and could be one of the factor for lower sensitivity of serological tests in East Africa (50). Most importantly, anti-leishmanial antibodies do not play any role in antigen-specific IFN-γ or IL-10 production in whole blood of active VL patients (unpublished data).

TARGETED THERAPY AND IMMUNOTHERAPY

In the absence of human vaccine and effective vector control measures, chemotherapy is the only option for treatment and control of VL. Several hundred comparative and prospective cohort studies on therapies for leishmaniasis have been published (**Table 1**). Increasing evidence of drug unresponsiveness and resistance has raised concern to save the drugs, as the armory of anti-leishmanial drugs is limited. Reports of emerging resistance to Miltefosine, the newest and only oral anti-leishmanial drugs, which is the basis of VL elimination program, are particularly worrying (14); and makes VL management and elimination challenging. Drug discovery is struggling to prevent resistance, therefore changes in the drug policy are much needed step as on today. Reductions in VL morbidity and mortality will require the development and deployment of immune modulators in order to achieve the prophylactic or therapeutic goal; and also prevent the transmission of *Leishmania* from human to sand fly. One of the most interesting approaches currently being explored is immunotherapy and targeted therapy [reviewed in Ref. (51)]. Targeted therapies act by blocking essential biochemical or signaling pathways that are indispensable for *Leishmania* parasite growth and survival, however, immunotherapy involves the use of biological molecules or compounds to modulate immune responses in combination with drugs. Over the last two decades, various approaches of immunotherapies or targeted therapies have been developed and applied in the treatment of human leishmaniasis (**Table 2**). The strengths and weaknesses of such therapies suggest that both approaches might have complimentary roles in VL treatment, and combination could prove synergistic. Because targeted therapies can induce rapid parasite clearance, with a consequent decrease in *Leishmania* associated immune-suppression, they might afford a favorable window for immunotherapy to improve the efficacy of treatment.

TARGETING HOST IMMUNITY BY ANTI-LEISHMANIAL DRUGS/MOLECULES

Within the mammalian host, *parasites* reside as amastigotes in phagocytic cells such as polymorphonuclear neutrophils (PMN), macrophages, and dendritic cells (DCs). Therefore, it is important to identify an immunomodulatory compound with leishmanicidal properties capable of activating phagocytic cells. Following entry of *Leishmania* parasite into the mammalian host, PMNs are thought to be the first effector cells recruited to the site of infection within 24 h, implying that they possibly serve as host cells for *Leishmania* parasites in the very early phase of infection (67). Neutrophils being inherently short-lived and apoptotic, are usually cleared without triggering activation of macrophages (67), while *Leishmania* parasites are known to delay neutrophils apoptosis,

Table 2 | Immunotherapy of human leishmaniasis.

Country	Year	Immunotherapeutic agent	Chemotherapeutic agent	No. of patients	Disease/ parasite	Treatment efficacy	Reference
India	1995	IFN-γ	Sb^v	16	VL	87%	(52)
Brazil	1990	IFN-γ	Sb^v	17	VL	82.3%	(53)
Brazil	2005	GM-CSF	Sb^v	05	CL	100% Cure	(54)
Brazil	2006	Killed *L. amazonensis* + BCG	Glucantime	47	ACL	87%	(55)
Brazil	2006	Mixed antigens[a]	–	06	MCL	76–94%	(56)
Brazil	2002	Killed *L. amazonensis*	Meglumine	47	ACL	100%	(57)
Argentina	2011	Killed *L. amazonensis* + BCG	–	01	ACL	High	(58)
Peru	2007	Imiquimod	Sb^v	07	CL	72%	(59)
Kenya	1993	IFN-γ	Sb^v	10	VL	75%	(60)
Sudan	2008	Alum/ALM + BCG	Sb^v	15	PKDL	87%	(61)
Iran	2006	Imiquimod	Glucantime	59	CL	44.1%	(62)
Uzbekistan	1993	Leukinferon (i.m.)	Monomycin	50	CL	High	(63)
Venezuela	1990–1999	Pasteurized *L. braziliensis* + BCG	–	5341	CL	91.2–98.7%	(64)
Venezuela	1994–2000	Mixture antigens[b]	Sb^v	87	CL	Moderate	(65)
Venezuela	2004	Pasteurized *L. braziliensis* + BCG	–	07	MCL, DCL	100%	(66)

VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; PKDL, post kala-azar dermal leishmaniasis; BCG, bacillus Calmette–Guerin; Sb, sodium stiboguconate; IFN-γ, interferon-γ; mixture antigens

[a]: TSA, thiol-specific antioxidant; LmSTI1, L. major stress inducible protein 1; LeIF, Leishmania elongation initiation factor; Lbhsp83, Leishmania heat shock protein 83; GM-CSF, granulocyte macrophage colony-stimulating factor; mixture antigens

[b]: amastigotes from L. (L.)amazonensis (La), L. (L.)venezuelensis (Lv), L. (V.)brasiliensis (Lb), and L. (L.)chagasi (Lch) Tosyl-Lysyl Chloromethyl-ketone (TLCK) treated and Non-idet P-40(NP-40) extracted (VT).

possibly by interfering with production of reactive oxygen species (ROS) (68, 69). Therefore, it would be logical and important to search an anti-leishmanial compound capable of generating an oxidative burst within *Leishmania* infected neutrophils to effectively eliminate parasites. Berberine chloride has been one of the compounds recently reported to enhance the apoptosis of *L. donovani*-infected neutrophils via modulation of the MAP kinase pathways (70).

Leishmania parasites that enter into macrophages via the uptake of infected, apoptotic PMNs may survive and multiply effectively (67). Since, macrophages have ability to kill parasite upon activation, *Leishmania* parasites overcome these macrophage activation and recognition by creating an anti-inflammatory milieu, beneficial for parasites survival. It has been reported that the amount of TGF-β secreted by macrophages following uptake of infected PMNs is higher than after direct uptake of *L. major* promastigotes (67), suggests that uptake of infected, apoptotic PMNs are responsible for creation of this environment within macrophages. Therefore, targeting pathogens residing in neutrophils should be taken into consideration when designing targeted novel anti-leishmanial compounds, as neutrophils harbor and transport parasites. For example, antimonials (sodium stibogluconate) increase the phagocytic capacity of neutrophils along with increased production of superoxide (71), unfortunately the loss of efficacy of antimonials has occurred in the Indian subcontinent and thus raised concern to search another compounds. In fact, several strategies to interfere with macrophage signaling by parasites have been reported that favor its survival in host cells (72). Oghumu et al. have highlighted the role of STAT4 pathway in immunity to *L. donovani* infection and also reported the evidence that STAT4 is dispensable for antimonial-based chemotherapy (73). Furthermore, some of the other strategies employed by *Leishmania* to evade effector mechanisms of the host immune system are the recruitment of inhibitory $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) (74, 75), inhibition of macrophage phagosomal maturation (76), and inhibition of DC maturation (77). Receptors expressed on Treg or its corresponding ligands on effectors cells, such as glucocorticoid-induced TNF receptor family-related protein (GITR), PD-1, it is ligands programed cell death ligand-1 (PD-L1, B7-H1) or cytotoxic T lymphocyte antigen-4 (CTLA-4) could be also used as potential targets in future studies, as targeting these regulatory pathways has proven effective in experimental VL (78, 79).

CYTOKINE IMMUNOTHERAPY

Cytokines are the messengers of the immune system. They have autocrine and paracrine functions, so that they function locally or at a distance to suppress or enhance immunity. Attempts to identify cytokines that selectively induce Th1 responses might be useful in VL therapy. The evidence of the utility of cytokines as therapeutic use came from the studies by Murray et al., when an anti-IL-10 receptor monoclonal antibody (anti-IL-10R mAb) was reported to inflict parasite killing through an inducible nitric oxide synthase-dependent mechanism (80). Thus, immunostimulatory cytokines (e.g., IFN-γ, IL-12, GM-CSF) or antibodies that target suppressive/deactivating cytokines are being investigated or proposed as monotherapies or as combination therapies with Sb^v or other drugs. Combination therapy with recombinant human IFN-γ and pentavalent antimonials have been reported as stronger parasitological and clinical cure; compared with the drug alone in

VL patients from Brazil, Kenya, and India (53, 60, 81). Short course of IFN-γ is thought to be sufficient to activate macrophage and thereby accelerate parasitologic effect of Sb^v. IL-12 is another key cytokine inhibited by *Leishmania* parasites. Exogenous treatment with rIL-12 during *Leishmania* infection leads to resistance in susceptible mice (41), suggesting its important use in clinical outcome. However, suppression of other cytokines, including receptor fusion antagonists of IL-13, IL-4, and TGF-β inhibit parasite replication but only marginally affect parasite clearance without the induction of a synergistic effect with pentavalent antimonials (82). In a study, GM-CSF plus either with meglumine antimonite (54) or a mixture of *L. major* antigens (LmSTI1 + LeIf + HSP83) (56), was reported as being highly effective in treating American CL and MCL (**Table 2**).

IL-10: ROLE IN VL PATHOGENESIS AND IMMUNOTHERAPY

Visceral leishmaniasis pathogenesis has been associated to an overproduction of the regulatory cytokine, IL-10, which can promote parasite replication and disease progression. Several studies performed to characterize the immunologic effects of VL have focused on the role of IL-10 in the suppression of DC functions and rendering macrophages unresponsive to activation signals (83). Experimental models have demonstrated that IL-10 plays a central role in the pathogenesis and parasite growth in VL, as IL-10-deficient BALB/c and C57BL6 mice are highly resistant to *L. donovani* infection (84). Treatment of *L. donovani*-infected wild-type mice with a single dose anti-IL-10R mAb and daily low doses of Sb^v resulted in rapid control of the *L. donovani* infection and dramatically enhanced the therapeutic effects of Sb^v namely, an over 10-fold dose-sparing effect was observed with Sb^v and a shortened duration of treatment (85). In a separate study, single dose anti-IL-10R mAb (0.5 mg) treatment triggered a 63% liver parasite killing in *L. donovani*-infected BALB/c mice; moreover, when administered at a reduced dose (0.1 mg), the anti-IL-10 mAb enhanced the effect of Sb^v, also administered at a suboptimal dose (50 mg/kg), leading to a 72% liver parasite killing (82). Similar results were observed in *L. donovani*-infected BALB/c mice treated with a suboptimal single dose (0.1 mg) of an anti-IL-10R mAb and low-dose Amphotericin B (2 mg/kg total dose) (86). The combination therapy induced a 76% liver parasite killing, compared with a 16% observed with the anti-IL-10R mAb alone.

Elevated levels of IL-10 in serum as well as enhanced IL-10 mRNA expression in lesional tissue during active disease are a consistent finding in human VL [reviewed in Ref. (87)]. More recently, we have reported antigen stimulated IL-10 production in whole blood cells of VL patients and have shown a strong association of IL-27 and IL-21 with the up-regulation of the IL-10 response, and revealed the presence of both IFN-γ and IL-10 producing antigen-specific cells in the peripheral blood of VL patients (74, 88, 89). The findings have led to an underlying hypothesis that during active disease antigen-specific IL-10 producing T cells are activated under conditions that also drive strong and persistent Th1 responses, and the balance of these cells and the cytokines they produce favors the progression of disease. It has been shown that infected macrophages, Th1, Th2, $CD8^+$ T cells, and subsets Treg, of which naturally occurring $CD4^+CD25^+Foxp3^+$ Treg cells and antigen-inducible or adaptive Treg are the best defined, are all a potential source of IL-10 capable of suppressing *Leishmania*-specific immunity (90–92). Key findings have identified $CD4^+CD25^-Foxp3^-$ or adaptive Treg as the main source of both elevated IL-10 and IFN-γ in the spleen of VL patients (74). Furthermore, antigen driven IL-10 production has been difficult to detect in culture of peripheral blood mononuclear cells (PBMCs) (88, 93, 94). These findings are consistent with reports from a number of studies, which suggest that the immunologic defect in VL is characterized not by the complete absence of a potentially curative type 1 immune response, but by the co-expression of suppressive cytokines that compromise the leishmanicidal function and potency of the effector response in target organs, such as the spleen. A direct role for IL-10 in the pathology of VL is supported by studies demonstrating that IL-10 blockade can enhance IFN-γ responses (29, 95). More recently, we have demonstrated anti-parasitic effect of IL-10 blockade in human VL, showing that neutralization of IL-10 results in marked reduction of parasite number present in splenic aspirate cells (89). In continuation with these *ex vivo* supporting findings, Phase I study of anti-IL-10 mAb alone and in combination with AmBisome have been recently proposed for the human trial (clinicaltrial.gov) and this combination is expected to induce synergistic effects that contain the VL infection and immunopathology associated with the disease, while overcoming the threat of drug resistance and possibly achieving a chemotherapeutic dose-sparing effect that results in better efficacy and adherence to treatment. Importantly, demonstrating a therapeutic benefit from the IL-10 neutralization as a proof of concept will open the door to other strategies targeting the inhibition of IL-10 and other immunosuppressive factors.

DENDRITIC CELL-BASED IMMUNOTHERAPY

Another novel approach is the application of DCs for the induction of antigen-specific T cell immunity. The interaction of DCs and *Leishmania* parasites are complex and thought to be responsible for control of infection or progression of clinical disease (96). DCs play an important role in initial anti-*Leishmania* T cell responses and promoting their differentiation into memory T cell to achieve long lasting immunity, which makes them attractive candidates for potential synergy with immunotherapy [reviewed in Ref. (51)]. Interestingly, a C-type lectin receptor, DC-SIGN (DC-specific ICAM-3-grabbing non-integrin), which is exclusively expressed on tissue monocyte-derived DCs, has been shown to favor parasite survival by binding with distinct *Leishmania* species. It is then suggested that this receptor could also be taken into consideration as therapeutic target for both visceral and cutaneous leishmaniasis (97).

Dendritic cells based immunotherapy combined with antimony-based chemotherapy has been shown very effective against murine VL (98). Bone marrow derived DCs pulsed with soluble *L. donovani* antigen when given in combination with antimonials has been shown to reduce both hepatic and splenic parasite burden significantly (51). Thus, the future of DC-based immunotherapy appears promising and it could be looked upon as a prospective vaccine against VL.

CHALLENGES AND FUTURE DIRECTIONS

Treatments that enhance immune responses to fight against diseases are of significant clinical interest. A possible approach to overcome some of the challenges associated with the management

and treatment of VL is the use of immune based combination therapy (99), which has been proven successful in other parasitic diseases, such as malaria, tuberculosis, and leprosy (100). A combination of drugs with different modes of action could eliminate the potential for drug resistance and induce a chemotherapeutic dose-sparing effect, since the mechanism for resistance would be different for each drug (100). One drug could target the parasite itself, while a second drug or compound could modulate the immune system of the host (101–103). Likewise, the combination of drugs with different half-lives could provide a synergistic effect in the timing and exposure of the parasite to the different drug levels (100, 103).

Although, considerable progress on VL treatment has been made over the past years, we still have a limited understanding of the precise immune mechanism underlying human VL. One of the major problems in translating discoveries from disease models into treatments for humans is the risk that potential treatment strategies do not work on human cells in the same way as they do in the experimental model. Second and most important key issue for immunotherapy or targeted therapies is whether intensified anti-*Leishmanial* effects can be achieved without a corresponding increase in serious toxicities, as immunomodulatory agents that provoke an immune response may also pose a risk of severe sensitization, which might be anticipated to increase allergic reactions and lead to reduction in treatment efficacy.

Cytokine (e.g., IL-10) has therapeutically been used as a recombinant protein (i.e., a large molecule), which is quite expensive to produce. It can be only administered by injection, which is also quite inconvenient for the patient. It will then be important to ensure that the cost associated with cytokine immunotherapy must be less than conventional treatment and reach to the populations that need it most. Another better approaches could be to target the molecules acting downstream of the cytokine receptors or signal transduction. The problems in such cases are the specificity, as the known cytokine signaling pathways are shared by different cytokines. Therefore, problems and side effects associated with the use of cytokine therapy have to be addressed properly before its clinical application.

Although, these observations strongly support immunotherapy as a promising alternative to conventional chemotherapy against VL, big challenge remains to ensure long term maintenance of response and safety of treatments with biologic agents.

CONCLUSION

Each VL patients represents our failure to prevent leishmaniasis, and each death represents our failure to treat soon enough. Until VL elimination has been achieved, drug treatment will remain crucial to prevent complications and death from VL. There is an urgent need for innovative and effective alternative therapies against VL. Understanding of crucial cellular pathways that promote *Leishmania* parasite growth and maintenance together with the development of compounds or agents that specifically inhibit these pathways has offered a new era for anti-leishmanial therapy. The use of immunotherapy and targeted therapy could aid in addressing some of the current challenges associated with the management and treatment of VL, namely, minimizing resistance to currently available drugs, improving the therapeutic index, decreasing the dose or length of treatment, and reducing the cost of therapy. With the emergence of targeted delivery systems and technology to block the IL-10 transcription and other relevant molecules involved in the IL-10 signaling (e.g., STAT3), a new era of molecular targeting of regulatory cytokines is on the horizon.

ACKNOWLEDGMENTS

This work was supported by Extramural Program of the National Institute of Allergy and Infectious Diseases (NIAID), National Institute of Health (TMRC Grant No. P50AI074321). Authors thank Ms. Neetu Singh and Mr. Shashi Bhushan, Research Scholar of Department of Medicine, Banaras Hindu University for helpful comments and suggestions in the preparation of the manuscript. Om Prakash Singh thanks CSIR, New Delhi for providing Senior Research Fellowship.

REFERENCES

1. Desjeux P. Leishmaniasis. Public health aspects and control. *Clin Dermatol* (1996) **14**:417–23. doi:10.1016/0738-081X(96)00057-0
2. World Health Organization. *Eliminating Visceral Leishmaniasis: A Multi-Pronged Approach*. Available at: http://www.who.int/tdr/news/2011/vl-elimination/en/
3. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) **7**:e35671. doi:10.1371/journal.pone.0035671
4. Addy M, Nandy A. Ten years of kala-azar in West Bengal, part I. Did post-kala-azar dermal leishmaniasis initiate the outbreak in 24-parganas? *Bull World Health Organ* (1992) **70**:341–6.
5. Bern C, Hightower AW, Chowdhury R, Ali M, Amann J, Wagatsuma Y, et al. Risk factors for kala-azar in Bangladesh. *Emerg Infect Dis* (2005) **11**:655–62. doi:10.3201/eid1105.040718
6. Alvar J, Yactayo S, Bern C. Leishmaniasis and poverty. *Trends Parasitol* (2006) **22**:552–7. doi:10.1016/j.pt.2006.09.004
7. Meheus F, Boelaert M, Baltussen R, Sundar S. Costs of patient management of visceral leishmaniasis in Muzaffarpur, Bihar, India. *Trop Med Int Health* (2006) **11**:1715–24. doi:10.1111/j.1365-3156.2006.01732.x
8. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. *Clin Microbiol Rev* (2006) **19**:111–26. doi:10.1128/CMR.19.1.111-126.2006
9. Sundar S, More DK, Singh MK, Singh VP, Sharma S, Makharia A, et al. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clin Infect Dis* (2000) **31**:1104–7. doi:10.1086/318121
10. Pandey BD, Pandey K, Kaneko O, Yanagi T, Hirayama K. Relapse of visceral leishmaniasis after miltefosine treatment in a Nepalese patient. *Am J Trop Med Hyg* (2009) **80**:580–2.
11. Sundar S, Rai M. Treatment of visceral leishmaniasis. *Expert Opin Pharmacother* (2005) **6**:2821–9. doi:10.1517/14656566.6.16.2821
12. Jha TK. Drug unresponsiveness & combination therapy for kala-azar. *Indian J Med Res* (2006) **123**:389–98.
13. Sundar S, Chakravarty J, Agarwal D, Rai M, Murray HW. Single-dose liposomal amphotericin B for visceral leishmaniasis in India. *N Engl J Med* (2010) **11**:504–12. doi:10.1056/NEJMoa0903627
14. Sundar S, Singh A. What steps can be taken to counter the increasing failure of miltefosine to treat visceral leishmaniasis? *Expert Rev Anti Infect Ther* (2013) **11**:117–9. doi:10.1586/eri.12.170
15. Sundar S, Singh A, Rai M, Prajapati VK, Singh AK, Ostyn B, et al. Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clin Infect Dis* (2012) **55**:543–50. doi:10.1093/cid/cis474
16. Croft SL, Olliaro P. Leishmaniasis chemotherapy – challenges and opportunities. *Clin Microbiol Infect* (2011) **17**:1478–83. doi:10.1111/j.1469-0691.2011.03630.x
17. Singh N, Singh RT, Sundar S. Novel mechanism of drug resistance in kala azar field isolates. *J Infect Dis* (2003) **188**:600–7. doi:10.1086/377133
18. Sundar S, Chakravarty J, Rai VK, Agrawal N, Singh SP, Chauhan V, et al. Amphotericin B treatment for Indian visceral leishmaniasis: response to 15 daily versus alternate-day infusions. *Clin Infect Dis* (2007) **45**:556–61. doi:10.1086/520665

19. Sundar S, Kumar K, Chakravarty J, Agrawal D, Agrawal S, Chhabra A, et al. Cure of antimony-unresponsive Indian post-kala-azar dermal leishmaniasis with oral miltefosine. *Trans R Soc Trop Med Hyg* (2006) **100**:698–700. doi:10.1016/j.trstmh.2005.09.015
20. Jhingran A, Chawla B, Saxena S, Barrett MP, Madhubala R. Paromomycin: uptake and resistance in *Leishmania donovani*. *Mol Biochem Parasitol* (2009) **164**:111–7. doi:10.1016/j.molbiopara.2008.12.007
21. Sundar S, Jha TK, Thakur CP, Sinha PK, Bhattacharya SK. Injectable paromomycin for visceral leishmaniasis in India. *N Engl J Med* (2007) **356**:2571–81. doi:10.1056/NEJMoa066536
22. Sundar S, Chakravarty J. Paromomycin in the treatment of leishmaniasis. *Expert Opin Investig Drugs* (2008) **17**:787–94. doi:10.1517/13543784.17.5.787
23. Das VN, Ranjan A, Sinha AN, Verma N, Lal CS, Gupta AK, et al. A randomized clinical trial of low dosage combination of pentamidine and allopurinol in the treatment of antimony unresponsive cases of visceral leishmaniasis. *J Assoc Physicians India* (2001) **49**:609–13.
24. Das VN, Siddiqui NA, Pandey K, Singh VP, Topno RK, Singh D, et al. A controlled, randomized nonblinded clinical trial to assess the efficacy of amphotericin B deoxycholate as compared to pentamidine for the treatment of antimony unresponsive visceral leishmaniasis cases in Bihar, India. *Ther Clin Risk Manag* (2009) **5**:117–24. doi:10.2147/TCRM.S3581
25. Das A, Ali N. Vaccine development against *Leishmania donovani*. *Front Immunol* (2012) **3**:99. doi:10.3389/fimmu.2012.00099
26. Singh OP, Stober CB, Singh AK, Blackwell JM, Sundar S. Cytokine responses to novel antigens in an Indian population living in an area endemic for visceral leishmaniasis. *PLoS Negl Trop Dis* (2012) **6**:e1874. doi:10.1371/journal.pntd.0001874
27. Janeway CA, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* (2002) **20**:197–216. doi:10.1146/annurev.immunol.20.083001.084359
28. Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat Rev Immunol* (2002) **2**:845–58. doi:10.1038/nri933
29. Ghalib HW, Piuvezam MR, Skeiky YA, Siddig M, Hashim FA, el-Hassan AM, et al. Interleukin 10 production correlates with pathology in human *Leishmania donovani* infections. *J Clin Invest* (1993) **92**:324–9. doi:10.1172/JCI116570
30. Karp CL, el-Safi SH, Wynn TA, Satti MM, Kordofani AM, Hashim FA, et al. In vivo cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-gamma. *J Clin Invest* (1993) **91**:1644–8. doi:10.1172/JCI116372
31. Kenney RT, Sacks DL, Gam AA, Murray HW, Sundar S. Splenic cytokine responses in Indian kala-azar before and after treatment. *J Infect Dis* (1998) **177**:815–8. doi:10.1086/517817
32. Bhattacharya P, Ali N. Involvement and interactions of different immune cells and their cytokines in human visceral leishmaniasis. *Rev Soc Bras Med Trop* (2013) **46**:128–34. doi:10.1590/0037-8682-0022-2012
33. Cummings HE, Tuladhar R, Satoskar A. Cytokines and their STATs in cutaneous and visceral leishmaniasis. *J Biomed Biotechnol* (2010) **2010**:294389. doi:10.1155/2010/294389
34. Basu A, Chakrabarti G, Saha A, Bandyopadhyay S. Modulation of CD11C+ splenic dendritic cell functions in murine visceral leishmaniasis: correlation with parasite replication in the spleen. *Immunology* (2000) **99**:305–13. doi:10.1046/j.1365-2567.2000.00939.x
35. Schwarz T, Remer KA, Nahrendorf W, Masic A, Siewe L, Müller W, et al. T cell-derived IL-10 determines leishmaniasis disease outcome and is suppressed by a dendritic cell based vaccine. *PLoS Pathog* (2013) **9**:e1003476. doi:10.1371/journal.ppat.1003476
36. Bacellar O, Brodskyn C, Guerreiro J, Barral-Netto M, Costa CH, Coffman RL, et al. Interleukin-12 restores interferon-gamma production and cytotoxic responses in visceral leishmaniasis. *J Infect Dis* (1996) **173**:1515–8. doi:10.1093/infdis/173.6.1515
37. Ghalib HW, Whittle JA, Kubin M, Hashim FA, el-Hassan AM, Grabstein KH, et al. IL-12 enhances Th1-type responses in human *Leishmania donovani* infections. *J Immunol* (1995) **154**:4623–9.
38. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med* (1989) **170**(3):827–45. doi:10.1084/jem.170.3.827
39. Scharton TM, Scott P. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to *Leishmania major* in mice. *J Exp Med* (1993) **178**:567–77. doi:10.1084/jem.178.2.567
40. Trinchieri G. Interleukin-12 and its role in the generation of TH1 cells. *Immunol Today* (1993) **14**(7):335–8. doi:10.1016/0167-5699(93)90230-I
41. Heinzel FP, Schoenhaut DS, Rerko RM, Rosser LE, Gately MK. Recombinant interleukin 12 cures mice infected with *Leishmania major*. *J Exp Med* (1993) **177**:1505–9. doi:10.1084/jem.177.5.1505
42. Murray HW, Hariprashad J, Coffman RL. Behavior of visceral *Leishmania donovani* in an experimentally induced T helper cell 2 (Th2)-associated response model. *J Exp Med* (1997) **185**:867–74. doi:10.1084/jem.185.5.867
43. Ahuja SS, Reddick RL, Sato N, Montalbo E, Kostecki V, Zhao W, et al. Dendritic cell (DC)-based anti-infective strategies: DCs engineered to secrete IL-12 are a potent vaccine in a murine model of an intracellular infection. *J Immunol* (1999) **163**:3890–7.
44. Alexander CE, Kaye PM, Engwerda CR. CD95 is required for the early control of parasite burden in the liver of *Leishmania donovani*-infected mice. *Eur J Immunol* (2001) **31**:1199–210. doi:10.1002/1521-4141(200104)31:4<1199::AID-IMMU1199>3.0.CO;2-6
45. De Almeida Silva L, Romero HD, Prata A, Costa RT, Nascimento E, Carvalho SF, et al. Immunologic tests in patients after clinical cure of visceral leishmaniasis. *Am J Trop Med Hyg* (2006) **75**:739–43.
46. Hailu A. Pre- and post-treatment antibody levels in visceral leishmaniasis. *Trans R Soc Trop Med Hyg* (1990) **84**:673–5. doi:10.1016/0035-9203(90)90141-Z
47. Gidwani K, Picado A, Ostyn B, Singh SP, Kumar R, Khanal B, et al. Persistence of *Leishmania donovani* antibodies in past visceral leishmaniasis cases in India. *Clin Vaccine Immunol* (2010) **18**:346–8. doi:10.1128/CVI.00473-10
48. Hoerauf A, Solbach W, Lohoff M, Rollinghoff M. The Xid defect determines an improved clinical course of murine leishmaniasis in susceptible mice. *Int Immunol* (1994) **6**:1117–24. doi:10.1093/intimm/6.8.1117
49. Hasker E, Malaviya P, Gidwani K, Picado A, Ostyn B, Kansal S, et al. Strong association between serological status and probability of progression to clinical visceral leishmaniasis in prospective cohort studies in India and Nepal. *PLoS Negl Trop Dis* (2014) **8**(1):e2657. doi:10.1371/journal.pntd.0002657
50. Bhattacharyya T, Bowes DE, El-Safi S, Sundar S, Falconar AK, Singh OP, et al. Significantly lower anti-*Leishmania* IgG responses in Sudanese versus Indian visceral leishmaniasis. *PLoS Negl Trop Dis* (2014) **8**(2):e2675. doi:10.1371/journal.pntd.0002675
51. El-On J. Current status and perspectives of the immunotherapy of leishmaniasis. *Isr Med Assoc J* (2009) **11**:623–8.
52. Sundar S, Rosenkaimer F, Lesser ML, Murray HW. Immunochemotherapy for a systemic intracellular infection: accelerated response using interferon-gamma in visceral leishmaniasis. *J Infect Dis* (1995) **171**:992–6. doi:10.1093/infdis/171.4.992
53. Badaro R, Falcoff E, Badaro FS, Carvalho EM, Pedral-Sampaio D, Barral A, et al. Treatment of visceral leishmaniasis with pentavalent antimony and interferon gamma. *N Engl J Med* (1990) **322**:16–21. doi:10.1056/NEJM199001043220104
54. Almeida RP, Brito J, Machado PL, De Jesus AR, Schriefer A, Guimarães LH, et al. Successful treatment of refractory cutaneous leishmaniasis with GM-CSF and antimonials. *Am J Trop Med Hyg* (2005) **73**:79–81.
55. Mayrink W, Botelho AC, Magalhães PA, Batista SM, Lima Ade O, Genaro O, et al. Immunotherapy, immunochemotherapy, and chemotherapy for American cutaneous leishmaniasis treatment. *Rev Soc Bras Med Trop* (2006) **39**:14–21. doi:10.1590/S0037-86822006000100003
56. Badaro R, Lobo I, Munos A, Netto EM, Modabber F, Campos-Neto A, et al. Immunotherapy for drug-refractory mucosal leishmaniasis. *J Infect Dis* (2006) **194**:1151–9. doi:10.1086/507708
57. Machado-Pinto J, Pinto J, da Costa CA, Genaro O, Marques MJ, Modabber F, et al. Immunochemotherapy for cutaneous leishmaniasis: a controlled trial using killed *Leishmania (Leishmania) amazonensis* vaccine plus antimonial. *Int J Dermatol* (2002) **41**:73–8. doi:10.1046/j.1365-4362.2002.01336.x
58. García Bustos MF, Barrio AB, Parodi Ramoneda CM, Ramos F, Mora MC, Convit J, et al. Immunological correlates of cure in the first American cutaneous leishmaniasis patient treated by immunotherapy in Argentina. A case report. *Invest Clin* (2013) **52**:365–75.

59. Arevalo I, Tulliano G, Quispe A, Spaeth G, Matlashewski G, Llanos-Cuentas A, et al. Role of imiquimod and parenteral meglumine antimoniate in the initial treatment of cutaneous leishmaniasis. *Clin Infect Dis* (2007) **44**:1549–54. doi:10.1086/518172
60. Squires KE, Rosenkaimer F, Sherwood JA, Forni AL, Were JB, Murray HW. Immunochemotherapy for visceral leishmaniasis: a controlled pilot trial of antimony versus antimony plus interferon-gamma. *Am J Trop Med Hyg* (1993) **48**:666–9.
61. Musa AM, Khalil EA, Mahgoub FA, Elgawi SH, Modabber F, Elkadaru AE, et al. Immunochemotherapy of persistent post-kala-azar dermal leishmaniasis: a novel approach to treatment. *Trans R Soc Trop Med Hyg* (2008) **102**:58–63. doi:10.1016/j.trstmh.2007.08.006
62. Firooz A, Khamesipour A, Ghoorchi MH, Nassiri-Kashani M, Eskandari SE, Khatami A, et al. Imiquimod in combination with meglumine antimoniate for cutaneous leishmaniasis: a randomized assessor-blind controlled trial. *Arch Dermatol* (2006) **142**:1575–9. doi:10.1001/archderm.142.12.1575
63. Sergiev VP, Shuikina EE, Kuznetsov VP, Beliaev DL, Fediainova IE, Norbadalov MT, et al. The use of leukinferon in treating zoonotic cutaneous leishmaniasis. 2. Patient treatment. *Med Parazitol (Mosk)* (1993) (1):42–3.
64. Convit J, Ulrich M, Zerpa O, Borges R, Aranzazu N, Valera M, et al. Immunotherapy of American cutaneous leishmaniasis in Venezuela during the period 1990-99. *Trans R Soc Trop Med Hyg* (2003) **97**:469–72. doi:10.1016/S0035-9203(03)90093-9
65. O'Daly JA, Spinetti HM, Gleason J, Rodríguez MB. Clinical and immunological analysis of cutaneous leishmaniasis before and after different treatments. *J Parasitol Res* (2013) **2013**:657016. doi:10.1155/2013/657016
66. Convit J, Ulrich M, Polegre MA, Avila A, Rodríguez N, Mazzedo MI, et al. Therapy of Venezuelan patients with severe mucocutaneous or early lesions of diffuse cutaneous leishmaniasis with a vaccine containing pasteurized *Leishmania* promastigotes and bacillus Calmette-Guerin: preliminary report. *Mem Inst Oswaldo Cruz* (2004) **99**:57–62. doi:10.1590/S0074-02762004000100010
67. Laskay T, van Zandbergen G, Solbach W. Neutrophil granulocytes as host cells and transport vehicles for intracellular pathogens: apoptosis as infection-promoting factor. *Immunobiology* (2008) **213**:183–91. doi:10.1016/j.imbio.2007.11.010
68. Aga E, Katschinski DM, van Zandbergen G, Laufs H, Hansen B, Müller K, et al. Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J Immunol* (2002) **169**:898–905. doi:10.4049/jimmunol.169.2.898
69. Laufs H, Müller K, Fleischer J, Reiling N, Jahnke N, Jensenius JC, et al. Intracellular survival of *Leishmania major* in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. *Infect Immun* (2002) **70**:826–35. doi:10.1128/IAI.70.2.826-835.2002
70. Saha P, Bhattacharjee S, Sarkar A, Manna A, Majumder S, Chatterjee M. Berberine chloride mediates its anti-leishmanial activity via differential regulation of the mitogen activated protein kinase pathway in macrophages. *PLoS One* (2011) **6**:e18467. doi:10.1371/journal.pone.0018467
71. Muniz-Junqueira MI, de Paula-Coelho VN. Meglumine antimonate directly increases phagocytosis, superoxide anion and TNF-alpha production, but only via TNF-alpha it indirectly increases nitric oxide production by phagocytes of healthy individuals, in vitro. *Int Immunopharmacol* (2008) **8**:1633–8. doi:10.1016/j.intimp.2008.07.011
72. Shadab M, Ali N. Evasion of host defence by *Leishmania donovani*: subversion of signaling pathways. *Mol Biol Int* (2011) **2011**:343961. doi:10.4061/2011/343961
73. Oghumu S, Gupta G, Snider HM, Varikuti S, Terrazas CA, Papenfuss TL, et al. STAT4 is critical for immunity but not for antileishmanial activity of antimonials in experimental visceral leishmaniasis. *Eur J Immunol* (2013) **44**(2):450–9. doi:10.1002/eji.201343477
74. Nylén S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D. Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. *J Exp Med* (2007) **204**:805–17. doi:10.1084/jem.20061141
75. Peters N, Sacks D. Immune privilege in sites of chronic infection: *Leishmania* and regulatory T cells. *Immunol Rev* (2006) **213**:159–79. doi:10.1111/j.1600-065X.2006.00432.x
76. Olivier M, Gregory DJ, Forget G. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clin Microbiol Rev* (2005) **18**:293–305. doi:10.1128/CMR.18.2.293-305.2005
77. Brandonisio O, Spinelli R, Pepe M. Dendritic cells in *Leishmania* infection. *Microbes Infect* (2004) **6**:1402–9. doi:10.1016/j.micinf.2004.10.004
78. Joshi T, Rodriguez S, Perovic V, Cockburn IA, Stager S. B7-H1 blockade increases survival of dysfunctional CD8 (+) T cells and confers protection against *Leishmania donovani* infections. *PLoS Pathog* (2009) **5**(5):e1000431. doi:10.1371/journal.ppat.1000431
79. Murray HW, Lu CM, Brooks EB, Fichtl RE, DeVecchio JL, Heinzel FP. Modulation of T-cell costimulation as immunotherapy or immunochemotherapy in experimental visceral leishmaniasis. *Infect Immun* (2003) **71**:6453–62. doi:10.1128/IAI.71.11.6453-6462.2003
80. Murray HW, Moreira AL, Lu CM, DeVecchio JL, Matsuhashi M, Ma X, et al. Determinants of response to interleukin-10 receptor blockade immunotherapy in experimental visceral leishmaniasis. *J Infect Dis* (2003) **188**:458–64. doi:10.1086/376510
81. Sundar S, Murray HW. Effect of treatment with interferon-gamma alone in visceral leishmaniasis. *J Infect Dis* (1995) **172**:1627–9. doi:10.1093/infdis/172.6.1627
82. Murray HW, Flanders KC, Donaldson DD, Sypek JP, Gotwals PJ, Liu J, et al. Antagonizing deactivating cytokines to enhance host defense and chemotherapy in experimental visceral leishmaniasis. *Infect Immun* (2005) **73**:3903–11. doi:10.1128/IAI.73.7.3903-3911.2005
83. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* (2001) **19**:683–765. doi:10.1146/annurev.immunol.19.1.683
84. Murphy ML, Wille U, Villegas EN, Hunter CA, Farrell JP. IL-10 mediates susceptibility to *Leishmania donovani* infection. *Eur J Immunol* (2001) **31**:2848–56. doi:10.1002/1521-4141(2001010)31:10<2848::AID-IMMU2848>3.0.CO;2-T
85. Murray HW. Interleukin 10 receptor blockade – pentavalent antimony treatment in experimental visceral leishmaniasis. *Acta Trop* (2005) **93**:295–301. doi:10.1016/j.actatropica.2004.11.008
86. Murray HW, Brooks EB, DeVecchio JL, Heinzel FP. Immunoenhancement combined with amphotericin B as treatment for experimental visceral leishmaniasis. *Antimicrob Agents Chemother* (2003) **47**:2513–7. doi:10.1128/AAC.47.8.2513-2517.2003
87. Nylen S, Sacks D. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. *Trends Immunol* (2007) **28**:378–84. doi:10.1016/j.it.2007.07.004
88. Ansari NA, Kumar R, Gautam S, Nylén S, Singh OP, Sundar S, et al. IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral leishmaniasis. *J Immunol* (2011) **186**:3977–85. doi:10.4049/jimmunol.1003588
89. Gautam S, Kumar R, Maurya R, Nylén S, Ansari N, Rai M, et al. IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. *J Infect Dis* (2011) **204**:1134–7. doi:10.1093/infdis/jir461
90. Anderson CF, Oukka M, Kuchroo VJ, Sacks D. CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis. *J Exp Med* (2007) **204**:285–97. doi:10.1084/jem.20061886
91. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* (2002) **420**:502–7. doi:10.1038/nature01152
92. Holaday BJ. Role of CD8+ T cells in endogenous interleukin-10 secretion associated with visceral leishmaniasis. *Mem Inst Oswaldo Cruz* (2000) **95**:217–20. doi:10.1590/S0074-02762000000200013
93. Singh OP, Gidwani K, Kumar R, Nylén S, Jones SL, Boelaert M, et al. Reassessment of immune correlates in human visceral leishmaniasis as defined by cytokine release in whole blood. *Clin Vaccine Immunol* (2012) **19**:961–6. doi:10.1128/CVI.00143-12
94. Singh OP, Sundar S. Whole blood assay and visceral leishmaniasis: challenges and promises. *Immunobiology* (2014) **219**:323–8. doi:10.1016/j.imbio.2014.01.005
95. Carvalho EM, Bacellar O, Brownell C, Regis T, Coffman RL, Reed SG. Restoration of IFN-gamma production and lymphocyte proliferation in visceral leishmaniasis. *J Immunol* (1994) **152**:5949–56.
96. Moll H. Dendritic cells and host resistance to infection. *Cell Microbiol* (2003) **5**(8):493–500. doi:10.1046/j.1462-5822.2003.00291.x
97. Colmenares M, Corbi AL, Turco SJ, Rivas L. The dendritic cell receptor DC-SIGN discriminates among species and life cycle forms of

Leishmania. *J Immunol* (2004) **172**:1186–90. doi:10.4049/jimmunol.172.2.1186

98. Ghosh M, Pal C, Ray M, Maitra S, Mandal L, Bandyopadhyay S. Dendritic cell-based immunotherapy combined with antimony-based chemotherapy cures established murine visceral leishmaniasis. *J Immunol* (2003) **170**:5625–9. doi:10.4049/jimmunol.170.11.5625
99. Meheus F, Balasegaram M, Olliaro P, Sundar S, Rijal S, Faiz MA, et al. Cost-effectiveness analysis of combination therapies for visceral leishmaniasis in the Indian subcontinent. *PLoS Negl Trop Dis* (2010) **4**(9):e818. doi:10.1371/journal.pntd.0000818
100. Bryceson A. A policy for leishmaniasis with respect to the prevention and control of drug resistance. *Trop Med Int Health* (2001) **6**:928–34. doi:10.1046/j.1365-3156.2001.00795.x
101. Davis AJ, Murray HW, Handman E. Drugs against leishmaniasis: a synergy of technology and partnerships. *Trends Parasitol* (2004) **20**:73–6. doi:10.1016/j.pt.2003.11.006
102. Smith AC, Yardley V, Rhodes J, Croft SL. Activity of the novel immunomodulatory compound tucaresol against experimental visceral leishmaniasis. *Antimicrob Agents Chemother* (2000) **44**:1494–8. doi:10.1128/AAC.44.6.1494-1498.2000
103. Sundar S, Chatterjee M. Visceral leishmaniasis – current therapeutic modalities. *Indian J Med Res* (2006) **123**:345–52.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 January 2014; accepted: 07 June 2014; published online: 26 June 2014.
*Citation: Singh OP and Sundar S (2014) Immunotherapy and targeted therapies in treatment of visceral leishmaniasis: current status and future prospects. Front. Immunol. **5**:296. doi: 10.3389/fimmu.2014.00296*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 05 May 2014
doi: 10.3389/fimmu.2014.00193

Exploring the role of medicinal plant-based immunomodulators for effective therapy of leishmaniasis

Garima Chouhan[1], Mohammad Islamuddin[1], Dinkar Sahal[2] and Farhat Afrin[1]*

[1] *Parasite Immunology Laboratory, Department of Biotechnology, Jamia Hamdard (Hamdard University), New Delhi, India*
[2] *Malaria Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India*

Edited by:
Nahid Ali, Indian Institute of Chemical Biology, India

Reviewed by:
Ennio De Gregorio, Novartis Vaccines and Diagnostics, Italy
Angamuthu Selvapandiyan, Institute of Molecular Medicine, India

***Correspondence:**
Farhat Afrin, Parasite Immunology Laboratory, Department of Biotechnology, Jamia Hamdard (Hamdard University), New Delhi 110062, India
e-mail: afrin_farhat@yahoo.co.in

Leishmaniasis is a pestilent affliction that importunately needs better therapeutics necessitated by the absence of effective vaccine, emergence as HIV co-infection, and the dread of debilitating chemotherapy. The *Leishmania* parasites incapacitate host macrophages by preventing the formation of phagolysosomes, impeding antigen presentation to T cells, leading to suppression of cell-mediated immunity. An ideal approach to cure leishmaniasis includes administration of antileishmanial compounds that can concomitantly establish an effective Th1 response via restoration of requisite signaling between macrophages and T cells, for subsequent activation of macrophages to eliminate intracellular amastigotes. Plants have provided an opulent treasure of biomolecules that have fueled the discovery of antileishmanial drugs. Modulation of immune functions using medicinal plants and their products has emerged as an effective therapeutic strategy. Herein, we review the plant extracts and natural products that have resulted in therapeutic polarization of host immunity to cure leishmaniasis. These immunostimulatory phytochemicals as source of potential antileishmanials may provide new strategies to combat leishmaniasis, alone or as adjunct modality.

Keywords: medicinal plants, immunomodulators, leishmaniasis, antileishmanial, phytochemicals

INTRODUCTION

Leishmaniasis is a neglected; usually poverty-associated complex vector-borne disease that is caused by more than 21 species of parasites belonging to the order Kinetoplastida and family Trypanosomatidae. The disease manifests into different clinical indexes depending on the parasite tropism and ranges from self-healing cutaneous lesions, to malign mucocutaneous leishmaniasis and fatal visceral manifestations. The disease is endemic in 98 countries, where 10–12 million people are afflicted worldwide with 1.5–2.5 million new cases, death toll of 70,000 and 350 million at risk of developing the infection (1–3). Since *Leishmania* parasites reside and multiply within the parasitophorous vacuoles of macrophages, failure of host immunity to contain the infection results in immunosuppression. Thus, the host becomes susceptible to various secondary infections including HIV (4, 5).

The antiquated therapeutic modalities for leishmaniasis are crippled because of variable efficacy, drug resistance, and pronounced side effects. Even the known antileishmanial drugs such as amphotericin B (AmB), sodium stibogluconate (SSG), and miltefosine exert their antileishmanial effect via host immunomodulation [(6) and references therein]. Modulation of the host immune response via generation of antileishmanial vaccine would certainly be a propitious step in leishmaniasis control, but is impeded by the digenetic life cycle of *Leishmania*, and antigenic diversity among different *Leishmania* species, making prospects of a cross-protective vaccine a distant future (7, 8). Thus, in the absence of any vaccine, a quintessential approach to control leishmaniasis shall be based on discovery of drugs from alternative sources that can directly kill the parasite as well as activate sentinels of immune system for clearance of the pathogen. Herein, we further elaborate the mechanisms employed by *Leishmania* parasites to evade host immune responses, lacunae in current chemotherapy and discuss potential role of natural immunomodulators in antileishmanial therapy.

LEISHMANIA PARASITE-EVASION FROM HOST IMMUNE DEFENSES

MODULATION OF NEUTROPHIL FUNCTIONS

Within 24 h of *Leishmania* infection, neutrophils are recruited to the site of infection, serving as early and transitory host to *Leishmania* promastigotes (9). Although exact mechanisms underlying the recruitment of neutrophils remain unclear, the role of both parasites or vector-derived molecules is speculated (9, 10). The role of neutrophils in *Leishmania* infection is variable, *Leishmania* species specific, and also depends on the host genetics (11–14). Although neutrophils are responsible for early containment of different *Leishmania* species (15–18), they play equally pivotal role in harboring the parasites till they reach their evolutionary destined host cells, i.e., macrophages. *Leishmania* promastigotes deviously modulate neutrophil phagocytic functions in more than one way. Internalized *Leishmania* promastigotes block the production of CXC-chemokine interferon gamma (IFN-γ) inducible protein-10, which results in decreased recruitment and activation of natural killer (NK) cells and Th1 cells (10). *Leishmania donovani* promastigotes have been shown to induce NETs (fibrous traps of DNA, histones, and proteins) in which they get trapped but escape their microbicidal activity by aid of lipophosphoglycan (LPG) (19). *Leishmania* parasites also extend the life span

of neutrophils and delay their apoptosis by various mechanisms (20, 21). Since ingestion of apoptotic neutrophils by macrophages does not trigger macrophage microbicidal defenses, it creates safe passage for stealth entry of *Leishmania* parasites. The theory that neutrophils act as Trojan horses is well perceived in case of *L. donovani* (10, 22), but there is also evidence that *Leishmania* parasites escape neutrophils before infecting the macrophages (23), a Trojan rabbit strategy where viable promastigotes hide in the shadow of apoptotic neutrophils (9).

ENTRY INTO MACROPHAGES

In their quest for survival, *Leishmania* parasites face the arduous challenge to gain entry inside the macrophages and silence their impeccable defenses. The parasites express a wide array of ligands on their surfaces, which interact with a variety of macrophage receptors. Some of the key receptors that mediate promastigote–macrophage binding include the receptors for complement, fibronectin, mannose–fucose, and other sugars [(24) and references therein]. Interestingly, the foremost ligands employed by parasite for its phagocytic uptake are not encoded by the parasite itself; instead, parasite gets ingested into the macrophages via opsonin-dependent pathways. The sharp-witted *Leishmania* parasites not only circumvent complement-mediated lysis but also modulate the complement system for their active uptake inside the macrophages. C3bi and C3b are two major complement system components that bind to promastigote surface (25–28) and facilitate their intracellular uptake via CR3 and CR1, respectively. The uptake via CR3 is more advantageous since internalization via these receptors does not result in oxidative burst and also suppresses the secretion of IL-12 and other pro-inflammatory signals, thus hampering the initiation of cell-mediated immunity (CMI) (29, 30).

ESTABLISHING INFECTION IN MACROPHAGES

Once inside the macrophage phagosomes, promastigotes create an intracellular niche for their survival by silencing the macrophages through multifarious schemes. Predominantly, *Leishmania* parasites retard phagosome maturation, delay enodosome–phagosome fusion, inhibit hydrolytic enzymes in phagolysosomes, prevent generation of reactive-nitrogen and -oxygen species, suppress antigen presentation, and repress pro-inflammatory cytokine production. LPG present on the surface of *Leishmania* parasites retards phagosome maturation by inducing Cdc42- and Rac1 (Rho family GTPases, F-actin regulators)-dependent F-actin accumulation, which also involves inhibition of PKCα leading to impaired recruitment of LAMP-1 and rab7 (31–34). It has been reported earlier that incorporation of LPG selectively into one of the leaflets of lipid bilayers of phagosome membrane alters its biophysical properties making it less fusogenic [(31) and references therein]. LPG impairs the acquisition of vesicular proton-ATPase, which is involved in acidification of phagolysosomes (35). *Leishmania* parasites either decimate or suppress expression of major histocompatibility complex (36–38). *Leishmania* parasites also interfere with protein expression of their host cells (39), suppress the secretion of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-6, IL-12), and induce secretion of anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) via modulation of host cell signaling (40–42). Thus, establishment of *Leishmania* infection involves complex in-depth interactions between a vast repertoire of immunostimulating and immunosuppressive molecules that finally determine a species-dependent outcome of infection.

THERAPEUTIC MODALITIES

The pentavalent antimonials, SSG and Meglumine Antimoniate (MA), have been employed in treatment of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) for more than 60 years. The use of SSG and MA as first-line treatment for VL has been already abandoned after failure rates of ~65% in endemic regions of Bihar, India. Antimonials show variable efficacy against CL and VL, and accompanying severe side effects have demerited their use. The second line of drug AmB is now employed to treat the antimony-resistant patients but the need of hospitalization, prolonged duration of treatment, and infusion-related side effects are drawbacks. These constraints have now been overcome by lipid formulations of AmB that prevent tissue retention, thereby reducing the toxicity and promoting preferential uptake by reticuloendothelial cells that harbor the parasites. However, cost of liposomal AmB is a serious limitation and frequent noxious effects and drug resistance associated with pentamidine has also led to its withdrawal. Paromomycin has been registered for use in India against VL but is oto- and nephrotoxic. Miltefosine, the first oral drug, is of limited use because of its teratogenicity (43–45).

NATURAL IMMUNOMODULATORS: ROLE IN ANTILEISHMANIAL THERAPY

The drug discovery against leishmaniasis has been more reliant on therapeutic switching rather than discovery of novel drugs. Since elevation of host immunity is critical in parallel to the drug-mediated killing of *Leishmania* parasites, the antileishmanial arsenal may be benefited by antileishmanials that can prong a bifurcated attack, i.e., elimination of the parasites as well as restoration of CMI. The potential of immunomodulators in treating experimental leishmaniasis gained momentum with the discovery of antileishmanial activity of imiquimod (46, 47), an agonist for toll-like receptor 7, which is present on macrophages and dendritic cells (DC) and promotes the development of Th1 immune response [(48) and references therein]. Several other synthetic compounds such as S_2 complex (an organic complex of copper chloride, ascorbic acid, and nicotinamide) (49), acetyl salicylic acid (50), and immunomodulatory peptide from cystatin (51) have been demonstrated to possess dual immune-modulating and antileishmanial activities.

Various herbal formulations and plant secondary metabolites such as flavonoids, isoflavonoids, saponins, alkaloids, sesquiterpenes, polysaccharides, tannins, indoles, and glucans are known to be immunomodulatory in different diseases (52, 53). Among natural resources, plants have been most extensively explored for bioactive leishmanicidal and immunomodulatory compounds. Plant extracts contain a plethora of biomolecules that can naturally kill *Leishmania* parasites and also exert immunostimulatory properties, on otherwise depressed immune system during the diseased state, as has been extensively reviewed in **Table 1**.

In brief, the studies examining immunomodulatory effect of bioactive plant extracts or compounds have reported skewing of

Table 1 | Immunomodulatory antileishmanial plant extracts or purified molecules thereof.

Plant extracts/purified compounds/secondary metabolites/herbal medicines	*Leishmania* strain	Concentration (*in vitro*)/dose regimen (*in vivo*)	Immunomodulatory mechanism	Reference
Asparagus racemosus (whole plant)	*L. donovani* (Dd8)	[b]650 mg/kg b.w. +cisplatin (5 mg/kg b.w.) for 5 days i.p.	↑ INF-γ, IL-2, IgG2a ↓ IL-4, IL-10, IgG1 Induced DTH response	Sachdeva et al. (54)
Allium sativum in combination with *Tridax procumbens*	*Leishmania major* [Hd-18-(MHET/MX/97/Hd-18)]	[b]40 mg/kg b.w. (1:1) i.p. daily for 2 weeks	↑ IgG2a/IgG1 ratio	Gamboa-Leon et al. (55)
Galactomannan (isolated from seeds of *Mimosa scabrella*)	*Leishmania amazonensis* (MHOM/BR173/M2269)	250 μg/ml	↑ IL-1β, IL-6 and NO production TNF-α and IL-10 levels unaffected	Adriazola et al. (56)
[a]Licarin A (neolignan, plant secondary metabolite)	*L. major* (MHOM/IL/1980/FN)	5 and 20 μg/ml	↓ IL-6 and IL-10 No significant alterations in TNF-α and NO levels	Néris et al. (57)
Niranthrin (lignan isolated from aerial parts of *Phyllanthus amarus*)	*L. donovani* (MHOM/IN/1983/AG83)	[b]5 and 10 mg/kg b.w. twice for 3 weeks	↑ NO, ROS, iNOS Induced lymphoproliferation ↑ IFN-γ, TNF-α, and IL-12p70 ↑ IgG2a levels ↓ IL-10 and TGF-β No change in IL-4 expression and IgG1	Chowdhury et al. (58)
[a]Berberine chloride (quaternary isoquinoline alkaloid)	*L. donovani* isolate (NS2)	2.5 and 10 μM	↑ NO production Activated iNOS ↑ mRNA expression of IL-12p40 ↓ IL-10 Upregulated p38 MAPK pathway	Saha et al. (59)
Picroliv (iridoid glycoside mixture from *Picrorhiza kurroa*) in combination with fluconazole and miltefosine	*L. donovani* (MHOM/IN/80/Dd8)	Picroliv (10 mg/kg) + Fluconazole (50 mg/kg) + Miltefosine (5 mg/kg b.w.) in hamsters	Induced lymphoproliferation ↑ ROS, hydrogen peroxide, RNS ↑ Phagocytic activity	Shakya et al. (60)
Spiranthera odoratissima (fruit hexane extract and its alkaloid Skimmianine)	*Leishmania braziliensis*	1.6 μg/ml	↑ NO production ↓ IL-10 production	Dos Santos et al. (61)
Echium amoenum (flowers-aqueous and alcoholic extracts)	*L. major* (MRHO/IR/75/ER)	[b]250, 750, and 3750 mg/kg b.w.	↑ IFN-γ Induced lymphoproliferation IL-4 levels unaffected	Hosseini and Abolhassani (62)
A. sativum (aqueous extract)	*L. major* (MRHO/IR/75/ER)	37 mg/ml	↑ INF-γ and iNOS mRNA expression levels	Gharavi et al. (63)
A.sativum (aqueous extract)	*L. major* (MRHO/IR/75/ER)	37 mg/ml	↑ IL-12 ↓ IL-10	Gharavi et al. (64)
[a]Artemisinin (sesquiterpene lactone from *Artemisia annua*)	*L. donovani*	10 and 25 μM [b]10 and 25 mg/kg b.w.	↑ NO production ↑ IL-12 and IFN-γ	Sen et al. (65)
Kalanchoe pinnata (leaves-aqueous extract)	*Leishmania chagasi*	[b]400 mg/kg b.w. by intragastric gavage from day 1–29 of infection	Depressed serum IgG levels ↓ IL-4, INF-γ ↑ NO production	Gomes et al. (66)

(Continued)

Table 1 | Continued

Plant extracts/purified compounds/secondary metabolites/herbal medicines	*Leishmania* strain	Concentration (*in vitro*)/dose regimen (*in vivo*)	Immunomodulatory mechanism	Reference
Warburgia ugandensis, Psiadia punctulata, and *Chasmanthera dependens* (bark-aqueous extract)	*L. major* (IDU/KE/83 = NLB-144)	1000 μg/ml	↑ NO production	Githinji et al. (67)
Himatanthus sucuuba latex	*L. amazonensis* (WHOM/BR/75/Josefa strain)	200 μg/ml	↑ TNF-α and NO production ↓ TGF-β	Soares et al. (68)
A.sativum (methanolic extract)	*L. donovani* (NLB065)	250 μg/ml	↑ NO production	Wabwoba et al. (69)
Xylopia discreta (leaf methanolic extract and essential oil)	*L. panamensis* (MHOM/CO/87/UA140)	Different concentrations	↑ Monocyte chemoattractant protein-1 (MCP-1) expression	López et al. (70)
[a]Quassin (one of the quassinoids isolated from *Quassia amara*)	*L. donovani* (MHOM/IN/1983/AG83)	25 μg/ml	↑ iNOS2 expression ↑ TNF-α, and IL-12p70 ↓ TGF-β and IL-10	Bhattacharjee et al. (71)
A.sativum (aqueous extract)	*Leishmania mexicana* (MNYC/BZ/62/M379)	37 μg/ml [b]20 and 60 mg/kg b.w. for 2 weeks i.p.	↑ NO production ↑ IFN-γ	Gamboa-León et al. (72)
Pelargonium sidoides (aqueous-ethanolic formulation of roots and methanol insoluble fraction of this extract)	*L. major*	50 μg/ml	↑ iNOS activity ↑ IFN-γ, IL-12, IL-18 mRNA levels	Trun et al. (73)
[a]Plant polyphenols (Tannins and structurally related compounds)	*L. major* and *L. donovani* promastigotes	Different concentrations	Moderate effect on NO production ↑ TNF and INF like activities	Kolodziej and Kiderlen (74)
Desmodium gangeticum (Aminoglucosyl glycerolipid and Cerebroside)	*L. donovani*	100 μg/ml	↑ NO production	Mishra et al. (75)
Canova medication (*Aconitum napellus, Arsenium album, Bryonia alba,* and *Thuya occidentalis)*	*L. amazonensis* (MHOM/BR/73/M2269)	20 and 40%	↑ NO production	Pereira et al. (76)
Croton cajucara (Essential oil)	*L. amazonensis* (Raimundo strain, MHOM/BR/76/Ma-5)	1, 1.5, and 0.2 ng/ml	↑ NO production	do Socorro et al. (77)
A.sativum extract	*L. major* (MRHO/IR/76/ER)	[b]Garlic (20 mg/kg b.w.) +glucantime (60 mg/kg b.w.) daily for 2 weeks	↑ IFN-γ and IL-2 ↓IL-4 and IL-10	Ghazanfari et al. (78)

[a] *Synthetic molecules of plant origin.*

[b] *Studies carried out in BALB/c mice.*

immune response from Th2 (diseased state) to Th1 (cure) by causing the up- or downregulation of pro-inflammatory (activating Th1) and anti-inflammatory (promoting Th2) cytokines, respectively. The most commonly assessed immunomodulatory parameter for parasite clearance is stimulation of nitric oxide (NO). NO is the principle effector molecule in killing of *Leishmania* amastigotes (79) and is either estimated directly as nitrite concentration in culture supernatant or indirectly by the changes in nitric oxide synthase (iNOS) gene expression levels. NO-mediated killing of *Leishmania* parasites by tannins and related compounds

has also been demonstrated (74). IL-12 is the central cytokine produced by DC, NK, and T cells, which activates macrophages to produce IFN-γ and TNF-α. Different plant secondary metabolites and extracts have induced IL-12 up-regulation (**Table 1**), indicating the worthy potential of natural resources. Macrophages also produce IL-18, which in synergism with IL-12 stimulates IFN-γ production and aids in parasite clearance (80, 81). *Pelargonium sidoides* extracts have been shown to increase mRNA levels of IL-18 in *Leishmania major* infection (73).

In parallel to stimulation of IL-12 and other pro-inflammatory cytokines, expression levels of IL-4, IL-10, and TGF-β have also been widely investigated (**Table 1**). IL-4, IL-10, and TGF-β inhibit the production of IFN-γ from macrophages. IL-4 is known to potently inhibit macrophage activation, but IL-10 plays a cardinal role in progression of both CL and VL. In both, murine and human VL, despite the production of adequate amounts of IFN-γ, the hosts are unable to mount an effective CMI response, and this host inefficiency is attributed to increased levels of IL-10. Kane and Mosser (82) demonstrated that host-derived IgG present on *Leishmania* amastigotes ligates to Fcγ receptors on inflammatory macrophages and modulates them to secrete IL-10 in high amounts in CL. The levels of these Th2 cytokines have been observed to decline along with successful recuperation of CMI after treatment with immunomodulatory extracts and molecules of natural origin (**Table 1**).

It can thus be well established that natural immunomodulators can skew the Th1–Th2 balance but pro- and anti-inflammatory cytokines play diverse and inter-regulatory roles. As also supported by Couper et al. (83), understanding the dynamics of Th1–Th2 paradigm has changed over the years and it is conceived that both Th1 and Th2 cells can mediate inflammation as well as aid parasite clearance. For instance, Néris et al. (57) reported that Licarin A, treated *L. major*-infected macrophages exhibit decline in IL-6 as well as IL-10 levels. IL-6 is a characteristic pro-inflammatory cytokine, which also negatively regulates Th1 differentiation and promotes $CD4^+$ Th2 differentiation mediated by IL-4 (84). However, as the study presented only the *in vitro* data, and also the pro- or anti-immunopotentiating effect can be dose dependent (85) further *in vivo* studies may throw proper light on mechanism of action of Licarin A.

CONCLUSION

The use of herbal preparations to modulate the immune response to cure or avert diseases has been described in traditional systems of Indian, Unani, and Chinese medicine. The natural substances with dual, antileishmanial, and immunomodulatory properties have been carefully evaluated, however, it should be noted that human leishmaniasis varies in immunological pattern from murine leishmaniasis. Thus, the natural immunomodulators need to be evaluated more systematically and specifically in terms of dosage, biodistribution, kinetics, and interactions with other drugs and their putative role in other co-infections should also be examined.

Nonetheless, the concept of using natural immunomodulators to treat parasitic infections including leishmaniasis holds mighty potential in achieving the control of this disease. These natural immunomodulatory molecules can serve as scaffolds for synthesis and discovery of new immunodrugs. Also, the use of natural immunomodulators in synergy with existing drugs may involve the functional manipulation of multiple molecular targets leading to improved therapeutic efficacy and reduced toxicity.

ACKNOWLEDGMENTS

This work received financial assistance from University Grants Commission (UGC), Indian Council of Medical Research (ICMR), and Department of Biotechnology, Government of India. Garima Chouhan and Mohammad Islamuddin are recipients of UGC-BSR (Basic Science Research) and ICMR-SRF (Senior Research Fellowship), respectively.

REFERENCES

1. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) **7**:e35671. doi:10.1371/journal.pone.0035671
2. Sharlow ER, Grogl M, Johnson J, Lazo JS. Anti-leishmanial drug discovery: rising to the challenges of a highly neglected disease. *Mol Interv* (2010) **10**:72–5. doi:10.1124/mi.10.2.4
3. Bern C, Courtenay O, Alvar J. Of cattle, sandflies and men: a systematic review of risk factor analyses for South Asian visceral leishmaniasis and implications for elimination. *PLoS Negl Trop Dis* (2010) **4**:e599. doi:10.1371/journal.pntd.0000599
4. Cota GF, de Sousa MR, Rabello A. Predictors of visceral leishmaniasis relapse in HIV-infected patients: a systematic review. *PLoS Negl Trop Dis* (2011) **5**:e1153. doi:10.1371/journal.pntd.0001153
5. Kumar R, Nylén S. Immunobiology of visceral leishmaniasis. *Front Immunol* (2012) **3**:251. doi:10.3389/fimmu.2012.00251
6. Saha P, Mukhopadhyay D, Chatterjee M. Immunomodulation by chemotherapeutics agents against Leishmaniasis. *Int Immunopharmacol* (2011) **11**:1668–79. doi:10.1016/j.intimp.2011.08.002
7. Kedzierski L. Leishmaniasis vaccine: where are we today? *J Glob Infect Dis* (2010) **2**:177–85. doi:10.4103/0974-777X.62881
8. Schallig HD, Oskam L. Review: molecular biological applications in the diagnosis and control of leishmaniasis and parasite identification. *Trop Med Int Health* (2002) **7**:641–51. doi:10.1046/j.1365-3156.2002.00911.x
9. Ritter U, Frischknecht F, van Zandbergen G. Are neutrophils important host cells for *Leishmania* parasites? *Trends Parasitol* (2009) **25**:505–10. doi:10.1016/j.pt.2009.08.003
10. van Zandbergen G, Hermann N, Laufs H, Solbach W, Laskay T. *Leishmania* promastigotes release a granulocyte chemotactic factor and induce interleukin-8 release but inhibit interferon-inducible 10 production by neutrophil granulocytes. *Infect Immun* (2002) **70**:4177–84. doi:10.1128/IAI.70.8.4177-4184.2002
11. Chen L, Zhang ZH, Watanabe T, Yamashita T, Kobayakawa T, Kaneko A, et al. The involvement of neutrophils in the resistance to *Leishmania major* infection in susceptible but not in resistance mice. *Parasitol Int* (2005) **54**:109–18. doi:10.1016/j.parint.2005.02.001
12. Lima GM, Vallochi AL, Silva UR, Bevilacqua EM, Kiffer MM, Abrahamsohn IA. The role of polymorphonuclear leukocytes in the resistance to cutaneous leishmaniasis. *Immunol Lett* (1998) **64**:145–51. doi:10.1016/S0165-2478(98)00099-6
13. Ribeiro-Gomes FL, Otero AC, Gomes NA, Moniz-De-Souza MC, Cysne-Finkelstein L, Arnholdt AC, et al. Macrophage interactions with neutrophils regulate *Leishmania major* infection. *J Immunol* (2004) **172**:4454–62. doi:10.4049/jimmunol.172.7.4454
14. Tacchini-Cottier F, Zweifel C, Belkaid Y, Mukankundiye C, Vasei M, Launois P, et al. An immunomodulatory function for neutrophils during a CD^+ Th2 response in BALB/c mice infected with *Leishmania major*. *J Immunol* (2000) **165**:2628–36. doi:10.4049/jimmunol.165.5.2628
15. Novais FO, Santiago RC, Báfica A, Khouri R, Afonso L, Borges VM, et al. Neutrophils and macrophages cooperate in host resistance against *Leishmania braziliensis* infection. *J Immunol* (2009) **183**:8088–98. doi:10.4049/jimmunol.0803720
16. Guimarães-Costa AB, Nascimento MT, Froment GS, Soares RP, Morgado FN, Conceição-Silva F, et al. *Leishmania amazonensis* promastigotes induce and

are killed by neutrophil extracellular traps. *Proc Natl Acad Sci U S A* (2009) **106**:6748–53. doi:10.1073/pnas.0900226106
17. McFarlane E, Perez C, Charmoy M, Allenbach C, Carter KC, Alexander J, et al. Neutrophils contribute to development of a protective immune response during onset of infection with *Leishmania donovani*. *Infect Immun* (2008) **76**:532–41. doi:10.1128/IAI.01388-07
18. Pearson RD, Steigbigel RT. Phagocytosis and killing of the protozoan *Leishmania donovani* by human polymorphonuclear leukocytes. *J Immunol* (1981) **127**:1438–43.
19. Gabriel C, McMaster WR, Girard D, Descoteaux A. *Leishmania donovani* promastigotes evade the antimicrobial activity of neutrophil extracellular traps. *J Immunol* (2010) **185**:4319–27. doi:10.4049/jimmunol.1000893
20. Aga E, Katschinski DM, van Zandbergen G, Laufs H, Hansen B, Müller K, et al. Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J Immunol* (2002) **169**:898–905. doi:10.4049/jimmunol.169.2.898
21. Sarkar A, Aga E, Bussmeyer U, Bhattacharyya A, Möller S, Hellberg L, et al. Infection of neutrophil granulocytes with *Leishmania major* activates ERK 1/2 and modulates multiple apoptotic pathways to inhibit apoptosis. *Med Microbiol Immunol* (2013) **202**:25–35. doi:10.1007/s00430-012-0246-1
22. Gueirard P, Laplante A, Rondeau C, Milon G, Desjardins M. Trafficking of *Leishmania donovani* promastigotes in non-lytic compartments in neutrophils enables the subsequent transfer of parasites to macrophages. *Cell Microbiol* (2008) **10**:100–11. doi:10.1111/j.1462-5822.2007.01018.x
23. Peters NC, Egen JG, Secundino N, Debrabant A, Kimblin N, Kamhawi S, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science* (2008) **321**:970–4. doi:10.1126/science.1159194
24. Mosser DM, Miles SA. Avoidance of innate immune mechanisms by the protozoan parasite, *Leishmania* spp. In: Denkers E, Gazzinelli R, editors. *Protozoans in Macrophages*. Landes Bioscience (2007). p. 118–29.
25. Puentes SM, Dwyer DM, Bates PA, Joiner KA. Binding and release of C3 from *Leishmania donovani* promastigotes during incubation in normal human serum. *J Immunol* (1989) **143**:3743–9.
26. Hermoso T, Fishelson Z, Becker SI, Hirschberg K, Jaffe CL. Leishmanial protein kinases phosphorylate components of the complement system. *EMBO J* (1991) **10**:4061–7.
27. Brittingham A, Morrison CJ, McMaster WR, McGwire BS, Chang KP, Mosser DM. Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *J Immunol* (1995) **155**:3102–11.
28. Cunningham AC. Parasitic adaptive mechanisms in infection by *Leishmania*. *Exp Mol Pathol* (2002) **72**:132–41. doi:10.1006/exmp.2002.2418
29. Ehlers MR. CR3: a general purpose adhesion-recognition receptor for innate immunity. *Microbes Infect* (2000) **2**:289–94. doi:10.1016/S1286-4579(00)00299-9
30. Gasque P. Complement: a unique innate immune sensor for danger signals. *Mol Immunol* (2004) **41**:1089–98. doi:10.1016/j.molimm.2004.06.011
31. Holm A, Tejle K, Magnusson KE, Descoteaux A, Rasmusson B. *Leishmania donovani* lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKC alpha and defective phagosome maturation. *Cell Microbiol* (2001) **3**:439–47. doi:10.1046/j.1462-5822.2001.00127.x
32. Lerm M, Holm A, Seiron A, Särndahl E, Magnusson KE, Rasmusson B. *Leishmania donovani* requires functional Cdc42 and Rac1 to prevent phagosomal maturation. *Infect Immun* (2006) **74**:2613–8. doi:10.1128/IAI.74.5.2613-2618.2006
33. Scianimanico S, Desrosiers M, Dermine JF, Méresse S, Descoteaux A, Desjardins M. Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by *Leishmamnia donovani* promastigotes. *Cell Microbiol* (1999) **1**:19–32. doi:10.1046/j.1462-5822.1999.00002.x
34. Lodge R, Descoteaux A. *Leishmania donovani* induce periphagosomal F-actin accumulation through retention of the GTPase Cdc42. *Cell Microbiol* (2005) **7**:1647–58. doi:10.1111/j.1462-5822.2005.00582.x
35. Vinet AF, Fukuda M, Turco SJ, Descoteaux A. The *Leishmania donovani* lipophosphoglycan excludes the vesicular proton-ATPase from phagosomes by impairing the recruitment of synaptotagmin V. *PLoS Pathog* (2009) **5**:e1000628. doi:10.1371/journal.ppat.1000628
36. Courret N, Frehel C, Prina E, Lang T, Antoine JC. Kinetics of the intracellular differentiation of *Leishmania amazonensis* and internalization of host MHC molecules by the intermediate parasite stages. *Parasitology* (2001) **122**:263–79. doi:10.1017/S0031182001007387
37. Fruth U, Solioz N, Louis JA. *Leishmania major* interferes with antigen presentation by infected macrophages. *J Immunol* (1993) **150**:1857–64.
38. Kwan WC, McMaster WR, Wong N, Reiner NE. Inhibition of expression of major histocompatibility complex class II molecules in macrophages infected with *Leishmania donovani* occurs at the level of gene transcription via a cyclic AMP-independent mechanism. *Infect Immun* (1992) **60**:2115–20.
39. Thi EP, Lambertz U, Reiner NE. Sleeping with the enemy: how intracellular pathogens cope with a macrophage lifestyle. *PLoS Pathog* (2012) **8**:e1002551. doi:10.1371/journal.ppat.1002551
40. Cameron P, McGachy A, Anderson M, Paul A, Coombs GH, Mottram JC, et al. Inhibition of lipopolysaccharides-induced macrophage IL-12 production by *Leishmania mexicana* amastigotes: the role of cysteine peptidase and the NF-kappaB signalling pathway. *J Immunol* (2004) **173**:3297–304. doi:10.4049/jimmunol.173.5.3297
41. Gantt KR, Schultz-Cherry S, Rodriguez N, Jeronimo SM, Nascimento ET, Goldman TL, et al. Activation of TGF-β by *Leishmania chagasi*: importance for parasite survival in macrophages. *J Immunol* (2003) **170**:2613–20. doi:10.4049/jimmunol.170.5.2613
42. Kima PE. The amastigote forms of *Leishmania* are experts at exploiting host cell processes to establish infection and persist. *Int J Parasitol* (2007) **37**:1087–96. doi:10.1016/j.ijpara.2007.04.007
43. Singh S, Sivakumar R. Challenges and new discoveries in the treatment of leishmaniasis. *J Infect Chemother* (2004) **10**:307–15. doi:10.1007/s10156-004-0348-9
44. Monzote L. Current treatment of leishmaniasis: a review. *Open Antimicrob Agents J* (2009) **1**:9–19.
45. Richard JV, Werbovetz KA. New antileishmanial candidates and lead compounds. *Curr Opin Chem Biol* (2010) **14**:447–55. doi:10.1016/j.cbpa.2010.03.023
46. Buates S, Matlashewski G. Treatment of experimental leishmaniasis with the immunomodulators imiquimod and S-28463: efficacy and mode of action. *J Infect Dis* (1999) **179**:1485–94. doi:10.1086/314782
47. Arevalo I, Ward B, Miller R, Meng TC, Najar E, Alvarez E, et al. Successful treatment of drug-resistant cutaneous leishmaniasis in humans by use of imiquimod, an immunomodulator. *Clin Infect Dis* (2001) **33**:1847–51. doi:10.1086/324161
48. Miranda-Verastegui C, Lianos-Cuentas A, Arevalo I, Ward BJ, Matlashewski G. Randomized, double-blind clinical trial of topical imiquimod 5% with parenteral meglumine antmoniate in the treatment of cutaneous leishmaniasis in Peru. *Clin Infect Dis* (2005) **40**:1395–403. doi:10.1086/429238
49. Al-Mulla Hummadi YM, Najim RA, Al-Bashir NM. *Leishmania major* and *Leishmania tropica*: I the *in vitro* effects of an immunomodulator, S2-complex. *Exp Parasitol* (2005) **111**:47–54. doi:10.1016/j.exppara.2005.04.007
50. Nahrevanian H, Jalalian M, Farahmand M, Assmar M, Rastaghi AE, Sayyah M. Inhibition of murine systemic leishmaniasis by acetyl salicylic acid via nitric oxide immunomodulation. *Iran J Parasitol* (2012) **7**:21–8.
51. Mukherjee S, Ukil A, Das PK. Immunomodulatory peptide from cystatin, a natural cysteine protease inhibitor, against leishmaniasis as a model macrophage disease. *Antimicrob Agents Chemother* (2007) **51**:1700–7. doi:10.1128/AAC.01555-06
52. Shukla S, Bajpai VK, Kim M. Plants as potential sources of natural immunomodulators. *Rev Environ Sci Biotechnol* (2014) **13**:17–33. doi:10.1007/s11157-012-9303-x
53. Patwardhan B, Gautam M. Botanical immunodrugs: scope and opportunities. *Drug Discov Today* (2005) **10**:495–502. doi:10.1016/S1359-6446(04)03357-4
54. Sachdeva H, Sehgal R, Kaur S. *Asparagus racemosus* ameliorates cisplatin induced toxicities and augments its antileishmanial activity by immunomodulation *in vivo*. Parasitol Int (2014) **63**:21–30. doi:10.1016/j.parint.2013.09.016
55. Gamboa-Leon R, Vera-Ku M, Peraza-Sanchez SR, Ku-chulim C, Horta-Baas A, Rosado-Vallado M. Antileishmanial activity of a mixture of *Tridax procumbens* and *Allium sativum* in mice. *Parasite* (2014) **21**:15. doi:10.1051/parasite/2014016
56. Adriazola IO, Amaral AE, Amorim JC, Correia BL, Petkowicz CL, Mercê AL, et al. Macrophages activation and leishmanial activity by galactomannan and its oxovanadium (IV/V) complex *in vitro*. *J Inorg Biochem* (2014) **132**:45–51. doi:10.1016/j.jinorgbio.2013.09.017
57. Néris PL, Caldas JP, Rodrigues YK, Amorim FM, Leite JA, Rodrigues-Mascarenhas S, et al. Neolignan Licarin A presents effect against *Leishmania* (*Leishmania*) *major* associated with immunomodulation *in vitro*. *Exp Parasitol* (2013) **135**:307–13. doi:10.1016/j.exppara.2013.07.007
58. Chowdhury S, Mukherjee T, Mukhopadhyay R, Mukherjee B, Sengupta S, Chattopadhyay S, et al. The lignan niranthin poisons *Leishmania donovani*

topoisomerase IB and favours a Th1 immune response in mice. *EMBO Mol Med* (2012) **4**:1126–43. doi:10.1002/emmm.201201316
59. Saha P, Bhattacharjee S, Sarkar A, Manna A, Majumder S, Chatterjee M. Berberine chloride mediates its anti-leishmanial activity via differential regulation of the mitogen activated proteinkinase pathway in macrophages. *PLoS One* (2011) **6**:e18467. doi:10.1371/journal.pone.0018467
60. Shakya N, Sane SA, Gupta S. Antileishmanial efficacy of fluconazole and miltefosine in combination with an immunomodulator picroliv. *Parasitol Res* (2011) **108**:793–800. doi:10.1007/s00436-010-2230-2
61. Dos Santos RA, Batista J Jr, Rosa SI, Torquato HF, Bassi CL, Ribeiro TA, et al. Leishmanicidal effect of *Spiranthera odoratissima* (Rutaceae) and its isolated alkaloid skimmianine occurs by a nitric oxide dependent mechanism. *Parasitology* (2011) **138**:1224–33. doi:10.1017/S0031182011001168
62. Hosseini N, Abolhassani M. Immunomodulatory properties of borage (*Echium amoenum*) on Balb/c mice infected with *Leishmania major*. *J Clin Immunol* (2011) **31**:465–71. doi:10.1007/s10875-010-9502-6
63. Gharavi M, Nobakht M, Khademvatan S, Bandani E, Bakhshayesh M, Roozbehani M. The effect of garlic extract on expression of INF-γ and iNOS genes in macrophages infected with *Leishmania major*. *Iran J Parasitol* (2011) **6**:74–81.
64. Gharavi M, Nobakht M, Khademvatan S, Fani F, Bakhshayesh M, Roozbehani M. The effect of aqueous garlic extract on interleukin-12 and 10 levels in *Leishmania major* (MRHO/IR/75/ER) infected macrophages. *Iran J Public Health* (2011) **40**:105–11.
65. Sen R, Ganguly S, Saha P, Chatterjee M. Efficacy of artemisinin in experimental visceral leishmaniasis. *Int J Antimicrob Agents* (2010) **36**:43–9. doi:10.1016/j.ijantimicag.2010.03.008
66. Gomes DC, Muzitano MF, Costa SS, Rossi-Bergmann B. Effectiveness of the immunomodulatory extract of *Kalanchoe pinnata* against murine visceral leishmaniasis. *Parasitology* (2010) **137**:613–8. doi:10.1017/S0031182009991405
67. Githinji EK, Irungu LW, Tonui WK, Rukunga GM, Mutai C, Muthaura CN, et al. *In vitro* effects of *Warburgia ugandensis*, *Psiadia punctulata* and *Chasmanthera dependes* on *Leishmania major* promastigotes. *Afr J Tradit Complement Altern Med* (2010) **7**:264–75.
68. Soares DC, Andrade AL, Delorenzi JC, Silva JR, Freire-de-Lima L, Falcão CA, et al. Leishmanicidal activity of *Himatanthus sucuuba* latex against *Leishmania amazonensis*. *Parasitol Int* (2010) **59**:173–7. doi:10.1016/j.parint.2010.01.002
69. Wabwoba BW, Anjili CO, Ngeiywa MM, Ngure PK, Kingondu EM, Ingonga J, et al. Experimental chemotherapy with *Allium sativum* (Liliaceae) methanolic extract in rodents infected with *Leishmania major* and *Leishmania donovani*. *J Vector Borne Dis* (2010) **47**:160–7.
70. López R, Cuca LE, Delgado G. Antileishmanial and immunomodulatory activity of *Xylopia discreta*. *Parasite Immunol* (2009) **31**:623–30. doi:10.1111/j.1365-3024.2009.01134.x
71. Bhattacharjee S, Gupta G, Bhattacharya P, Mukherjee A, Mujumdar SB, Pal A, et al. Quassin alters the immunological patterns of murine macrophages through generation of nitric oxide to exert antileishmanial activity. *J Antimicrob Chemother* (2008) **63**:317–24. doi:10.1093/jac/dkn479
72. Gamboa-León MR, Aranda-González I, Mut-Martín M, García-Miss MR, Dumonteil E. *In vivo* and *in vitro* control of *Leishmania mexicana* due to garlic-induced NO production. *Scand J Immunol* (2007) **66**:508–14. doi:10.1111/j.1365-3083.2007.02000.x
73. Trun W, Kiderlen AF, Kolodziej H. Nitric oxide synthase and cytokines gene expression analyses in *Leishmania*-infected RAW 264.7 cells treated with an extract of *Pelargonium sidoides* (Eps 7630). *Phytomedicine* (2006) **13**:570–5. doi:10.1016/j.phymed.2005.07.004
74. Kolodziej H, Kiderlen AF. Antileishmanial activity and immune modulatory effects of tannins and related compounds on *Leishmania* parasitised RAW 264.7 cells. *Phytochemistry* (2005) **66**:2056–71. doi:10.1016/j.phytochem.2005.01.011
75. Mishra PK, Singh N, Ahmad G, Dube A, Maurya R. Glycolipids and other constituents from *Desmodium gangeticum* with antileishmanial and immunomodulatory activities. *Bioorg Med Chem Lett* (2005) **15**:4543–6. doi:10.1016/j.bmcl.2005.07.020
76. Pereira WK, Lonardoni MV, Grespan R, Caparroz-Assef SM, Cuman RK, Bersani-Amado CA. Immunomodulatory effect of Canova medication on experimental *Leishmania amazonensis* infection. *J Infect* (2005) **51**:157–64. doi:10.1016/j.jinf.2004.09.009
77. do Socorro S, Rosa Mdo S, Mendonça-Filho RR, Bizzo HR, de Almeida Rodrigues I, Soares RM, et al. Antileishmanial activity of a linalool-rich essential oil from *Croton cajucara*. *Antimicrob Agents Chemother* (2003) **47**:1895–901. doi:10.1128/AAC.47.6.1895-1901.2003
78. Ghazanfari T, Hassan ZM, Ebtekar M, Ahmadiani A, Naderi G, Azar A. Garlic induces a shift in cytokine pattern in *Leishmania major*-infected BALB/c mice. *Scand J Immunol* (2000) **52**:491–5. doi:10.1046/j.1365-3083.2000.00803.x
79. Ukil A, Biswas A, Das T, Das PK. 18 Beta-glycyrrhetinic acid triggers curative Th1 response and nitric oxide up-regulation in experimental visceral leishmaniasis associated with the activation of NF-kappa B. *J Immunol* (2005) **175**:1161–9. doi:10.4049/jimmunol.175.2.1161
80. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* (1995) **13**:251–76. doi:10.1146/annurev.iy.13.040195.001343
81. Munder M, Mallo M, Eichmann K, Modolell M. Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: a novel pathway of autocrine macrophages activation. *J Exp Med* (1998) **187**:2103–8. doi:10.1084/jem.187.12.2103
82. Kane MM, Mosser DM. The role of IL-10 in promoting disease progression in leishmaniasis. *J Immunol* (2001) **166**:1141–7. doi:10.4049/jimmunol.166.2.1141
83. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol* (2008) **180**:5771–7.
84. Diehl S, Anguita J, Hoffmeyer A, Zapton T, Ihle JN, Fikrig E, et al. Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. *Immunity* (2000) **13**:805–15. doi:10.1016/S1074-7613(00)00078-9
85. Spelman K, Burns J, Nichols D, Winters N, Ottersberg S, Tenborg M. Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. *Altern Med Rev* (2006) **11**:128–50.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 March 2014; accepted: 18 April 2014; published online: 05 May 2014.
Citation: Chouhan G, Islamuddin M, Sahal D and Afrin F (2014) Exploring the role of medicinal plant-based immunomodulators for effective therapy of leishmaniasis. Front. Immunol. 5:193. doi: 10.3389/fimmu.2014.00193
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 11 June 2014
doi: 10.3389/fimmu.2014.00273

Leishmania donovani nucleoside hydrolase terminal domains in cross-protective immunotherapy against *Leishmania amazonensis* murine infection

Dirlei Nico[1], Daniele Crespo Gomes[1], Iam Palatnik-de-Sousa[2], Alexandre Morrot[3], Marcos Palatnik[4] and Clarisa Beatriz Palatnik-de-Sousa[1]*

[1] Laboratório de Biologia e Bioquímica de Leishmania, Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[2] Programa de Pós Graduação em Metrologia, Laboratório de Biometrologia, Pontifícia Universidade Católica do Rio de Janeiro, Rio de Janeiro, Brazil
[3] Laboratório de Imunologia, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[4] Programa de Pós Graduação em Clínica Médica, Faculdade de Medicina, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Edited by:
Nahid Ali, Indian Institute of Chemical Biology, India

Reviewed by:
Hira Nakhasi, Food and Drug Administration, USA
Benjamin M. J. Owens, University of Oxford, UK

***Correspondence:**
Clarisa Beatriz Palatnik-de-Sousa, Laboratório de Biologia e Bioquímica de Leishmania, Instituto de Microbiologia Paulo de Góes, CCS, Universidade Federal do Rio de Janeiro, Avda. Carlos Chagas 373, Caixa Postal 68040, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, Rio de Janeiro CEP 21941-902, Brazil
e-mail: immgcpa@micro.ufrj.br

Nucleoside hydrolases of the *Leishmania* genus are vital enzymes for the replication of the DNA and conserved phylogenetic markers of the parasites. *Leishmania donovani* nucleoside hydrolase (NH36) induced a main $CD4^+$ T cell driven protective response against *L. chagasi* infection in mice which is directed against its C-terminal domain. In this study, we used the three recombinant domains of NH36: N-terminal domain (F1, amino acids 1–103), central domain (F2 aminoacids 104–198), and C-terminal domain (F3 amino acids 199–314) in combination with saponin and assayed their immunotherapeutic effect on Balb/c mice previously infected with *L. amazonensis*. We identified that the F1 and F3 peptides determined strong cross-immunotherapeutic effects, reducing the size of footpad lesions to 48 and 64%, and the parasite load in footpads to 82.6 and 81%, respectively. The F3 peptide induced the strongest anti-NH36 antibody response and intradermal response (IDR) against *L. amazonenis* and a high secretion of IFN-γ and TNF-α with reduced levels of IL-10. The F1 vaccine, induced similar increases of IgG2b antibodies and IFN-γ and TNF-α levels, but no IDR and no reduction of IL-10. The multiparameter flow cytometry analysis was used to assess the immune response after immunotherapy and disclosed that the degree of the immunotherapeutic effect is predicted by the frequencies of the $CD4^+$ and $CD8^+$ T cells producing IL-2 or TNF-α or both. Total frequencies and frequencies of double-cytokine CD4 T cell producers were enhanced by F1 and F3 vaccines. Collectively, our multifunctional analysis disclosed that immunotherapeutic protection improved as the CD4 responses progressed from 1+ to 2+, in the case of the F1 and F3 vaccines, and as the CD8 responses changed qualitatively from 1+ to 3+, mainly in the case of the F1 vaccine, providing new correlates of immunotherapeutic protection against cutaneous leishmaniasis in mice based on T-helper TH1 and $CD8^+$ mediated immune responses.

Keywords: visceral leishmaniasis, cutaneous leishmaniasis, diffuse cutaneous leishmaniasis, cross-immunotherapy, nucleoside hydrolases, recombinant vaccines

INTRODUCTION

Leishmaniasis is a complex of vector-borne protozoan diseases the etiological agents of which belong to the *Leishmania* genus. The global incidence and prevalence of leishmaniasis is increasing. The main clinical syndromes of leishmaniasis are: cutaneous (CL), diffuse cutaneous (DCL), mucocutaneous (MCL), and visceral (VL) (1). While CL accounts for approximately 0.7–1.2 million cases per year, which is more than 50% of the new cases of leishmaniasis (2). Most of the CL cases occur in the Mediterranean (85,555 cases/year), the Americas (66,941 cases/year), and the Middle East to Central Asia (61,013 cases/year) (2). The 10 countries with the highest estimated number of cases are: Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru and together they account for 70–75% of the estimated global incidence of CL (2). The disease causes skin ulcers at the site of the sand-fly bite, usually on exposed parts of the body, such as the face, neck, arms, and legs and develops an active T cell mediated immune response that plays a pivotal role in the processes in the cure or in the aggravation of the disease (3). VL, on the other hand, has approximately 0.2–0.4 million new cases per year (2) and is the most severe clinical syndrome of leishmaniasis characterized by hepato-splenomegaly, malaise, cachexia, fever, hypergammaglobulinemia, anemia, and the progressive suppression of the T cell mediated immune response. If left untreated, the disease has a high mortality rate mainly due to immunosuppression and secondary infections. Indeed, anergy to leishmanial antigens and negative skin tests have been reported in cases of VL caused by *Leishmania donovani* and *L. infantum/chagasi* (4–6),

and DCL caused by *L. amazonensis* (7) while a strong TH1 pro-inflammatory response has been detected in cases of CL (8) and MCL caused by *L. braziliensis* (9).

Since the chemotherapy of leishmaniasis is highly toxic and the few available therapeutic drugs are only partially effective (10, 11), due to an increase in the resistance of parasites to antibiotics, a protective vaccine would be important not only for prophylaxis but also for the immunotherapy of the disease. The success of immunotherapy in the control of human CL leishmaniasis with the use of crude parasite vaccines combined to BCG has been reported since the 80s (12–14). Furthermore, immunochemotherapy against human CL leishmaniasis has been reported to reduce the time of chemotherapy needed to cure this disease in humans, thus decreasing its toxicity (15).

Since the epidemics of VL and CL are spreading on a worldwide scale, even overlapping in some areas, and no human vaccine is available yet, the development of a bivalent vaccine for the control of tegumentary and VL leishmaniasis is highly recommended. Consequently, we believe that the search for cross-protective antigens is mandatory. Recently, we developed the first licensed second generation vaccine against canine VL leishmaniasis (Leishmune®), which contains the fucose–mannose ligand (FML) antigen of *L. donovani* in formulation with saponin (16–19), is a transmission blocking vaccine (18, 19) and has already determined a reduction in the incidence of the human and canine disease in Brazilian endemic areas (20). Prophylactic vaccination of dogs with Leishmune® promoted increases in the production of NO, IgG2 antibodies against FML and *L. chagasi*, intradermal reactions and proportions of $CD8^+$ lymphocytes, which secrete more IFN-γ than IL-4 (21, 22) expressing a selective pro-inflammatory pattern (IFN-γ/NO) (23). The early and persistent activation of neutrophils and monocytes have also been described (23). This increase in proportions of $CD8^+$ T cells is expected for the QS21 saponin adjuvant of Leishmune® (24) and this was also described in the Leishmune® immunotherapy assays against naturally (25) and experimentally acquired canine VL leishmaniasis (26). Furthermore, the sustained or increased proportions of $CD4^+$ and CD21-B lymphocytes (25, 26) and the reduced $CD4^+/CD25^+$ T cell counts (27) have also been described in Leishmune® vaccinated dogs.

Leishmune® canine immunotherapy, on the other hand, reduced the number of deaths and the clinical and parasitological signs of canine VL and, when used for immunochemotherapy with allopurinol, amphotericin, and enrofloxacin, promoted the sterile cure (28).

QS21 and deacylated saponins of *Quillaja saponaria* are the adjuvants of the Leishmune® vaccine (29). The QS21 Stimulon 1 saponin (Agenus) is also the adjuvant currently being studied in 17 human clinical programs, including four Phase 3 anti-Malaria assays, by GlaxoSmithKline. The anti-Malaria vaccine, called the RTS,S or Mosquirix, indeed contains the *P. falciparum* cir-cumsporozoite (CS) protein central tandem repeat and carboxy-terminal regions fused to the amino-terminus of the S antigen of hepatitis B virus (HBsAg) (30) and the AS01 adjuvant, which is composed of QS21 Stimulon in combination with monophosphoryl Lipid A (31). The RTS,S/AS01 vaccine co-administered with EPI vaccines provided modest protection against both clinical and severe malaria in young infants (32).

The main component of the FML antigen is the nucleoside hydrolase of *L. donovani* (NH36), which was the only FML component specifically recognized by the sera of patients with human VL leishmaniasis (33). NH36 is not only a vital enzyme which cleaves exogenous nucleosides to release pyrimidines or purines for the DNA synthesis and further replication of the parasite (34, 35), but also a strong antigen (36) present in the early stages of the parasite infection. It fulfills the requirements for a cross-protective antigen of a *Leishmania* vaccine perfectly since it is a strong phylogenetic marker *Leishmania* (37, 38) that shares high identity with the sequences of the nucleoside hydrolases of *L. major* (95%) (39), *L. mexicana* (93%), *L. chagasi* (99%), *L. infantum* (99%), *L. tropica* (97%), and *L. braziliensis* (84%) (40). This fact explains why a vaccine containing NH36, in its native form, reduced the infection by *L. donovani* (41) in mice and was characterized as an *L. major* exo-antigen (42), and in its recombinant or DNA formulations, protected mice against challenge with *L. chagasi*, *L. mexicana* (43, 44), *L. amazonensis* (45), and *L. major* (42), and dogs against challenge with *L. chagasi* (46). The DNA-NH36 vaccine induced a TH1 immune response related to the IFN-γ expression by $CD4^+$ T cells, which led to an 88% prophylactic protection against VL (43), 65–81% against tegumentary leishmaniasis (42, 43, 45) and 91% immunotherapy against VL leishmaniasis in the mouse model (47). Also, higher proportions of $CD4^+$-NH36-specific lymphocytes and higher levels of IFN-γ and IL-2 were found in *L. chagasi* infected dogs treated with NH36-DNA vaccine (46).

We recently obtained three recombinant fragment proteins representing the whole sequence of NH36: amino acids 1–103 (F1, N-terminal domain), 104–198 (F2, central domain), and 199–314 (F3, C-terminal domain) and used them in a mouse vaccination against *L. chagasi* infection in order to map the domain of NH36, which is the target of the adaptive immunity (48). Protection against *L. chagasi* infection in mice was determined by the C-terminal domain of NH36, which induced a main $CD4^+$ T cell mediated response with a minor contribution of $CD8^+$ T cells. Protection induced by this C-terminal peptide was superior to that induced by the whole protein. Vaccination with the C-terminal determined the increases of antibody titers (IgM, IgG2a, IgG1, and IgG2b), frequencies of $CD4^+$ T lymphocytes, and levels of IFN-γ in the splenocyte supernatants. The proportions of $CD4^+$ and $CD8^+$ T lymphocytes generating IFN-γ were higher than those generating IL-10. Antibodies of Leishmune® vaccinated dogs showed the most potent reactivity against the epitopes of the C-terminal domain. The intradermal response (IDR) against *L. donovani* antigen and the increase of TNF-α, when compared to IL-10, expressed by $CD4^+$ lymphocytes were very good correlates of vaccine induced immunity (48). Important epitopes for mice (48), human, and dog B cells (49) were also recently demonstrated in the sequence of the C-terminal domain.

In the search for cross-protection for CL leishmaniasis, we further vaccinated mice with the NH36 domains and challenged them with *L. amazonensis* (48). Different from the absolute dominance of the C-terminal domain in immune protection to VL, the most severe syndrome (1), preliminary results suggest that protection

against CL by *L. amazonensis* is mediated by the C-terminal and the N-terminal domain in similar proportions (48).

In the present work, we studied the immunotherapeutic effect of NH36 or its peptide components in a formulation with saponin, on mice infection by *L. amazonensis*, in order to assess which of the NH36 domains deserves to be considered as components in a future cross-therapeutic vaccine for leishmaniasis. We identified that the N-terminal and C-terminal domains of NH36 induced strong curative effects which improved, as the CD4 T cell responses shifted from single- to double-cytokine producers (TNF-α^+-IL-2^+), and, in the case of the N-terminal domain vaccine, as the CD8 T cell responses shifted qualitatively from single- to triple-cytokine producers (TNF-α^+-IL-2^+-IFN-γ^+).

MATERIALS AND METHODS

ETHICAL STATEMENTS

All experiments were reviewed and approved by the Animal Care and Use Committee of the Instituto de Biofisica Carlos Chagas Fo.-UFRJ (CAUAP-CONCEA, Brazil, IMPPG-016) and were performed according to the guidelines of the National Institutes of Health, USA. We made all efforts to minimize animal suffering.

NUCLEOSIDE HYDROLASE-NH36 DOMAINS

The sequence of DNA and amino acids of NH36 is deposited in the EMBL, GenBank™, and DDJB data bases, access number AY007193. NH36 is composed of 314 amino acids. The three peptide domains of NH36 codifying, respectively, for the amino acids 1–103 (F1), 104–198 (F2), and 199–314 (F3) were cloned in the pET28b plasmid and were expressed and chromatographed as previously described (48). A preliminary molecular model was obtained through homology modeling using the Modeller9.10 software and the data of the nucleoside hydrolase from *L. major* template (RCSB PDB code: 1EZR; Crystal structure of nucleoside hydrolase of *L. major*) (50). It is important to note that the model shown in this investigation is preliminary and not a final, optimized model. The predicted epitopes for MHC class II-IAd and IEd, haplotype H2d $CD4^+$ T cells, MHC class I Ld-$CD8^+$ T cells, and B cells were plotted in the C-terminal and N-terminal moieties of the model (48). Additionally, the analysis of the solvent accessible surface area of the C-terminal, central, and N-terminal sections of the tetramer was performed using the PyMol 1.3 software.

IMMUNOTHERAPEUTIC VACCINATION IN *LEISHMANIA AMAZONENSIS* INFECTED MICE

Two-month-old Balb/c mice (female) were infected with 10^5 *L. amazonensis* (pH 8 strain) metacyclic promastigotes isolated from hamsters and maintained in Schneider's medium in the right hind footpads (45). The evolution of lesions was monitored weekly with a caliper apparatus (Mitutoyo) and the swelling of the non-infected contra-lateral left footpads were subtracted. Six weeks after infection groups of mice received three doses of 100 μg of NH36, F1, F2, or F3 recombinant proteins and 100 μg of SIGMA saponin (NH36sap, F1sap, F2sap, and F3sap vaccines, respectively), at weekly intervals, in the back by the sc route, while the control group was treated with saline solution. At 9 weeks after infection sera were collected for the assay of anti-NH36 antibodies in an ELISA assay and the IDR against *L. amazonensis* (pH 8) lysate (IDR) was determined in the footpads as described previously (48). Mice were euthanized with CO_2 and their cellular immune response was assessed by intracellular staining (ICS), multiparameter cytometry analysis of splenocytes (51, 52), and by a cytokine-ELISA assay of the splenocytes supernatants. The total number of parasites in the footpad lesions was determined after sacrifice by Real Time PCR as previously described (53) using primers for *L. chagasi* on DNA isolated from infective promastigotes of *L. amazonensis* (pH 8) obtained from hamsters footpads (48).

ASSESSMENT OF THE CELLULAR IMMUNE RESPONSE

The cellular immune response was assessed using 10^6 splenocytes that had been cultured in RPMI for 72 h *in vitro* at 37°C and 5% CO_2 in the presence or absence of 5 μg of NH36. The multiparameter analysis (51, 52) was carried out to assess the intracellular production of IL-2, TNF-α, and IFN-γ cytokines by $CD4^+$ and $CD8^+$ T lymphocytes. For this evaluation, the cells were treated with brefeldin (SIGMA) at a final concentration of 10 μg/ml, incubated for an additional 4 h, and then stained with rat anti-mouse CD4FITC (clone GK1.5) and CD8FITC (clone 53–6.7) monoclonal antibodies (R&D systems, Inc.) and further stained with IFN-γAPC, IL-2-PerCP-Cy5.5, and TNF-αPE monoclonal antibodies (BD-Pharmingen) as described before (48). For the ICS methods, 100,000 lymphocytes were acquired using a BD FACScalibur apparatus. Data were analyzed using the Cell Quest program. The secretion of cytokines was also evaluated in the supernatants of splenocytes by an ELISA assay as previously described (48).

STATISTICAL ANALYSIS

The Kruskal Wallis and Mann Whitney non-parametrical tests were used for comparison of means and the two-tailed Pearson bivariate analysis for the assessment of the correlation coefficient (GraphPad Prism 6 for Windows).

RESULTS

To understand more clearly how the peptide domains F1, F2, and F3 are distributed along the whole of the NH36 molecule, we obtained the preliminary model of the nucleoside hydrolase-NH36 by homology to the model of the nucleoside hydrolase of *L. major* (**Figure 1**). Our aim was only to illustrate the molecule. The image of the molecule shows its tetramer composition, with four identical subunits. The solvent accessible surface area was computed and this disclosed the distribution of the F1, F2, and F3 domains (**Figure 1A**). This tridimensional surface model of the tetramer revealed that the F3 (C-terminal domain) is the domain with the largest area of exposed surface (29,507,002 Å) (**Figure 1A**). This is followed by the F1 (N-terminal domain) with an area of 27,132,781 Å. The F2 (central domain) has the smallest surface area (19,931,451 Å) and is therefore the least exposed domain (**Figure 1A**). The detailed monomer (**Figure 4B**) shows the F3 as the most exposed peptide, followed by the less exposed F1, while the F2 fragment (central domain), on the other hand, is apparently more hidden (**Figure 4B**). The sequence of F3 includes three predicted epitopes for $CD4^+$ T cells (**Figure 4C**) and three epitopes for antibodies (**Figure 4D**) while the F1 shows two epitopes for $CD4^+$, one epitope for $CD8^+$ T cells (**Figure 4C**), and two epitopes for antibodies (**Figure 4D**).

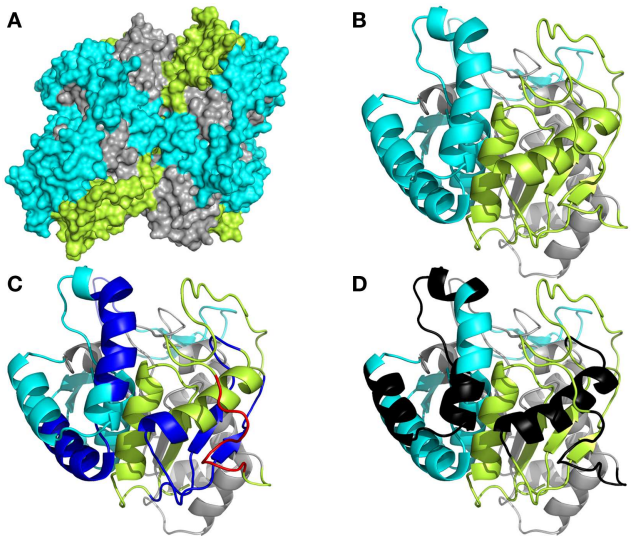


FIGURE 1 | Spatial distribution of epitopes in the monomer of *Leishmania donovani* nucleoside hydrolase-NH36. **(A)** Illustration of the tridimensional surface model of the NH36 tetramer obtained by homology modeling to the sequence of the nucleoside hydrolase of *L. major*. **(B)** Monomer of *L. donovani* NH36 with the sequences of the N-terminal (F1, amino acids 1–103 in lime green), central (F2, amino acids 104–198 in gray), and C-terminal (F3, amino acids 199–314 in cyan) moieties. **(C)** MHC class II-IAd and IEd, haplotype H2d CD4$^+$ T cell epitopes (dark blue), and of MHC class I Ld-CD8$^+$ T cell predicted epitopes (red) of the C-terminal and N-terminal moieties. **(D)** Epitopes for B cells on the C-terminal and N-terminal moieties (black).

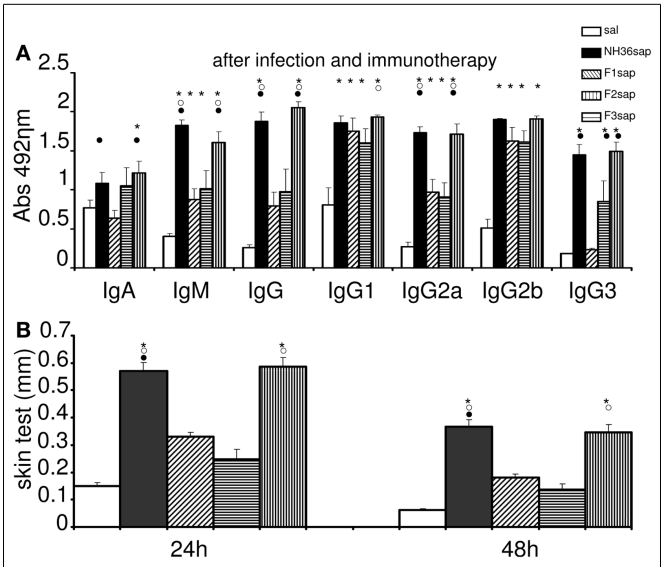


FIGURE 2 | Therapeutic vaccination, anti-NH36 antibodies, and intradermal response to *L. amazonenis*. Six weeks after infection with 10^5 metacyclic promastigotes of *L. amazonensis* in the footpads, Balb/c mice were further vaccinated with three subcutaneous doses of NH36sap, F1sap, F2sap, or F3sap at weekly intervals. Bars represent the mean ± SE of the absorbance values of anti-NH36 antibodies from 1/100 diluted serum **(A)** and intradermal response to the promastigote lysate of *L. amazonensis* (24 and 48 h after antigen injection) **(B)** of two independent experiments with $n = 6$–7 mice per treatment performed after complete vaccination. $^*p < 0.05$ different from the saline control; $^\circ p < 0.05$ different from the F2sap vaccine; •from the F1sap vaccine.

We also studied the immunotherapeutic effect of the NH36, F1, F2, and F3-saponin vaccines in mice previously infected with *L. amazonensis*. On week 6 after infection, when significant differences between the sizes of infected and the contra-lateral uninfected footpads were already detected, three doses of each vaccine were injected with weekly intervals. Sera samples were obtained and analyzed for anti-NH36 antibodies 1 week 9 after completing vaccination schedule (**Figure 2A**). Significant variations were detected for all antibody classes and subtypes ($p < 0.0001$). The F3sap vaccine induced levels of anti-NH36 IgA, IgM, IgG, and IgG2a antibodies as high as the NH36 vaccine and of IgG1 antibodies higher than the F2sap vaccine indicating that the main NH36 B cell epitopes involved in immunotherapy are located in the C-terminal moiety of NH36. The F1 vaccine, on the other hand, induced only IgG2b levels higher than saline controls and compatible with all other vaccines (**Figure 2A**).

After immunotherapy, the IDR specific response against *L. amazonensis* lysate was predominant in the F3 vaccinated mice, which showed an IDR as high as the one induced by the NH36 vaccine (**Figure 2B**). The other peptide vaccines were not different from the saline treated controls which exhibited, as expected for CL leishmaniasis, a positive and mild IDR reaction of 0.15 mm at 24 h and 0.06 mm at 48 h (**Figure 2B**). This result points out the pre-dominance of the epitopes present at the C-terminal domain in the generation of a cellular immune response to *L. amazonensis* infection.

As a further measure of the therapeutic effect, we compared lesion development and parasite burden on week 9 after challenge. Significant differences between treatments were detected in the size of the footpad lesions along the time ($p < 0.0001$). The NH36sap ($p < 0.001$), F1sap ($p < 0.05$), and F3sap vaccines ($p < 0.001$) reduced the size of footpad lesions, along the time, to a similar extent if compared to the untreated infected saline controls. The F3sap vaccine also showed to be more therapeutic than the F2sap vaccine ($p < 0.05$) (not shown). When looking in detail at the individual footpad sizes on week 9 (**Figure 3A**), it was possible to observe that the best therapeutic effect was detected in the F3sap vaccinated mice, whose mean lesion size (0.23 mm) was 64% ($p < 0.001$) lower than that of the saline controls (0.64 mm) and 48% ($p < 0.05$) lower than that of the F2sap vaccine group (0.44 mm) (**Figure 3A**). The sizes of footpad lesions at week 9 were significantly correlated to the number of *L. amazonensis* parasites in lesions quantified by RTPCR, which disclosed that only the N-terminal and C-terminal domains reduced to 82.6% ($p < 0.006$) and 81% ($p < 0.021$), respectively, the number of parasites in lesions when compared to the control animals (**Figure 3B**). No difference in parasite load was detected between both vaccines ($p < 0.05$). Mice treated with F2sap, on the other hand, showed no decrease in parasite load when compared to the untreated controls ($p > 0.05$) (**Figures 3A,B**). None of the animals in the saline control or F2 vaccine group showed a total absence of parasites, however, three animals of the F1 and NH36 vaccines, and two

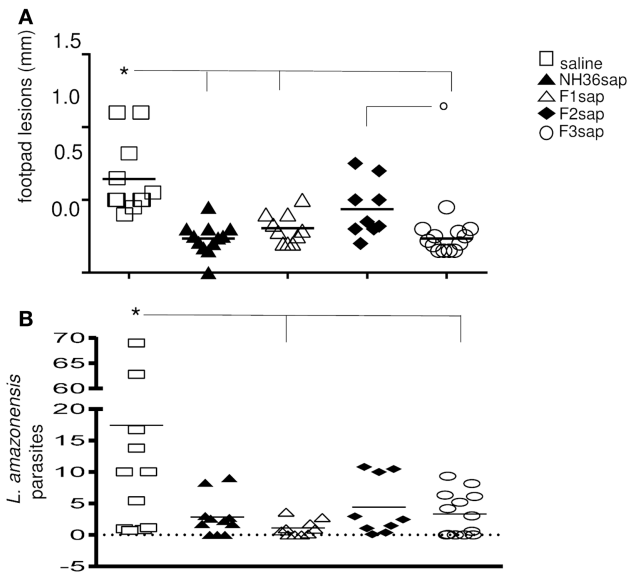


FIGURE 3 | Therapeutic effect of NH36 vaccines on *L. amazonensis* infection. **(A)** Individual sizes of footpad lesions 9 weeks after infection with 10^5 infective promastigotes of *L. amazonensis* and further treated with NH36, F1, F2, and F3 vaccines formulated in saponin. Results represent the mean + SE of the footpad measurements of two independent experiments (six to seven animals per treatment in each experiment). $*p < 0.05$ different from the saline control and $^{\circ}p < 0.05$ different from the F2sap vaccine. **(B)** *Leishmania amazonensis* promastigotes in the footpad lesions as determined by Real Time PCR. *Lines indicate significant differences compared to the saline control ($p < 0.05$).

animals of the F3 vaccine showed zero parasites in their footpad lesions.

Furthermore, the cytokine levels secreted to the splenocytes supernatants after stimulation with NH36 were measured (**Figure 4**) and significant variations among treatments were detected for the secretion of IFN-γ ($p < 0.001$), TNF-α ($p < 0.001$), and IL-10 ($p < 0.01$) (**Figures 4A–C**). The NH36sap, F1sap, and F3sap vaccines induced increased levels of IFN-γ above the saline controls ($p < 0.01$ for each vaccine) (**Figure 4A**) while TNF-α was increased by the F3sap ($p < 0.05$) and F1sap ($p < 0.05$) above the F2sap vaccine (**Figure 4B**). Additionally, only the NH36sap ($p < 0.01$) and F3sap ($p < 0.05$) vaccines expressed lower levels of IL-10 than the saline controls (**Figure 4C**). Therefore, while the F3 vaccine promoted a TH1 therapeutic response with high secretion of the pro-inflammatory cytokines IFN-γ and TNF-α and low levels of the regulatory cytokine IL-10, the F1 vaccine, differently, induced the increase of IFN-γ and TNF-α (**Figures 4A,B**) but no decrease however, of the IL-10 levels (**Figure 4C**). The analysis of the IFN-γ/IL-10 ratio (**Figure 4D**) disclosed also that the F2sap vaccine did not differ from the saline control. The higher ratios were seen in animals treated with the NH36 and F3 vaccines, followed by the F1sap vaccine (**Figure 4D**). The TNF-α/IL-10 ratios, on the other hand were only enhanced by the NH36sap and F3sap vaccine above the levels of the saline controls and F2sap vaccine (**Figure 4E**).

Based on the requirements of IFN-γ and the roles of TNF-α and IL-2 as effector cytokines that mediate protection, we assessed the frequency of NH36-specific IFN-γ, IL-2-, and TNF-α-producing $CD4^+$ T cells after immunotherapy treatment by multiparameter cytometry analysis. We initially assessed the total frequencies of $CD4^+$-T cells producing IFN-γ, IL-2-, and TNF-α, which summarize the frequency of cells that produce each particular cytokine alone (single producers), and together with one more (double producers) and two other cytokines (triple cytokines). On week 9 after infection, significant differences between treatments in the total frequencies of TNF-α ($p = 0.0077$) and IL-2-producing-$CD4^+$ T cells of the spleens ($p = 0.0035$) were found (**Figure 5A**). The total frequencies of $CD4^+$ T cells producing TNF-α and IL-2 (**Figure 5A**) were significantly increased above their saline controls and reached 35–36%, in the case of F1sap, and 28% in the case of the F3sap vaccine, while the NH36 vaccine increased only the IL-2-producing cells to 29%. In agreement with that, the proportion of $CD4^+$ T cells producing TNF-α^+-IL-2^+ was increased to 33 and 27%, by the F1sap and F3sap vaccines, respectively (**Figure 5B**). On the other hand, the frequencies of IL-2^+ and TNF-α single cytokine producer $CD4^+$ T cells were increased significantly, only by the F3sap vaccine to 14 and 12%, respectively (**Figure 5B**).

In contrast to the lack of correlation seen by measuring the total frequencies of $CD4^+$IFN-γ^+ producing cells alone or in combination with other cytokines (**Figures 5A,B**), which collectively developed frequencies below 1%, our analysis showed a high correlation between the frequency of multifunctional (IL-2, TNF-α, TNF-α-IL-2) $CD4^+$ T cells and the degree of protection. The sizes of footpad lesions (**Figure 3A**), which were positively correlated with the number of parasites (**Figure 3B**) ($R = 0.7239$, $p < 0.001$), were negatively correlated to the total frequencies of $CD4^+$-IL-2^+ ($R = -0.3063$; $p = 0.0243$), $CD4^+$-TNF-α^+ ($R = -0.2847$; $p = 0.0369$), $CD4^+$-IL-2^+-TNF-α^+ ($R = -0.2964$; $p = 0.0295$) and of the $CD4^+$-IL-2^+ ($R = -0.3611$; $p = 0.0068$) single cytokine producer T cell populations (**Figures 5A,B**).

Differences in the quality of the response between vaccine groups are represented pictorially by pie charts (**Figure 5C**). Quantifying the fraction of the total cytokine response comprising three (3+), any two (2+), or any one (1+) cytokine, we found that over a half of the $CD4^+$-responses in untreated controls, NH36sap and F3sap vaccines were 1+ cells, while 65% of the response in F1sap and F2sap vaccines were 2+ cells.

Remarkably, and despite the low global frequency of triple-cytokine and of IFN-γ producing $CD4^+$ T cells (**Figures 5A,B**), we noted a progressive 3,165 and 3,473-fold increase in the median fluorescence intensity (MFI) for IFN-γ from $CD4^+$ T cells that secrete all the three cytokines compared with single cytokine-producing $CD4^+$ T cells (**Figure 5D**) only in the animals treated with the F1sap and the F3 vaccines.

On the other hand, the multiparameter analysis of the NH36-specific $CD8^+$ T cell population, disclosed that the total frequencies of IL-2-producing cells were enhanced to 19, 15, and 20%, respectively, by the NH36, the F1sap, and the F3sap vaccines (**Figure 6A**). The frequency of IL-2^+ single cytokine producer $CD8^+$ T cells was increased (**Figure 6B**) above controls and to 10%, by the NH36 vaccine. In correlation with that, the multifunctional analysis revealed that only the increase

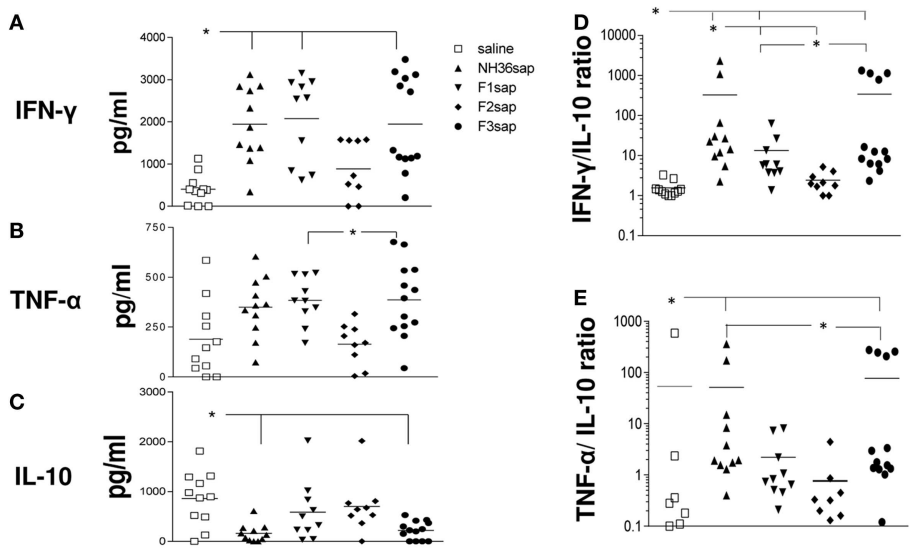


FIGURE 4 | Cytokine expression. After euthanasia, the secretion of IFN-γ, TNF-α, and IL-10 were evaluated by an ELISA assay, in the supernatants of splenocytes, which had been incubated with NH36 for 72 h. Results in **(A–C)** are presented as means and individual levels of secreted cytokines, expressed as picogram per milliliter, of two independent experiments (six to seven mice per treatment in each experiment) and as the IFN-γ/IL-10 **(D)** and TNF-α/IL-10 **(E)** ratios. *Significant differences between treatments.

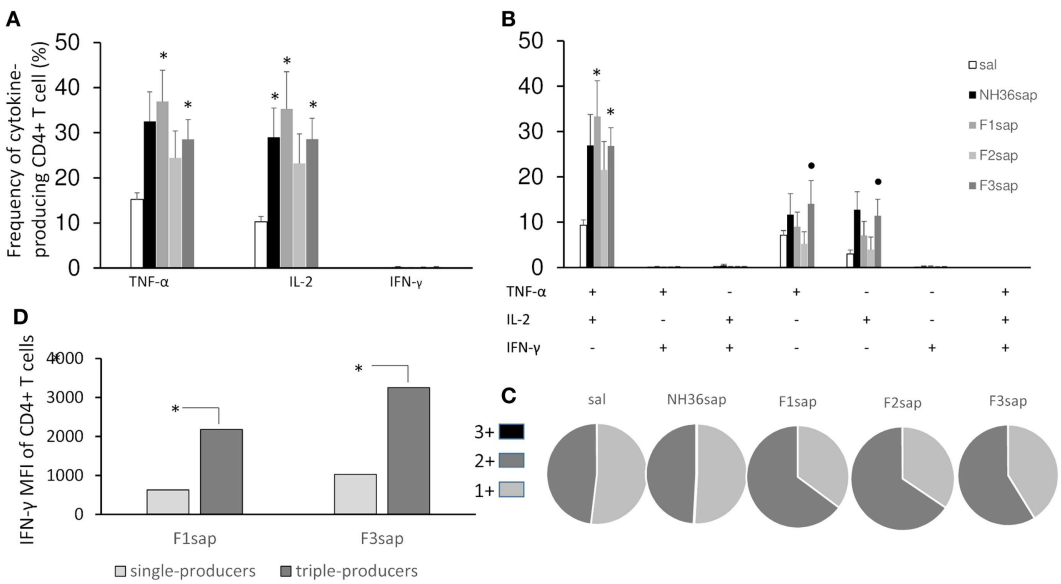


FIGURE 5 | Multifunctional analysis discloses the magnitude and quality of the CD4$^+$ T cell response. NH36-specific cytokine production from CD4$^+$ T cells of spleens of immunotherapy treated and control mice 9 weeks after infection **(A–C)**. Multiparameter flow cytometry was used to determine **(A)** the total frequency of IFN-γ-, IL-2-, or TNF-α-producing CD4$^+$ T cells, **(B)** the frequency of cells expressing each of the seven possible combinations of IFN-γ, IL-2, and TNF-α, **(C)** the magnitude of the IFN-γ secretion expressed by its median intensity fluorescence (MFI) in single- and triple-cytokine CD4$^+$ T cell producers and **(D)** the fraction of the total response comprising cells expressing all three cytokines (3+), any two cytokines (2+), or any one cytokine (1+). Results shown as the mean ± SE of two independent experiments with $n = 6$–7 in each experiment. *Significant differences from saline treated controls, •significant differences from the F2sap vaccine.

of the CD8$^+$ T cells producing IL-2 or TNF-α or both were predictive of the therapeutic effect of vaccination. Indeed, the total frequencies of CD8$^+$-IL-2$^+$ ($R = -0.4575$; $p = 0.004$ and $R = -0.4363$; $p = 0.0292$), CD8$^+$-IL-2$^+$-TNF-α$^+$ ($R = -0.2795$; $p = 0.0407$ and $R = -0.3820$; $p = 0.0500$) and the CD8$^+$-IL-2$^+$ single cytokine populations ($R = -0.3716$; $p = 0.0057$ and

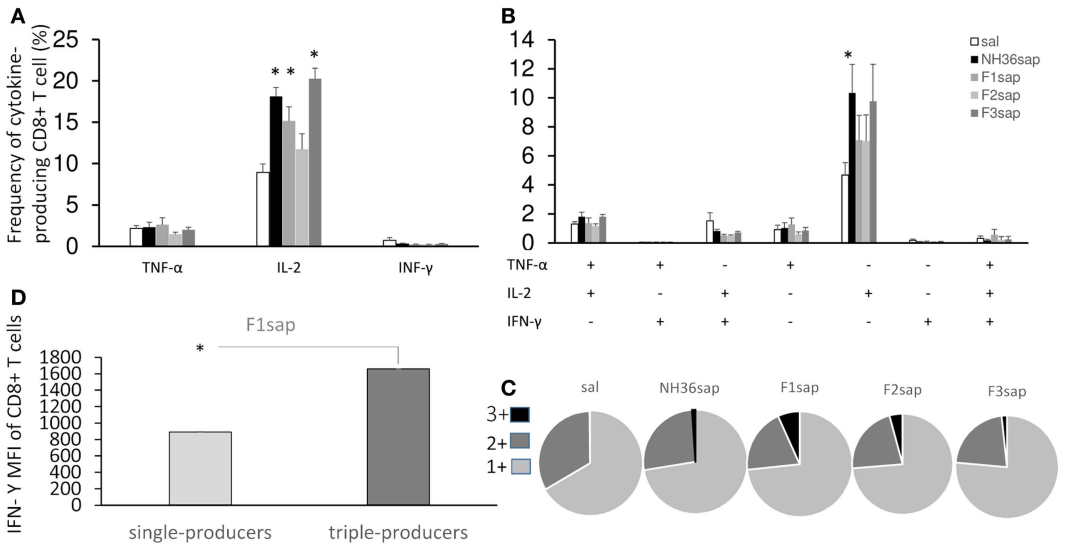


FIGURE 6 | Multifunctional analysis discloses the magnitude and quality of the CD8$^+$ T cell response. NH36-specific cytokine production from CD8$^+$ T cells of spleens of immunotherapy treated and control mice 9 weeks after infection **(A–C)**. Multiparameter flow cytometry was used to determine **(A)** the total frequency of IFN-γ-, IL-2-, or TNF-α-producing CD8$^+$ T cells, **(B)** the frequency of cells expressing each of the seven possible combinations of IFN-γ, IL-2, and TNF-α, **(C)** the magnitude of the IFN-γ secretion expressed by its median intensity fluorescence (MFI) in single cytokine and triple-cytokine CD8$^+$ T cell producers and **(D)** the fraction of the total response comprising cells expressing all three cytokines (3+), any two cytokines (2+), or any one cytokine (1+). Results shown as the mean ± SE of two independent experiments with $n = 6$–7 in each experiment. *Significant differences from saline treated controls.

$R = -0.5367$; $p = 0.0057$) were negatively correlated to the sizes of footpad lesions and the number of parasites in lesions, respectively.

Furthermore, quantifying the fraction of the total cytokine response of CD8 T cells comprising three (3+), any two (2+), or any one (1+) cytokine (**Figure 6C**), we found that while almost no triple labeled cells were detected in the saline controls (mean = 0.31%), this proportion increased in all vaccinated groups and exhibited the highest values in the F1sap vaccinated mice (6.75%). We also found that 72–76% of the response to all treatments was 1+ cell and from 20 to 32% was 2+ cell. The proportion of the 3+ labeled CD8+ T cells increased therefore at the expense of the 2+ cell population (**Figure 6C**).

Regarding the magnitude of the immune response and in agreement with the highest frequency of triple-producers cells in F1sap vaccinated mice (**Figure 6C**), we noted a 1,859-fold increase in MFI for IFN-γ in CD8$^+$ T cells that secrete all the three cytokines when compared to the single cytokine-producing CD8$^+$ T cells (**Figure 6D**), only in mice treated with the F1sap vaccine (**Figure 6D**).

As an alternative method to calculate the magnitude of the response, the iMFI values were additionally obtained by multiplying the frequency of the single cytokine producer CD4$^+$ T cells and their MFI of single cytokine producers (**Figure 7A**). The F3 vaccine enhanced the iMFI-IL-2 and, together with the NH36 vaccine, also the iMFI-TNF-α values over the respective saline controls. There was not any significant variation in the magnitude of the response (iMFI) of CD8$^+$ T cells for any cytokine by any of the vaccines (**Figure 7B**).

Collectively, our multifunctional analysis revealed that the immunotherapy treatment with NH36 peptide vaccines determined that the IL-2, TNF-α, and TNF-α-IL-2-CD4$^+$ and -CD8$^+$ T cells were predictive of protection and immunotherapeutic potential of the vaccines and that protection improved as the CD4 responses shifted from 1+ to 2+ and the CD8 responses shifted qualitatively from 1+ to 3+.

DISCUSSION

Several epitopes for T cell lymphocytes and antibodies where predicted along the whole sequence of NH36 but they have different levels of immunogenicity in prophylaxis against *L. chagasi* infection (48). The calculation of the surface area of the NH36 model revealed that the sequences of the F3 and F1 peptides are the most exposed and this suggests they have a greater availability for lysosome or proteaimmunosome enzymes, and hence, the enhanced probability of being presented by the MHC receptors. The F3 peptide, which has the highest number of predicted epitopes for CD4$^+$ T cells and antibodies (48), has the largest surface area and, is the target of the strongest cellular and humoral immune response against *L. amazonensis* (in this investigation) and *L. chagasi* (48). On the other hand, the lower access of the F2 domain to the surface area explains its lower immunogenicity in the *L. chagasi* (48) and *L. amazonensis* infection models, despite the prediction of one epitope for CD4$^+$, two for CD8$^+$ T cells, and two for antibodies in its sequence (48).

After immunotherapy of *L. amazonensis* infection, only the F3 vaccine stands out as the most potent inducer of IgG and IgG2a anti-NH36 antibodies, while the IgG2b and IgG1 antibodies were equally enhanced by the F1, F2, and F3 vaccines. Interestingly, the F1 vaccine was less capable than the F3 vaccine in sustaining the IgG2a response. Additionally, the F2 vaccine induced an increase

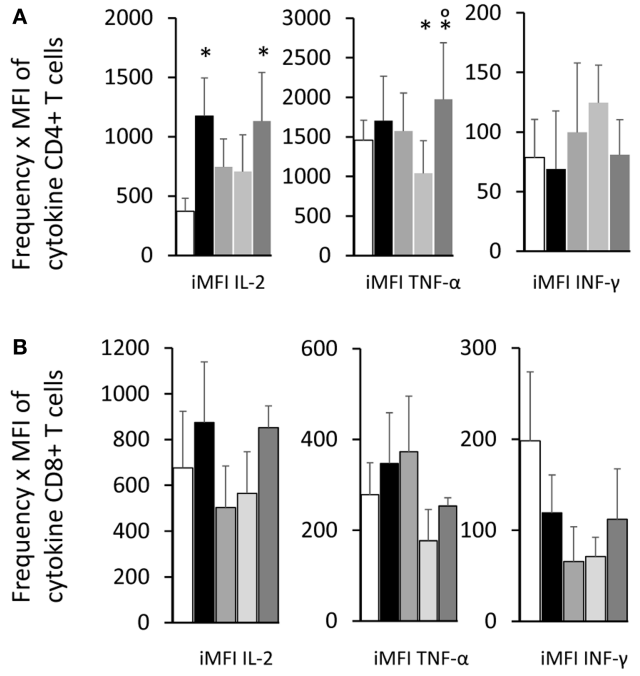


FIGURE 7 | Total functional response of the single cytokine producer T cells. By multiplying the frequency by the MFI of the single cytokine producer T cells, we calculated the iMFI that reflects the total functional response of the population. Single cytokine producer $CD4^+$ **(A)** and $CD8^+$ T cells **(B)**. Results shown as the mean ± SE of two independent experiments with $n = 6$–7 in each experiment. *Significant differences from saline treated controls.

in IgG1 and IgG2b antibodies, which indicates the advancement of infection and is not correlated to therapeutic protection. We conclude that the most important epitopes for anti-NH36 antibodies generated after immunotherapy of *L. amazonensis* or prophylaxis against *L. chagasi* infection (48) are located in F3. F3 is then the target of the anti-*Leishmania* cross-specific humoral response of mice (48), and the antibody target of humans and dogs with VL (49) and of dogs vaccinated with Leishmune® (17). Since the antibodies generated by the Leishmune® vaccine in dogs reacted mostly with the F3 epitopes (48) and block the transmission of VL in the insect vector (18, 19), the identification of these cross-reactive immunogenic sequences in F3 might also help in blocking the transmission of CL.

The IDR to the lysate of *L. amazonensis* after immunotherapy was enhanced by the F3 and NH36 vaccines, similarly to what was detected before and after infection by *L. chagasi* (48). IDR is a well known correlate of protection that is expected to be absent in patients with VL (6) and DCL (7), who show immunosuppression, but present in cured individuals (6, 7), or after generation of vaccine protection (16, 17, 48, 54, 55) or in patients with CL caused by *L. braziliensis*, which, on the contrary, show a strong TH1 response (8). In the selection of candidates for clinical trials of vaccines against CL, IDR is the main criteria for exclusion, as it indicates sensitization due to previous contact with the parasite (56). The description of the F3 vaccine and the NH36 vaccine as good enhancers of the IDR response of mice infected with *L. amazonensis* infection is important for the future development of defined cross-protective vaccines since: (1) *L. amazonensis* causes both CL and DCL (2, 7) individuals with DCL are commonly anergic, showing diminished or absent immune responses to *Leishmania* antigens (3, 7) and (3) the single human vaccine licensed for immunotherapy of CL leishmaniasis is based on a *L. amazonensis* crude vaccine (15).

Additionally, as described for mice prophylaxis against VL and CL (48) the F3 vaccine was the most therapeutic against *L. amazonensis*, reducing the size of footpad lesions and the parasite load. A significant, although different, therapeutic effect was induced by the F1 vaccine. While both vaccines induced high secretion of the pro-inflammatory cytokines IFN-γ and TNF-α by splenocytes, only the F3 vaccine exhibited the typical TH1 response with reduced levels of IL-10. The epitope prediction programs disclosed the existence of three epitopes for $CD4^+$ in the F3 and two in the F1 sequences, respectively. The $CD8^+$ T cell epitope prediction program disclosed the highest affinity for the YPPEFKTKL epitope in F1 and no epitope in F3 (48). Accordingly and as described for VL (48), an *in vivo* depletion assay recently demonstrated that, protection against *L. amazonensis* infection is mediated by a TH1 $CD4^+$ T cell driven response to F3 and a $CD8^+$ T cell mediated response to the F1 domain (57).

In agreement with the above mentioned responses, Seder et al. (51), when describing immune correlates for vaccine-elicited protection against CL, stated that a $CD4^+$ T-Helper 1-type response is considered necessary and even sufficient for infection by *L. major*, while $CD8^+$ T cells are considered to have an important role in protection following natural infection and may be important for optimizing vaccine efficacy. Their model involves the earliest single secretion of TNF-α and of IL-2, followed by the development of double producers (TNF-α and IL-2) and by the later triple-producers of IFN-γ-TNF-α-IL-2 that can persist as memory or effector $CD4^+$ T cells. In agreement with that our work revealed as a correlate of protection, the increase of total frequencies of TNF-α and IL-2, single and double producers of IL-2-TNF $CD4^+$ T cells while the work of Darrah et al. (52) indicated the triple-positive $CD4^+$ T cells. This fact could suggest that the MML of *L. major* live vaccines promotes a more mature condition of immune protection. However, while Darrah et al. (52) described the correlation between the triple-cytokine producers and protection only post-vaccination, our analysis disclosed the correlates after challenge with *L. amazonensis* and immunotherapy. Indeed, there is no description of immune correlates for the protection by the MML vaccine after challenge with *L. major* (52). It is worth noting that it is more difficult to generate protection and disclose the immune correlates after the establishment of infection than before. Furthermore, while Darrah et al. (52), used cells of draining lymph nodes of C57BL/6 mice, inoculated with *L. major* intradermally in the ear, we used splenocytes of Balb/c mice inoculated with *L. amazonensis* in the footpads. Our approach reveals the state of the systemic cellular immunity. The different antigen and adjuvant composition of the vaccines could also account for the differences.

In contrast to Darrah et al. (52), we did not find a correlation between the frequencies of IFN-γ^+-producer cells and protection, and their frequencies were very low. In agreement with Darrah

et al. (52), however, after immunotherapy with the F3 and F1 vaccines, we observed a progressive increase in the MFI values of IFN-γ as the degree of functionality increased from single- to triple-cytokine producers, indicating that IFN-γ might also represent a contribution to the cellular immune response against *L. amazonensis*. Another reason for the detection of low frequencies of IFN-γ-$CD4^+$ producers by ICS might be the time of *in vitro* incubation. After 72 h, the IFN-γ might have already been secreted and therefore would no longer be inside the cells. The detection of increased amount of IFN-γ in the supernatants of the same cells, of mice treated with the NH36, F1, or F3 vaccines confirms that hypothesis. Furthermore, the time of *in vitro* incubation might also be the reason for the higher frequencies of $CD4^+$ (30–40%) and $CD8^+$ cytokine producers cells (20%). In the work of Darrah et al. (52), *in vitro* incubation lasted for 2 h only and the frequencies of T cells ranged from 0 to 1%.

An important role of the $CD8^+$ T cells in protection against CL (51, 58) and VL (59) has been reported. Although the NH36 vaccine induced a $CD4^+$ T cell mediated protection or therapy against VL in mice (43, 47) and dogs (46, 60), the recombinant NH36-saponin vaccine showed equal contributions of $CD4^+$ and $CD8^+$ T cells in protection for mice against VL (48) with the F3 being responsible for the $CD4^+$ response to VL (48) and CL and the F1 being responsible for the main $CD8^+$ T cell driven protection against *L. amazonensis* (57).

According to Seder et al. (51), following activation, the naïve $CD8^+$ T cells fully differentiate into activated effector $CD8^+$ T cells that secrete IFN-γ, most with cytolytic activity, which can further differentiate into $CD8^+$ T effect memory cells (T_{EM}) secreting IFN-γ–TNF-α, either directly, or after a step of conversion, to $CD8^+$ central memory cells (T_{CM}) which are triple–cytokine producers (IFN-γ^+-TNF-α^+-IL-2^+). Therefore, the induction of IL-2 in $CD8^+$ T cells is detected at a later time and is lost in chronic infections (51). In contrast to $CD4^+$ T cells, it is considered very rare to find $CD8^+$ T_{EM} cells that produce IL-2. The enhanced ability of $CD8^+$ T_{CM} cells to produce IL-2 has been shown to confer improved protection compared with $CD8^+$ T_{EM} cells against a systemic viral challenge (61). Our results of immunotherapy of *L. amazonensis* infection with the F1-saponin vaccine gain relevance since frequencies of total and single IL-2^+ $CD8^+$ T cell producers were significantly increased, were predictive of the therapeutic effect and the percentages of triple-cytokine producers were also increased. We recently demonstrated that protection against *L. amazonensis* infection is mediated by the $CD8^+$ T cell response induced by the F1 vaccine (57). Williams et al. (62) showed the IL-2 signaling to pathogen-specific $CD8^+$ T cells is required for the generation of robust secondary responses, programing the development of $CD8^+$ memory T cells capable of full secondary expansion. Our results suggest that the F1 domain, which contains the highest affinity epitope of the NH36 for $CD8^+$ T cells (48), might be important for the development of $CD8^+$ T_{CM} cells (51), which through the high secretion of IL-2, or TNF or IL-2-TNF actively contribute to the cure of the established infection. The intensity of IFN-γ secretion by triple-producers, in our investigation, also proved to be above the levels of single producers, indicating the progressive increase in the MFI values of IFN-γ in F1sap treated mice as the degree of functionality increased.

The F1sap vaccine was also a determinant in the increased secretion of IFN-γ by $CD4^+$ triple-cytokine producers, in the increased frequencies of total and double TNF-α and IL-2 producers and in the secretion of IFN-γ and TNF-α into the splenocyte supernatants indicating the induction of a TH1 response. However, mice treated with the F1 vaccine also showed a high secretion of IL-10 by splenocytes. While in VL IL-10 is considered to be the marker of the severe immunosuppressive disease (5, 63), IL-10 in human CL has been shown to be related to the pathology of the disease as well as the control of the parasite (64). Recently, the frequency and functional capacity of Tregs were evaluated in chronical patients with CL and in asymptomatic subjects (65). Although, the chronical patients presented higher frequencies of Tregs in peripheral blood and higher expression of *FOXP3* at leishmanin skin test sites, their $CD4^+CD25^+$ cells were less capable of suppressing antigen specific IFN-γ secretion by effector cells compared with asymptomatic infected individuals. At the end of the treatment, both the frequency of $CD4^+CD25^{hi}CD127^-$ cells and their capacity to inhibit proliferation and IFN-γ secretion increased and coincided with healing of CL lesions suggesting that the restored IL-10 secretion by Tregs was involved in the cure of the disease (65). The authors suggested that the Tregs impaired function was evidence of pathogenesis of CL and Treg subsets would be relevant in designing immunotherapeutic strategies for recalcitrant dermal leishmaniasis (65). $CD4^+CD25^+$ regulatory T cells have also been shown to restrain pathogenic responses during *L. amazonensis* infection (66). The simultaneous induction of an immunotherapeutic effect and the increase in the secretion of IL-10 determined by the F1 peptide might also be related to the stimulation of Treg subsets and to the presence of epitopes for Tregs along its sequence.

In our investigation, a significant decrease of IL-10 levels was found in the supernatants of whole splenocytes of F3 vaccinated mice. A population of $IFNy^+$-producing $CD4^+$ T cells that also produce IL-10 has been identified in VL as a feature of T cell differentiation (67). Expanded numbers of these cells were associated with disease progression (67). Conventional $CD11c^{hi}$ DCs that produce both IL-10 and IL-27 It have also been shown to promote the production of IL-10 by these effector $CD4^+$ T cells (67). In our investigation, besides $CD4^+$ T cells, DCs could also be the source of the IL-10 secretion detected in splenocyte supernatants. These types of DCs were also present in our mice model of CL infection. If that is the case, we could assume that immunotherapy with the F3 peptide formulated with saponin, could promote the direct shifting of DCs away from an IL-10 producing phenotype, which is more frequent in the untreated controls, to a pro-Th1 IL-12 producing phenotype, with reduced IL-10 secretion (67).

An alternative source of IL-10, in *L. amazonensis* infected mice, could be natural killer (NK) cells. In mice infected with *L. donovani*, NK cells are found in the spleen and liver hepatic granulomas (68). They are responsible for suppressing the host resistance to the parasite, through the secretion of IL-10, which is present in early infection. In mice with an established infection, the IL-10 mRNA acquires more stability and IL-10 secretion by NK is enhanced (68). In the context of CL leishmaniasis, IL-10 has been shown to be essential for *L. major* persistence (69). NK cells were also more frequent in relapsed than in cured cases of mucosal leishmaniasis

and a decrease in NK cells and in IL-10 levels was observed after therapy (70).

A few other antigens have been proposed as potential synthetic vaccines against leishmaniasis (59, 71–73). The kmp-11 protein of *L. donovani* has epitopes recognized by human $CD8^+$ lymphocytes and by many different HLA receptors (59). The Leish110f fusion protein of *L. major*, on the other hand, induced mice protection mediated by $CD4^+$ lymphocytes (72). Recently, an adenovirus based vaccine comprising a synthetic HASPB gene composed of 10 repeats, linked to the KMP-11 gene, was obtained and assayed in the therapy of *L. donovani* infected mice therapeutics (73). The synthetic gene was cloned using humanized codons. The immunogenicity increased if the vaccine was administered in the footpads instead of subcutaneously. A detailed study of the contribution of the epitopes of HASPB protein was performed. After therapeutic vaccination, the IgG1 and IgG2a antibody responses were enhanced and IFN-γ-$CD8^+$ T cell response, mainly to HASPB, became apparent. Interestingly, a single dose of the vaccine reduced the parasite growth in spleens by 66% (73).

Immunotherapy for the treatment of human VL leishmaniasis has recently been recommended (74). The C-terminal and N-terminal domains of NH36 of *L. donovani* are the basis of the strong immunotherapeutic effect against *L. amazonenis* infection. Our findings contribute to the design of defined vaccines for cross-protection against CL leishmaniasis.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Clarisa Beatriz Palatnik-de-Sousa, Dirlei Nico. Acquisition, analysis, and interpretation of data: Dirlei Nico, Daniele Crespo Gomes, Iam Palatnik-de-Sousa, Alexandre Morrot, Marcos Palatnik, Clarisa Beatriz Palatnik-de-Sousa. Wrote the paper: Clarisa Beatriz Palatnik-de-Sousa. Final approval of the last version of the manuscript to be published: Clarisa Beatriz Palatnik-de-Sousa, Dirlei Nico, Daniele Crespo Gomes, Iam Palatnik-de-Sousa, Alexandre Morrot, Marcos Palatnik.

ACKNOWLEDGMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ, Fellowships 301215-2007-3, 302039/2010-4, 559756/2010-0 and grant 404400/2012-4) and by Fundação de Amparo à Pesquisa do Estado de Rio de Janeiro (FAPERJ, grants 102733/2008 and 102957/2011, and Fellowships E-26/102415/2010 and E-26/110535/2010). David Straker is gratefully acknowledged for the English language revision of this manuscript and Professors Rafael Linden and Pedro Geraldo Pascutti, of the Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, for their important contribution on the design of the molecular model.

REFERENCES

1. Kedzierski L, Zhu Y, Handman E. *Leishmania* vaccines: progress and problems. *Parasitology* (2006) **133**(Suppl S):87–112. doi:10.1017/S0031182006001831
2. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) **7**(5):e35671. doi:10.1371/journal.pone.0035671
3. Da-Cruz AM, Bittar R, Mattos M, Oliveira-Neto MP, Nogueira R, Pinho-Ribeiro V, et al. T-cell-mediated immune responses in patients with cutaneous or mucosal leishmaniasis: long-term evaluation after therapy. *Clin Diagn Lab Immunol* (2002) **9**(2):251–6.
4. Das A, Ali N. Vaccine development against *Leishmania donovani*. *Front Immunol* (2012) **3**:99. doi:10.3389/fimmu.2012.00099
5. Ganguly S, Das NK, Panja M, Pal S, Modak D, Rahaman M, et al. Increased levels of interleukin-10 and IgG3 are hallmarks of Indian post-kala-azar dermal leishmaniasis. *J Infect Dis* (2008) **197**(12):1762–71. doi:10.1086/588387
6. Stober CB, Jeronimo SM, Pontes NN, Miller EN, Blackwell JM. Cytokine responses to novel antigens in a peri-urban population in Brazil exposed to *Leishmania infantum chagasi*. *Am J Trop Med Hyg* (2012) **87**(4):663–70. doi:10.4269/ajtmh.2012.12-0180
7. Silveira FT, Lainson R, Corbett CE. Further observations on clinical, histopathological, and immunological features of borderline disseminated cutaneous leishmaniasis caused by *Leishmania (Leishmania) amazonensis*. *Mem Inst Oswaldo Cruz* (2005) **100**(5):525–34. doi:10.1590/S0074-02762005000500013
8. Schnorr D, Muniz AC, Passos S, Guimaraes LH, Lago EL, Bacellar O, et al. IFN-γ production to *Leishmania* antigen supplements the *Leishmania* skin test in identifying exposure to *L. braziliensis* infection. *PLoS Negl Trop Dis* (2012) **6**(12):e1947. doi:10.1371/journal.pntd.0001947
9. Carvalho AM, Magalhães A, Carvalho LP, Bacellar O, Scott P, Carvalho EM. Immunologic response and memory T cells in subjects cured of tegumentary leishmaniasis. *BMC Infect Dis* (2013) **13**:529. doi:10.1186/1471-2334-13-529
10. Sundar S. Drug resistance in Indian visceral leishmaniasis. *Trop Med Int Health* (2001) **6**(11):849–54. doi:10.1046/j.1365-3156.2001.00778.x
11. Maltezou HC. Drug resistance in visceral leishmaniasis. *J Biomed Biotechnol* (2010) **2010**:617521. doi:10.1155/2010/617521
12. Convit J, Castellanos PL, Ulrich M, Castés M, Rondón A, Pinardi ME, et al. Immunotherapy of localized, intermediate, and diffuse forms of American cutaneous leishmaniasis. *J Infect Dis* (1989) **160**(1):104–15. doi:10.1093/infdis/160.1.104
13. Castes M, Moros Z, Martinez A, Trujillo D, Castellanos PL, Rondon AJ, et al. Cell-mediated immunity in localized cutaneous leishmaniasis patients before and after treatment with immunotherapy or chemotherapy. *Parasite Immunol* (1989) **11**(3):211–22. doi:10.1111/j.1365-3024.1989.tb00660.x
14. Cabrera M, Blackwell JM, Castes M, Trujillo D, Convit J, Shaw MA. Immunotherapy with live BCG plus heat killed *Leishmania* induces a T helper 1-like response in American cutaneous leishmaniasis patients. *Parasite Immunol* (2000) **22**(2):73–9. doi:10.1046/j.1365-3024.2000.00278.x
15. Mayrink W, Botelho AC, Magalhães PA, Batista SM, Lima Ade O, Genaro O, et al. Immunotherapy, immunochemotherapy and chemotherapy for American cutaneous leishmaniasis treatment. *Rev Soc Bras Med Trop* (2006) **39**(1):14–21. doi:10.1590/S0037-86822006000100003
16. Silva VO, Borja-Cabrera GP, Correia Pontes NN, Paraguai de Souza E, Luz KG, Palatnik M, et al. A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (São Gonçalo do Amarante, RN). *Vaccine* (2000) **19**(9–10):1082–92.
17. Borja-Cabrera GP, Santos FN, Bauer FS, Parra LE, Menz I, Morgado AA, et al. Immunogenicity assay of the Leishmune® vaccine against canine visceral leishmaniasis in Brazil. *Vaccine* (2008) **26**(39):4991–7. doi:10.1016/j.vaccine.2008.07.029
18. Palatnik-de-Sousa CB, Barbosa AF, Oliveira SM, Nico D, Bernardo RR, Santos WR, et al. The FML-vaccine against canine visceral leishmaniasis: from the second generation to the synthetic vaccine. *Expert Rev Vaccines* (2008) **7**(6):833–51. doi:10.1586/14760584.7.6.833
19. Saraiva EM, de Figueiredo Barbosa A, Sanots FN, Borja-Cabrera GP, Nico D, Souza LP, et al. The FML-vaccine (Leishmune®) against canine visceral leishmaniasis: a transmission blocking vaccine. *Vaccine* (2006) **24**(13):2423–31. doi:10.1016/j.vaccine.2005.11.061
20. Palatnik-de-Sousa CB, Silva-Antunes I, Morgado Ade A, Menz I, Palatnik M, Lavor C. Decrease of the incidence of human and canine visceral leishmaniasis after dog vaccination with Leishmune® in Brazilian endemic areas. *Vaccine* (2009) **27**(27):3505–12. doi:10.1016/j.vaccine.2009.03.045
21. Araújo MS, de Andrade RA, Vianna LR, Mayrink W, Reis AB, Sathler-Avelar R, et al. Despite Leishvaccine and Leishmune trigger distinct immune profiles, their ability to activate phagocytes and CD8+ T-cells support their high-quality immunogenic potential against canine visceral leishmaniasis. *Vaccine* (2008) **26**(18):2211–4. doi:10.1016/j.vaccine.2008.02.044
22. Araújo MS, de Andrade RA, Sathler-Avelar R, Teixeira-Carvalho A, Andrade MC, Vianna LR, et al. T-cell-derived cytokines, nitric oxide production by

peripheral blood monocytes and seric anti-*Leishmania (Leishmania) chagasi* IgG subclass patterns following immunization against canine visceral leishmaniasis using Leishvaccine and Leishmune. *Vaccine* (2009) **27**(7):1008–17. doi:10.1016/j.vaccine.2008.11.104

23. Araújo MS, de Andrade RA, Sathler-Avelar R, Magalhães CP, Carvalho AT, Andrade MC, et al. Immunological changes in canine peripheral blood leukocytes triggered by immunization with first or second generation vaccines against canine visceral leishmaniasis. *Vet Immunol Immunopathol* (2011) **141**(1–2):64–75. doi:10.1016/j.vetimm.2011.02.007
24. Marciani DJ. Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. *Drug Discov Today* (2003) **8**(20):934–43. doi:10.1016/S1359-6446(03)02864-2
25. Borja-Cabrera GP, Cruz Mendes A, Paraguai de Souza E, Hashimoto Okada LY, de A Trivellato FA, Kawasaki JK, et al. Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. *Vaccine* (2004) **22**(17–18):2234–43. doi:10.1016/j.vaccine.2003.11.039
26. Santos FN, Borja-Cabrera GP, Miyashiro LM, Grechi J, Reis AB, Moreira MA, et al. Immunotherapy against experimental canine visceral leishmaniasis with the saponin enriched-Leishmune vaccine. *Vaccine* (2007) **25**(33):6176–90. doi:10.1016/j.vaccine.2007.06.005
27. de Lima VM, Ikeda FA, Rossi CN, Feitosa MM, Vasconcelos RO, Nunes CM, et al. Diminished CD4+/CD25+ T cell and increased IFN-gamma levels occur in dogs vaccinated with Leishmune in an endemic area for visceral leishmaniasis. *Vet Immunol Immunopathol* (2010) **135**(3–4):296–302. doi:10.1016/j.vetimm.2009.12.008
28. Borja-Cabrera GP, Santos FN, Santos FB, Trivellato FA, Kawasaki JK, Costa AC, et al. Immunotherapy with the saponin enriched-Leishmune vaccine versus immunochemotherapy in dogs with natural canine visceral leishmaniasis. *Vaccine* (2010) **28**(3):597–603. doi:10.1016/j.vaccine.2009.09.071
29. Oliveira-Freitas E, Casas CP, Borja-Cabrera GP, Santos FN, Nico D, Souza LO, et al. Acylated and deacylated saponins of *Quillaja saponaria* mixture as adjuvants for the FML-vaccine against visceral leishmaniasis. *Vaccine* (2006) **24**(18):3909–20. doi:10.1016/j.vaccine.2006.02.034
30. Leach A, Vekemans J, Lievens M, Ofori-Anyinam O, Cahill C, Owusu-Agyei S, et al. Design of a phase III multicenter trial to evaluate the efficacy of the RTS,S/AS01 malaria vaccine in children across diverse transmission settings in Africa. *Malar J* (2011) **10**:224. doi:10.1186/1475-2875-10-224
31. Asante KP, Abdulla S, Agnandji S, Lyimo J, Vekemans J, Soulanoudjingar S, et al. Safety and efficacy of the RTS,S/AS01E candidate malaria vaccine given with expanded-programme-on-immunisation vaccines: 19 month follow-up of a randomised, open-label, phase 2 trial. *Lancet Infect Dis* (2011) **11**(10):741–9. doi:10.1016/S1473-3099(11)70100-1
32. RTS,S Clinical Trials Partnership; Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BG, et al. A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. *N Engl J Med* (2012) **367**:2284–95. doi:10.1056/NEJMoa1208394
33. Palatnik de Sousa CB, Gomes EM, de Souza EP, dos Santos WR, de Macedo SR, de Medeiros LV, et al. The FML (fucose mannose ligand) of *Leishmania donovani*: a new tool in diagnosis, prognosis, transfusional control and vaccination against human kala-azar. *Rev Soc Bras Med Trop* (1996) **29**(2):153–63.
34. Iovane E, Giabbai B, Muzzolini L, Matafora V, Fornili A, Minici C, et al. Structural basis for substrate specificity in group I nucleoside hydrolases. *Biochemistry* (2008) **47**(15):4418–26. doi:10.1021/bi702448s
35. Versées W, Goeminne A, Berg M, Vandemeulebroucke A, Haemers A, Augustyn K, et al. Crystal structures of *T. vivax* nucleoside hydrolase in complex with new potent and specific inhibitors. *Biochim Biophys Acta* (2009) **1794**(6):953–60. doi:10.1016/j.bbapap.2009.02.011
36. Santana DM, Borja-Cabrera GP, Paraguai de Souza E, Sturm NR, Palatnik de Sousa CB, Campbell DA. Nucleoside hydrolase from *Leishmania (L.) donovani* is an antigen diagnostic for visceral leishmaniasis. *Mol Biochem Parasitol* (2002) **120**(2):315–9. doi:10.1016/S0166-6851(02)00010-5
37. Lukes J, Mauricio IL, Schönian G, Dujardin JC, Soteriadou K, Dedet JP, et al. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *Proc Nat Acad Sci U S A* (2007) **104**(22):9375–80. doi:10.1073/pnas.0703678104
38. Mauricio IL, Yeo M, Baghaei M, Doto D, Pratlong F, Zemanova E, et al. Towards multilocus sequence typing of the *Leishmania donovani* complex: resolving genotypes and haplotypes for five polymorphic metabolic enzymes (ASAT, GPI, NH1, NH2, PGD). *Int J Parasitol* (2006) **36**(7):757–69. doi:10.1016/j.ijpara.2006.03.006
39. Cui L, Rajasekariah GR, Martin SK. A nonspecific nucleoside hydrolase from *Leishmania donovani*: implications for purine salvage by the parasite. *Gene* (2001) **280**(1–2):153–62. doi:10.1016/S0378-1119(01)00768-5
40. Blast-Basic Local Alignment Search Tool. *National Institute of Heath (NIH)* (2014). Available from: http://blast.ncbi.nlm.nih.gov/Blast.cgi
41. Paraguai de Souza E, Bernardo RR, Palatnik M, Palatnik de Sousa CB. Vaccination of Balb/c mice against experimental visceral leishmaniasis with the GP36 glycoprotein antigen of *Leishmania donovani*. *Vaccine* (2001) **19**(23–24):3104–15. doi:10.1016/S0264-410X(01)00031-7
42. Al-Wabel MA, Tonui WK, Cui L, Martin SK, Titus RG. Protection of susceptible BALB/c mice from challenge with *Leishmania major* by nucleoside hydrolase, a soluble exo-antigen of *Leishmania*. *Am J Trop Med Hyg* (2007) **77**(6):1060–5.
43. Aguilar-Be I, Zardo RS, Paraguai de Souza E, Borja-Cabrera GP, Rosado-Vallado M, Mut-Martin M, et al. Cross-protective efficacy of a prophylactic *Leishmania donovani* DNA vaccine against visceral and cutaneous murine leishmaniasis. *Infect Immun* (2005) **73**(2):812–9. doi:10.1128/IAI.73.2.812-819.2005
44. Chalé-Balboa WG, Mut-Martin M, Ramirez-Sierra MJ, Garcia-Miss MR, Dumonteil E. A combination DNA vaccine encoding nucleoside hydrolase 36 and glycoprotein 63 protects female but not male hamsters against *Leishmania mexicana*. *Parasite* (2009) **16**(3):227–30. doi:10.1051/parasite/2009163227
45. Souza LOP, Palatnik de Sousa CB. The nucleoside hydrolase DNA vaccine VR1012NH36 in prophylactic vaccination against mice tegumentar leishmaniasis. *Proc Vaccinol* (2009) **1**(1):120–3. doi:10.1016/j.provac.2009.07.022
46. Borja-Cabrera GP, Santos FB, Picillo E, Gravino E, Manna L, Palatnik de Sousa CB. Nucleoside hydrolase DNA vaccine against canine visceral leishmaniasis. *Proc Vaccinol* (2009) **1**(1):104–9. doi:10.1016/j.provac.2009.07.019
47. Gamboa-León R, Paraguai de Souza E, Borja Cabrera GP, Santos FN, Miyashiro LM, Pinheiro RO, et al. Immunotherapy against visceral leishmaniasis with the nucleoside hydrolase-DNA vaccine of *L. donovani*. *Vaccine* (2007) **24**(22):4863–73. doi:10.1016/j.vaccine.2006.03.005
48. Nico D, Claser C, Travassos LR, Palatnik M, Soares IS, Rodrigues MM, et al. Adaptive immunity against *Leishmania* nucleoside hydrolase maps its C-terminal domain as the target of the CD4+ T cell-driven protective response. *PLoS Negl Trop Dis* (2010) **4**(11):e866. doi:10.1371/journal.pntd.0000866
49. Costa MM, Penido M, dos Santos MS, Doro D, de Freitas E, Michalik MS, et al. Improved canine and human visceral leishmaniasis immunodiagnosis using combinations of synthetic peptides in enzyme-linked immunosorbent assay. *PLoS Negl Trop Dis* (2012) **6**(5):e1622. doi:10.1371/journal.pntd.0001622
50. Shi W, Schramm VL, Almo SC. Nucleoside hydrolase from *Leishmania major*. Cloning, expression, catalytic properties, transition state inhibitors, and the 2.5-å crystal structure. *J Biol Chem* (1999) **274**(30):21114–20. doi:10.1074/jbc.274.30.21114
51. Seder RA, Darrah PA, Roederer M. T cell quality in memory and protection: implications for vaccine design. *Nat Rev* (2008) **8**(4):247–58. doi:10.1038/nri2274
52. Darrah PA, Patel DT, De Luca PM, Lindsay RWB, Davey DF, Flynn BG, et al. Multifunctional T_H1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat Med* (2007) **13**(7):843–50. doi:10.1038/nm1592
53. Manna L, Reale S, Viola E, Vitale F, Manzillo VF, Pavone LM, et al. *Leishmania* DNA load and cytokine expression levels in asymptomatic naturally infected dogs. *Vet Parasitol* (2006) **142**(3–4):271–80. doi:10.1016/j.vetpar.2006.06.028
54. Kamil AA, Khalil EA, Musa AM, Modabber F, Mukhtar MM, Ibrahim ME, et al. Alum-precipitated autoclaved *Leishmania major* plus bacille Calmette-Guérrin, a candidate vaccine for visceral leishmaniasis: safety, skin-delayed type hypersensitivity response and dose finding in healthy volunteers. *Trans R Soc Trop Med Hyg* (2003) **97**(3):365–8. doi:10.1016/S0035-9203(03)90171-4
55. Khalil EA, El Hassan AM, Zijlstra EE, Mukhtar MM, Ghalib HW, Musa B, et al. Autoclaved *Leishmania major* vaccine for prevention of visceral leishmaniasis: a randomised, double-blind, BCG-controlled trial in Sudan. *Lancet* (2000) **356**(9241):1565–9. doi:10.1016/S0140-6736(00)03128-7
56. Antunes CM, Mayrink W, Magalhaes PA, Costa CA, Melo MN, Dias M, et al. Controlled field trials of a vaccine against New World cutaneous leishmaniasis. *Int J Epidemiol* (1986) **15**(4):572–80. doi:10.1093/ije/15.4.572
57. Nico D, Gomes DC, Alves-Silva MV, Freitas EO, Rodrigues MM, Palatnik M, et al. Cross-protective immunity to *Leishmania amazonensis* is mediated by CD4+ and CD8+-epitopes of *Leishmania donovani* nucleoside hydrolase terminal domains. *Front Immunol* (2014) **5**:189. doi:10.3389/fimmu.2014.00189

58. Rodrigues MM, Boscardin SB, Vasconcelos JR, Hiyane MI, Salay G, Soares IS. Importance of CD8 T cell-mediated immune response during intracellular parasitic infections and its implications for the development of effective vaccines. *An Acad Bras Cienc* (2003) **75**(4):443–68. doi:10.1590/S0001-37652003000400005
59. Basu R, Roy S, Walden P. HLA class I-restricted T cell epitopes of the kinetoplastid membrane protein-11 presented by *Leishmania donovani*-infected human macrophages. *J Infect Dis* (2007) **195**(9):1373–80. doi:10.1086/513439
60. Borja-Cabrera GP, Santos FB, Nico D, Gravino AE, Manna L, Palatnik M, et al. The Leishmune®'s nucleoside hydrolase DNA vaccine as an aid inimmunotherapy of canine visceral leishmaniasis. *Proc Vaccinol* (2012) **6**:64–73. doi:10.1016/j.provac.2012.04.009
61. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* (2003) **77**(8):4911–27. doi:10.1128/JVI.77.8.4911-4927.2003
62. Williams MA, Tyznik AJ, Bevan MJ. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* (2006) **441**(7095):890–3. doi:10.1038/nature04790
63. Gama ME, Gomes CM, Silveira FT, Laurenti MD, Gonçalves Eda G, Silva AR, et al. Severe visceral leishmaniasis in children: the relationship between cytokine patterns and clinical features. *Rev Soc Bras Med Trop* (2013) **46**(6):741–5. doi:10.1590/0037-8682-0203-2013
64. Costa DL, Guimarães LH, Cardoso TM, Queiroz A, Lago E, Roselino AM, et al. Characterization of regulatory T cell (Treg) function in patients infected with *Leishmania braziliensis*. *Hum Immunol* (2013) **74**(12):1491–500. doi:10.1016/j.humimm.2013.08.269
65. Rodriguez-Pinto D, Navas A, Blanco VM, Ramírez L, Garcerant D, Cruz A, et al. Regulatory T cells in the pathogenesis and healing of chronic human dermal leishmaniasis caused by *Leishmania* (Viannia) species. *PLoS Negl Trop Dis* (2012) **6**(4):e1627. doi:10.1371/journal.pntd.0001627
66. Ji J, Masterson J, Sun J, Soong L. CD4 CD25 regulatory T cells restrain pathogenic responses during *Leishmania amazonensis* infection. *J Immunol* (2005) **174**(11):7147–53. doi:10.4049/jimmunol.174.11.7147
67. Owens BM, Beattie L, Moore JW, Brown N, Mann JL, Dalton JE, et al. IL-10-producing Th1 cells and disease progression are regulated by distinct $CD11c^{+}$ cell populations during visceral leishmaniasis. *PLoS Pathog* (2012) **8**(7):e1002827. doi:10.1371/journal.ppat.1002827
68. Maroof A, Beattie L, Zubairi S, Svensson M, Stager S, Kaye PM. Posttranscriptional regulation of II10 gene expression allows natural killer cells to express immunoregulatory function. *Immunity* (2008) **29**(2):295–305. doi:10.1016/j.immuni.2008.06.012
69. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* (2002) **420**(6915):502–7. doi:10.1038/nature01152
70. Tuon FF, Gomes-Silva A, Da-Cruz AM, Duarte MI, Neto VA, Amato VS. Local immunological factors associated with recurrence of mucosal leishmaniasis. *Clin Immunol* (2008) **128**(3):442–6. doi:10.1016/j.clim.2008.05.007
71. Palatnik-de-Sousa CB. Vaccines for leishmaniasis in the fore coming 25 years. *Vaccine* (2008) **26**(4):1709–24. doi:10.1016/j.vaccine.2008.01.023
72. Bertholet S, Goto Y, Carter L, Bhatia A, Howard RF, Carter D, et al. Optimized subunit vaccine protects against experimental leishmaniasis. *Vaccine* (2009) **27**(50):7036–45. doi:10.1016/j.vaccine.2009.09.066
73. Maroof A, Brown N, Smith B, Hodgkinson MR, Maxwell A, Losch FO, et al. Therapeutic vaccination with recombinant adenovirus reduces splenic parasite burden in experimental visceral leishmaniasis. *J Infect Dis* (2012) **205**(5):853–63. doi:10.1093/infdis/jir842
74. Musa AM, Noazin S, Khalil EA, Modabber F. Immunological stimulation for the treatment of leishmaniasis: a modality worthy of serious consideration. *Trans R Soc Trop Med Hyg* (2010) **104**(1):1–2. doi:10.1016/j.trstmh.2009.07.026

Conflict of Interest Statement: The authors have declared that there are no competing interests. This work is related to the pendent Patent PI1015788-3, INPI, Brazil.

Received: 28 February 2014; accepted: 27 May 2014; published online: 11 June 2014.
*Citation: Nico D, Gomes DC, Palatnik-de-Sousa I, Morrot A, Palatnik M and Palatnik-de-Sousa CB (2014) Leishmania donovani nucleoside hydrolase terminal domains in cross-protective immunotherapy against Leishmania amazonensis murine infection. Front. Immunol. **5**:273. doi: 10.3389/fimmu.2014.00273*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 22 August 2014
doi: 10.3389/fimmu.2014.00380

Visceral leishmaniasis: advancements in vaccine development via classical and molecular approaches

***Sumit Joshi*[1], *Keerti Rawat*[1], *Narendra Kumar Yadav*[1], *Vikash Kumar*[2], *Mohammad Imran Siddiqi*[2] *and Anuradha Dube*[1]*

[1] *Division of Parasitology, Central Drug Research Institute, Lucknow, India*
[2] *Division of Molecular and Structural Biology, Central Drug Research Institute, Lucknow, India*

Edited by:
Jesus G. Valenzuela, National Institute of Allergy and Infectious Diseases, USA

Reviewed by:
Regis Bernardo Brandim Gomes, Centro de Pesquisas Gonçalo Moniz-Fiocruz, Brazil
Daniela De Melo Resende, Universidade Federal de Ouro Preto, Brazil

***Correspondence:**
Anuradha Dube, LSN 201, Leishmania Laboratory, Division of Parasitology, CSIR-Central Drug Research Institute, Sector 10, Janakipuram Extension, Sitapur Road, Lucknow 226031, India
e-mail: anuradha_dube@hotmail.com

Visceral leishmaniasis (VL) or kala-azar, a vector-borne protozoan disease, shows endemicity in larger areas of the tropical, subtropical and the Mediterranean countries. WHO report suggested that an annual incidence of VL is nearly 200,000 to 400,000 cases, resulting in 20,000 to 30,000 deaths per year. Treatment with available anti-leishmanial drugs are not cost effective, with varied efficacies and higher relapse rate, which poses a major challenge to current kala-azar control program in Indian subcontinent. Therefore, a vaccine against VL is imperative and knowing the fact that recovered individuals developed lifelong immunity against re-infection, it is feasible. Vaccine development program, though time taking, has recently gained momentum with the emergence of omic era, i.e., from genomics to immunomics. Classical as well as molecular methodologies have been overtaken with alternative strategies wherein proteomics based knowledge combined with computational techniques (immunoinformatics) speed up the identification and detailed characterization of new antigens for potential vaccine candidates. This may eventually help in the designing of polyvalent synthetic and recombinant chimeric vaccines as an effective intervention measures to control the disease in endemic areas. This review focuses on such newer approaches being utilized for vaccine development against VL.

Keywords: visceral leishmaniasis, recombinant vaccines, DNA vaccines, mutant vaccines, synthetic peptide vaccines

VISCERAL LEISHMANIASIS: AN UNSOLVED PROBLEM

Visceral leishmaniasis (VL), synonymously known as kala-azar, is caused by obligate intra-macrophage protozoan parasite and is characterized by both diversity and complexity (1). The disease is prevalent in larger areas of tropical, subtropical, and the Mediterranean countries. As per WHO report, nearly 200,000 to 400,000 new cases of VL (with an average duration of several months to more than one year) occur annually with 20,000 to 30,000 deaths per year (http://www.who.int/mediacentre/factsheets/fs375/en/), which is lesser than by malaria among parasitic diseases, although its exact impact has been underestimated as an exact number of cases were never recorded. Ninety percent of the VL cases occur in Bangladesh, Brazil, India, Nepal, and Sudan. In India, 80% VL cases were only from the state of Bihar (2). A sharp ascent in the prevalence of disease is directly related to environmental changes and migration of non-immune people in endemic areas (3). Occurrence of HIV–*Leishmania* co-infection has placed VL as category-1 disease by WHO (4). The arthropod vector – female phlebotomine sandflies, nocturnal, and telmophagous, are responsible for the transmittance of the disease. Two species – *Leishmania donovani donovani* (in East Africa and the Indian subcontinent) and *L. donovani infantum* (in the Mediterranean region of Europe, North Africa, and Latin America) are the main causative organisms for VL (5). The parasite bears two distinct life forms: promastigote, a flagellar form, found in the gut of the vector, which is inoculated into the dermis where it is internalized by dendritic cells and the macrophages and eventually is transformed into an aflagellated amastigote form, which thrives and multiply within the phagolysosomes through a complex parasite–host interaction (6). Current control strategies for VL rely on anti-leishmanial drugs such as pentavalent antimonials, amphotericin B (AmB), miltefosine, paromomycin, etc., but they are far from satisfactory because of their cost, toxicity as well as unpleasant side effects, longer dose schedule with variable efficacies (7). The situation has further worsened with the emergence of resistance against current anti-leishmanial drugs in various regions of endemicity (8). Hence, in the present situation, there is an urgent need to develop an effective vaccine against VL. Although vaccination against VL has received limited attention as compared to cutaneous leishmaniasis (CL), till date, there is no commercial vaccine against any human parasitic disease including leishmaniasis (9). The fact that healing and recovery from the active infection protects individuals from re-infection specifies the possibility of a vaccine against VL (1). An effective vaccine against the disease must rely on the generation of a strong T-cell immunity (10). Both innate (macrophages and neutrophils) as well as adaptive (B-cells, T-cells, and dendritic cells) immune response plays a significant role against *Leishmania* infection where macrophages play the critical role. It has been a consensus for a long time that a Th1 dominant response instead of Th2 promotes IFN-γ production, which activates macrophages to kill parasites via nitric oxide (NO) production, ultimately leading to reduction in parasitic burden (4). The cytokine production

and cytotoxic activity by CD8+ T-cells also contribute to the disease outcome in *Leishmania* infection. Initially, CD8+ T-cells were thought to play a role only during re-infection, however, they were also shown to be crucial in controlling the primary infection by skewing the responses toward Th1-type. Effector CD4+ T-cells allow activation of macrophages through various cytokines and are required for optimal host response to infection (11) whereas cytotoxic CD8+ T-cells play a role in parasite clearance with the generation of memory responses (12).

As *Leishmania* parasite follows a digenetic life cycle it results in significant antigenic diversity, which ultimately hampered the passage of vaccine development against VL, therefore, the knowledge of such antigenic diversity is of utmost importance (13). Researchers have utilized several approaches for identification of potential antigens, which can be targeted as suitable vaccine candidate (**Figure 1**). Among them, proteomics attract the most since it addresses several unanswered questions related to microbial pathogens, including its development, evolution, and pathogenicity. Proteomic studies revealed several proteins, which are seen as potential vaccine targets offering varied levels of protection in different animal models. Recent advancement in computational biology further simplifies our knowledge regarding the in-depth study of parasite. T-cell epitope prediction via bioinformatics analysis of protein sequences has been proposed as another alternative for rationale vaccine development (14). The concept that CD8+ T lymphocytes could be important in protection and long-lasting resistance to infection has opened up a new strategy in *Leishmania* vaccine design known as "polytope vaccine" (15). Its major advantages include greater potency, can be controlled better, can be designed to break tolerance, can overcome safety concerns associated with entire organisms or proteins, etc.

CLASSICAL APPROACHES TO *LEISHMANIA* VACCINE DEVELOPMENT

LIVE/KILLED WHOLE PARASITE VACCINE

Cutaneous leishmaniasis remained the focus point for earlier attempts for vaccination made in the Middle East due to the fact

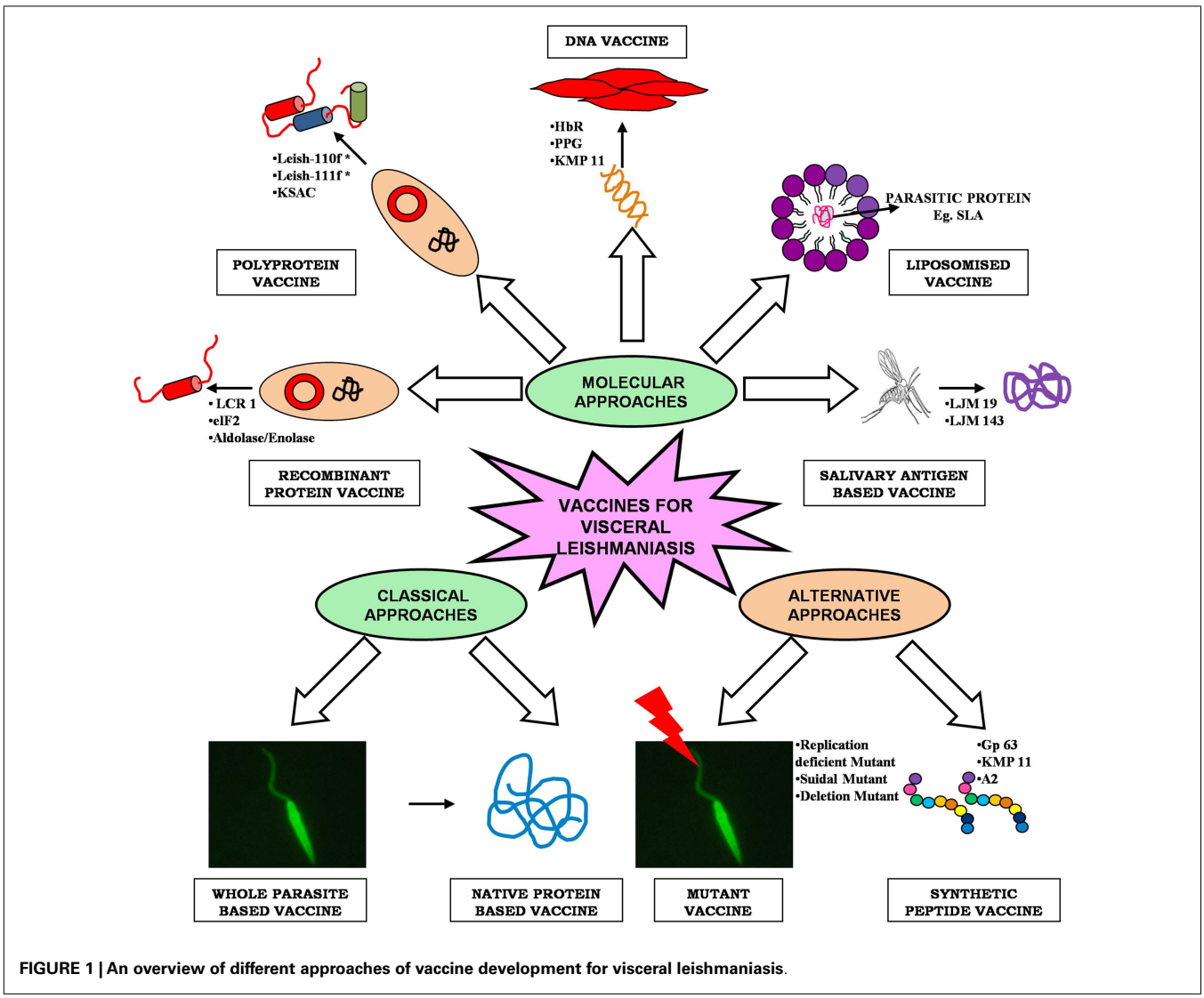


FIGURE 1 | An overview of different approaches of vaccine development for visceral leishmaniasis.

that people who had their skin lesions healed up were protected lifelong from re-infection. Leishmanization (LZ), the deliberate inoculation of virulent parasite from the exudate of a cutaneous lesion to uninfected individuals, was successfully practiced in Western and South-Western Asia, which offers a strong immunity among individuals through the formation of self-healing lesions (16). As the researchers started culturing promastigote form of parasite in artificial media, the concept of live vaccination came into existence. A number of large-scale vaccination trials were conducted during the 1970s and 1980s in Israel, Iran, and the Soviet Union with a higher success rate. However, standardization and quality control are the major issues associated with live vaccines because parasites used for LZ losses its infectivity due to repeated sub-culturing. Therefore, the focus of vaccine development program was shifted toward killed organisms in the early 90s (17). This concept was abandoned for many years due to the conflicting results obtained in the 40s. However, the vaccination trial conducted in a Brazilian population showed excellent protection with up-regulation of IFN-γ and absence of IL-4, an indicator of long-lasting Th1-type immune response (18, 19). Use of whole killed parasites with or without adjuvant was proposed for both therapeutic as well as for prophylactic purposes (20).

Knowing the fact that deliberate infection of *L. major* to naive people could confer protection against subsequent VL (21) several attempts utilizing this approach was also initiated for the development of vaccine against VL. In this direction, autoclaved *L. major* (ALM) along with BCG was evaluated for its cross protection against VL (**Table 1**). Dube et al. (22) assessed its protective potential against *L. donovani* challenge in Indian langur monkeys in single as well as triple dose schedules where triple dose schedule was found to be more effective. Immunogenicity of the ALM + BCG vaccine was further enhanced by adsorbing ALM to alum (aluminum hydroxide), which resulted in successful vaccination against *L. donovani* infection in Indian langur monkeys (23). Encouraged with these results Khalil et al. (24) performed a double-blind randomized trial with ALM ± BCG in human subjects against VL in Sudan. None of the evidences showed that ALM + BCG offered significant protective immunity as compared to BCG alone. Here also, the addition of alum improved the immunogenicity of ALM, when administered intradermally (i.d.) at different doses in healthy volunteers from a non-endemic area of Sudan. Results indicated toward the safety of the vaccine mixture, which induced strong delayed type hypersensitivity (DTH) reaction with minimal side effects (25). A similar trial was conducted against canine leishmaniasis in Iran wherein a single injection of alum-ALM + BCG was found to be protective to the tune of 69.3% (26). Killed *Leishmania* can also be given therapeutically in combination with antimonial therapy in order to enhance cure rates and to reduce incidence of relapse (27). However De Luca et al. (28), advocated that autoclaving lowers the immunogenicity of the parasite as it destroys most of the immunogenic proteins. As such Breton et al. (29), applied another approach where they utilized *L. tarentolae*, a non-pathogenic species, to immunize BALB/c mice and found a significant protective immune response after single peritoneal injection against *L. donovani* challenge.

Though, whole parasite vaccine (either live/killed or attenuated one) offered vast array of antigens to the host immune system that induced both protective as well as non-protective responses (94), recent advent in our knowledge about the immunobiology of the *Leishmania* infection provided probable explanations for the failure of the first generation vaccines, which further insisted for the development of newer vaccination strategies against VL. A variety of different molecules were identified from parasite based on their abundance, surface localization, T-cell clones, screening of antigen pools/expression libraries with sera of infected animals and humans, which was further evaluated as suitable vaccine candidates leading to the production of a number of experimental vaccines against different forms of leishmaniasis over past few decades (95). In case of VL, extensive vaccination studies have not been possible due to unavailability of an appropriate animal model. Although, golden hamsters and dogs were utilized for studying the immunobiology of *L. donovani* and *L. infantum*, respectively, lack of immunological reagents and assays needed for the characterization of immune responses makes inconclusive study. In such case, a mouse model of VL has been extensively utilized since it exhibit organ-specific pathology in the liver and spleen.

PROTEIN FRACTIONS BASED VACCINE

Selection of suitable vaccine candidates seems to be a difficult task due to the multitude of antigens that has been evaluated with varied success rate depending on their formulation and the type of animal model used (20). Complete protection has not been achieved so far due to the complexity of the parasite, which generates poly-specific response (96). Therefore, different fractions of the parasite in the form of crude preparations were tested as vaccine preparation in order to draw any conclusive results (**Table 1**). Jaffe et al. (38) demonstrated that mice receiving promastigote-derived membrane protein dp72 yielded a 81.1% reduction in the liver parasitemia as compared with the adjuvant controls, but there has been no further advance on the use of this antigen for the development of vaccines. Another membranous protein, FML, a glycoprotein mixture, of *L. donovani* in combination with saponin was assessed as vaccine in mice, hamster, and dog models of VL and found to be protective (39–42). Lemesre et al. (43) and Bourdoiseau et al. (44) utilized naturally excretory/secretory (ES) antigens of *L. infantum* promastigotes (*Li*ESAp) and found them to be protective in dogs against experimental *L. infantum* infections. Mutiso et al. (37) delivered sonicated antigen of *L. donovani* i.d. with alum-BCG (AlBCG), MISA, or monophosphoryl lipid A (MPLA) in vervet monkeys against homologous challenge and concluded that *L. donovani* sonicated antigen containing MISA is safe and is associated with protective immune response.

A recent meta-analysis of different vaccination trials using these classical approaches had shown the lack of efficacy of these vaccines in clinical trials (97). Also, the efficacy of LZ has not been shown against VL (98). Standardization and quality control are the major problems associated with LZ, which limit its practicality and acceptability (10). Genetic variation and polymorphism in *Leishmania* isolates also deject this approach (99). In case of fraction based vaccines, there are issues related to purity and yield of immunogenic protein. All these lead to explore alternate approaches for generation of better vaccine.

Table 1 | Summary of vaccines evaluated against visceral leishmaniasis.

Vaccine delivery	Antigen	Species used	Challenge with	Host system	Remarks	Reference
(1) WHOLE PARASITE						
(a) Killed	ALM ± BCG	*L. major*	*L. donovani*	Indian langur	Triple dose is more effective than single dose	Dube et al. (22)
				Human	Poor efficacy (6%)	Khalil et al. (24)
	Alum-ALM + BCG			Indian langur	Single dose is effective; increased IFN-γ production	Misra et al. (23)
				Human	Protective; induced strong DTH response	Kamil et al. (25)
			L. infantum	Dog	Moderate efficacy (69.3%)	Mohebali et al. (26)
(b) Live-attenuated	BT1 deleted parasite	*L. donovani*	*L. donovani*	BALB/c mice	Protective immunity; increased IFN-γ production	Papadopoulou et al. (30)
	SIR2 single allele deletion	*L. infantum*	*L. infantum*		High IFN-γ/IL-10 ratio with increased NO production; protective immunity	Silvestre et al. (31)
	Non-pathogenic strain expressing *L. donovani* A2 antigen	*L. tarentolae*	*L. infantum*		Protective response with high level of IFN-γ production	Mizbani et al. (32)
	Amastigote-specific protein p27	*L. donovani*	*L. donovani*, *L. major*, and *L. braziliensis*		Significant reduction in parasite burden, Th1-type response	Dey et al. (33)
	Suicidal mutant	*L. amazonensis*	*L. donovani*	Hamster	Effective cellular immunity; increased iNOS expression and IFN-γ, IL-12 production	Kumari et al. (34)
	Replication deficient centrin gene	*L. donovani*	*L. donovani* and *L. brazilensis*	BALB/c mice and Hamster	Protective immunity with increased level of IFN-γ, IL-2, and TNF-α producing cells	Selvapandiyan et al. (35)
			L. infantum	Beagle dog	High immunogenicity; increased secretion of IFN-γ, TNF-α, IL-12, and decreased production of IL-4	Fiuza et al. (36)
(2) NATIVE PROTEIN OF PARASITE						
Parasite fraction	Sonicated antigen+ AIBCG/MISA/MPLA	*L. donovani*	*L. donovani*	Vervet Monkey	Good protection; elicit IFN-γ production	Mutiso et al. (37)
Membrane protein	Dp72 and gp70-2			BALB/c mice	Dp 72 showed 81.1% efficacy; gp70-2 is non-protective	Jaffe et al. (38)
	FML + saponin			Mice	84.4% Protection	Palatnik et al. (39)
				Hamster	Protective	Palatnik et al. (40)
				Mice	Increase in IgG2 and decrease in parasite load by 88%	Santos et al. (41)
			L. donovani and *L. chagasi*	Dog	Effective protection; cellular and humoral response	Saraiva et al. (42)

(Continued)

Table 1 | Continued

Vaccine delivery	Antigen	Species used	Challenge with	Host system	Remarks	Reference
Secretory protein	LiESAp	*L. infantum*	*L. infantum*	Beagle dog	Protective; high level of IFN-γ and low level of IL-4 with increased NO production	Lemesre et al. (43)
					Humoral response with cell-mediated immunity	Bourdoiseau et al. (44)
(3) RECOMBINANT PROTEIN OF PARASITE						
Membrane protein	LCR 1	*L. chagasi*	*L. chagasi*	BALB/c mice	Partial protection with increased IFN-γ production but not IL-4, IL-5, and IL-10	Wilson et al. (45)
	HASPB1	*L. donovani*	*L. donovani*	Mice	Protective (70 and 90%); increased IL-12 production by dendritic cells	Stager et al. (12)
	A2			Beagle dog	Partial protection with increased IgG and IFN-γ production; low IL-10 level	Fernandes et al. (46)
Soluble protein	F14	*L. donovani*	*L. donovani*	Golden hamster	Partial protection; increased level of IFN-γ	Bhardwaj et al. (47)
	eIF2				Protective (65%); increased level of IFN-γ, IL-12, TNF-α, IgG2, and down-regulation of IL-4, IL-10, TGF-β	Kushawaha et al. (48)
	P45				Protective (85%); increased level of IFN-γ, IL-12, TNF-α, iNOS, and decreased TGF-β, IL-4	Gupta et al. (49)
	PDI				Protective (90%); increased level of IFN-γ, TFN-α, IL-12, and IgG2	Kushawaha et al. (50)
	TPI				Protective (90%); increased level of IFN-γ, TFN-α, IL-12, IgG2, and down-regulation of IL-10, IL-4	Kushawaha et al. (51)
	TPR				Good efficacy (~60%); increased iNOS, IFN-γ, IL-12, TNF-α, and downregulation of IL-4, IL-10, and TGF-β	Khare et al. (52)
	Aldolase and enolase				Increased expression of iNOS, IFN-γ, TNF-α, and IL-12 with down-regulation of TGF-β, IL-4, and IL-10	Gupta et al. (53)
	Ribosomal protein + saponin	*L. infantum*	*L. chagasi*	BALB/c mice	Increased production of IFN-γ, IL-12, and GM-CSF	Chavez-Fumagalli et al. (54)
	Hypothetical amastigote-specific protein		*L. infantum*	BALB/c mice	Protective; increased level of IFN-γ, IL-12, GM-CSF, and down-regulation of IL-4, IL-10	Martins et al. (55)
Secretory protein	Secretory serine protease	*L. donovani*	*L. donovani*	BALB/c mice	Exhibit significant protection with lower parasite burden	Choudhury et al. (56)

(Continued)

Table 1 | Continued

Vaccine delivery	Antigen	Species used	Challenge with	Host system	Remarks	Reference
	LiESAp-MDP	*L. chagasi*	*L. infantum*	Dog	Efficacy (92%); increased IgG2, NO, and IFN-γ production	Lemesre et al. (57)
(4) POLYPROTEIN						
	Q protein	*L. infantum*	*L. infantum*	Dog	Protective (90%); positive DTH response	Molano et al. (58)
				BALB/c mice	Induced significant protection with long-lasting IgG response	Parody et al. (59)
	Leish-111f	*L. major* and *L. braziliensis*	*L. infantum*	Beagle dog	No protection	Gradoni et al. (60)
				Mice and hamster	Decreased parasite load (99.6%); strong Th1 response (increased IFN-γ, IL-2, TNF-α)	Coler et al. (61)
				Dog	Protection	Trigo et al. (62)
	Leish-110f	*L. major*	*L. infantum*	Dog	Protective with increased IFN-γ, TNF-α, and IL-2	Bertholet et al. (63)
	KSAC	*L. infantum* or *L. donovani*	*L. infantum*	C57BL/6 mice	Protective Th1-type response	Goto et al. (64)
(5) DNA OF PARASITE						
	A2 DNA	*L. donovani*	*L. donovani*	Mice	Significant protection with increased IFN-γ production	Ghosh et al. (65)
	P36LACK			Mice	Strong Th1-type response (IFN-γ); non-protective	Melby et al. (66)
	ORFF			BALB/c mice	Significant protection (80%) with increased IFN-γ expression	Sukumaran et al. (67)
	KMP-11			Hamster	Mixed Th1/Th2 response; protective with up-regulation of IFN-γ, TNF-α, and IL-12 and down-regulation of IL-10	Basu et al. (68)
				BALB/c mice	Protective; mixed Th1/Th2 response (enhanced IFN-γ and depressed IL-4 production)	Bhaumik et al. (69)
	H2A, H2B, H3, H4, and p36 (LACK)			Dog	Partial protection; elicit type 1 cellular response (IFN-γ)	Saldarriaga et al. (70)
	γGCS			Mice	Protective immunity; production of specific IgG1 and IgG2a antibodies; enhanced granuloma formation	Carter et al. (71)
	UBQ-ORFF			Mice	Protective; higher levels of IL-12 and IFN-γ and the low levels of IL-4 and IL-10	Sharma and Madhubala (72)

(Continued)

Table 1 | Continued

Vaccine delivery	Antigen	Species used	Challenge with	Host system	Remarks	Reference
	PPG			Hamster	Efficacy about 80% with increased IFN-γ, TNF-α, IL-12, and decreased IL-4, IL-10, TGF-β	Samant et al. (73)
	HbR			BALB/c mice and hamster	Complete protection; increased Th1 response (IFN-γ, TNF-α, IL-12) with down-regulation of IL-4 and IL-10	Guha et al. (74)
	p36 LACK	*L. infantum*	*L. chagasi*	BALB/c mice	Non-protective(IL-10 production); no reduction in parasite load (both liver and spleen)	Marques-da-Silva et al. (75)
	PapLe22			Dog	Downregulate Th2-type response and reduces parasite burden by 50%	Fragaki et al. (76)
	P36 LACK			Mice	Protective immunity; significantly increased IFN-γ and IL-4 with decreased IL-10 production	Gomes et al. (77)
	H2A, H2B, H3, and H4			BALB/c mice	No protection	Carrion et al. (78)
	Purified FML, rNH36, and NH36 DNA	*L. donovani*	*L. chagasi* and *L. mexicana*	BALB/c mice	Significant protection with 88% reduction in parasite load; Th1-type response	Aguilar-Be et al. (79)
	VR1012-NH36		*L. chagasi*	BALB/c mice	Protective (77%); reduction in parasite burden (91%)	Gamboa-Leon et al. (80)
	A2 and NH	*L. chagasi*	*L. chagasi*	BALB/c mice	Protective response (only A2) with increased IFN-γ and decreased IL-4 and IL-10 production	Zanin et al. (81)
(6) RECOMBINANT PROTEIN + DNA						
	ORFF (HPB)	*L. donovani*	*L. donovani*	BALB/c mice	Protective; reduction in parasite load (75–80%) with increased IgG2a and IFN-γ production	Tewary et al. (82)
	GP63 as heterologous prime boost (HPB)				Enhanced IFN-γ, IL-12, NO, IgG2a/IgG1 ratio, and reduced IL-4 and IL-10	Mazumder et al. (83)
	Virus expressing LACK antigen (WRp36 or MVAp36)	*L. infantum*	*L. infantum*	BALB/c mice	Protective; significant level of IFN-γ and TNF-α	Dondji et al. (84)
	LACK			Dog	Moderate protection (60%); increased level of IL-4 and IFN-γ	Ramiro et al. (85)
	Type I (*cpb*) and II (*cpa*)			BALB/c mice	Protective; strong Th1 response (higher level of IFN-γ/IL-5 ratio)	Rafati et al. (86)

(Continued)

Table 1 | Continued

Vaccine delivery	Antigen	Species used	Challenge with	Host system	Remarks	Reference
	CP type I and II		*L. donovani*	Dog	Increased IFN-γ expression and IgG, IgG2 level with strong DTH response	Rafati et al. (87)
(7) LIPOSOMISED DELIVERY OF PARASITE PROTEINS						
	Liposomised *L. donovani* antigens	*L. donovani*	*L. donovani*	BALB/c mice	Induced both Th1 and Th2-type responses with high level of IgG2a, IgG2b, and IgG1	Afrin et al. (88)
	pDNA + SLA				Protective; potentiate Th1 response and downregulate Th2 response	Mazumder et al. (89)
	GP63 in stable cationic liposomes				Up-regulation of IFN-γ and down-regulation of IL-4; mixed Th1/Th2-type response	Bhowmick et al. (90)
	BM-DCs pulsed with H1	*L. infantum*	*L. infantum*		Increased level of IFN-γ and IgG2a/IgG1 ratio; decreased level of IL-10	Agallou et al. (91)
(8) SALIVARY PROTEIN OF VECTOR						
	LJM19	*Lutzomyia longipalpis*	*L. infantum chagasi*	Golden hamster	Protective; high IFN-γ/TGF-β ratio and increased iNOS expression	Gomes et al. (92)
	LJM143 and LJM17		*L. infantum*	Beagle dog	Strong Th1-type response with IFN-γ and IL-12 expression	Collin et al. (93)

ALM, autoclaved L. major; BCG, Mycobacterium bovis bacillus Calmette Guerin; BT1, biopterin transporter; SIR2, silent information regulatory 2; AIBCG, alum-BCG; MISA, montanide ISA 720; MPLA, monophosphoryl lipid A; dp72, L. donovani promastigote antigen of 72 kDa; FML, fucose–mannose ligand; SLA, soluble leishmanial antigens; LiESAp, L. infantum excreted-secreted antigen purified; HASPB1, hydrophilic acylated surface protein B1; eIF-2, elongation factor-2; PDI, protein disulfide isomerase; TPI, triose phosphate isomerase; MDP, muramyl dipeptide; UBQ-ORFF, ubiquitin open reading frame F; KMP-11, kinetoplastid membrane protein-11; NH, nucleoside hydrolase; LACK, Leishmania homolog of receptors for activated C-kinase; γGCS, gamma-glutamyl cysteine synthetase; PPG, proteophosphoglycan; HPB, heterologous prime boost; HbR, hemoglobin receptor; CP, cysteine proteinase; BM-DCs, bone marrow-dendritic cells; TPR, trypanothione reductase.

MOLECULAR APPROACHES TO *LEISHMANIA* VACCINE DEVELOPMENT

RECOMBINANT PROTEIN VACCINE

With the advancement in recombinant DNA technology, several leishmanial molecules, either species or life cycle stage specific, were extensively studied as a promising vaccine candidate in the form of recombinant proteins. The major advantages associated with these proteins are in terms of purity as well as yield. Numerous proteins were examined against the cutaneous form of diseases, which were later examined against VL when found suitable. LCR1, A2, HASPB1 are the major membrane protein, which was made recombinant and were tested against experimental VL. Wilson et al. (45) identified specific parasite antigens LCR1 from the amastigote stage of the *L. chagasi* that stimulate IFN-γ production and provided partial protection against homologous challenge directing its possible utility in a subunit vaccine. Stager et al. (12) confirmed the role of recombinant hydrophilic acylated surface protein B1 (HASPB1) in protection against *L. donovani* challenge in mice. Fernandes et al. (46) investigated the protective immunity of recombinant A2 protein with saponin against *L. chagasi* infection in dogs where partial protection was noticed with significantly increased IFN-γ and low IL-10 levels (**Table 1**).

However, several proteins from the soluble fractions of promastigotes stage were also found to be a potent Th1 stimulatory by Kumari et al. (100, 101), which were further developed as recombinant molecules such as protein disulfide isomerase (PDI), triose phosphate isomerase (TPI), elongation factor-2 (elF-2), aldolase, enolase, P45, trypanothione reductase (TPR), etc. Kushawaha et al. (48, 50, 51) studied the immunogenicity of LelF-2, TPI, and PDI of *L. donovani* in PBMCs of cured *Leishmania*-infected patients and hamsters where they found Thl-type cytokine profile (production of IFN-γ, IL-12, and TNF-α but not IL-4 or IL-10) with a remarkable increase in IgG2 and considerable protection. Gupta et al. (49, 53) reported p45, enolase, and aldolase as a potential vaccine candidate with considerable prophylactic efficacy to the tune of 85–90% with an increased mRNA expression of iNOS, IFN-γ, TNF-α, and IL-12 and decrease in TGF-β and IL-4. Vaccination with rLdTPR + BCG provided considerably good

prophylactic efficacy (∼60%) against *L. donovani* challenge in hamsters well supported by the increased inducible NO synthase mRNA transcript and Th1-type cytokines IFN-γ, IL-12 and TNF-α and downregulation of IL-4, IL-10 and TGF-β (52). Several other proteins from soluble lysate were also evaluated as recombinant vaccines against VL. For example, recombinant F14 and ribosomal proteins offered partial protection in hamster and BALB/c mice against *L. donovani/L. chagasi* challenges (47, 54). Among the proteins from amastigote stage, recently, a hypothetical *Leishmania* amastigote-specific protein (LiHyp1) was reported to offer protection via IL-12-dependent production of IFN-γ mainly by CD4+ T-cells (55).

Fewer recombinant ES molecules like cysteine proteinases, serine proteases, etc., were also tested as potential vaccine molecule against experimental VL. Lemesre et al. (57) combined ES antigens of *Li*ESAp with muramyl dipeptide (MDP) and found 100% protection in dogs with increased IgG2 and IFN-γ level against homologous challenge. *In vivo* studies of Choudhury et al. (56) in BALB/c mice confirmed serine protease as a potential vaccine candidate.

POLYPROTEIN VACCINE

Due to the genetic polymorphism in the mammalian immune system, a multicomponent vaccine thought to elicit a better protective immune response (64). Therefore, multicomponent or polyprotein preparations such as Q protein, Leish-111f, Leish-110f, KSAC, etc., came into existence that had been demonstrated to afford better protection against experimental VL. Among these, Q protein containing five genetically fused antigenic determinants from Lip2a, Lip2b, H2A, and P0 proteins, was initially assessed along with either BCG or CpG-ODN in mice and dogs (58, 59) against *L. donovani* challenge. Results showed 90% protection with Q protein + BCG in dogs with strong DTH response while Q protein + CpG-ODN motifs were able to induce a long-lasting IgG response in mice. Lately, a phase III trial was conducted in dogs with another potent single polyprotein – Leish-111f, composed of *L. major* homolog of eukaryotic thiol specific antioxidant (TSA), the *L. major* stress-inducible protein-1 (LmSTI-1), and the *L. braziliensis* elongation and initiation factor (LeIF), which was found to be ineffective against *L. infantum* challenge (60). However, when Leish-111f was combined with adjuvant MPLA-stable emulsion (MPL-SE) a significant protection was achieved against experimental *L. infantum* infection in mice and hamsters (61) as well as in dogs (62) with reduction in parasite burden and a cytokine profile indicative of Th1-type immune response. Later on, a new formulation of Leish-111f vaccine – viz Leish-110f was prepared after removal of His-tag, due to the manufacturing and regulatory purposes (102) and was evaluated for its prophylactic potential with different adjuvants [natural (MPL-SE) or synthetic (EM005) toll-like receptor 4 agonists]. This vaccine was also found to be protective, generating good humoral and cellular responses (63). Another defined polyprotein vaccine – KSAC utilizing four proteins, namely, kinetoplastid membrane protein-11 (KMP-11), SMT, A2, and CPB was developed against VL which, along with MPL was found to be immunogenic and offer significant protection against *L. infantum* challenge in mice (64).

Among all these polyprotein vaccines, Leish-110f is under clinical trial in Indian population and the outcome of this vaccination trial is yet to be seen.

DNA VACCINES

Besides proteins, DNA had also been extensively utilized as a means of vaccine delivery, which reformed the area of vaccinology. Here, genes encoding the target proteins are cloned into a mammalian expression vector, which is injected either intradermally or intramuscularly leading to induction of Th1 responses, resulting in strong cytotoxic T-cell immunity. Safety, stability, long-term protection, ease of administration, and cost effectiveness are the major issues associated with this form of vaccine delivery. Several molecules were evaluated using this approach such as A2, PapLe22, P36LACK, ORFF, KMP-11 proteophosphoglycan (PPG), etc., in different animal models with significant level of protection. A2 (65) and ORFF (67) when administered as a DNA vaccine were found to be significantly protective in BALB/c mice against VL, which induced both humoral and cellular immune responses. However, mice immunized with truncated 24-kDa LACK antigen, which, though, generated a robust parasite-specific Th1 immune response (IFN-γ but not IL-4), did not confer any protection in BALB/c mice (66). PapLe22, another protein, was assessed in the golden hamster by Fragaki et al. (76) experienced down-regulation of Th2 response and half reduction of parasitic episodes in blood circulation. The potential of a p36 (LACK) DNA vaccine was evaluated in BALB/c mice against *L. chagasi* wherein no reduction in parasite load (liver and spleen both) was observed, possibly due to IL-10 production (75). On the other hand, Aguilar-Be et al. (79) reported significant protection with the NH36-DNA vaccine against *L. chagasi* in BALB/c mice with 88% reduction in parasite load and with two to fivefold increase in IFN-γ producing CD4+ T-cells confirming Th1-type immune response. Further, Gamboa-Leon et al. (80) used garlic extract with NH36-DNA vaccine, which did not reduce parasite load, but increased survival (100%) with non-specific enhancement of IFN-γ. In an another interesting study, the efficacy of intranasal (i.n.) vaccination with pCIneo-LACK against VL in BALB/c mice was assessed wherein significant reduction in parasite burden was noticed in both liver and spleen along with significantly increased IFN-γ and IL-4 level with decreased IL-10 production (77). Basu et al. (68) and Bhaumik et al. (69) utilized KMP-11 for DNA vaccine in hamsters and BALB/c mice, respectively, where they found significant protection with the mixed Th1/Th2 response (surge of IFN-γ, TNF-α, and IL-12 with extreme down-regulation of IL-10). In another study by Samant et al. (73), vaccination with DNA-encoding N-terminal domain of the PPG gene in golden hamsters yielded 80% protection against the *L. donovani* challenge with generation of Th1 type of immune response. Recently, Guha et al. (74) showed that immunization with hemoglobin receptor (HbR)–DNA induces complete protection against virulent *L. donovani* infection in both BALB/c mice and hamsters with an up-regulation of IFN-γ, IL-12, and TNF-α with concomitant down-regulation of IL-10 and IL-4.

Several enzymes related to protection against oxidative stress were also shown to be better vaccine targets in *Leishmania* as well as in other parasitic diseases. Carter et al. (71) and Sharma and Madhubala (72) vaccinated mice with pVAXγGCS (gamma-glutamyl

cysteine synthetase) and UBQ-ORFF, respectively, which resulted in a protective response to increased levels of IL-12 and IFN-γ and the lower levels of IL-4 and IL-10 confirming Th1-type response.

Several workers utilized different antigens in the combinatorial approach in order to enhance the efficacy and protective response of different antigens. Zanin et al. (81) immunized mice with a NH/A2 DNA vaccine resulted in increased IFN-γ, IL-4, and IL-10 levels associated with edema and increased parasite loads. Das et al. (103) very recently have developed a DNA vaccine using conserved proteins from various *Leishmania* species and found to be immunogenic inducing CD4+ and CD8+ T-cell responses in genetically diverse human populations of different endemic regions.

HETEROLOGOUS PRIME BOOST VACCINE

Different researchers utilized another strategy known as heterologous DNA-prime protein-boost (HPB) approach for some VL vaccine antigens such as ORFF, cysteine proteinases, GP63, etc., which have also shown success but are yet to reach the level of clinical trials. Ramiro et al. (85) observed 60% protection in dogs immunized with DNA-LACK prime/rVV-LACK boost against *L. infantum* challenge. Since the immune response in a canine model differs significantly from murine and human hosts, Dondji et al. (84) and Tewary et al. (82) conducted similar studies using the murine intradermal model for VL and found comparable levels of protection. With another combination of cysteine proteinases DNA/protein along with ORFF DNA/protein against experimental VL, Rafati et al. (86, 87) observed that vaccination mainly elicited antigen-specific IgG2a antibodies, suggesting the induction of a Th1 immune response. Very recently, Mazumder et al. (83) evaluated a membrane protein, GP63 in BALB/c mice and found robust cellular and humoral responses correlating with durable protection against *L. donovani* challenge.

LIPOSOMISED DELIVERY OF PARASITE PROTEIN

Liposome formulations have been adopted as a drug delivery system against *Leishmania* infection so as to induce an elevated immune response owing to their adjuvant property (104) thus can offer a new approach to the development of VL vaccines wherein it may induce a sustained Th1 immune response. This approach using *L. donovani* promastigote membrane antigens (LAg) encapsulated in positively charged liposomes were found to induce significant protection against experimental VL by Afrin et al. (88). Later, a study conducted by Mazumder et al. (89) showed increase in protective efficacy in animal against homologous challenge with *L. donovani* when vaccinated with both soluble leishmanial antigens (SLA) and non-coding plasmid DNA (pDNA) bearing immunostimulatory sequences (ISS), co-entrapped in cationic liposomes. In another study, using liposomised recombinant membranous protein – GP63 of *L. donovani*, there was a long-term protection against VL in BALB/c mice (90). Recently, vaccination with bone marrow-derived dendritic cells (BM-DCs) – a new delivery system, pulsed with *L. infantum* histone H1 against homologous challenge, Agallou et al. (91) demonstrated antigen-specific splenocyte proliferation with increased IFN-γ and decreased IL-10 production confirming Th1-type immune response.

SANDFLY'S SALIVARY ANTIGEN AS VACCINE

Salivary proteins of vector-sandfly also fetch attraction as a suitable anti-VL vaccine candidates. They received little attention in spite of the fact that salivary proteins from the vector are also delivered to the host during natural transmission of the pathogen and sometimes found immunomodulatory for the host (20). Several salivary proteins of *Phlebotomus* spp. and *Lutzomyia* spp. such as PpSP15, maxadilan, LJM17, LJM19, and LJM143 have been reported as potent immunogens inducing lymphocytic infiltration with up-regulation of IFN-γ and IL-12 (92, 93). Although, these proteins conferred protection against CL (105, 106) they were also assessed for their immunogenicity as well as a protective response against VL. LJM19, an 11 kDa protein, was found to be protective with higher expression of IFN-γ and a strong DTH response in a hamster model (92). Similarly, immunization with other two salivary proteins – LJL143 and LJM17 generated strong Th1 responses in dogs with distinct cellular infiltration of CD3 + lymphocytes and macrophages (93). Therefore, these proteins may further be explored in conjunction with potent parasite proteins for vaccination studies.

Despite these different approaches offer a variable degree of efficacy, several problems still hampers its feasibility due to variations in immunogenicity and due to genetic variation in host as well as in pathogen (99). Therefore, despite of numerous recombinant proteins that have been suggested as potential vaccine candidates, to date barely few have reached to clinical trials (107). Similarly, DNA vaccine faces problems in terms of demonstration of safety and efficacy in humans in clinical trial (99).

NEWER ALTERNATIVE STRATEGIES FOR DEVELOPING ANTI-LEISHMANIAL VACCINE

LIVE MUTANT VACCINE

Attenuation of virulent *Leishmania* parasites through defined genetic alteration is a new area in vaccine research since the perception of vaccination suggests that the more similar a vaccine is to the natural disease, better is the generation of protective immune response (108). Poor long-term immunity is the major issue with various recombinant vaccines tested so far while whole cell killed vaccines showed variable efficacy. Consequently, live-attenuated vaccine attracts the immunologists, since, it offers a complete milieu of antigens to the antigen presenting cells (APCs), therefore, providing an optimal polarization of CD4+ T-cells, resulting in better immune response (109). Also, they assure persistence of antigen that may allow the generation of antigen-specific effector and memory cells, which react immediately following infection (110). However, till date, only limited attenuated strains have been tested with various outcomes. Earlier construct generated by gene replacement was *dhfr-ts* – and *lpg2* – mutants of *L. major* and *L. mexicana* (111) that were excluded as future *Leishmania* vaccines due to some inherent problem, but still they did open the door for live-attenuated vaccine against VL. Papadopoulou et al. (30) inactivated the *L. donovani* biopterin transporter BT1 by gene disruption that elicits protective immunity in mice against a *L. donovani* challenge (**Table 1**). However, Silvestre et al. (31) inactivated one allele of SIR2 in *L. infantum*, which elicits complete protection in BALB/c mice with generation of specific anti-*Leishmania* IgG antibody subclasses and increased IFN-γ/IL-10 ratio indicating both

type 1 and type 2 responses. Mizbani et al. (32) stably expressed the *L. donovani* A2 antigen in *L. tarentolae* to check its protective efficacy in BALB/c mice against *L. infantum*. Results showed increased production of IFN-γ followed by reducing levels of IL-5 when administered intraperitoneally indicates potential Th1 immune response. In contrast, intravenous injection elicited a Th2-type response, characterized by higher levels of IL-5 and high humoral immune response, resulting in a less efficient protection.

Recent investigations have established that tumor cells treated *in vitro* by photodynamic therapy (PDT) can be used for generating potent vaccines against cancers of the same origin. *Leishmania*, naturally residing in the phagolysosomes of macrophages, is a suitable carrier for vaccine delivery. Genetic complementation of *Leishmania* to partially rectify their defective heme-biosynthesis renders them inducible with delta-aminolevulinate to develop porphyria for selective photolysis, leaving infected host cells unscathed. Delivery of released "vaccines" to APCs is thus expected to enhance immune response, while their self-destruction presents added advantages of safety. Such suicidal *L. amazonensis* was found to confer immunoprophylaxis and immunotherapy on hamsters against *L. donovani* (34).

Centrin, a growth regulated gene was deleted from the amastigote stage of the *L. donovani* parasite and was subjected to evaluation of its prophylactic potential (112). The *LdCen*$^{-/-}$ parasite was found to be safe and protective in mice and hamsters against virulent challenge (35) and is under exploration for further development as potential vaccine against VL. Fiuza et al. (36) presented an immunogenicity profile of LdCen$^{-/-}$ in dogs and showed increased antibody production and amplified lymphoproliferative response. Further, LdCen$^{-/-}$ vaccinated dogs showed higher frequencies of activated CD4+ and CD8+ T-cells, IFN-γ production by CD8+ T-cells, increased secretion of TNF-α and IL-12/IL-23p40 and decreased secretion of IL-4. Very recently, Dey et al. (33) have demonstrated another knock out – Ldp27 (−/−) parasites to be safe and can provide protective immunity against both homologous and heterologous challenge with stimulation of both Th1-type CD4+ and CD8+ T-cells. Since, effector T-cell population requires continuous stimulation for excellent protection; it can be well accomplished through live-attenuated vaccines. Although, there are certain issues associated with these vaccines such as probable reversal to virulence, reactivation in immune compromised individuals, manufacturing considerations, restraint to their usage in clinical studies due to the presence of antibiotic resistance genes used as selective markers during the steps of gene deletion, etc., the two-step approach, i.e., gene deletion with parasite selection and excision of the antibiotic gene cassette offers a promising way toward the generation of a safe live-attenuated vaccine. Thus, all these approaches pave the way for the development of newer generation of vaccine, which would rather be safer, provide long-lasting immunity and meet both scientific as well as regulatory standards.

SYNTHETIC PEPTIDE VACCINE

Recent developments in blending of bioinformatics with vaccinology has revolutionized and expedited this area. Sequencing of large number of pathogen genome and increase in nucleotide and protein sequence databases accelerate the pace of vaccine development program. Although, killed or attenuated parasites are utilized for most of the existing vaccines, protective immune response is more often triggered by small amino acid sequence (peptides). More recent bioinformatic approaches utilizes number of algorithms for predicting epitopes, HLA-binding, transporter of antigen processing (TAP) affinity, proteasomal cleavage, etc., in order to explore the use of peptide epitopes with the highest probability of inducing protective immune responses. Generation of synthetic polyvalent peptide vaccine requires better understanding of T- and B-cell epitopes in the microorganism's proteins and their interaction with major histocompatibility (MHC) or HLA complexes. The basis of using such peptide epitopes arises from the screening of hundreds of overlapping synthetic peptides, which revealed that only a small number of regions in a protein are immunogenic and capable of provoking humoral as well as cellular immune responses. Synthetic peptide vaccines offer several advantages over other vaccine types like absence of any potentially infectious material, ability to include multiple epitopes, minimization of the amount and complexity of an antigen, economical scale up and decreased chance of stimulating a response against self-antigens.

T-cell epitopes are presented on APC surface where they interact with MHC molecules in order to induce immune response. They can be categorized as conformational or linear, depending on their structure and integration with the paratope. One of the key issues in T-cell epitope prediction is the prediction of MHC binding as it is considered a pre-requisite for T-cell recognition. All T-cell epitopes are good MHC binders, but not all good MHC binders are T-cell epitopes. For epitope prediction, generally two methods are adopted, first, sequence based that analyze protein sequences and second, structure based method using three-dimensional protein structures. Whether the predicted epitopes interact with paratope or not can also be assessed by using computational tools, which determines protein–protein interactions that helps in designing novel vaccines. Several strategies such as genomic databases, evolutionary relationships, three-dimensional structure of proteins, presence of specific protein domains, primary structure of proteins, etc., have been applied to knowhow novel interacting partners in order to validate the presumed interactions. Due to the availability of epitope mapping and binding prediction algorithms, several workers have applied different bioinformatic approaches to design synthetic peptide vaccines against several parasitic diseases. In case of malaria, there have been nine clinical trials from 2000 to 2009 utilizing synthetic peptide vaccines, which target the pre-erythrocytic and erythrocytic stages of the *Plasmodium falciparum*, with encouraging results (113). Similarly, this approach has also been utilized in other parasitic diseases such as *Toxoplasma* (114), *Trypanosoma* (115), etc.

In case of *Leishmania*, several proteins like glycoprotein 63 (GP63), KMP-11, amastigote virulence factor (A2), lipophosphoglycan (LPG), cysteine proteinase, etc., both from promastigote as well as amastigote form were screened for determination of potential antigenic peptides for generation of peptide vaccine (**Table 2**).

Glycoprotein 63

GP63 also known as leishmanolysin, is the most widely studied protein, which is highly conserved among all leishmanial species.

Table 2 | Summary of peptide vaccines evaluated against leishmaniasis.

Protein(s)	Spp. used	Epitopes (no. of amino acid residues)	Prediction tool(s) utilized	Challenge with	Dose and route	Host system	Immune response	Reference
GP63	*L. major*	PT 1–4; PT 6–8 (12–16 residues)	Predictive algorithm	*L. major*	100 μg (each) + 8% poloxamer 407; SC	BALB/c mice	Proliferation of $CD4^+$ Th1 sub-set cells PT3 showed immunoprotection	Jardim et al. (116)
		24 Partially overlapping peptides (12–35 residues)	AMPHI algorithm	*L. major*	100 μg + 100 μg *C. parvum*/entrapped within liposomes; SC/IV	CBA and BALB/c mice	Induction of T-cell response; classical DTH reactivity and secretion of IL-2 and IFN-γ p146-171 and p467-482 induces significant host-resistance	Yang et al. (117)
		P154 and P467 (16 residues)	AMPHI algorithm	*L. major*	50 μg; IP or SC	CBA mice	ThI type cytokine responses Secretion of IL-2, IFN-γ, and GM-CSF	Frankenburg et al. (118)
		PT3 (16 residues)	Predictive algorithm	*L. major*	100 μg + 8% poloxamer 407; SC	BALB/c mice	Long-lasting protection	Spitzer et al. (119)
		MHC class II – restricted peptides (AAR, AAP, ASR) (15 residues)	SYFPETHI		100 μg emulsified in 1:1 dilution with IFA; SC	FVB/N-DR1 transgenic mice	High levels of Th1-type immune response and significant level of IFN-γ	Rezvan (120)
	L. mexicana/ *L. major*	HLA-A2 peptides (9 residues)	SYFPETHI		100 μg + 140μg HAP-B (helper peptide) + 50 μl IFA; SC	HHDII and BALB/c mice	Induction of CTL responses Up-regulation of IFN-γ	Rezvan et al. (121)
	L. donovani	P1–P4 (9–18 residues)	EpiMatrix		100 μg of each peptide	Human PBMCs	Moderate increase in IFN-γ	Elfaki et al. (122)
KMP-11	*L. donovani*	84 Overlapping peptides (9 residues)	SYFPEITHI	*L. donovani*	44 μg/ml (each)	$CD8^+$ T-cells from human PBMCs	Trigger interferon-γ secretion by $CD8^+$ T-cells	Basu et al. (123)

(Continued)

Table 2 | Continued

Protein(s)	Spp. used	Epitopes (no. of amino acid residues)	Prediction tool(s) utilized	Challenge with	Dose and route	Host system	Immune response	Reference
A2	*L. donovani*	MHC class I binding peptide (CD8) and B-cell epitope (B-1) 2 MHC class II binding epitopes (CD4-1 and CD4-2; 17 residues each)	BIMAS and ProtScale	*L. chagasi*	CFSE (20 μm) cells pulsed for 30 min at 37°C + A2-specific peptide + CFSE (1 μm each), and injected at 4×10^7 cells/mouse; IV	BALB/c and C57BL/6 mice	Induction of both IFN-γ secreting $CD4^+$ T and $CD8^+$ T-cells as well as cytolytic $CD8^+$ T-cells	Resende et al. (124)
CPB, CPC LmsTI-1, TSA, LeIF, and LPG-3	*L. major*	18 HLA-A*0201 restricted peptides (9 residues)	SYFPEITHI, BIMAS, EpiJen, Rankpep, nHLApred, NetCTL, and Multipred	*L. major*	10 μg/ml	Human PBMCs	Induces $CD8^+$ T-cell response	Seyed et al. (125)

GP63, glycoprotein 63; KMP-11, kinetoplastid membrane protein-11; A2, amastigote virulence factor; CPB, type I cysteine proteinase; CPC, type III cysteine peptidase; LmsTI-1, L. major stress-inducible protein; TSA, thiol specific antioxidant; LeIF, elongation initiation factor-2 alpha subunit; LPG-3, lipophosphoglycan biosynthetic protein; CPA, cysteine peptidase A.

This zinc metalloprotease is expressed well both in promastigote as well as in amastigote form and implicated in a number of mechanisms related to parasite virulence. Also, proteinase activity of leishmanolysin results in increased resistance to complement-mediated lysis. All these make it an attractive vaccine candidate. As early as in 1990, Jardim et al. (116) utilized primary structure of GP63 to delineate the structures of 7 T-cell epitopes (12–16 residues), which stimulate the proliferation of CD4+ cells. One of these synthetic antigens (with adjuvant) showed proliferation of the Thl subset when inoculated subcutaneously and provided immunoprotection against two species of *Leishmania* parasites. Eleven T-cell epitopes out of 24 partially overlapping peptides (12–35 residues) of GP63 of *L. major* have been identified and their prophylactic efficacy was assessed in CBA and BALB/c mice against *L. major* challenge. These epitopes induce a T-cell response suggesting GP63 as a dominant T-cell inducer *in vivo*. There is a clear segregation of the antigenicity and the immunogenicity of the peptides; only 3 of the 11 stimulatory peptides were able to induce a T-cell response as well as being recognized by T-cells from recovered mice. Frankenburg et al. (118) also tested two peptides representing predicted T-cell epitopes of GP63 of *L. major* in vaccines tested in murine model of CL. Either subcutaneous (s.c.) or intraperitoneal (i.p.) immunization in saline with a peptide representing GP63 amino acids 467–482 (p467) significantly protected CBA mice against the development of severe cutaneous lesions only when the peptide was intrinsically adjuvanted by covalently adding a lauryl cysteine moiety (LC-p467) to its amino terminus during synthesis. A single synthetic T-cell epitope (PT3) was obtained from the histidine zinc-binding region of GP63 and was utilized in a vaccine trial using two virulent strains of *L. major* by Spitzer et al. (119). A single s.c. injection of PT3 with poloxamer 407 protected BALB/c mice for 10 months. Protection was similar for both strains, which manifest different disease sequelae. Elfaki et al. (122) used EpiMatrix algorithm to select putative T-cell epitopes of *L. donovani* GP63 in order to assess their immunogenicity *in vitro*. They found significant reduction in IL-10 level in all individual peptides as compared with unstimulated controls. Also, pooled peptides showed moderate increase in IFN-γ level in some volunteers while individual peptides did not show significant difference from negative controls. Similarly, four HLA-A2 peptides of *L. mexicana/major* GP63 were predicted by SYFPETHI and tested in HHD II mice. Results revealed immunogenicity for three of four peptides predicted for HLA-A2 with induction of CTL responses detected by standard 4-h cytotoxicity assay and significant up-regulation of IFN-γ. When HHDII mice were injected i.m with *L. mexicana* GP63 cDNA and splenocytes were restimulated with blasts loaded with the immunogenic peptides, two of the peptides induced significant level of IFN-γ detected by ELISA (121). Recently, three MHC class II – restricted peptides (AAR, AAP, and ASR) from *L. major* GP63 protein were predicted by SYFPEITHI and tested in FVB/N-DR1 transgenic mice. AAR produced high levels of Th1-type immune response as well as IFN-γ (120).

Kinetoplastid membrane protein-11

An 11 kDa highly conserved protein exclusively present in parasite cell membrane, differentially expressed more in amastigotes

than in promastigotes, which further increases during metacyclogenesis, plays crucial role in host–parasite interaction (126). Basu et al. (123) scanned the entire sequence of KMP-11 of *Leishmania* with overlapping nonapeptides to decipher the role of CD8+ T-cells in defense against infection and in the cure of the disease. Thirty peptides that specifically trigger interferon-γ secretion by human CD8+ T-cells were identified. Four T-cell lines with specificities for different peptides recognize *Leishmania*-infected autologous macrophages, which prove that KMP-11 is processed and presented via the MHC class I pathway of infected cells.

A2 protein

It is a member of amastigote stage-specific protein family, identified in *L. donovani*, required for the survival of amastigotes in visceral organs of mammalian host (127). It consists of multiple copies of a decameric amino acid repeat thus ranges from 45 to 100 kDa inducing a strong Th1 immune response thus conferring partial protection against natural infection. Resende et al. (124) predicted hydrophilic, class I and II MHC-binding synthetic peptides recognized by A2-specific antibodies, CD8+ T and CD4+ T-cells, respectively. Immunization of BALB/c mice with adenovirus expressing A2 (AdA2) resulted in low antibody response, contrasting with high levels of IFN-γ producing CD4+ T and CD8+ T-cells specific for A2. Further, A2-specific CD8+ T-cells from immunized mice were capable of lysing sensitized target cells *in vivo*. They further demonstrated an association of A2-specific T-cell responses and reduced parasitism in both liver and spleen from mice immunized with AdA2 and challenged with *L. (L.) chagasi*. Six *L. major* antigens (CPB, CPC, LmsTI-1, TSA, LeIF, and LPG-3) were screened for potential CD8+ T-cell activating 9-mer epitopes presented by HLA-A*0201. Specific response to LmsTI-1 and LPG-3-related peptides presented in HLA-A*0201 was demonstrated (125). Recently, Agallou et al. (128) analyzed eight peptides from four known antigenic *L. infantum* proteins, i.e., cysteine peptidase A (CPA), histone H1, KMP-11, and *Leishmania* eukaryotic initiation factor (LeIF) for their immunogenicity in BALB/c mice where they found that CPA_p2, CPA_p3, LeIF_p3, and LeIF_p6 induced IFN-γ producing CD4+ T-cells indicating a Th1-type response. In addition, CPA_p2, CPA_p3, and H1_p1 also induced CD8+ T-cells.

CONCLUDING REMARKS

For effective intervention measures to control VL in endemic areas, it is imperative to design a vaccine, which is the most economical way of controlling infectious diseases. An ideal vaccine involves suitable vaccine candidates, ought to offer long-lasting immunity, which is the prime pre-requisite for evaluating the efficacy of a vaccine. Although researchers utilize different approaches for designing vaccines against VL, they still face several challenges either due to heterogeneity of the human population or due to unusual host evasive mechanisms of parasite. The key step in vaccine designing is the identification of most appropriate vaccine candidate, which is found to be a time consuming and labor-intensive task. Therefore, efforts were made for rationale and faster identification of potential antigens. With the emergence of immunoinformatics, peptide-based vaccines attract the most due its several merits. These vaccines should include promiscuous T-cell epitopes derived from the potential Th1 stimulatory proteins of *L. donovani*, which expands host protective immune responses.

REFERENCES

1. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* (2007) **5**:873–82. doi:10.1038/nrmicro1748
2. Hasker E, Singh SP, Malaviya P, Picado A, Gidwani K, Singh RP, et al. Visceral leishmaniasis in rural Bihar, India. *Emerg Infect Dis* (2012) **18**(10):1662–4. doi:10.3201/eid1810.111083
3. Desjeux P. Leishmaniasis. *Nat Rev Microbiol* (2004) **2**:692–3. doi:10.1038/nrmicro981
4. Piscopo T, Azzopardi M. Leishmaniasis. *Postgrad Med J* (2006) **83**:649–57. doi:10.1136/pgmj.2006.047340corr1
5. Lukes J, Mauricio IL, Schonian G, Dujardin JC, Soteriadou K, Dedet JP, et al. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *Proc Natl Acad Sci U S A* (2007) **104**:9375–80. doi:10.1073/pnas.0703678104
6. Lodge R, Diallo TO, Descoteaux A. *Leishmania donovani* lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane. *Cell Microbiol* (2006) **8**:1922–31. doi:10.1111/j.1462-5822.2006.00758.x
7. Kedzierski L. Leishmaniasis vaccine: where are we today? *J Glob Infect Dis* (2010) **2**(2):177–85. doi:10.4103/0974-777X.62881
8. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. *Clin Microbiol Rev* (2006) **19**:111–26. doi:10.1128/CMR.19.1.111-126.2006
9. Mutiso JM, Macharia JC, Kiio MN, Ichagichu JM, Rikoi H, Gicheru MM. Development of *Leishmania* vaccines: predicting the future from past and present experience. *J Biomed Res* (2013) **27**(2):85–102. doi:10.7555/JBR.27.20120064
10. Kamhawi S, Oliveira F, Valenzuela JG. Using humans to make a human leishmaniasis vaccine. *Sci Transl Med* (2014) **6**:234fs18. doi:10.1126/scitranslmed.3009118
11. Kharazmi A, Kemp K, Ismail A, Gasim S, Gaafar A, Kurtzhals JA, et al. T-cell response in human leishmaniasis. *Immunol Lett* (1999) **65**:105–8. doi:10.1016/S0165-2478(98)00132-1
12. Stager S, Smith DF, Kaye PM. Immunization with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. *J Immunol* (2000) **165**:7064–71. doi:10.4049/jimmunol.165.12.7064
13. Kumari S, Kumar A, Samant M, Sundar S, Singh N, Dube A. Proteomic approaches for discovery of new targets for vaccine and therapeutics against visceral leishmaniasis. *Proteomics Clin Appl* (2008) **3**:372–86. doi:10.1002/prca.200780017
14. Lundegaard C, Lund O, Kesmir C, Brunak S, Nielsen M. Modeling the adaptive immune system: predictions and simulations. *Bioinformatics* (2007) **23**(24):3265–75. doi:10.1093/bioinformatics/btm471
15. Palatnik de Sousa CB. Vaccines for leishmaniasis in the fore coming 25 years. *Vaccine* (2008) **26**:1709–24. doi:10.1016/j.vaccine.2008.01.023
16. Bray RS, Modabber F. The history of leishmaniasis. In: Gilles HM, editor. *Protozoal Diseases*. New York, NY: Hodder Arnold Publisher (2000). p. 414–9.
17. Handman E. Leishmaniasis: current status of vaccine development. *Clin Microbiol Rev* (2001) **14**:229–43. doi:10.1128/CMR.14.2.229-243.2001
18. Pessoa SB. Segunda nota sobre a vacinacao preventiva na leishmaniose tegumentar Americana com leptomones mortas. *Rev Paul Med* (1941) **19**:106.
19. Pessoa SB, Pestana BR. Enseaio sobre vacinacao preventive na leishmaniose tegumentar americana com germenes mortos. *Arq Hig Saude Publ* (1941) **6**:141–7.
20. Basyoni MM. *Leishmania* vaccines updates. *PUJ* (2012) **5**(1):1–10.
21. Zijlstra EE, El-Hassan AM, Ismael A, Ghalib HW. Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and post-kala-azar dermal leishmaniasis. *Am J Trop Med Hyg* (1994) **51**:826–36.
22. Dube A, Sharma P, Srivastava JK, Misra A, Naik S, Katiyar JC. Vaccination of langur monkeys (*Presbytis entellus*) against *Leishmania donovani* with autoclaved *L. major* plus BCG. *Parasitology* (1998) **116**:219–21. doi:10.1017/S0031182097002175

23. Misra A, Dube A, Srivastava B, Sharma P, Srivastava JK, Katiyar JC, et al. Successful vaccination against *Leishmania donovani* infection in Indian langur using alum-precipitated autoclaved *Leishmania major* with BCG. *Vaccine* (2001) **19**:3485–92. doi:10.1016/S0264-410X(01)00058-5
24. Khalil EA, El-Hassan AM, Zijlstra EE, Mukhtar MM, Ghalib HW, Musa B, et al. Autoclaved *Leishmania major* vaccine for prevention of visceral leishmaniasis: a randomised, double-blind, BCG-controlled trial in Sudan. *Lancet* (2000) **356**:1565–9. doi:10.1016/S0140-6736(00)03128-7
25. Kamil AA, Khalil EA, Musa AM, Modabber F, Mukhtar MM, Ibrahim ME, et al. Alum-precipitated autoclaved *Leishmania major* plus *Bacillus* Calmette-Guerrin, a candidate vaccine for visceral leishmaniasis: safety, skin-delayed type hypersensitivity response and dose finding in healthy volunteers. *Trans R Soc Trop Med Hyg* (2003) **97**(3):365–8. doi:10.1016/S0035-9203(03)90171-4
26. Mohebali M, Khamesipour A, Mobedi I, Zarei Z, Hashemi-Fesharki R. Double blind randomized efficacy field trial of alum precipitated autoclaved *Leishmania major* vaccine mixed with BCG against canine visceral leishmaniasis in Meshkin-Shahr district, I. R. Iran. *Vaccine* (2004) **22**:4097–100. doi:10.1016/j.vaccine.2004.03.058
27. Musa AM, Khalil EA, Mahgoub FA, Elgawi SH, Modabber F, Elkadaru AE, et al. Immunochemotherapy of persistent post-kala-azar dermal leishmaniasis: a novel approach to treatment. *Trans R Soc Trop Med Hyg* (2008) **102**:58–63. doi:10.1016/j.trstmh.2007.08.006
28. De Luca PM, Mayrink W, Alves CR, Coutinho SG, Oliveira MP, Bertho AL, et al. Evaluation of the stability and immunogenicity of autoclaved and nonautoclaved preparations of a vaccine against American tegumentary leishmaniasis. *Vaccine* (1999) **17**:1179–85. doi:10.1016/S0264-410X(98)00338-7
29. Breton M, Tremblay MJ, Ouellette M, Papadopoulou B. Live nonpathogenic parasitic vector as a candidate vaccine against visceral leishmaniasis. *Infect Immun* (2005) **73**:6372–82. doi:10.1128/IAI.73.10.6372-6382.2005
30. Papadopoulou B, Roy G, Breton M, Kundig C, Dumas C, Fillion I, et al. Reduced Infectivity of a *Leishmania donovani* biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. *Infect Immun* (2002) **70**:62–8. doi:10.1128/IAI.70.1.62-68.2002
31. Silvestre R, Cordeiro-da-Silva A, Santarém N, Vergnes B, Sereno D, Ouaissi A. SIR2-deficient *Leishmania infantum* induces a defined IFN-γ/IL-10 pattern that correlates with protection. *J Immunol* (2007) **179**:3161–70. doi:10.4049/jimmunol.179.5.3161
32. Mizbani A, Taheri T, Zahedifard F, Taslimi Y, Azizi H, Azadmanesh K, et al. Recombinant *Leishmania tarentolae* expressing the A2 virulence gene as a novel candidate vaccine against visceral leishmaniasis. *Vaccine* (2009) **28**:53–62. doi:10.1016/j.vaccine.2009.09.114
33. Dey R, Dagur PK, Selvapandiyan A, McCoy JP, Salotra P, Duncan R, et al. Live attenuated *Leishmania donovani* p27 gene knockout parasites are nonpathogenic and elicit long-term protective immunity in BALB/c mice. *J Immunol* (2013) **190**(5):2138–49. doi:10.4049/jimmunol.1202801
34. Kumari S, Samant M, Khare P, Misra P, Dutta S, Kolli BK, et al. Photodynamic vaccination of hamsters with inducible suicidal mutants of *Leishmania amazonensis* elicits immunity against visceral leishmaniasis. *Eur J Immunol* (2009) **39**:178–91. doi:10.1002/eji.200838389
35. Selvapandiyan A, Dey R, Nylen R, Duncan R, Sacks D, Nakasi HL. Intracellular replication-deficient *Leishmania donovani* induces long lasting protective immunity against visceral leishmaniasis. *J Immunol* (2009) **183**:1813–20. doi:10.4049/jimmunol.0900276
36. Fiuza JA, Santiago HC, Selvapandiyan A, Gannavaram S, Ricci ND, Bueno LL, et al. Induction of immunogenicity by live attenuated *Leishmania donovani* centrin deleted parasites in dogs. *Vaccine* (2013) **31**:1785–92. doi:10.1016/j.vaccine.2013.01.048
37. Mutiso JM, Macharia JC, Taracha E, Gicheru MM. *Leishmania donovani* whole cell antigen delivered with adjuvants protects against visceral leishmaniasis in vervet monkeys (*Chlorocebus aethiops*). *J Biomed Res* (2012) **26**:8–16. doi:10.1016/S1674-8301(12)60002-5
38. Jaffe CL, Rachamim N, Sarfstein R. Characterization of two proteins from *Leishmania donovani* and their use for vaccination against visceral leishmaniasis. *J Immunol* (1990) **144**:699–706.
39. Palatnik de Sousa CB, Paraguai de Souza E, Gomes EM, Borojevic R. Experimental murine *Leishmania donovani* infection: immunoprotection by the fucose-mannose ligand (FML). *Braz J Med Biol Res* (1994) **27**:547–55.
40. Palatnik de Sousa CB, Moreno MB, Paraguai de Souza E, Borojevic R. The FML-vaccine (fucose-mannose ligand) protects hamsters from experimental kala-azar. *J Braz Assoc Adv Sci* (1994) **46**:290–6.
41. Santos WR, Paraguai de Souza E, Palatnik M, Palatnik de Sousa CB. Vaccination of Swiss albino mice against experimental visceral leishmaniasis with the FML antigen of *Leishmania donovani*. *Vaccine* (1999) **17**:2554–61. doi:10.1016/S0264-410X(99)00058-4
42. Saraiva EM, de Figueiredo Barbosa A, Santos FN, Borja-Cabrera GP, Nico D, Souza LO, et al. The FML-vaccine (Leishmune) against canine visceral leishmaniasis: a transmission blocking vaccine. *Vaccine* (2006) **24**:2423–31. doi:10.1016/j.vaccine.2005.11.061
43. Lemesre JL, Holzmuller P, Cavaleyra M, Goncalves RB, Hottin G, Papierok G. Protection against experimental visceral leishmaniasis infection in dogs immunized with purified excreted secreted antigens of *Leishmania infantum* promastigotes. *Vaccine* (2005) **23**:2825–40. doi:10.1016/j.vaccine.2004.11.061
44. Bourdoiseau G, Hugnet C, Goncalves RB. Effective humoral and cellular immunoprotective responses in LiESAp-MDP vaccinated protected dogs. *Vet Immunol Immunopathol* (2009) **128**:71–8. doi:10.1016/j.vetimm.2008.10.309
45. Wilson ME, Young BM, Andersen KP, Weinstock JV, Metwali A, Ali KM, et al. A recombinant *Leishmania chagasi* antigen that stimulates cellular immune responses in infected mice. *Infect Immun* (1995) **63**:2062–9.
46. Fernandes AP, Costa MM, Coelho EA, Michalick MS, de Freitas E, Melo MN, et al. Protective immunity against challenge with *Leishmania chagasi* in beagle dogs vaccinated with recombinant A2 protein. *Vaccine* (2008) **26**:5888–95. doi:10.1016/j.vaccine.2008.05.095
47. Bhardwaj S, Vasishta RK, Arora SK. Vaccination with a novel recombinant *Leishmania* antigen plus MPL provides partial protection against *L. donovani* challenge in experimental model of visceral leishmaniasis. *Exp Parasitol* (2009) **121**:29–37. doi:10.1016/j.exppara.2008.09.019
48. Kushawaha PK, Gupta R, Sundar S, Sahasrabuddhe AA, Dube A. Elongation factor-2, a Th1 stimulatory protein of *Leishmania donovani*, generates strong IFN-γ and IL-12 response in cured *Leishmania*-infected patients/hamsters and protects hamsters against *Leishmania* challenge. *J Immunol* (2011) **187**:6417–27. doi:10.4049/jimmunol.1102081
49. Gupta R, Kushawaha PK, Tripathi CD, Sundar S, Dube A. A novel recombinant *Leishmania donovani* p45, a partial coding region of methionine aminopeptidase, generates protective immunity by inducing a Th1 stimulatory response against experimental visceral leishmaniasis. *Int J Parasitol* (2012) **42**(5):429–35. doi:10.1016/j.ijpara.2012.02.013
50. Kushawaha PK, Gupta R, Tripathi CD, Khare P, Jaiswal AK, Sundar S, et al. *Leishmania donovani* triose phosphate isomerase: a potential vaccine target against visceral leishmaniasis. *PLoS One* (2012) **7**:e45766. doi:10.1371/journal.pone.0045766
51. Kushawaha PK, Gupta R, Tripathi CD, Sundar S, Dube A. Evaluation of *Leishmania donovani* protein disulfide isomerase as a potential immunogenic protein/vaccine candidate against visceral leishmaniasis. *PLoS One* (2012) **7**:e35670. doi:10.1371/journal.pone.0035670
52. Khare P, Jaiswal AK, Tripathi CD, Joshi S, Sundar S, Dube A. Efficacy of *Leishmania donovani* trypanothione reductase,identified as a potent Th1 stimulatory protein, for its immunogenicity and prophylactic potential against experimental visceral leishmaniasis. *Parasitol Res* (2014) **113**:851–62. doi:10.1007/s00436-013-3716-5
53. Gupta R, Kumar V, Kushawaha PK, Tripathi CP, Joshi S, Sahasrabuddhe AA, et al. Characterization of glycolytic enzymes – rAldolase and rEnolase of *Leishmania donovani*, identified as Th1 stimulatory proteins, for their immunogenicity and immunoprophylactic efficacies against experimental visceral leishmaniasis. *PLoS One* (2014) **9**(1):e86073. doi:10.1371/journal.pone.0086073
54. Chavez-Fumagalli MA, Costa MA, Oliveira DM, Ramırez L, Costa LE, Duarte MC, et al. Vaccination with the *Leishmania infantum* ribosomal proteins induces protection in BALB/c mice against *Leishmania chagasi* and *Leishmania amazonensis* challenge. *Microbes Infect* (2010) **12**:967–77. doi:10.1016/j.micinf.2010.06.008
55. Martins VT, Chavez-Fumagalli MA, Costa LE, Canavac AM, Lage PS, Duarte MC, et al. Antigenicity and protective efficacy of a *Leishmania* amastigote specific protein, member of the super-oxygenase family, against visceral leishmaniasis. *PLoS Negl Trop Dis* (2013) **7**(3):e2148. doi:10.1371/journal.pntd.0002148

56. Choudhury R, Das P, Bhaumik SK, De T, Chakraborti T. *In situ* immunolocalization and stage-dependent expression of a secretory serine protease in *Leishmania donovani* and its role as a vaccine candidate. *Clin Vaccine Immunol* (2010) **17**:660–7. doi:10.1128/CVI.00358-09
57. Lemesre JL, Holzmuller P, Goncalves RB, Bourdoiseau G, Hugnet C, Cavaleyra M, et al. Long-lasting protection against canine visceral leishmaniasis using the LiESAp-MDP vaccine in endemic areas of France: double-blind randomised efficacy field trial. *Vaccine* (2007) **25**:4223–34. doi:10.1016/j.vaccine.2007.02.083
58. Molano I, Garcıa Alonso M, Miron C. A *Leishmania infantum* multicomponent antigenic protein mixed with live BCG confers protection to dogs experimentally infected with *L. infantum*. *Vet Immunol Immunopathol* (2003) **92**:1–13. doi:10.1016/S0165-2427(02)00315-X
59. Parody N, Soto M, Requena JM, Lonso CA. Adjuvant guided polarization of the immune humoral response against a protective multicomponent antigenic protein (Q) from *Leishmania infantum*. A CpG +Q mix protects Balb/c mice from infection. *Parasite Immunol* (2004) **26**:283–93. doi:10.1111/j.0141-9838.2004.00711.x
60. Gradoni L, Foglia Manzillo V, Pagano A, Piantedosi D, De Luna R, Gramiccia M, et al. Failure of a multi-subunit recombinant *Leishmania* vaccine (MML) to protect dogs from *Leishmania infantum* infection and to prevent disease progression in infected animals. *Vaccine* (2005) **23**:5245–51. doi:10.1016/j.vaccine.2005.07.001
61. Coler RN, Yasuyuki G, Bogatzki L, Raman V, Reed SG. Leish-111f, a recombinant polyprotein vaccine that protects against visceral leishmaniasis by elicitation of CD4+ T cells. *Infect Immun* (2007) **75**:4648–54. doi:10.1128/IAI.00394-07
62. Trigo J, Abbehusen M, Netto EM, Nakatani M, Pedral-Sampaio G, de Jesus RS. Treatment of canine visceral leishmaniasis by the vaccine Leish-111f +MPL-SE. *Vaccine* (2010) **28**:3333–40. doi:10.1016/j.vaccine.2010.02.089
63. Bertholet S, Goto Y, Carter L. Optimized subunit vaccine protects against experimental leishmaniasis. *Vaccine* (2009) **23**:7036–45. doi:10.1016/j.vaccine.2009.09.066
64. Goto Y, Bhatia A, Raman VS, Liang H, Mohamath R, Picone AF, et al. KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. *Clin Vaccine Immunol* (2011) **18**:1118–24. doi:10.1128/CVI.05024-11
65. Ghosh A, Zhang WW, Matlashewski G. Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against *Leishmania donovani* infections. *Vaccine* (2001) **20**:59–66. doi:10.1016/S0264-410X(01)00322-X
66. Melby PC, Yang J, Zhao W, Perez LE, Cheng J. *Leishmania donovani* p36 (LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. *Infect Immun* (2001) **69**:4719–25. doi:10.1128/IAI.69.8.4719-4725.2001
67. Sukumaran B, Tewary P, Saxena S, Madhubala R. Vaccination with DNA encoding ORFF antigen confers protective immunity in mice infected with *Leishmania donovani*. *Vaccine* (2003) **21**:1292–9. doi:10.1016/S0264-410X(02)00352-3
68. Basu R, Bhaumik S, Basu JM, Naskar K, De T, Roy S. Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial sensitive and resistant strains of *Leishmania donovani* that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. *J Immunol* (2005) **174**:7160–71. doi:10.4049/jimmunol.174.11.7160
69. Bhaumik S, Basu R, Sen S, Naskar K, Roy S. KMP-11 DNA immunization significantly protects against *L. donovani* infection but requires exogenous IL-12 as an adjuvant for comparable protection against *L. major*. *Vaccine* (2009) **27**:1306–16. doi:10.1016/j.vaccine.2008.12.053
70. Saldarriaga OA, Travi BL, Park W, Perez LE, Melby PC. Immunogenicity of a multicomponent DNA vaccine against visceral leishmaniasis in dogs. *Vaccine* (2006) **24**:1928–40. doi:10.1016/j.vaccine.2005.10.052
71. Carter KC, Henriquez FL, Campbell SA, Roberts CW, Nok A, Mullen AB, et al. DNA vaccination against the parasite enzyme gamma glutamyl cysteine synthetase confers protection against *Leishmania donovani* infection. *Vaccine* (2007) **25**:4502–9. doi:10.1016/j.vaccine.2007.03.014
72. Sharma A, Madhubala R. Ubiquitin conjugation of open reading frame F DNA vaccine leads to enhanced cell-mediated immune response and induces protection against both antimony-susceptible and -resistant strains of *Leishmania donovani*. *J Immunol* (2009) **183**:7719–31. doi:10.4049/jimmunol.0900132
73. Samant M, Gupta R, Kumari S, Misra P, Khare P, Kushawaha PK, et al. Immunization with the DNA-encoding N-terminal domain of proteophosphoglycan of *Leishmania donovani* generates Th1-type immunoprotective response against experimental visceral leishmaniasis. *J Immunol* (2009) **183**:470–9. doi:10.4049/jimmunol.0900265
74. Guha R, Gupta D, Rastogi R, Vikram R, Krishnmurthy G, Bimal S, et al. Vaccination with *Leishmania* hemoglobin receptor-encoding DNA protects against visceral leishmaniasis. *Sci Transl Med* (2013) **5**(202):202ra121. doi:10.1126/scitranslmed.3006406
75. Marques-da-Silva EA, Coelho EA, Gomes DC, Vilela MC, Masioli CZ, Tavares CA, et al. Intramuscular immunization with p36(LACK) DNA vaccine induces IFN-γ production but does not protect BALB/c mice against *Leishmania chagasi* intravenous challenge. *Parasitol Res* (2005) **98**(1):67–74. doi:10.1007/s00436-005-0008-8
76. Fragaki K, Suffia I, Ferrua B, Rousseau D, Le Fichoux Y, Kubar J. Immunisation with DNA encoding *Leishmania infantum* protein papLe22 decreases the frequency of parasitemic episodes in infected hamsters. *Vaccine* (2001) **19**:1701–9. doi:10.1016/S0264-410X(00)00398-4
77. Gomes DC, Pinto EF, de Melo LD, Lima WP, Larraga V, Lopes UG, et al. Intranasal delivery of naked DNA encoding the LACK antigen leads to protective immunity against visceral leishmaniasis in mice. *Vaccine* (2007) **25**(12):2168–72. doi:10.1016/j.vaccine.2006.11.060
78. Carrion J, Folgueira C, Alonso C. Immunization strategies against visceral leishmaniasis with the nucleosomal histones of *Leishmania infantum* encoded in DNA vaccine or pulsed in dendritic cells. *Vaccine* (2008) **26**:2537–44. doi:10.1016/j.vaccine.2008.03.003
79. Aguilar-Be I, da Silva Zardo R, Paraguai de Souza E, Borja-Cabrera GP, Rosado-Vallado M, Mut-Martin M, et al. Cross-protective efficacy of a prophylactic *Leishmania donovani* DNA vaccine against visceral and cutaneous murine leishmaniasis. *Infect Immun* (2005) **73**(2):812–9. doi:10.1128/IAI.73.2.812-819.2005
80. Gamboa-Leon R, Paraguaide Souza E, Borja-Cabrera GP, Santos FN, Myashiro LM, Pinheiro RO, et al. Immunotherapy against visceral leishmaniasis with the nucleoside hydrolase DNA vaccine of *Leishmania donovani*. *Vaccine* (2006) **24**:4863–73. doi:10.1016/j.vaccine.2006.03.005
81. Zanin FH, Coelho EA, Tavares CA, Marques-da-Silva EA, Costa MM, Rezende SA, et al. Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against *Leishmania chagasi* and *Leishmania amazonensis* experimental infections. *Microbes Infect* (2007) **9**:1070–7. doi:10.1016/j.micinf.2007.05.012
82. Tewary P, Jain M, Sahani MH, Saxena S, Madhubala R. A heterologous prime-boost vaccination regimen using ORFF DNA and recombinant ORFF protein confers protective immunity against experimental visceral leishmaniasis. *J Infect Dis* (2005) **191**:2130–7. doi:10.1086/430348
83. Mazumder S, Maji M, Das A, Ali N. Potency, efficacy and durability of DNA/DNA, DNA/protein and protein/protein based vaccination using GP63 against *Leishmania donovani* in BALB/c mice. *PLoS One* (2011) **6**:e14644. doi:10.1371/journal.pone.0014644
84. Dondji B, Perez-Jimenez E, Goldsmith-Pestana K, Esteban M, McMahon-Pratt D. Heterologous prime – boost vaccination with the LACK antigen protects against murine visceral leishmaniasis. *Infect Immun* (2005) **73**:5286–9. doi:10.1128/IAI.73.8.5286-5289.2005
85. Ramiro MJ, Zarate JJ, Hanke T, Rodriguez D, Rodriguez JR, Esteban M, et al. Protection in dogs against visceral leishmaniasis caused by *Leishmania infantum* is achieved by immunization with a heterologous prime-boost regime using DNA vaccine and *vaccinia* recombinant vectors expressing LACK. *Vaccine* (2003) **21**:2474–84. doi:10.1016/S0264-410X(03)00032-X
86. Rafati S, Zahedifard F, Nazgouee F. Prime boost vaccination using cysteine proteinases type I and II of *Leishmania infantum* confers protective immunity in murine visceral leishmaniasis. *Vaccine* (2006) **24**:2169–75. doi:10.1016/j.vaccine.2005.11.011
87. Rafati S, Nakhaee A, Taheri T, Taslimi Y, Darabi H, Eravani D, et al. Protective vaccination against experimental canine visceral leishmaniasis using a combination of DNA and protein immunization with cysteine proteinases type I and II of *L. infantum*. *Vaccine* (2005) **23**(28):3716–25. doi:10.1016/j.vaccine.2005.02.009
88. Afrin F, Rajesh R, Anam K, Gopinath M, Pal S, Ali N. Characterization of *Leishmania donovani* antigens encapsulated in liposomes that induce

protective immunity in BALB/c mice. *Infect Immun* (2002) **70**:6697–706. doi:10.1128/IAI.70.12.6697-6706.2002
89. Mazumder S, Ravindran R, Banerjee A, Ali N. Non-coding pDNA bearing immunostimulatory sequences co-entrapped with *Leishmania* antigens in cationic liposomes elicits almost complete protection against experimental visceral leishmaniasis in BALB/c mice. *Vaccine* (2007) **25**:8771–81. doi:10.1016/j.vaccine.2007.10.028
90. Bhowmick S, Ravindran R, Ali N. GP63 in stable cationic liposomes confers sustained vaccine immunity to susceptible BALB/c mice infected with *Leishmania donovani*. *Infect Immun* (2008) **76**:1003–15. doi:10.1128/IAI.00611-07
91. Agallou M, Smirlis D, Soteriadou KP, Karagouni E. Vaccination with *Leishmania* histone H1-pulsed dendritic cells confers protection in murine visceral leishmaniasis. *Vaccine* (2012) **30**:5086–93. doi:10.1016/j.vaccine.2012.05.075
92. Gomes R, Teixeira C, Teixeira MJ, Oliveira F, Menezes MJ, Silva C, et al. Immunity to a salivary protein of a sandfly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. *Proc Natl Acad Sci U S A* (2008) **105**:7845–50. doi:10.1073/pnas.0712153105
93. Collin N, Gomes R, Teixeira C, Cheng L, Laughinghouse A, Ward JM, et al. Sand fly salivary proteins induce strong cellular immunity in a natural reservoir of visceral leishmaniasis with adverse consequences for *Leishmania*. *PLoS Pathog* (2009) **5**(5):e1000441. doi:10.1371/journal.ppat.1000441
94. Reis AB, Giunchetti RC, Carrillo E, Martins-Filho OA, Moreno J. Immunity to Leishmania and the rational search for vaccines against canine leishmaniasis. *Trends Parasitol* (2010) **26**(7):341–9. doi:10.1016/j.pt.2010.04.005
95. Coler RN, Reed SG. Second-generation vaccines against leishmaniasis. *Trends Parasitol* (2005) **21**:244–9. doi:10.1016/j.pt.2005.03.006
96. Campos-Neto A, Porrozzi R, Greeson K, Coler RN, Webb JR, Seiky YA, et al. Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. *Infect Immun* (2001) **69**:4103–8. doi:10.1128/IAI.69.6.4103-4108.2001
97. Noazin S, Modabber F, Khamesipour A, Smith PG, Moulton LH, Nasseri K, et al. First generation leishmaniasis vaccines: a review of field efficacy trials. *Vaccine* (2008) **26**:6759–67. doi:10.1016/j.vaccine.2008.09.085
98. Kaye P, Scott P. Leishmaniasis: complexity at the host pathogen interface. *Nat Rev Microbiol* (2011) **9**:604–15. doi:10.1038/nrmicro2608
99. Kumar R, Engwerda C. Vaccines to prevent leishmaniasis. *Clin Trans Immunol* (2014) **3**:e13. doi:10.1038/cti.2014.4
100. Kumari S, Samant M, Khare P, Sundar S, Sinha S, Dube A. Induction of Th1-type cellular responses in cured/exposed *Leishmania*-infected patients and hamsters against polyproteins of soluble *Leishmania donovani* promastigotes ranging from 89.9 to 97.1kDa. *Vaccine* (2008) **26**:4813–8. doi:10.1016/j.vaccine.2008.06.102
101. Kumari S, Samant M, Misra P, Khare P, Sisodia B, Shasany AK, et al. Th1-stimulatory polyproteins of soluble *Leishmania donovani* promastigotes ranging from 89.9 to 97.1kDa offers long-lasting protection against experimental visceral leishmaniasis. *Vaccine* (2008) **26**:5700–11. doi:10.1016/j.vaccine.2008.08.021
102. Vedvick TS, Carter L, Garner M, Goto Y, Bertholet S, Reed SG, et al. An improved manufacturing process for a recombinant polyprotein vaccine. *Biopharm Inter* (2008) **January**(Supplement):14–22.
103. Das S, Freier A, Boussoffara T, Das S, Oswald D, Losch FO, et al. Modular multiantigen T cell epitope-enriched DNA vaccine against human leishmaniasis. *Sci Transl Med* (2014) **6**:234ra56. doi:10.1126/scitranslmed.3008222
104. Bhowmick S, Mazumdar T, Sinha R, Ali N. Comparison of liposome based antigen delivery systems for protection against *Leishmania donovani*. *J Control Release* (2010) **141**:199–207. doi:10.1016/j.jconrel.2009.09.018
105. Valenzuela JG, Belkaid Y, Garfield MK, Mendez S, Kamhawi S, Rowton ED, et al. Toward a defined anti-*Leishmania* vaccine targeting vector antigens: characterization of a protective salivary protein. *J Exp Med* (2001) **194**(3):331–42. doi:10.1084/jem.194.3.331
106. Morris RV, Shoemaker CB, David JR, Lanzaro GC, Titus RG. Sandfly maxadilan exacerbates infection with *Leishmania major* and vaccinating against it protects against *L. major* infection. *J Immunol* (2001) **167**:5226–30. doi:10.4049/jimmunol.167.9.5226
107. Duthie MS, Reed SG. The emergence of defined subunit vaccines for the prevention of leishmaniasis. *Curr Trop Med Rep* (2014) **1**(3):154–62. doi:10.1007/s40475-014-0024-9
108. Silvestre R, Cordeiro-da-Silva A, Ouaissi A. Live attenuated *Leishmania* vaccines: a potential strategic alternative. *Arch Immunol Ther Exp (Warsz)* (2008) **56**(2):123–6. doi:10.1007/s00005-008-0010-9
109. Sporri R, Reis e Sousa C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol* (2005) **6**:163–70. doi:10.1038/ni1162
110. Foulds KE, Wu CY, Seder RA. Th1 memory: implications for vaccine development. *Immunol Rev* (2006) **211**:58–66. doi:10.1111/j.0105-2896.2006.00400.x
111. Titus RG, Gueiros-Filho FJ, De Freitas LA, Beverley SM. Development of a safe live *Leishmania* vaccine line by gene replacement. *Proc Natl Acad Sci U S A* (1995) **92**(22):10267–71. doi:10.1073/pnas.92.22.10267
112. Selvapandiyan A, Debrabant A, Duncan R. Centrin gene disruption impairs stage-specific basal body duplication and cell cycle progression in *Leishmania*. *J Bio Chem* (2004) **279**(24):25703–10. doi:10.1074/jbc.M402794200
113. Nardin E. The past decade in malaria synthetic peptide vaccine clinical trials. *Hum Vaccin* (2010) **6**(1):27–38. doi:10.4161/hv.6.1.9601
114. Wang Y, Wang G, Zhang D, Yin H, Wang M. Screening and identification of novel B cell epitopes of *Toxoplasma gondii* SAG1. *Parasit Vectors* (2013) **6**:125. doi:10.1186/1756-3305-6-125
115. Serna C, Lara JA, Rodrigues SP, Marques AF, Almeida IC, Maldonado RA. A synthetic peptide from *Trypanosoma cruzi* mucin-like associated surface protein as candidate for a vaccine against Chagas disease. *Vaccine* (2014) **32**:3525–32. doi:10.1016/j.vaccine.2014.04.026
116. Jardim A, Alexander J, Teh HS, Ou D, Olafson RW. Immunoprotective *Leishmania major* synthetic T-cell epitopes. *J Exp Med* (1990) **172**:645–8. doi:10.1084/jem.172.2.645
117. Yang DM, Rogers MV, Liew FY. Identification and characterization of host-protective T-cell epitopes of a major surface glycoprotein (GP63) from *Leishmania major*. *J Immunol* (1991) **72**:3–9.
118. Frankenburg S, Axelrod O, Kutner S, Greenblatt CL, Klaus SN, Pirak EA, et al. Effective immunization of mice against cutaneous leishmaniasis using an intrinsically adjuvanted synthetic lipopeptide vaccine. *Vaccine* (1996) **14**(9):923–9. doi:10.1016/0264-410X(95)00245-V
119. Spitzer N, Jardim A, Lippert D, Olafson RW. Long-term protection of mice against *Leishmania major* with a synthetic peptide vaccine. *Vaccine* (1999) **17**:1298–300. doi:10.1016/S0264-410X(98)00363-6
120. Rezvan H. Immunogenicity of HLA-DR1 restricted peptides derived from *Leishmania major* GP63 using FVB/N-DR1 transgenic mouse model. *Iran J Parasitol* (2013) **8**(2):273–9.
121. Rezvan H, Rees R, Ali SA. Immunogenicity of MHC class I peptides derived from *Leishmania mexicana* GP63 in HLA-A2.1 transgenic (HHDII) and BALB/C mouse models. *Iran J Parasitol* (2012) **7**(4):27–40.
122. Elfaki ME, Khalil EA, Groot AS, Musa AM, Gutierrez A, Younis BM, et al. Immunogenicity and immune modulatory effects of *in silico* predicted *L. donovani* candidate peptide vaccines. *Hum Vaccin Immunother* (2012) **8**(12):1769–74. doi:10.4161/hv.21881
123. Basu R, Roy S, Walden P. HLA class I-restricted T cell epitopes of the kinetoplastid membrane protein-11 presented by *Leishmania donovani*-infected human macrophages. *J Infect Dis* (2007) **195**:1373–80. doi:10.1086/513439
124. Resende DM, Caetano BC, Dutra MS, Penido ML, Abrantes CF, Verly RM, et al. Epitope mapping and protective immunity elicited by adenovirus expressing the *Leishmania* amastigote specific A2 antigen: correlation with IFN-γ and cytolytic activity by CD8+ T cells. *Vaccine* (2008) **26**:4585–93. doi:10.1016/j.vaccine.2008.05.091
125. Seyed N, Zahedifard F, Safaiyan S, Gholami E, Doustdari F, Azadmanesh K, et al. *In silico* analysis of six known *Leishmania major* antigens and *in vitro* evaluation of specific epitopes eliciting HLA-A2 restricted CD8 T cell response. *PLoS Negl Trop Dis* (2011) **5**(9):e1295. doi:10.1371/journal.pntd.0001295
126. Matos DC, Faccioli LA, Cysne-Finkelstein L, Luca PM, Corte-Real S, Armoa GR, et al. Kinetoplastid membrane protein-11 is present in promastigotes and amastigotes of *Leishmania amazonensis* and its surface expression increases during metacyclogenesis. *Mem Inst Oswaldo Cruz* (2010) **105**:341–7. doi:10.1590/S0074-02762010000300018
127. Charest H, Matlashewski G. Developmental gene expression in *Leishmania donovani*: differential cloning and analysis of an amastigote-stage-specific gene. *Mol Cell Biol* (1994) **14**:2975–84.

128. Agallou M, Athanasiou E, Koutsoni O, Dotsika E, Karagouni E. Experimental validation of multi-epitope peptides including promising MHC class I- and II-restricted epitopes of four known *Leishmania infantum* proteins. *Front Immunol* (2014) **5**:268. doi:10.3389/fimmu.2014.00268

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 March 2014; accepted: 24 July 2014; published online: 22 August 2014.

*Citation: Joshi S, Rawat K, Yadav NK, Kumar V, Siddiqi MI and Dube A (2014) Visceral leishmaniasis: advancements in vaccine development via classical and molecular approaches. Front. Immunol. **5**:380. doi: 10.3389/fimmu.2014.00380*

This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 10 June 2014
doi: 10.3389/fimmu.2014.00260

Leishmania spp. proteome data sets: a comprehensive resource for vaccine development to target visceral leishmaniasis

Toni Aebischer*

Agents of Mycoses, Parasitoses and Mycobacterioses, Robert Koch-Institut, Berlin, Germany

Edited by:
Alexandre Barbosa Reis, Universidade Federal de Ouro Preto, Brazil

Reviewed by:
William Castro-Borges, Universidade Federal de Ouro Preto, Brazil
Eduardo Ferraz Coelho, Universidade Federal de Minas Gerais, Brazil

***Correspondence:**
Toni Aebischer, FG16 Erreger von Pilz- und Parasiteninfektionen und Mykobakteriosen, Robert Koch-Institut, Berlin 13353, Germany
e-mail: aebischera@rki.de

Visceral leishmaniasis is a neglected infectious disease caused primarily by *Leishmania donovani* and *Leishmania infantum* protozoan parasites. A significant number of infections take a fatal course. Drug therapy is available but still costly and parasites resistant to first line drugs are observed. Despite many years of trial no commercial vaccine is available to date. However, development of a cost effective, needle-independent vaccine remains a high priority. Reverse vaccinology has attracted much attention since the term has been coined and the approach tested by Rappuoli and colleagues. This *in silico* selection of antigens from genomic and proteomic data sets was also adapted to aim at developing an anti-*Leishmania* vaccine. Here, an analysis of the efforts is attempted and the challenges to be overcome by these endeavors are discussed. Strategies that led to successful identification of antigens will be illustrated. Furthermore, these efforts are viewed in the context of anticipated modes of action of effective anti-*Leishmania* immune responses to highlight possible advantages and shortcomings.

Keywords: proteome, T cell antigen receptor, vaccine, kinetoplastida, major histocompatibility complex antigens

INTRODUCTION

A cure or effective prophylaxis for visceral leishmaniasis (VL) also known as Kala azar is a prioritized objective in global efforts directed toward improving the situation for people at risk of and patients suffering from *Leishmania*-infections (1, 2). The problem of VL is grave as it is thought to be second only to malaria in terms of fatal infections (3). Therapy is one option to help the individual patient but on its own is unlikely to offer a lasting solution to manage the public health problem because of emerging resistance to available drugs (4). Vaccines are therefore considered a desirable, cost effective strategy complement (5).

There is encouraging evidence that vaccination against VL should be possible. Immunity is thought to depend on a protective cellular immune response requiring CD4 as well as CD8 T cells that activate leishmanicidal mechanisms in host phagocytes (6, 7) since their suppression correlates with disease (8, 9). Epidemiological data suggest that the majority of infections are in fact controlled and do not lead to disease. For example, the KalaNet study reported an estimate of only 1 in 10 infections leading to disease in India and Nepal where more than 50% of globally recorded fatal VL cases occur (10, 11). In addition, there is the paradigmatic example of lifelong protection against cutaneous leishmaniasis through the century old practice of Leishmanization. This deliberate infection of a non-immune person with virulent parasites (12) has been implemented in the immunization programs of soldiers of several armies in the Middle East but has been discontinued because of the risk of uncontrolled disease in a fraction of vaccines (13) and problems with vaccine strain stability (14). The protective efficacy against subsequent infection afforded by a healed primary infection due to Leishmanization in the majority of cases fostered the development of attenuated live parasites (15–18) or parasites not pathogenic to humans (19) as vaccines also against VL. This approach works remarkably well in rodent models of disease and may be a very promising approach to control VL where this is fueled by a zoonotic cycle.

For human use, subunit vaccines based on selected parasite antigens, however, would offer a more defined and more stable alternative (20). But, major obstacles to their successful development exist and these are on the one hand the identification of the most effective antigens and on the other hand their formulation. Formulation relates to selecting adjuvants and/or delivery systems such as recombinant viruses (2, 21, 22) or bacteria (23, 24) and exploitation or engineering of immune-modulating agents and properties to induce protective antigen-specific CD4 and CD8 T cells. Although our understanding of what makes a protective response in humans remains sketchy (8, 9, 25), there is no reason to object to the idea that this can be achieved through vaccine formulation if selected *Leishmania*-antigens were fit for purpose.

In the post-genomic era, the approach to antigen selection and vaccine development has been revolutionized. The term reverse vaccinology has been coined by Rappuoli and colleagues (26) at the turn of the millennium to designate the process. The idea is simple and is about exploiting genomic and other -omics data sets to filter out relevant gene products *in silico*. Selection proceeds through an algorithm that is developed "backwards" starting from a known or anticipated mode of action of the vaccine. This has been impressively successful for the development of novel anti-Meningococcal Serotype B vaccines because (a) the mode of action was known and allowed to develop a straight forward *in vitro*

screening assay based on lysis-mediating antibodies and (b) this assay was scalable and had high throughput capacity (26, 27). The Reverse Vaccinology approach has also been adapted to identify potential vaccine protein antigens against leishmaniasis and the combined search terms "reverse vaccinology" and "*leishmania*" retrieve five publications from PubMed as of March 10th 2014 (24, 28–32). Reverse Vaccinology when adapted to VL will aim at identifying vaccine antigens that induce protective CD4 and CD8 T cells (24, 28, 30).

In the following, I will try to critically assess the adaptation of the Reverse Vaccinology approach to the development of an anti-VL vaccine. However, before doing so, I will summarize in a bullet point way features of the cell biology of *Leishmania*-infection and of MHC class I and II dependent antigen-presentation in the context of this infection. The aim is to distil scenarios that allow identification of process-relevant steps through which reverse vaccinology may be improved. The reader will quickly note that this comes at a price. This is the deliberate simplification of our understanding of the parasite's intracellular life style.

BULLET POINT STYLE SUMMARY OF THE CELL BIOLOGY OF *LEISHMANIA* SPP. INFECTIONS

- Disease-causing *Leishmania* replicate in the form of amastigotes in a membrane-delimited intracellular habitat of host phagocytes (33).
- The habitat has the characteristics of a late endosome/early lysosome, i.e., a relatively low pH with numerous proteases such as cathepsins and other hydrolases present (34, 35).
- The parasites' habitat is in communication with the host cell's endocytic compartments via fusion and fission of vesicles (36, 37).
- Parasite protein secretion can occur via the classical, signal peptide-dependent pathways or, as recently favored, via the release of exosomes (38, 39).

BULLET POINT STYLE SUMMARY OF ANTIGEN-PRESENTATION BY *LEISHMANIA*-INFECTED HOST CELLS

- Parasite proteins are processed for presentation by proteolysis inside vesicles and it is within a vesicular compartment that peptides form complexes with MHC class I and II histocompatibility antigens (37, 40).
- The so-called cross-presentation, i.e., formation of parasite peptide – MHC class I complexes does not involve proteasomal cleavage (41).
- Proteins secreted via the classical route or located on the surface of the parasite are more efficiently presented to stimulate CD4 and CD8 T cells (40, 42, 43).
- The major antigen-presenting cells initiating the immune response are dendritic cells (44, 45) while infected macrophages are likely the most frequent antigen-presenting cell during infection (46, 47).
- Macrophages need to be activated, e.g., through cytokines such as IFN-γ to express MHC class II molecules, a prerequisite to present antigens to CD4 T cells (48, 49).
- Only a minority of infected macrophages seems to interact with *Leishmania*-specific T cells *in vivo* (46).

BULLET POINT STYLE SUMMARY OF PROCESSES AND MOLECULE NUMBERS RELEVANT FOR ANTIGEN-PRESENTATION

- Mature dendritic cells express up to 10^6–10^7 MHC Class II and 10^5 MHC I molecules per cell (50, 51).
- Mature dendritic cell "fix" a surface MHC class II-peptide complex repertoire to present an immunological snap shot to interacting T cells (52).
- Activated macrophages express 10^5–10^6 MHC Class II and 10^5 MHC I molecules per cell and these are undergoing turn over and recycling (53).
- Immature dendritic cells and Macrophages constantly cycle MHC–peptide complexes from cell surface through endocytic peptide loading enabling compartments back to the surface allowing peptide sampling over time (54).
- Cells display two populations of MHC–peptide complexes, one with a fast off rate of the peptide ligand and one with slow off-rates, a property that in combination with dynamic sampling is a mechanism to enrich for the thermodynamically most stable MHC–peptide complexes for presentation (55).
- Estimates of the number of cognate MHC–peptide complexes required for successful T cell stimulations vary from a single complex (56) to several hundred (57) and a number in the order of 10^2 is a reasonable estimate (58).
- Amastigotes yield ~$2–4 \times 10^{-12}$ g of protein per cell that corresponds to $3–5 \times 10^7$ protein molecules per parasite assuming an average size of ~50 kDa per molecule (40, 59).
- *Leishmania* genomes encode some 8200 distinct proteins (60), which are predicted to encode nearly 3×10^5 MHC class I epitopes with binding capacity for MHC even when only a single MHC class I allele is considered (30).
- The average number of predicted epitopes per protein is thus >36 hence $>10^9$ epitope molecules are likely to be generated from a single parasite if all proteins were processed.
- Parasite proteins may become accessible for the presentation machinery either through parasite lysis, directed release (through exosomes or via classical secretion) or surface exposure and hydrolytic release.

ALGORITHMS OF REVERSE VACCINOLOGY TO IDENTIFY CANDIDATE PROTEINS FOR ANTI-*LEISHMANIA* VACCINE DEVELOPMENT

The most puristic Reverse Vaccinology algorithms to identify candidate vaccine antigens adapted for leishmaniasis proceeded stepwise from genome to T cell epitope prediction (28, 30). For example, Herrera-Najera et al. (30) based their algorithm on the condition that a vaccine protects through induction of CD8 T cells recognizing a parasite protein-derived epitope in the context of MHC class I molecules. In a first step, they analyzed the complete genome for encoding peptides predicted to have MHC–ligand properties (for selected mouse H-2 class I alleles) using a sliding window of 8–11mer amino acids over the entire open reading frames and adapting a filter to account for proteasome-processing preferences implemented in the RankPep software. This identified ~3×10^5 candidate epitopes. To reduce this number, a stringent but arbitrary threshold of the binding score to MHC was introduced resulting in 250 candidate peptides. In step 2 of the process,

these candidates were further analyzed using different T cell epitope prediction algorithms. A set of 78 epitopes was predicted by all or nearly all software. In step 3, the 78 epitopes were compared to the predicted proteomes of putative hosts based on mouse and human genome data, the rationale being to reduce the risk of inducing autoimmune reactions. In this step, it was considered satisfactory that none of the selected peptide-epitopes had >80% identity with a host protein. However, there were peptides with lower identity, i.e., with up to 9 of 11 amino acids identical. Step 4 checked for conservation of the candidate protein containing the epitope(s) in different *Leishmania* spp. and other kinetoplastids. The authors noted that their algorithm did not identify any of the known, experimentally validated candidates. These failed the arbitrarily set stringent threshold for the MHC-binding score in step 1.

An alternative algorithm based on the same idea of vaccine mode of action was developed by John et al. (28). In this case, additional characteristics of a vaccine antigen were assumed and used for filtering. In step 1, subcellular localization was analyzed using PSORT and TMHMM software, respectively, and used to enrich for 903 proteins with predictions for plasma membrane localization or secretion and with counter-selection of proteins with more than one predicted transmembrane domain. This list was purged in step 2 of proteins showing homology to murine or human host proteins leaving 553 candidates in the basket. Selection step 3 analyzed the presence of MHC class I binding and step 4 of MHC class II binding peptides using several programs. Unfortunately, the adopted thresholds that reduced the number to 19 candidates were not described. This final set was tested again for similarity to host self-epitopes but this did not reduce the number further. As before, no experimentally identified protein antigen has passed this selection process.

While both of these approaches identified potentially immunogenic epitopes [in fact immunogenicity was demonstrated in the case of Ref. (30)], the fact that these algorithms did not identify any of the experimentally tested vaccine proteins/epitopes (which is not the same as the ideal vaccine antigen) is worrisome. What is missing?

REFLECTIONS ON IMPROVING REVERSE VACCINOLOGY APPROACHES FOR THE PREDICTION OF CANDIDATE ANTIGENS FOR A VACCINE AGAINST LEISHMANIASIS

The working hypothesis that the success of a vaccine to prevent or treat VL in humans will rely on the induction of CD4 and CD8 T cells is valid. However, individual steps in the algorithms aiming at antigen identification need to be scrutinized on the one hand for the validity of underlying concepts and logic and on the other hand for their effectiveness as selecting filters. Since the above-mentioned studies offer recent examples, I shall follow steps as proposed in their algorithms for illustration.

Herrera-Najera et al. (30) started with predicting MHC-binding peptides considering the proteasomal pathway of peptide generation. While there is evidence against involvement of the proteasome for cross-presentation of parasite-delivered antigens (41), there is currently no evidence in support of it. Thus, this filter may neither be necessary nor instructive. MHC-binding peptide prediction highlighted nearly 3×10^5 candidates. Thus, every ORF is likely to encode more than one candidate hence the filter lacks efficiency. An arbitrary threshold as introduced can seemingly provide filtering capacity but will quickly become too stringent since in the said example it excluded all experimentally identified candidates. The next step involved selection based on T cell epitope predicting algorithms. This filtering is highly error prone and probably superfluous as the T cell receptor is an explorative, adaptive molecule that can recognize epitope variants (61). Because of this, the advantage of implementing this step can be questioned. In addition, there is little evidence from many other areas of its predicting power.

Both *in silico* Reverse Vaccinology algorithms discussed added then an additional step of counter-selection at the epitope stage by testing for molecular mimicry of proteins of putative host species. In theory, this is totally reasonable. In practice, this is either insensitive [see Ref. (30)] or seems impossible since cognate interaction of MHC–peptide complexes with TCRs is not as specific as previously thought and, where analyzed, the sequence space allowing mimicry is extensive (62). The intricacies of this have been reviewed recently in the context of cancer-cell specific epitopes and provide instructive insight (63). In conclusion, T cell epitope prediction may have no and selection against host proteins very limited practical value.

Does this mean that genome and other genomics information offers no opportunities of adapting the Reverse Vaccinology approach to our field? This view may be too pessimistic. The algorithm proposed by John et al. (28) enriched for proteins predicted to be secreted or surface localized. Reasons for this are that these two topologies will facilitate access for the MHC processing and loading machineries from living, actively replicating parasites. This assumption is founded on experimental evidence since phagocytes infected with parasites genetically engineered to secrete or surface expose trackable antigens were more readily presenting the antigens (40, 42). However, evidence that this situation is the prevailing or most relevant mode of antigen-delivery for presentation *in vivo* is still scarce. In fact recent data from *in vivo* tracing approaches suggest that control of infection and healing involves engagement of only a minority of infected or parasite-exposed cells with protective T cells (47, 64). Also, there is evidence that major normally secreted antigens are relatively resistant to proteolytic processing, as shown for the highly abundant secreted proteophosphoglycans of *Leishmania mexicana* (65). This is probably no surprise since parasite products secreted into the phagolysosomal compartment should have evolved this property to preserve their function. Nonetheless, under the assumption that a productively infected cell is the most relevant antigen-presenting cell in these infections, filtering candidate antigens for secreted or surface exposed localization remains reasonable.

Alternative scenarios of antigen-presentation that should be considered are host cells that become activated under pro-inflammatory conditions to kill the parasites or cells in which a fraction of parasites may undergo spontaneous lysis, e.g., due to faulty replication. In these cases, the entire set of parasite proteins will ultimately become available for processing and presentation. Of note, from the point of view, which set of proteins will be presented these modes are also akin to a scenario where antigens reach the processing machinery via the recently proposed secretion pathway involving exosome release by live parasite. Antigens

accessible to the processing machinery in these situations are similar because the proteome of exosomes largely overlaps that of the abundant protein set present in whole parasite lysates [e.g., compare data from Ref. (39, 66)].

In all these situations, I would argue that relative protein abundance is the single most important parameter for candidate antigen selection and is of a high practical value. The algorithms discussed so far did not take relative protein abundance into account. Instead they assumed equivalence of all predicted proteins with respect to their chance to being successfully processed and loaded onto MHC molecules. Not having considered abundance may be an additional reason why none of the experimentally identified candidate antigens were within the set identified by purely bioinformatics approaches. Fortunately proteome data sets reporting about relative abundance of proteins are available and these resources are permanently expanding (67–73) although improvements to the reporting of quantitative aspects of proteome data would be desirable.

In the following, I would like to analyze the potential of integrating quantitative proteome information with a quantitative view of the presentation process (see also bullet point style summaries above) into an algorithm of Reverse Vaccinology. If we accept that in principle each parasite protein contains functional MHC I and II binding peptides and, thus, potential T cell epitopes, we may simply base our estimates on the number of protein molecules per parasite ($\sim 5 \times 10^7$ molecules). Similarly, if we agree that both CD4 and CD8 cells are relevant for protection, we can base our analysis on the number of MHC class I molecules expressed per antigen-presenting cell ($\sim 10^5$ molecules) since this is thought to be lower than the number of class II molecules, hence can be considered the limiting peptide receptor species. To illustrate the next steps, I will base my arguments on a data set published by our group. We aimed at identifying the relative abundance of proteins in amastigotes of *L. mexicana* based on a label free method that deduces a protein abundance index (emPAI) for each protein in a data set (66, 74). The reason for this choice is simply that equivalent data is not easily accessible in other comparable proteome data sets. When parasite proteins are ranked according to their emPAI value, it is quickly realized that proteins encoded by less than 50 and 200 genes contribute more than 25 and 50% of the total parasite protein content in terms of mass (**Figure 1**).

These relative values can be expressed as number of molecules per parasite taking into consideration the respective molecular weight and the total protein content per parasite (~4 pg). Thus, the copy number per cell of proteins detected in current proteomic analyses ranges from a few million to a couple of 100 molecules. MHC–peptide complex formation, however, ultimately follows the law of mass, hence abundant molecules have a greater chance of becoming processed and ensuing peptides bound to the MHC-binding groove. The simplest version of predicting the chance of a protein to be successful in this respect is to calculate an expected value for how often this may be the case if 10^5 MHC molecules are allowed to dip into the compartment where the peptides are formed and pick a peptide (remember as a further simplification, we equal 1 protein to 1 epitope). The expected value of MHC–peptide complexes for each protein in the data set can be plotted in an ordered way according to protein abundance, which produces

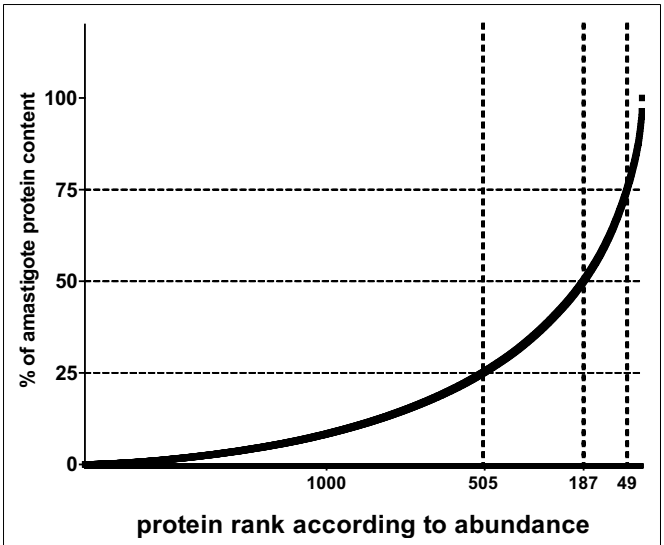


FIGURE 1 | Relative contribution of individual proteins to total protein content of *Leishmania* amastigotes. For illustration, the contribution of each of 1764 proteins detected by shot gun proteomics in *L. mexicana* amastigotes (66) is expressed as percent to total protein mass and values plotted in ordered fashion for each protein. Numbers on *X*-axes show the rank of the *n*th protein at the thresholds of 75, 50, and 25% of total mass.

an S-shaped curve (**Figure 2**). For candidate prediction purposes, it is then necessary to try to define rationally a threshold below which the chance of a peptide species to be bound by a stimulatory number of MHC molecules becomes negligible. One way to set this threshold is to adopt the number of surface MHC–epitope complexes required for stimulating T cells as defined by immunologists. As mentioned before a reasonable estimate for this is in the order of 100 complexes, which is indicated by a horizontal line in **Figure 2**. The expected number of MHC-peptides was calculated for experimentally validated, naturally immunogenic proteins and, indeed, for the majority the expected number is above this threshold (**Figure 2**; green shaded area of plot). A complementary way to define the threshold is by extrapolation of experimental data on individual parasite proteins that were assayed either in vaccination studies or in T cell stimulation tests. Importantly, there is experimental evidence for a lower boundary of the protein copy number per cell value at which infected macrophages do no longer stimulate the respective antigen-specific CD4 T cells (40). This threshold is indicated as a blue dotted line in **Figure 2**.

The presented approach is easily expanded or adapted to additional proteomic data sets when information on relative protein abundance becomes available. It reveals not only the likely reason why most experimentally studied antigens were immuno- and antigenic but also defines a large number of additional candidates. In contrast, the majority of the candidates predicted purely by bioinformatics (28, 30) were not in the proteome data set. This may indeed indicate that their respective copy number per parasite was below detection levels of the method (which is then likely to be also below the detection level of the MHC presentation machinery). However, this conclusion has to be drawn with caution as the likelihood of detecting the protein by proteomics can be reduced

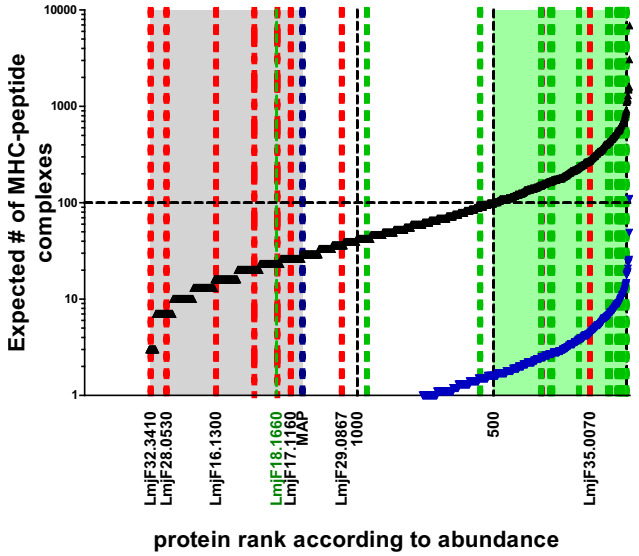


FIGURE 2 | Expected number of individual MHC–peptide complexes depending on protein abundance. The black curve indicates expected number of complexes assuming protein copy number is most determining. Blue curve indicates lower boundary of the model basing expected number of complexes on the assumption that all protein are first degraded to peptides. The likelihood of complex formation for a peptide derived from the average size protein (52 kDa) is thus reduced 36-fold [i.e., the average number of predicted epitopes deducted from Herrera-Najera et al. (30)]. Green shaded area in plot: proteins above the threshold of 100 MHC–peptide complexes when sampled by 10^5 MHC molecules assuming one binding peptide per protein. Area shaded in gray: proteins with ranks below that of lysosomal membrane acid phosphatase (MAP; blue dotted line) for which the corresponding molecule number per parasite was experimentally shown to be non-stimulatory for T cells. Green dotted lines indicate ranks of proteins with experimental evidence of T cell recognition (in ascending order GRP78, HSP83, Histone H-2, STI-1, CSP-B, Glu synthetase, ATP synthase, LACK, LeIF, TSA, gp63, KMP-11, HSP20, 60S ribosomal protein, nucleoside hydrolase, amastin, SMT, and γ-glutamylcysteine synthetase = LmjF18.1660). Blue dotted line indicates lysosomal membrane acid phosphatase (MAP) for which the corresponding molecule number per parasite was experimentally shown to be non-stimulatory for T cells. Red dotted lines refer to the rank of proteins identified *in silico* to contain candidate epitopes by Herrera-Najera et al. (30) (again in ascending order, LmjF35.0070, LmjF29.0867, LmjF17.1160, LmjF16.1300, LmjF28.0530, and LmjF32.3410), or John et al. (28) (red stippled line: PI-3 Kinase like protein, lipase).

for technical reasons, which is the case, e.g., for integral membrane proteins [see also Ref. (66)]. The latter however can be reasonably well-predicted through bioinformatics analysis.

Of course an algorithm as presented above, that integrates protein abundance to derive the set of likely immunogenic and hence vaccine candidate proteins, is simplistic. But, its advantages are its practical value and high flexibility since any change in parameters can be easily accommodated. Changing parameters will essentially only re-position the threshold value for the effective number of MHC–peptide complexes. For example, the threshold may change if dynamic sampling of the peptide pool by recycling MHC is integrated over the time of an infection cycle. In this case, peptide off-rates from MHC–peptide complexes may be a valuable, bioinformatically accessible factor to improve the algorithm. It has been shown that kinetic stability of MHC–peptide complexes is probably the single most important determinant that defines immunodominant T cell epitopes (75). Furthermore, dynamic exchange of weakly binding peptides for more stably bound peptides has been shown to occur upon MHC-peptide recycling from and to the plasma membrane (76). Thus, in theory the algorithm for ranking candidates may include a weighting factor based on predicted peptide off-rates from their MHC receptors. This factor may be multiplied by protein/peptide abundance to derive an "effective concentration" of a particular peptide. A high effective concentration may be the reason underlying the efficacy of leishmanial γ-glutamylcysteine synthetase as an effective vaccine in animal models of *Leishmania donovani* infection (77, 78). Alternatively, this antigen may be more abundantly expressed in *L. donovani* than suggested by the data derived from *L. mexicana* that were used here for illustration. Consistent with the latter idea, the same γ-glutamylcysteine synthetase-based vaccines were less effective against *L. mexicana* (79). Unfortunately, experimental data on an exemplary set of antigens to derive such a weighting factor are lacking and given the uncertainties associated with MHC-peptide ligand predicting algorithms the practical value of such a factor is currently difficult to assess.

In summary, developing an algorithm to adapt Reverse Vaccinology for the identification of antigens for anti-VL vaccine should include as a first step quantitative aspects of protein expression and incorporate the growing resource of proteomic data sets. On its own, however, this approach still leaves one with some 500 candidates. Selection against epitopes with homology to host proteins is certainly advisable but one should be aware of its limitations and the gargantuan dimension of its unknowns due to the fact that T cells recognize a sequence space (63). If adopted, the definition of the immunological self should probably include commensals (80). Thus, selection against peptides with homology to host proteins seems on the one hand not rigorous enough and, on the other hand, appears to adopt a functionally limited if not wrong concept of self. Nonetheless, integration of this information and data on predicted candidate antigen localization, MHC-peptide stability, conservation between parasites and selection of genus-specific antigens may all be criteria of practical value. It should be noted though that the latter two are common sense criteria but there is scarcely any experimental data (81) to validate them.

ACKNOWLEDGMENTS

This work was supported by grant Ae16/5-1 to Toni Aebischer from the Deutsche Forschungsgemeinschaft within the Priority program SPP1580.

REFERENCES

1. Singh B, Sundar S. Leishmaniasis: vaccine candidates and perspectives. *Vaccine* (2012) **30**(26):3834–42. doi:10.1016/j.vaccine.2012.03.068
2. Kedzierski L. Leishmaniasis vaccine: where are we today? *J Glob Infect Dis* (2010) **2**(2):177–85. doi:10.4103/0974-777X.62881
3. Mathers CD, Ezzati M, Lopez AD. Measuring the burden of neglected tropical diseases: the global burden of disease framework. *PLoS Negl Trop Dis* (2007) **1**(2):e114. doi:10.1371/journal.pntd.0000114
4. Olliaro PL, Guerin PJ, Gerstl S, Haaskjold AA, Rottingen JA, Sundar S. Treatment options for visceral leishmaniasis: a systematic review of clinical studies done

in India, 1980-2004. *Lancet Infect Dis* (2005) **5**(12):763–74. doi:10.1016/S1473-3099(05)70296-6
5. Lee BY, Bacon KM, Shah M, Kitchen SB, Connor DL, Slayton RB. The economic value of a visceral leishmaniasis vaccine in Bihar state, India. *Am J Trop Med Hyg* (2012) **86**(3):417–25. doi:10.4269/ajtmh.2012.10-0415
6. Fernandes AP, Coelho EA, Machado-Coelho GL, Grimaldi GJ, Gazzinelli RT. Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives. *Curr Opin Microbiol* (2012) **15**(4):476–85. doi:10.1016/j.mib.2012.05.002
7. Belkaid Y, Von SE, Mendez S, Lira R, Caler E, Bertholet S, et al. CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with *Leishmania major*. *J Immunol* (2002) **168**(8):3992–4000. doi:10.4049/jimmunol.168.8.3992
8. Gautam S, Kumar R, Singh N, Singh AK, Rai M, Sacks D, et al. CD8 T cell exhaustion in human visceral leishmaniasis. *J Infect Dis* (2014) **209**(2):290–9. doi:10.1093/infdis/jit401
9. Nylen S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D. Splenic accumulation of IL-10 mRNA in T cells distinct from $CD4^+CD25^+$ (Foxp3) regulatory T cells in human visceral leishmaniasis. *J Exp Med* (2007) **204**(4):805–17. doi:10.1084/jem.20061141
10. Stauch A, Sarkar RR, Picado A, Ostyn B, Sundar S, Rijal S, et al. Visceral leishmaniasis in the Indian subcontinent: modelling epidemiology and control. *PLoS Negl Trop Dis* (2011) **5**(11):e1405. doi:10.1371/journal.pntd.0001405
11. Ostyn B, Gidwani K, Khanal B, Picado A, Chappuis F, Singh SP, et al. Incidence of symptomatic and asymptomatic *Leishmania donovani* infections in high-endemic foci in India and Nepal: a prospective study. *PLoS Negl Trop Dis* (2011) **5**(10):e1284. doi:10.1371/journal.pntd.0001284
12. Nadim A, Javadian E, Tahvildar-Bidruni G, Ghorbani M. Effectiveness of leishmanization in the control of cutaneous leishmaniasis. *Bull Soc Pathol Exot Filiales* (1983) **76**(4):377–83.
13. Hosseini SM, Hatam GR, Ardehali S. Characterization of *Leishmania* isolated from unhealed lesions caused by leishmanization. *East Mediterr Health J* (2005) **11**(1–2):240–3.
14. Khamesipour A, Dowlati Y, Asilian A, Hashemi-Fesharki R, Javadi A, Noazin S, et al. Leishmanization: use of an old method for evaluation of candidate vaccines against leishmaniasis. *Vaccine* (2005) **23**(28):3642–8. doi:10.1016/j.vaccine.2005.02.015
15. Dey R, Dagur PK, Selvapandiyan A, McCoy JP, Salotra P, Duncan R, et al. Live attenuated *Leishmania donovani* p27 gene knockout parasites are nonpathogenic and elicit long-term protective immunity in BALB/c mice. *J Immunol* (2013) **190**(5):2138–49. doi:10.4049/jimmunol.1202801
16. Spath GF, Lye LF, Segawa H, Turco SJ, Beverley SM. Identification of a compensatory mutant (lpg2-REV) of *Leishmania major* able to survive as amastigotes within macrophages without LPG2-dependent glycoconjugates and its significance to virulence and immunization strategies. *Infect Immun* (2004) **72**(6):3622–7. doi:10.1128/IAI.72.6.3622-3627.2004
17. Titus RG, Gueiros-Filho FJ, de Freitas LA, Beverley SM. Development of a safe live *Leishmania* vaccine line by gene replacement. *Proc Natl Acad Sci U S A* (1995) **92**(22):10267–71. doi:10.1073/pnas.92.22.10267
18. Fiuza JA, Santiago Hda C, Selvapandiyan A, Gannavaram S, Ricci ND, Bueno LL, et al. Induction of immunogenicity by live attenuated *Leishmania donovani* centrin deleted parasites in dogs. *Vaccine* (2013) **31**(14):1785–92. doi:10.1016/j.vaccine.2013.01.048
19. Saljoughian N, Taheri T, Rafati S. Live vaccination tactics: possible approaches for controlling visceral leishmaniasis. *Front Immunol* (2014) **5**:134. doi:10.3389/fimmu.2014.00134
20. Coler RN, Reed SG. Second-generation vaccines against leishmaniasis. *Trends Parasitol* (2005) **21**(5):244–9. doi:10.1016/j.pt.2005.03.006
21. Darrah PA, Hegde ST, Patel DT, Lindsay RW, Chen L, Roederer M, et al. IL-10 production differentially influences the magnitude, quality, and protective capacity of Th1 responses depending on the vaccine platform. *J Exp Med* (2010) **207**(7):1421–33. doi:10.1084/jem.20092532
22. Maroof A, Brown N, Smith B, Hodgkinson MR, Maxwell A, Losch FO, et al. Therapeutic vaccination with recombinant adenovirus reduces splenic parasite burden in experimental visceral leishmaniasis. *J Infect Dis* (2012) **205**(5):853–63. doi:10.1093/infdis/jir842
23. Yang DM, Fairweather N, Button LL, McMaster WR, Kahl LP, Liew FY. Oral *Salmonella typhimurium* (AroA−) vaccine expressing a major leishmanial surface protein (gp63) preferentially induces T helper 1 cells and protective immunity against leishmaniasis. *J Immunol* (1990) **145**(7):2281–5.
24. Schroeder J, Aebischer T. Vaccines for leishmaniasis: from proteome to vaccine candidates. *Hum Vaccin* (2011) **7**:doi:10.4161/hv.7.0.14556
25. Singh OP, Gidwani K, Kumar R, Nylen S, Jones SL, Boelaert M, et al. Reassessment of immune correlates in human visceral leishmaniasis as defined by cytokine release in whole blood. *Clin Vaccine Immunol* (2012) **19**(6):961–6. doi:10.1128/CVI.00143-12
26. Rappuoli R. Reverse vaccinology. *Curr Opin Microbiol* (2000) **3**(5):445–50. doi:10.1016/S1369-5274(00)00119-3
27. Pizza M, Scarlato V, Masignani V, Giuliani MM, Arico B, Comanducci M, et al. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science* (2000) **287**(5459):1816–20. doi:10.1126/science.287.5459.1816
28. John L, John GJ, Kholia T. A reverse vaccinology approach for the identification of potential vaccine candidates from *Leishmania* spp. *Appl Biochem Biotechnol* (2012) **167**(5):1340–50. doi:10.1007/s12010-012-9649-0
29. Resende DM, Rezende AM, Oliveira NJ, Batista IC, Correa-Oliveira R, Reis AB, et al. An assessment on epitope prediction methods for protozoa genomes. *BMC Bioinformatics* (2012) **13**:309. doi:10.1186/1471-2105-13-309
30. Herrera-Najera C, Pina-Aguilar R, Xacur-Garcia F, Ramirez-Sierra MJ, Dumonteil E. Mining the *Leishmania* genome for novel antigens and vaccine candidates. *Proteomics* (2009) **9**(5):1293–301. doi:10.1002/pmic.200800533
31. Ouakad M, Bahi-Jaber N, Chenik M, Dellagi K, Louzir H. Selection of endogenous reference genes for gene expression analysis in *Leishmania major* developmental stages. *Parasitol Res* (2007) **101**(2):473–7. doi:10.1007/s00436-007-0491-1
32. Ben-Othman R, Guizani-Tabbane L, Dellagi K. *Leishmania* initially activates but subsequently down-regulates intracellular mitogen-activated protein kinases and nuclear factor-kappaB signaling in macrophages. *Mol Immunol* (2008) **45**(11):3222–9. doi:10.1016/j.molimm.2008.02.019
33. Antoine JC, Prina E, Jouanne C, Bongrand P. Parasitophorous vacuoles of *Leishmania amazonensis*-infected macrophages maintain an acidic pH. *Infect Immun* (1990) **58**(3):779–87.
34. Courret N, Frehel C, Gouhier N, Pouchelet M, Prina E, Roux P, et al. Biogenesis of *Leishmania*-harbouring parasitophorous vacuoles following phagocytosis of the metacyclic promastigote or amastigote stages of the parasites. *J Cell Sci* (2002) **115**:2303–16.
35. Burchmore RJ, Barrett MP. Life in vacuoles – nutrient acquisition by *Leishmania* amastigotes. *Int J Parasitol* (2001) **31**(12):1311–20. doi:10.1016/S0020-7519(01)00259-4
36. Collins HL, Schaible UE, Ernst JD, Russell DG. Transfer of phagocytosed particles to the parasitophorous vacuole of *Leishmania mexicana* is a transient phenomenon preceding the acquisition of annexin I by the phagosome. *J Cell Sci* (1997) **110**(Pt 2):191–200.
37. Muraille E, Cazareth J, Gounon P, Hoebeke J, Lippuner C, Soares H, et al. Visualization of peptide/MHC complexes at the surface and in the intracellular compartments of cells infected in vivo by *Leishmania major*. *PLoS Pathog* (2010) **6**(10):e1001154. doi:10.1371/journal.ppat.1001154
38. Lambertz U, Silverman JM, Nandan D, McMaster WR, Clos J, Foster LJ, et al. Secreted virulence factors and immune evasion in visceral leishmaniasis. *J Leukoc Biol* (2012) **91**(6):887–99. doi:10.1189/jlb.0611326
39. Silverman JM, Clos J, Horakova E, Wang AY, Wiesgigl M, Kelly I, et al. *Leishmania* exosomes modulate innate and adaptive immune responses through effects on monocytes and dendritic cells. *J Immunol* (2010) **185**(9):5011–22. doi:10.4049/jimmunol.1000541
40. Wolfram M, Fuchs M, Wiese M, Stierhof YD, Overath P. Antigen presentation by *Leishmania mexicana*-infected macrophages: activation of helper T cells by a model parasite antigen secreted into the parasitophorous vacuole or expressed on the amastigote surface. *Eur J Immunol* (1996) **26**(12):3153–62. doi:10.1002/eji.1830261248
41. Bertholet S, Goldszmid R, Morrot A, Debrabant A, Afrin F, Collazo-Custodio C, et al. *Leishmania* antigens are presented to CD8+ T cells by a transporter associated with antigen processing-independent pathway in vitro and in vivo. *J Immunol* (2006) **177**(6):3525–33. doi:10.4049/jimmunol.177.6.3525
42. Prickett S, Gray PM, Colpitts SL, Scott P, Kaye PM, Smith DF. In vivo recognition of ovalbumin expressed by transgenic *Leishmania* is determined by

its subcellular localization. *J Immunol* (2006) **176**(8):4826–33. doi:10.4049/jimmunol.176.8.4826
43. Bertholet S, Debrabant A, Afrin F, Caler E, Mendez S, Tabbara KS, et al. Antigen requirements for efficient priming of CD8+ T cells by *Leishmania major*-infected dendritic cells. *Infect Immun* (2005) **73**(10):6620–8. doi:10.1128/IAI.73.10.6620-6628.2005
44. Misslitz AC, Bonhagen K, Harbecke D, Lippuner C, Kamradt T, Aebischer T. Two waves of antigen-containing dendritic cells in vivo in experimental *Leishmania major* infection. *Eur J Immunol* (2004) **34**(3):715–25. doi:10.1002/eji.200324391
45. Schmid M, Wege AK, Ritter U. Characteristics of "Tip-DCs and MDSCs" and their potential role in leishmaniasis. *Front Microbiol* (2012) **3**:74. doi:10.3389/fmicb.2012.00074
46. Filipe-Santos O, Pescher P, Breart B, Lippuner C, Aebischer T, Glaichenhaus N, et al. A dynamic map of antigen recognition by CD4 T cells at the site of *Leishmania major* infection. *Cell Host Microbe* (2009) **6**(1):23–33. doi:10.1016/j.chom.2009.04.014
47. Muller AJ, Filipe-Santos O, Eberl G, Aebischer T, Spath GF, Bousso P. CD4+ T cells rely on a cytokine gradient to control intracellular pathogens beyond sites of antigen presentation. *Immunity* (2012) **37**(1):147–57. doi:10.1016/j.immuni.2012.05.015
48. Wolfram M, Ilg T, Mottram JC, Overath P. Antigen presentation by *Leishmania mexicana*-infected macrophages: activation of helper T cells specific for amastigote cysteine proteinases requires intracellular killing of the parasites. *Eur J Immunol* (1995) **25**(4):1094–100. doi:10.1002/eji.1830250435
49. Courret N, Prina E, Mougneau E, Saraiva EM, Sacks DL, Glaichenhaus N, et al. Presentation of the *Leishmania* antigen LACK by infected macrophages is dependent upon the virulence of the phagocytosed parasites. *Eur J Immunol* (1999) **29**(3):762–73. doi:10.1002/(SICI)1521-4141(199903)29:03<762::AID-IMMU762>3.0.CO;2-4
50. Inaba K, Turley S, Iyoda T, Yamaide F, Shimoyama S, Reis e Sousa C, et al. The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. *J Exp Med* (2000) **191**(6):927–36. doi:10.1084/jem.191.6.927
51. Santambrogio L, Sato AK, Fischer FR, Dorf ME, Stern LJ. Abundant empty class II MHC molecules on the surface of immature dendritic cells. *Proc Natl Acad Sci U S A* (1999) **96**(26):15050–5. doi:10.1073/pnas.96.26.15050
52. Pierre P, Turley SJ, Gatti E, Hull M, Meltzer J, Mirza A, et al. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* (1997) **388**(6644):787–92. doi:10.1038/42039
53. Yewdell JW, Reits E, Neefjes J. Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nat Rev Immunol* (2003) **3**(12):952–61. doi:10.1038/nri1250
54. Delamarre L, Holcombe H, Mellman I. Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is differentially regulated during dendritic cell maturation. *J Exp Med* (2003) **198**(1):111–22. doi:10.1084/jem.20021542
55. Su RC, Miller RG. Stability of surface H-2K(b), H-2D(b), and peptide-receptive H-2K(b) on splenocytes. *J Immunol* (2001) **167**(9):4869–77. doi:10.4049/jimmunol.167.9.4869
56. Sykulev Y, Joo M, Vturina I, Tsomides TJ, Eisen HN. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity* (1996) **4**(6):565–71. doi:10.1016/S1074-7613(00)80483-5
57. Kimachi K, Croft M, Grey HM. The minimal number of antigen-major histocompatibility complex class II complexes required for activation of naive and primed T cells. *Eur J Immunol* (1997) **27**(12):3310–7. doi:10.1002/eji.1830271230
58. Manz BN, Jackson BL, Petit RS, Dustin ML, Groves J. T-cell triggering thresholds are modulated by the number of antigen within individual T-cell receptor clusters. *Proc Natl Acad Sci U S A* (2011) **108**(22):9089–94. doi:10.1073/pnas.1018771108
59. Overath P, Aebischer T. Antigen presentation by macrophages harboring intravesicular pathogens. *Parasitol Today* (1999) **15**(8):325–32. doi:10.1016/S0169-4758(99)01473-8
60. Ivens AC, Peacock CS, Worthey EA, Murphy L, Aggarwal G, Berriman M, et al. The genome of the kinetoplastid parasite, *Leishmania major*. *Science* (2005) **309**(5733):436–42. doi:10.1126/science.1112680
61. Newell EW, Ely LK, Kruse AC, Reay PA, Rodriguez SN, Lin AE, et al. Structural basis of specificity and cross-reactivity in T cell receptors specific for cytochrome c-I-E(k). *J Immunol* (2011) **186**(10):5823–32. doi:10.4049/jimmunol.1100197
62. Sharav T, Wiesmuller KH, Walden P. Mimotope vaccines for cancer immunotherapy. *Vaccine* (2007) **25**(16):3032–7. doi:10.1016/j.vaccine.2007.01.033
63. Hastings KT. GILT: shaping the MHC class II-restricted peptidome and CD4 T cell-mediated immunity. *Front Immunol* (2013) **4**:429. doi:10.3389/fimmu.2013.00429
64. Olekhnovitch R, Ryffel B, Muller AJ, Bousso P. Collective nitric oxide production provides tissue-wide immunity during *Leishmania* infection. *J Clin Invest* (2014) **124**(4):1711–22. doi:10.1172/JCI72058
65. Aebischer T, Harbecke D, Ilg T. Proteophosphoglycan, a major secreted product of intracellular *Leishmania mexicana* amastigotes, is a poor B-cell antigen and does not elicit a specific conventional CD4+ T-cell response. *Infect Immun* (1999) **67**(10):5379–85.
66. Paape D, Barrios-Llerena ME, Le BT, Mackay L, Aebischer T. Gel free analysis of the proteome of intracellular *Leishmania mexicana*. *Mol Biochem Parasitol* (2010) **169**(2):108–14. doi:10.1016/j.molbiopara.2009.10.009
67. Nirujogi RS, Pawar H, Renuse S, Kumar P, Chavan S, Sathe G, et al. Moving from unsequenced to sequenced genome: reanalysis of the proteome of *Leishmania donovani*. *J Proteomics* (2014) **97**:48–61. doi:10.1016/j.jprot.2013.04.021
68. Pescher P, Blisnick T, Bastin P, Spath GF. Quantitative proteome profiling informs on phenotypic traits that adapt *Leishmania donovani* for axenic and intracellular proliferation. *Cell Microbiol* (2011) **13**(7):978–91. doi:10.1111/j.1462-5822.2011.01593.x
69. Paape D, Aebischer T. Contribution of proteomics of *Leishmania* spp. to the understanding of differentiation, drug resistance mechanisms, vaccine and drug development. *J Proteomics* (2011) **74**(9):1614–24. doi:10.1016/j.jprot.2011.05.005
70. Sardar AH, Kumar S, Kumar A, Purkait B, Das S, Sen A, et al. Proteome changes associated with *Leishmania donovani* promastigote adaptation to oxidative and nitrosative stresses. *J Proteomics* (2013) **81**:185–99. doi:10.1016/j.jprot.2013.01.011
71. Biyani N, Madhubala R. Quantitative proteomic profiling of the promastigotes and the intracellular amastigotes of *Leishmania donovani* isolates identifies novel proteins having a role in *Leishmania* differentiation and intracellular survival. *Biochim Biophys Acta* (2012) **1824**(12):1342–50. doi:10.1016/j.bbapap.2012.07.010
72. Singh AK, Roberts S, Ullman B, Madhubala R. A quantitative proteomic screen to identify potential drug resistance mechanism in alpha-difluoromethylornithine (DFMO) resistant *Leishmania donovani*. *J Proteomics* (2014) **102**:44–59. doi:10.1016/j.jprot.2014.02.030
73. Santarem N, Racine G, Silvestre R, Cordeiro-da-Silva A, Ouellette M. Exoproteome dynamics in *Leishmania infantum*. *J Proteomics* (2013) **84**:106–18. doi:10.1016/j.jprot.2013.03.012
74. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, et al. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol Cell Proteomics* (2005) **4**(9):1265–72. doi:10.1074/mcp.M500061-MCP200
75. Lazarski CA, Chaves FA, Jenks SA, Wu S, Richards KA, Weaver JM, et al. The kinetic stability of MHC class II:peptide complexes is a key parameter that dictates immunodominance. *Immunity* (2005) **23**(1):29–40. doi:10.1016/j.immuni.2005.05.009
76. Sijts AJ, Pamer EG. Enhanced intracellular dissociation of major histocompatibility complex class I-associated peptides: a mechanism for optimizing the spectrum of cell surface-presented cytotoxic T lymphocyte epitopes. *J Exp Med* (1997) **185**(8):1403–11. doi:10.1084/jem.185.8.1403
77. Carter KC, Henriquez FL, Campbell SA, Roberts CW, Nok A, Mullen AB, et al. DNA vaccination against the parasite enzyme gamma-glutamylcysteine synthetase confers protection against *Leishmania donovani* infection. *Vaccine* (2007) **25**(22):4502–9. doi:10.1016/j.vaccine.2007.03.014
78. Henriquez FL, Campbell SA, Roberts CW, Mullen AB, Burchmore R, Carter KC. Vaccination with recombinant *Leishmania donovani* gamma-glutamylcysteine

synthetase fusion protein protects against *L. donovani* infection. *J Parasitol* (2010) **96**(5):929–36. doi:10.1645/GE-2360.1

79. Campbell SA, Alawa J, Doro B, Henriquez FL, Roberts CW, Nok A, et al. Comparative assessment of a DNA and protein *Leishmania donovani* gamma glutamyl cysteine synthetase vaccine to cross-protect against murine cutaneous leishmaniasis caused by *L. major* or *L. mexicana* infection. *Vaccine* (2012) **30**(7):1357–63. doi:10.1016/j.vaccine.2011.12.067
80. Julia V, McSorley SS, Malherbe L, Breittmayer JP, Girard-Pipau F, Beck A, et al. Priming by microbial antigens from the intestinal flora determines the ability of CD4+ T cells to rapidly secrete IL-4 in BALB/c mice infected with *Leishmania major. J Immunol* (2000) **165**(10):5637–45. doi:10.4049/jimmunol.165.10.5637
81. Schroeder J, Brown N, Kaye P, Aebischer T. Single dose novel *Salmonella* vaccine enhances resistance against visceralizing *L. major* and *L. donovani* infection in susceptible BALB/c mice. *PLoS Negl Trop Dis* (2011) **5**(12):e1406. doi:10.1371/journal.pntd.0001406

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 March 2014; accepted: 19 May 2014; published online: 10 June 2014.
*Citation: Aebischer T (2014) Leishmania spp. proteome data sets: a comprehensive resource for vaccine development to target visceral leishmaniasis. Front. Immunol. **5**:260. doi: 10.3389/fimmu.2014.00260*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 01 May 2014
doi: 10.3389/fimmu.2014.00189

Cross-protective immunity to *Leishmania amazonensis* is mediated by CD4+ and CD8+ epitopes of *Leishmania donovani* nucleoside hydrolase terminal domains

Dirlei Nico[1], Daniele Crespo Gomes[1], Marcus Vinícius Alves-Silva[1], Elisangela Oliveira Freitas[1], Alexandre Morrot[2], Diana Bahia[3,4], Marcos Palatnik[5], Mauricio M. Rodrigues[6] and Clarisa B. Palatnik-de-Sousa[1]*

[1] *Laboratório de Biologia e Bioquímica de Leishmania, Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[2] *Laboratório de Imunologia, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[3] *Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil*
[4] *Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil*
[5] *Programa de Pós Graduação em Clínica Médica Faculdade de Medicina, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[6] *Departamento de Microbiologia, Imunologia e Parasitologia, Centro de Terapia Celular e Molecular, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil*

Edited by:
Nahid Ali, Indian Institute of Chemical Biology, India

Reviewed by:
Katsuyuki Yui, Nagasaki University, Japan
John William Greiner, National Institutes of Health, USA

****Correspondence:***
Clarisa B. Palatnik-de-Sousa, Laboratório de Biologia e Bioquímica de Leishmania, Instituto de Microbiologia Paulo de Góes, CCS, Universidade Federal do Rio de Janeiro, Avda. Carlos Chagas 373, Cidade Universitária, Ilha do Fundão, Caixa Postal 68040, Rio de Janeiro, Rio de Janeiro CEP 21941-902, Brazil
e-mail: immgcpa@micro.ufrj.br

The nucleoside hydrolase (NH) of *Leishmania donovani* (NH36) is a phylogenetic marker of high homology among *Leishmania* parasites. In mice and dog vaccination, NH36 induces a CD4+ T cell-driven protective response against *Leishmania chagasi* infection directed against its C-terminal domain (F3). The C-terminal and N-terminal domain vaccines also decreased the footpad lesion caused by *Leishmania amazonensis*. We studied the basis of the crossed immune response using recombinant generated peptides covering the whole NH36 sequence and saponin for mice prophylaxis against *L. amazonensis*. The F1 (amino acids 1–103) and F3 peptide (amino acids 199–314) vaccines enhanced the IgG and IgG2a anti-NH36 antibodies to similar levels. The F3 vaccine induced the strongest DTH response, the highest proportions of NH36-specific CD4+ and CD8+ T cells after challenge and the highest expression of IFN-γ and TNF-α. The F1 vaccine, on the other hand, induced a weaker but significant DTH response and a mild enhancement of IFN-γ and TNF-α levels. The *in vivo* depletion with anti-CD4 or CD8 monoclonal antibodies disclosed that cross-protection against *L. amazonensis* infection was mediated by a CD4+ T cell response directed against the C-terminal domain (75% of reduction of the size of footpad lesion) followed by a CD8+ T cell response against the N-terminal domain of NH36 (57% of reduction of footpad lesions). Both vaccines were capable of inducing long-term cross-immunity. The amino acid sequence of NH36 showed 93% identity to the sequence of the NH A34480 of *L. amazonensis*, which also showed the presence of completely conserved predicted epitopes for CD4+ and CD8+ T cells in F1 domain, and of CD4+ epitopes differing by a single amino acid, in F1 and F3 domains. The identification of the C-terminal and N-terminal domains as the targets of the immune response to NH36 in the model of *L. amazonensis* infection represents a basis for the rationale development of a bivalent vaccine against leishmaniasis.

Keywords: visceral leishmaniasis, cutaneous leishmaniasis, diffuse cutaneous leishmaniasis, cross-protection, prophylaxis, nucleoside hydrolases, recombinant vaccines

INTRODUCTION

Leishmaniasis is considered a severe public health problem with 12 million people currently infected, 350 million at risk (1, 2), and 4 clinical syndromes due to different *Leishmania* species: cutaneous (CL) (3–5), diffuse (DCL) (3), mucocutaneous (MCL), and visceral (VL). A bivalent vaccine that could generate protective immunity to the agents of the visceral and cutaneous syndromes would be economic and useful for the control of leishmaniasis (6) in countries where both diseases are endemic. First, second, and third generation vaccines have been developed against leishmaniasis (7, 8). Among the vaccines tested in the field, most are crude parasite vaccines against CL, with or without adjuvants (9, 10) that induced a maximum of 50% vaccine efficacy (9). The recombinant Leish-111f vaccine, on the other hand, was useful in the immunotherapy and immunochemotherapy of patients with CL and MCL (8) and in prophylaxis (11) but not in the therapy of canine VL (12). No human vaccine is available against VL.

The Leishmune® veterinary vaccine against canine VL (13–16) contributed to the reduction of the incidence of the human and canine diseases (17). Its main component is the nucleoside

hydrolase (NH) of *Leishmania donovani* (NH36) (18, 19). NHs release purines and pyrimidines from imported nucleosides, allow the synthesis of parasite DNA and its replication (20, 21) and are mandatory at the early infection. NH36 is a powerful antigen (22), a marker of the *Leishmania* genus (23, 24), which shows high homology to the sequences of NHs of other *Leishmania* species (25, 26), being thus a good candidate for a cross-protective bivalent *Leishmania* vaccine.

NH36 protected mice from *L. donovani* infection (27) and was identified among *Leishmania major* exo-antigens (28). As a genetic vaccine, it induced a TH1 immune response mediated by IFN-γ-producing CD4+ T cells (29) effective in mice prophylaxis against VL (30) and CL (28–31) and in mice (32) and dog immunotherapy against VL (33) indicating its potential use against both leishmaniasis.

Three recombinant peptides of NH36 representing the amino acids 1–103 (F1, N-terminal domain), 104–198 (F2, central domain), and 199–314 (F3, C-terminal domain) respectively, were generated and used to vaccinate mice (34). Protection against *Leishmania chagasi* was related to the C-terminal domain and was mainly mediated by a CD4+ T cell-driven response with a lower contribution of CD8+ T cells (34). Preliminary results indicated that, on other hand, both the C- and N-terminal domains determined the reduction of the size of footpad lesions of mice challenged with *Leishmania amazonensis* (34).

In this investigation, we aimed to study the cross-immunity generated by the peptide domains of NH36 of *L. donovani* used for prophylactic vaccination of mice against *L. amazonensis*. In order to study the generation of the humoral and cellular immune responses responsible for and to identify in this way, the immunogenic domains of NH36, which should be included in a potential future bivalent vaccine against VL and CL. We identified that the cross-protective efficacy responsible for protection against *L. amazonensis* was related to epitopes for CD4+ T cells of the C-terminal and epitopes for CD8+ T cells of the N-terminal domains of the NH, NH36.

MATERIALS AND METHODS

ETHICAL STATEMENTS

All mouse studies followed the guidelines set by the National Institute of Health, USA, the EU Directive 2010/63/EU, and the Institutional Animal Care and Use Committee approved the animal protocols (Biophysics Institute-UFRJ, Brazil, and protocol IMPPG-007). All procedures and euthanasia were performed under CO_2 anesthesia, and all efforts were made to minimize suffering.

RECOMBINANT PEPTIDES OF THE NH36 NUCLEOSIDE HYDROLASE OF *LEISHMANIA DONOVANI* AND HOMOLOGY TO NH OF *LEISHMANIA AMAZONENSIS*

NH36 is composed of 314 amino acids (EMBL, GenBank™, and DDJB data bases, access number AY007193). Three fragments of the NH36 antigen composed, respectively, of the amino acid sequences 1–103 (F1), 104–198 (F2), and 199–314 (F3) were cloned in the pET28b plasmid system (34) (Patent: INPI Brazil PI 1015788-3.PCT/BR2011/000411) and expressed in *Escherichia coli* Bl21DE3 cells and purified in a Ni-NTA column (Qiagen). The fractions containing highly purified recombinant protein were extensively dialyzed against PBS buffer and stored at −80°C. To improve protein expression, F2 was further cloned in the pET28a (34). For homology analysis, we used the sequence of *L. amazonensis* NH A34480 (Scaffold1680 15191–16135) (35). The sequence alignment was obtained using the BLASTP of the GenBank.

PROPHYLACTIC IMMUNIZATION, PARASITE CHALLENGE BY *L. AMAZONENSIS*, AND ASSESSMENT OF PROTECTION

Eight-week-old female Balb/c mice were vaccinated three times with 100 μg of NH36, F1, F2, or F3 recombinant proteins and 100 μg of SIGMA saponin (NH36sap, F1sap, F2sap, and F3sap vaccines, respectively) at weekly intervals, by the sc route. At week 4, mice were challenged in the right hind footpad with 10^5 *L. amazonensis* (PH 8 strain) metacyclic promastigotes (31), which had been isolated from hamsters and maintained in Schneider's axenic media supplemented with 10% fetal calf serum for one passage. The infected footpad thicknesses were measured weekly with a Mitutoyo apparatus and the thickness values of the non-infected left footpads were subtracted from them at each measure. Seven days after immunization and 6 weeks after infection, sera were collected for the anti-NH36 antibody assays and the intradermal response against *L. amazonensis* lysate (IDR) was measured in the footpads. Mice were sacrificed 6 weeks after challenge by euthanasia with carbon dioxide. The cellular immunity was assessed by flow cytometry analysis (FACS analysis), intracellular staining (ICS) of splenocytes, and cytokine-ELISA assays of splenocyte supernatants. For the assessment of long-term immunity, mice received the same immunization protocol but were challenged 1 month after the last vaccine dose. In these animals, cross-protection was evaluated by monitoring the sizes of footpad lesions and by determination of the parasite load in lesions after euthanasia by a limiting dilution assay as modified from de Oliveira Cardoso et al. (36).

DETECTION OF ANTIBODIES

Antibodies were measured in sera using an ELISA assay against NH36 recombinant proteins as previously described (34). The ELISA assay used 2 μg of NH36 per well (50 μl of a 40 μg/ml antigen solution) and goat anti-mouse IgG (Sigma) or goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA horseradish peroxidase conjugated antibodies (Southern, Biotechnology Associates, Birmingham, AL, USA) in a 1:1000 dilution in the blocking buffer. The reaction was developed with *O*-phenyldiamine (Sigma), interrupted with 1 N sulfuric acid, and monitored at 492 ηm. Each individual serum was analyzed in triplicate in double-blind tests. Positive and negative control sera were included in each test. Results were expressed as the mean of the absorbance values (492 ηm) of the 1/100 diluted sera of each animal.

ANALYSIS OF THE CELLULAR IMMUNITY

Intradermal response to leishmanial antigen (IDR)

The intradermal response against *L. amazonensis* lysate (IDR) was measured in the footpads. Briefly, mice were injected intradermally, in the right front footpad, with 10^7 freeze-thawed stationary phase *L. amazonensis* promastigotes in 0.1 ml sterile saline solution. The parasites were obtained as amastigotes aseptically

removed from *L. amazonensis* (PH 8 strain) infected hamster footpad lesions, transformed, and cultured in Schneider's axenic medium at 26°C until they reached the stationary phase of growth and were then disrupted by three consecutive freeze-and-thaw cycles using liquid Nitrogen. The footpad thicknesses were measured with a Mitutoyo apparatus, both before and at 0, 24, and 48 h after injection. Injecting each animal with 0.1 ml saline in the left front footpad served as control. At each measurement, the values of the saline control were subtracted from the reaction due to the *Leishmania* antigen.

Anti-NH36-specific T cell immunity

Spleens were aseptically removed and disrupted in NaCl saline solution (Sigma Co., USA) using a Petri dish and nylon mesh, suspended to 11 ml with lysis solution (NH_4Cl 8.29 g/l, $KHCO_3$ 1 g/l, and EDTA 37.2 mg/l) and further centrifuged at $400 \times g$ for 5 min at 4°C until total red blood cell removal. The pellet was further washed with saline solution by centrifugation, incubated with 3 ml RPMI supplemented with 10% fetal calf serum, 0.05 mM 2-mercaptoethanol and antibiotics (200 U/ml of penicillin and 200 μg/ml of streptomycin), counted in a hemocytometer chamber. For cytokine dosage, splenocytes were distributed in 96 well flat-bottomed plates (Nunc, Roskilde, Denmark) with each well containing 10^6 cells in a final volume of 200 μl and incubated, in the presence or absence of 5 μg of recombinant NH36 for 3 days at 37°C under a 5% CO_2 atmosphere. RPMI supplemented medium was added as negative control. After this period, supernatants were harvested, centrifuged at 14,000 rpm for 11 s, and further stored at −70°C until dosage. Secretion of IFN-γ, TNF-α, and IL-10 was evaluated in the supernatants by an ELISA assay, using the mouse IFN-γ, TNF-α, and IL-10 BD OptEIA ELISA Set II kits (BD Bioscience) according to the manufacturer's instructions. Splenocytes, after *in vitro* incubation, were processed for immunostaining with anti-CD4 (clone GK1.5) or anti-CD8-FITC (clone 53-6.7) monoclonal antibodies (R&D systems Inc.) and analyzed by flow cytometry analysis (FACS analysis) in a FACScalibur apparatus. Cells were analyzed by flow cytometry in a Becton Dickinson FACScalibur apparatus. Data were analyzed using the Win MDI program.

In vivo depletion of CD4+ or CD8+ T cells

Mice were vaccinated with three doses of F1sap and F3sap at weekly intervals were challenged with 10^5 *L. amazonensis* infective promastigotes, 10 days after complete vaccination. One week after complete vaccination and on week 6 after challenge, the IDR against *L. amazonensis* lysate was assayed. *In vivo* depletion was performed by treating groups of F1- and F3-vaccinated mice with GK1.5 or 53.6.7 rat IgG MAb on days 2, 4, and 6 before challenge and on day 14 after challenge. Mice were treated with 50 μl of ascitic fluid containing an approximate 5 mg/ml MAb concentration. Control mice received the F1sap or F3sap vaccines and 0.05 mL of rat serum ip, equivalent to 0.25 mg of IgG, or nude mice ascitic fluids containing 0.25 mg of anti-CD4+ and/or anti-CD8+ antibodies. As determined by FACS analyses, the efficacy of depletion of CD4+ or CD8+ spleen cells before challenge was of 99.94 or 96% in anti-CD4+ or anti-CD8+ treated mice, respectively. The efficacy of depletion treatment was monitored by the increase of the size of footpad lesions along the 6 weeks of experiment. In addition, the parasite load in lesions on week 6 was evaluated by a limiting dilution assay (36).

STATISTICAL ANALYSIS

Means were compared by Kruskall–Wallis and Mann–Whitney non-parametrical tests. For the levels of IFN-γ and TNF-α induced by the F1 vaccine, we also used the confidence interval (95% CI) (Analyze-it). Correlation coefficient analysis was determined on a Pearson bivariate, two tailed test of significance (GraphPad Prism 6). The values of R^2, which represents the fraction of the total variance in Y that can be explained by the variation in X, were obtained using linear regression analysis (Analyze-it).

RESULTS

Mice were immunized with NH36, F1, F2, or F3 proteins and saponin, challenged with infective promastigotes of *L. amazonensis* at 4 weeks and euthanized 6 weeks after challenge. After immunization (**Figure 1A**), the humoral response against the NH36 antigen assayed by ELISA disclosed higher IgM, IgG, IgG1, and IgG2a antibody levels in the mice sera of all vaccines when compared to saline controls ($p < 0.001$). The F3sap vaccine showed the best performance, inducing IgG and IgG2a levels as high as NH36sap. Both the F3 and the F1 vaccines induced similar levels of IgM to the NH36 vaccine while the IgG2b was only enhanced by the NH36 and the IgG3 by the F1 vaccine, respectively (**Figure 1A**). After challenge, significant differences were observed among IgG, IgG1, IgG2a, IgG2b, and IgG3 antibodies (<0.001, for all antibody types) (**Figure 1B**). While the NH36sap vaccine showed the highest levels of IgG and IgG3 antibodies, the F3sap was as strong as the NH36 vaccine in the IgG1, IgG2a, IgG2b subtypes. Differently from what

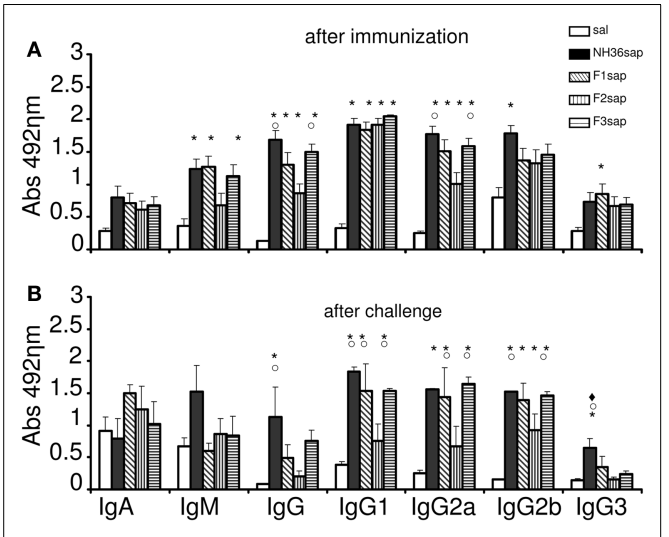


FIGURE 1 | Development of NH36-specific humoral immune response. Bars represent the mean ± SE of the absorbance values of anti-NH36 antibodies from 1/100 diluted serum of three independent experiments after immunization ($n = 3$ mice per treatment in each experiment) **(A)** and two independent experiments after challenge ($n = 7$ mice per treatment in each experiment) **(B)**. *$p < 0.05$ from the saline control; ○ $p < 0.05$ different from the F2sap vaccine; ◆ $p < 0.05$ different from F3sap.

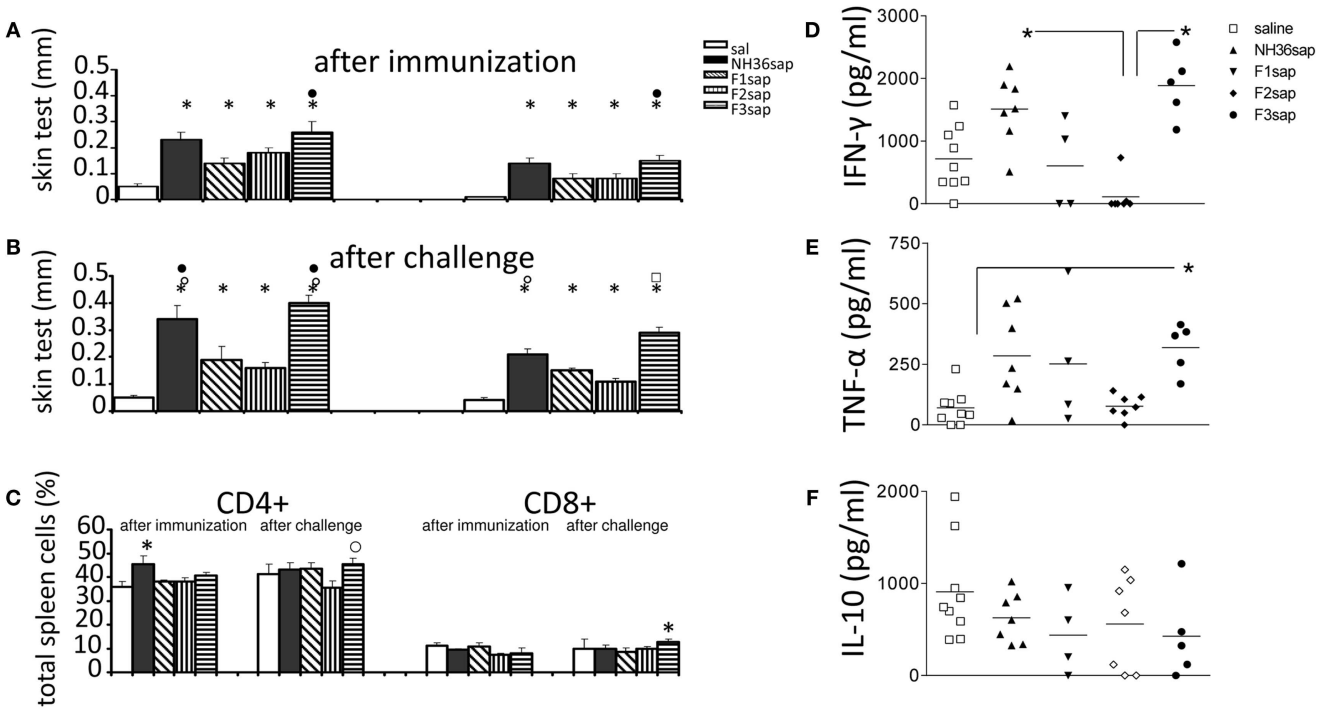


FIGURE 2 | Intradermal response to the leishmanial antigen, flow cytometry analysis, and ELISA of cytokines in supernatants of mice splenocytes. IDR after immunization **(A)** and after challenge **(B)** 24 h (left) and 48 h (right) after antigen injection. Splenocytes after *in vitro* culture for 3 days at 37°C and 5% CO_2 in the presence or absence of 5 μg/ml of recombinant NH36 and staining with anti-CD4 or anti-CD8 antibodies **(C)**. Results of two independent experiments with 9–10 mice per treatment group are shown as mean ± SE. *$p < 0.05$ from the saline controls, • the F1, ○ the F2, or ⍁ from all the other vaccines. Secretions of IFN-γ **(D)**, TNF-α **(E)**, and IL-10 **(F)** in the supernatant of splenocytes, after challenge, are expressed in picogram per milliliters. Horizontal bars represent the mean values of one experiment (four to nine mice per treatment). *Significant differences between groups.

was seen before infection (**Figure 1A**), after challenge, the F1sap and F3sap vaccines showed levels of IgG1 and IgG2a antibodies significantly increased above the F2sap vaccine (**Figure 1B**).

The cell-mediated immune response induced by immunization was initially assessed by the IDR to the *L. amazonensis* leishmanial antigen that was higher in all vaccinated animals than in controls prior to (**Figure 2A**) and after challenge (**Figure 2B**) ($p < 0.0001$ in both cases). After immunization, the F3sap vaccine induced higher footpad swelling than the F1sap vaccine. After challenge, the IDR responses were enhanced ($p = 0.049$ at 24 h and $p = 0.007$ at 48 h) mainly by the NH36sap, which was as potent as F3sap vaccine at 24 h after injection (**Figure 2B**). The preponderance of the F3sap vaccine was recovered 48 h after injection, when it induced the strongest intradermal reaction (**Figure 2B**). The proportions of anti-NH36-specific CD4+ and CD8+ lymphocytes in spleens were analyzed by FACS (**Figure 2C**). After immunization, the proportions of splenic CD4+ T cells of mice vaccinated with NH36 vaccine were higher than those of the saline controls. After challenge, and as expected for CL, the CD4+ proportions of saline control were sustained and only the F3 vaccine showed significantly increased proportions of NH36-specific CD4+ T cells over those of the F2 vaccine and of NH36-specific CD8+ T cells over the saline control (**Figure 2C**).

Six weeks after infection, the levels of cytokines were measured in supernatants of 10^6 splenocytes after 3 days of *in vitro* culture with the addition of 5 μg of recombinant NH36. The results shown in **Figures 2D–F** are already subtracted from the values obtained without RPMI medium without antigen stimulation. Both the NH36sap (mean = 1510.15 pg/ml) and the F3sap-vaccinated mice (mean = 1888.85 pg/ml) showed higher concentrations of IFN-γ ($p < 0.01$ for both vaccines) than the F2sap-vaccinated mice (mean = 111.21 pg/ml) (**Figure 2D**). The TNF-α expression was increased only by the F3sap vaccine (mean = 318.87 pg/ml) over the saline controls (mean = 70.45 pg/ml) ($p < 0.05$) (**Figure 2E**) while no differences were detected in the IL-10 expression (**Figure 2F**). The secretion of IFN-γ and TNF-α was strongly correlated ($p = 0.043$). The levels of IFN-γ and TNF-α induced by the F1 vaccine did not achieve a significant difference compared to the F2 vaccine. However, the mean for IFN-γ (607.19 pg/ml) of the F1sap group fell outside the CI95% of the F2sap group (−221.17 to 332.39 pg/ml) (**Figure 2D**) and the mean for TNF-α of the F1sap group (370.28 pg/ml) also fell outside the CI95% of the F2sap group (77.44–77.52 pg/ml) (**Figure 2E**). No significant differences were observed between the levels of IL-10 generated by any treatment (**Figure 2F**). The supernatants represented in **Figures 2D–F** correspond to the lymphocytes, after challenge, represented in **Figure 2C**. At this point, lymphocytes represent 56.36% of the total splenocytes in culture (43.70% average of CD4 T lymphocytes + 12.66% average of CD8 T lymphocytes).

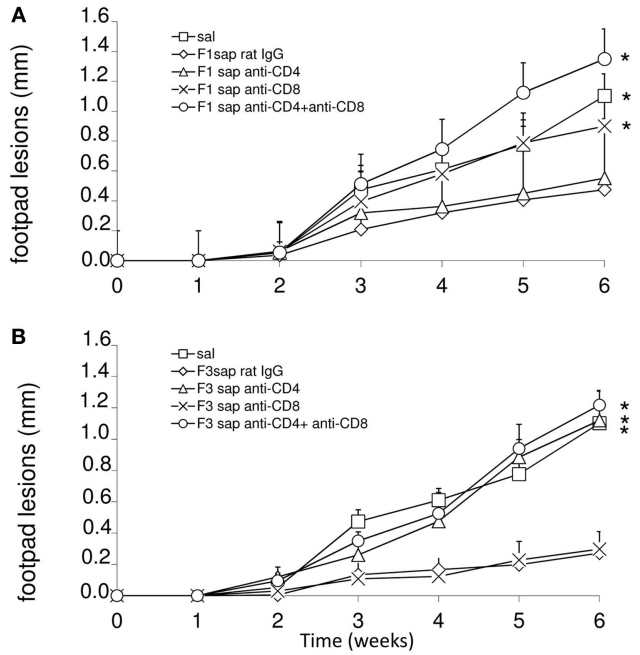


FIGURE 3 | *In vivo* depletion assay with anti-CD4 and anti-CD8 monoclonal antibodies. Mice were challenged with *L. amazonensis* after vaccination with F1sap **(A)** and F3sap **(B)** vaccines and treated with rat serum, anti-CD4+ or anti-CD8+, or the combination of anti-CD4 and anti-CD8 MAbs. Results are shown as the mean ± SD of the footpad measurements of one experiment (four to five animals per treatment) along the time. $^*p < 0.05$, different from the F1sap **(A)** and F3sap **(B)** vaccines.

To detail the importance of CD4 and CD8+ epitopes of the F3 and F1 domains in cross-protection to *L. amazonensis* infection, we performed an *in vivo* depletion assay with anti-CD4 and anti-CD8+ monoclonal antibodies using mice immunized with F1sap and F3sap vaccines and challenged. The evolution of the sizes of footpad lesions is summarized in **Figure 3**. Significant differences among treatments were detected at week 6 ($p < 0.0001$). When compared to saline control, the F1sap vaccine determined a 57% ($p = 0.008$) reduction of footpad lesions that was not blocked by the anti-CD4-Mab ($p = 0.413$) but that was abolished by treatment with anti-CD8 antibody ($p = 0.016$) (**Figure 3A**). On the other hand, when compared to the saline control, the F3sap vaccine (**Figure 3B**) determined a 75% ($p = 0.008$) reduction in footpad lesion that was blocked by anti-CD4+ antibodies ($p = 0.016$ compared to the F3sap vaccine) but not impaired by depletion with anti-CD8-Mab ($p = 0.730$ compared to the F3sap vaccine). Our results indicate that the reduction of the size of lesion generated by F1sap vaccine is mainly mediated by CD8 epitopes present in the sequence of the F1 domain while reduction of lesion size induced by the F3sap vaccine is related to the presence of CD4+ epitopes in the F3 domain.

This hypothesis was also supported by the analysis of IDR after challenge, which disclosed significant differences among treatments ($p < 0.0001$) both at 24 h (not shown) and 48 h after antigen injection (**Figure 4A**). IDR was increased above the saline controls, in mice vaccinated with F1sap ($p < 0.008$), treated or not with anti-CD4+ Mab ($p = 0.02$), but it was decreased after treatment with anti-CD8+ and both anti-CD4 and -CD8 antibodies (**Figure 4A**) suggesting that the IDR response enhancement is related to epitopes for CD8+ T cells located in the F1 domain. The F3sap vaccine, showed a stronger IDR than the F1sap vaccine ($p = 0.008$) (**Figure 4A**), that was abolished by anti-CD4 Mab but not anti-CD8 Mab suggesting that it was mainly mediated by CD4+ T cells with a partial contribution of CD8+ T lymphocytes. The size of footpad lesions on week 6 showed significant negative correlation to the results of intradermal response ($R = -0.79$; $p < 0.0001$; $R^2 = 0.63$ for IDR 24 h; and $R = -0.82$; $p < 0.0001$; $R^2 = 0.68$ for IDR at 48 h) confirming that IDR is a good correlate of protection.

In correlation with these results, the parasite load in footpad lesion, evaluated by a limiting dilution assay (**Figure 4B**), also disclosed that protection induced by F1sap was abolished in mice treated with anti-CD8 Mab ($p = 0.032$) while protection generated by the F3sap vaccine was absent in mice treated with anti-CD4 Mab ($p = 0.016$). When compared to the saline controls (514,850 promastigotes), 99.93% (513 promastigotes) and 99.90% (341 promastigotes) reductions in the number of parasites were determined by the F3 and the F1 vaccines, respectively. The $\log_{10}$ values of parasite load in footpads correlated significantly with the increase in IDR ($R = -0.6734$; $p < 0.0001$; $R^2 = 0.4534$) and with the decrease in footpad lesions ($R = 0.5994$; $p < 0.0001$; $R^2 = 0.3593$) confirming that NH36 vaccine generated cross-protection against cutaneous leishmaniasis is determined by CD8 epitopes of F1 domain and by CD4 epitopes in the F3 domain.

The secretion of IFN-γ ($R = -0.5518$; $p = 0.002$; $R^2 = 0.3045$) and TNF-α ($R = -0.4655$; $p = 0.011$; $R^2 = 0.2162$) was negatively correlated with the increase of footpads lesions sizes (not shown) and thus, were strong correlates of protection against *L. amazonensis* infection.

The superiority of the F3 over the NH36 vaccine was evident in many variables. We calculated the increment in the immunoprotective effect of the F3 vaccine taking into consideration all the variables that showed significant differences between the two formulations (**Table 1**). We found that the F3 vaccine developed a 40.40% higher average protective effect than the NH36 vaccine.

We further assessed the possible long-term cross-protection generated by the F3sap and F1sap vaccines in Balb/c mice that received three weekly interval vaccinations but that were challenged 1 month after the last vaccine dose. Significant reductions in the sizes of footpad lesions were achieved by vaccination with the F1sap (72%, $p = 0.0003$) and the F3sap vaccine (99.82%, $p = 0.0002$). Six weeks after challenge, the F3 vaccine reduced the lesions more than the F1 vaccine ($p = 0.002$) (**Figure 5A**). When compared to the saline controls ($p < 0.01$), the limiting dilution assay analysis disclosed also a 99.82% level of protection generated by the F3 vaccine (mean promastigotes = 757) followed by a 98.97% reduction (4531.25 promastigotes) due to the F1 vaccine (**Figure 5B**). Parasite reduction was more pronounced in the F3 than in the F1 vaccine treated mice ($p < 0.01$).

The alignment of the amino acid sequences of *L. donovani* NH36 and the recently identified, NH A34480 of *L. amazonensis,* is represented in **Figure 6**. Both proteins are composed of 314 amino acids and show 93% of identity (292 from 314 amino acids)

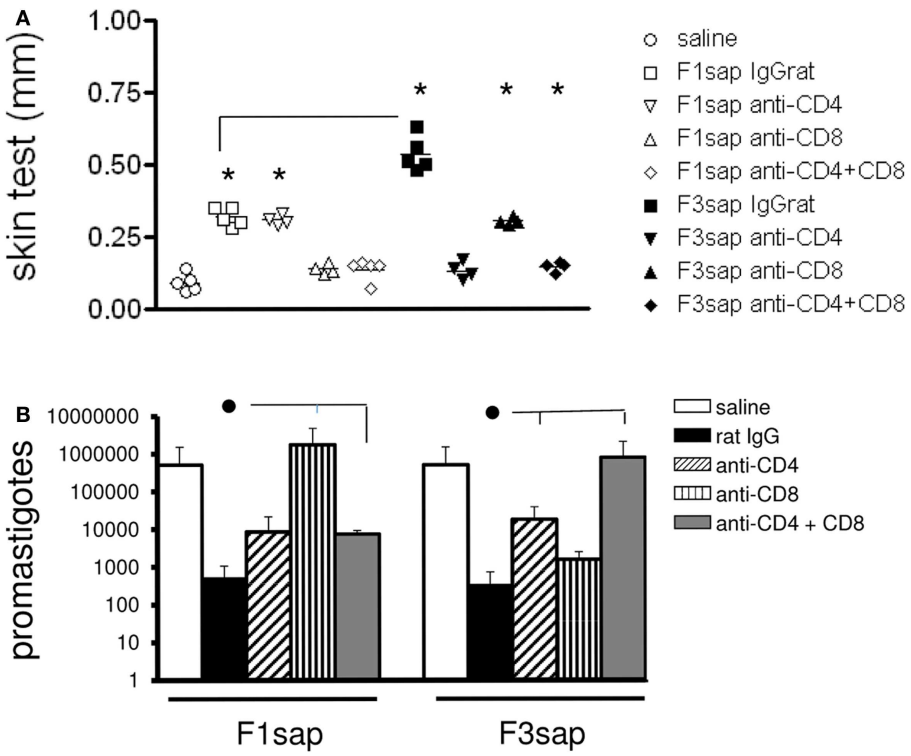


FIGURE 4 | Intradermal response and number of parasites in footpad lesions of mice submitted to an *in vivo* depletion assay with anti-CD4 and anti-CD8 monoclonal antibodies. The IDR to *Leishmania amazonensis* lysate was measured in F1sap- and F3sap-vaccinated animals that were challenged with *L. amazonensis* and treated with rat serum, anti-CD4 or anti-CD8, or the combination of anti-CD4+ and anti-CD8+ MAbs (A). IDR was measured 6 weeks after challenge and 48 h after antigen injection. Results of one experiment with four to five mice per treatment group are shown. $*p < 0.05$, different from the saline controls and horizontal lines represent significant differences between the two vaccines **(A)**. In the limiting dilution assay **(B)** bars represent the number of promastigotes ± SD in each treatment (one experiment with four to five mice per treatment). • Horizontal lines express significant differences from the F1sap- or F3sap-vaccinated treated with rat IgG only.

Table 1 | Superiority of the F3 peptide domains over the NH36 vaccine in prophylaxis against *L. amazonensis* infection.

Variable	F3	NH36	Enrichment (%)
IDR 48 h after challenge	0.290	0.210	27.58
INF-γ in supernatants	1888.85	1510.15	20.04
TNF-α in supernatants	322.47	284.95	11.64
Reduction of parasite load *L. amazonensis*	16.60	1.156	93.03
Mean + SD			40.40 + 27.77

Calculation was performed according to the following equation = (F3 − NH36/F3) values × 100 = protective effect increment.

with no gaps. Additionally, we show the identity of the sequences of the predicted epitopes for CD4+ and CD8+ T cells, in the F1 and F3 domains of both proteins (**Figure 6**). The first epitope for CD4+ and the epitope for CD8+ T cells of the F1 domain of the two *Leishmanias* are conserved showing total identity, while the second epitope for CD4+ T cell shows a difference only in the last amino acid. Indeed, Alanine (A) is present in *L. amazonensis* NH instead of the final threonine (T) of NH36 of *L. donovani*. Furthermore, a difference in only one amino acid was found in the sequences of the three epitopes for CD4+ lymphocytes of the F3 domain. In the first CD4+ epitope, glutamine (Q) is exchanged for glycine (G), in the second epitope, histidine (H) is substituted by asparagine (N), and in the third epitope, lysine (K) is replaced by glutamic acid (G) (**Figure 6**).

DISCUSSION

We were able to disclose the antigenic basis of NH36 of *L. donovani* in cross-protection to infection by *L. amazonensis*. Our results show that the global increase of the humoral and cellular immune response promoted by the F3sap vaccine and the increase of the antibody response, IFN-γ and TNF-α secretion by the F1sap vaccine determined the vaccine protection against the *L. amazonensis* challenge. We also demonstrated that the cellular immune response induced by the F3 peptide (C-terminal domain) against the *L. amazonensis* infection is superior to the one induced by the cognate NH36 protein suggesting that it holds the main NH36 sequences responsible for the TH1 immune response. The increased IFN-γ and TNF-α secretion in supernatants confirmed the predominance of the immunogenicity of the F3 peptide. On

the other hand, the F1 vaccine induced a weaker but significant DTH response and a mild enhancement of IFN-γ and TNF-α levels.

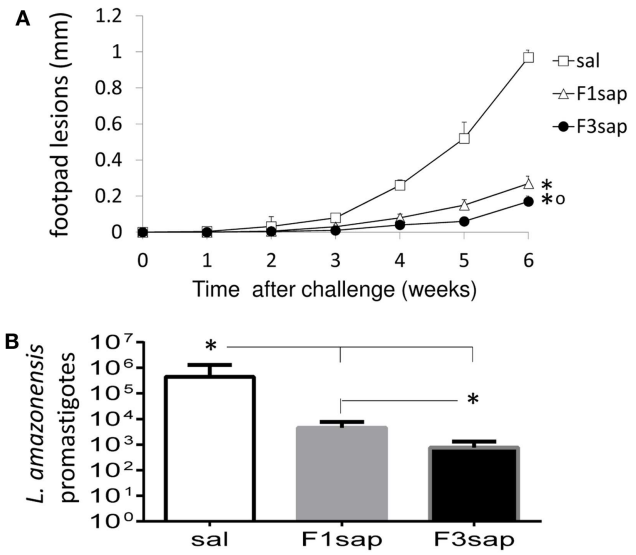


FIGURE 5 | Long-term cross-protection generated by the F3sap and F1sap vaccines. Balb/c mice were vaccinated with three doses of F1sap or F3sap with a weekly interval and challenged with *L. amazonensis* infective promastigotes, 30 days after the last immunization. The evolution of the sizes of footpad lesions **(A)** and the parasite load in lesions (limiting dilution assay) **(B)** were determined. Bars represent the mean ± SD of one experiment with 10 mice for each treatment. $*p < 0.05$ significant differences from the saline controls and ∘ from the F1sap vaccine.

In a previous work, we demonstrated that protection against *L. chagasi* generated by the NH36 vaccine is related to its C-terminal domain and is mediated mainly by a CD4+ T cell-driven response with a lower contribution of CD8+ T cells (34). Increases in IgM, IgG2a, IgG1, and IgG2b antibodies, CD4+ T cell proportions, IFN-γ secretion, ratios of IFN-γ/IL-10 producing CD4+ and CD8+ T cells, and percents of antibody binding inhibition by synthetic predicted epitopes were detected in F3-vaccinated mice. The increases in DTH and in ratios of TNFα/IL-10 CD4+ producing cells were however the strong correlates of protection, which was confirmed by *in vivo* depletion with monoclonal antibodies, algorithm predicted CD4 and CD8 epitopes and a pronounced decrease in parasite load (90.5–88.23%; $p = 0.011$) that was long-lasting. No decrease in parasite load was detected after vaccination with the N-domain of NH36, in spite of the induction of IFN-γ/IL-10 expression by CD4+ T cells after challenge. Both peptides reduced the size of footpad lesions, but only the C-domain reduced the parasite load of mice challenged with *L. amazonensis* (34).

In the present study, as detected in the previous investigation (34), the antibody response also indicated the predominance of the F3 followed by the F1 peptide. This occurred, in the *L. amazonensis* model, mainly after challenge. In the *L. chagasi* model (34), the F3 was the only peptide to induce levels of IgG and IgG2a antibodies as high as those of the NH36 vaccine. After *L. chagasi* challenge, the IgG2a levels were 34% higher in the F3sap than in the F1sap vaccine group. In the *L. amazonensis* model, both F3 and F1 peptides seem to have similar degrees of contribution to the humoral response. Antibodies to the F1 peptide were also increased in infected dogs after immunotherapy with the NH36 DNA vaccine (33). Coincidentally, two B cell epitopes for dog and human antibodies were identified along the sequence

```
Ld-NH36  1    MPRKIILDCDPGIDDAVAIFLAHGNPEVELLAITTVVGNQTLEKVTRNARLVADVAGIVG  60
              MP+KIILDCDPGIDDAVAIFLA+GNPE+ELLAITTVVGNQTLEKVTRNARLVADVAGIVG
La-NH    1    MPQKIILDCDPGIDDAVAIFLAYGNPEIELLAITTVVGNQTLEKVTRNARLVADVAGIVG  60

Ld-NH36  61   VPVAAGCTKPLVRGVRNASQIHGETGMGNVSYPPEFKTKLDGRHAVQLIIDLIMSHEPKT  120
              VPVAAGC KPLVRGVRNASQIHGETG+GNVSYPPEFKTKLD RHAVQLIIDLIMSHEPKT
La-NH    61   VPVAAGCAKPLVRGVRNASQIHGETGLGNVSYPPEFKTKLDSRHAVQLIIDLIMSHEPKT  120

Ld-NH36  121  ITLVPTGGLTNIAMAVRLEPRIVDRVKEVVLMGGGYHTGNASPVAEFNVFVDPEAAHIVF  180
              ITLVPTG LTNIAMAVRLEPRIV+RVK+VVLMGGGYHT NASPVAEFN+ VDPEAAHIVF
La-NH    121  ITLVPTGSLTNIAMAVRLEPRIVERVKKVVLMGGGYHTANASPVAEFNILVDPEAAHIVF  180

Ld-NH36  181  NESWNVTMVGLDLTHQALATPAVQKRVKEVGTKPAAFMLQILDFYTKVYEKERNTYATVH  240
              NESWNVTMVGLDLTHQALATPAV+KRVKEVGTKPAAFML ILDFYTKVYEK+R TYATVH
La-NH    181  NESWNVTMVGLDLTHQALATPAVRKRVKEVGTKPAAFMLGILDFYTKVYEKQRKTYATVH  240

Ld-NH36  241  DPCAVAYVIDPTVMTTEQVPVDIELNGALTTGMTVADFRYPRPKHCHTQVAVKLDFDKFW  300
              DPCAVAYVIDPTVMTTE+VPVDIELNGALTTGMTV DFRYPRPK+CHTQVAVKLDFDKFW
La-NH    241  DPCAVAYVIDPTVMTTERVPVDIELNGALTTGMTVTDFRYPRPKNCHTQVAVKLDFDKFW  300

Ld-NH36  301  CLVIDALKRIGDPQ  314
              CLVIDAL+RIG+P+
La-NH    301  CLVIDALERIGNPE  314
```

FIGURE 6 | Sequence analysis of nucleoside hydrolases of *Leishmania donovani* and *Leishmania amazonensis*. The sequences of the nucleoside hydrolases NH36 of *L. donovani* (Ld-NH36) and A34480 of *Leishmania amazonensis* (La-NH) were aligned using the BLASTP GenBank program. The line in the middle of the two sequences shows the amino acids share by the two NHs. The peptide sequence of MHC class II-IA^d and -IE^d, haplotype H^2 CD4+ T cell epitopes (34) are shown in black squares, on the F1 and F3 fragments. The amino acid sequence of MHC class I L^d-CD8+ T cell predicted epitope of the F1 fragment (34) is underlined in the gray square.

of NH36 (37). Peptide 17 (TPAVQKRVKEVGTKP) (37) overlaps with the epitope that we previously identified in the sequence of F3 (*AVQKRVKEVGTKP*AAFML) (34), which was responsible for the highest inhibition of antibody binding to NH36 (31.40%). Peptide 18 (TTVVGNQTLEKVT) (37) overlaps with the single antibody epitope that we previously identified in the F1 fragment (*NQTLEKVT*RNARLVADVAG) (34). Peptide 17 developed 100% sensitivity and 100% specificity against sera of canine VL and 100% sensitivity human VL samples (37). All these results suggest that the NH36 B epitopes are good candidates for immunodiagnosis of both visceral and cutaneous leishmaniasis (33, 34, 37) and that the F3 and F1 are good candidate for a bivalent vaccine.

Regarding the results of vaccination against the challenge by *L. chagasi* (34) or *L. amazonensis*, the IDR response and the increase of the proportions of lymphocytes after *in vitro* culture with NH36 showed similarities. In both models, the F3 vaccine was imunodominant, meaning that the strong contribution to protection against cutaneous leishmaniasis by the F1 peptide is not revealed by these variables.

Common protective effects of the F3 vaccine against the infections by *L. chagasi* (34) and *L. amazonensis* also include the increase of: DTH response, TNF-α expression over that of IFN-γ, levels of CD4+ and CD8+ NH36-specific splenocytes, and the impairment of the protective efficacy by depletion of the CD4+ T cells (34), which indicate that cross-protection is mediated by a TH1 response induced against CD4+ epitopes of F3. This is an outstanding property of the C-terminal domain of NH36 considering the difficulties to obtain CD4+ mediated immune protection against protozoa infections (38). The F1 vaccine, on the other hand, did not reduce the *L. chagasi* parasite load, despite the induction of the IFN-γ/IL-10 expression by CD4+ T cells (34), but reduced 57–99% of footpad lesions and parasite load, respectively, in *L. amazonensis* infection and this decrease was impaired by treatment with anti-CD8+ Mab. CD8 T cells have proved to be important in infection clearance promoting localized restricted lesions and being absent in lesions of diffuse cutaneous leishmaniasis patients (39). Thus, the identification of an antigen promoting a CD8 T cell-driven protection is worthy.

The *in vivo* depletion assay with anti-CD4 and anti-CD8 monoclonal antibodies disclosed that protection against *L. chagasi* infection induced by the NH36sap vaccine involved the function of CD4 and CD8+ lymphocytes (34). The CD4 protection was mainly related to the epitopes of F3 (34). The lack of efficacy of F1sap vaccine, a strong inducer of a CD8 T cell response, against *L. chagasi* infection, is explained by the importance of CD4+ T cell response in the immunosuppressive characteristic of VL (34). Indeed a 22% decrease in the CD4+ T cell proportions was detected in mice infected with *L. chagasi* while conversely, the CD4+ levels remained stable after *L. amazonensis* infection. Our results revealed that while the participation of CD4+ T cells is responsible for the protection against *L. chagasi* infection (34), the combined function of CD8+ T and CD4+ T cells is necessary for vaccine efficacy against infection with *L. amazonensis*, and this will be probably achieved by using the two peptide domains in vaccination against cutaneous leishmaniasis.

Therefore, while the F3 peptide hosts the immunodominant CD4+ epitopes necessary for protection against *L. chagasi* and *L. amazonensis*, the F1 peptide exerts a co-dominance in immunoprotection to *L. amazonensis* infection, which is mediated mostly by CD8+ epitopes. Interestingly, a high affinity epitope for CD8+ T cells (YPPEFKTKL) was described in our previous work inside the sequence of the F1 peptide (34).

Immunization with the F3 peptide exceeded in 36.73% the protective response induced by the cognate NH36 protein against *L. chagasi* (34) and in 40.40% the protection induced against *L. amazonensis*. These results indicate that vaccine formulations including F3 might show the best results against visceral leishmaniasis while a combination of F3 and F1, or a potential chimera might be needed for protection against both visceral and cutaneous leishmaniasis.

Our results also demonstrate the induction of long-term cross-protection by the F3 followed by the F1 vaccine. Indeed, strong reduction of lesion size and parasite load reduction were detected in mice challenged 1 month after vaccination suggesting that both vaccines are able to generate both effector and memory T cells responsible for the immunoprotective response.

Despite the many antigens tested for vaccination in laboratory models (7, 8) only a few are under analysis as tentative synthetic vaccines against *Leishmania* (40–44). The kmp-11 (40) and the amastigote A2 (43) contain units encoding CD8+ cytotoxic T lymphocyte epitopes while the polyprotein Leish110f (8, 41), the LACK158–173 peptide (42), the amastigote A2 antigen (43), and the MML-triple fusion *L. major* vaccine expressed in Adenovirus (44) trigger a Th1-biased CD4+ T cell response.

Since the NH36 function is mandatory at the early stages of the parasite infection and is a strong phylogenetic marker (24, 25) with significant homology to the sequences of NH of *L. major* (95%) (25), *L. chagasi* (99%), *Leishmania infantum* (99%), *Leishmania tropica* (97%), *Leishmania mexicana* (93%), *Leishmania braziliensis* (84%) (26), the achievement of high protection using the *L. donovani* NH36 vaccine against the challenge by *L. chagasi* was expected (34). The previous finding of cross-protection against *L. mexicana* induced by vaccination with NH36 supported this premise (29). Recently, the genome sequence of *L. amazonensis* was described (35) and the presence of the gene of NH A34480 was disclosed. We describe here that this gene shows 93% of identity to the sequence of NH36 of *L. donovani*. Additionally, we detected that the epitope for CD8+ T cells (34), and one epitope for CD4+ T cells of the F1 domain are completely conserved in *L. amazonensis* NH, while the other CD4+ epitopes of the F1 and F3 domains differ in a single amino acid, having the rest of their sequences preserved. These results reveal the structural basis of the demonstrated cross-immune protection induced by the *L. donovani* F1 and F3 vaccines in prophylaxis to the infection by *L. amazonensis*, and encourage us to pursue the development of a T cell epitope synthetic bivalent vaccine for prophylaxis against both leishmaniasis. The C-terminal and the N-terminal domains of NH36 could be potentially combined into a chimera, for the bivalent vaccine. Since NH of *L. donovani* also shares 68% identity with *Haemophilus influenzae* and 30% identity and conserved motifs with *Bacillus anthracis* (45) and NHs are also found in yeasts (46) and insect cells (47), the identification of shared NHs domains might allow the rational design development of cross-protective subunit or synthetic vaccines for

protection against multiple purine salvation pathway-dependent pathogens.

To our knowledge, this is the first case of a second-generation licensed vaccine to evolve DNA to a recombinant defined protein formulation that might be used in a potential bivalent vaccine against cutaneous and visceral leishmaniasis.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Clarisa B. Palatnik-de-Sousa, Dirlei Nico. Acquisition, analysis, and interpretation of data: Dirlei Nico, Daniele Crespo Gomes, Marcus Vinícius Alves-Silva, Elisangela Oliveira Freitas, Alexandre Morrot, Diana Bahia, Clarisa B. Palatnik-de-Sousa, Marcos Palatnik, Mauricio M. Rodrigues. Wrote the paper: Clarisa B. Palatnik-de-Sousa. Final approval of the last version of the manuscript to be published: Clarisa B. Palatnik-de-Sousa, Dirlei Nico, Daniele Crespo Gomes, Marcus Vinícius Alves-Silva, Elisangela Oliveira Freitas, Alexandre Morrot, Diana Bahia, Marcos Palatnik, Mauricio M. Rodrigues.

ACKNOWLEDGMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ, Fellowships 301215-2007-3, 302039/2010-4, 559756/2010-0 and grant 404400/2012-4) and by Fundação de Amparo à Pesquisa do Estado de Rio de Janeiro (FAPERJ, grants 102733/2008 and 102957/2011 and Fellowships E-26/102415/2010 and E-26/110535/2010). David Straker is gratefully acknowledged for the English language revision of this manuscript. We thank James Huntington for providing the use of the Analyze-it program.

REFERENCES

1. Kedzierski L. Leishmaniasis vaccine: where are we today? *J Glob Infect Dis* (2010) **2**(2):177–85. doi:10.4103/0974-777X.62881
2. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) **7**(5):e35671. doi:10.1371/journal.pone.0035671
3. Kedzierski L, Zhu Y, Handman E. *Leishmania* vaccines: progress and problems. *Parasitology* (2006) **133**(Suppl S):87–112. doi:10.1017/S0031182006001831
4. Lainson R, Shaw JJ. Evolution, classification and geographic distribution. 1st ed. In: Peters W, Killick Kendrick R, editors. *The Leishmaniases in Biology and Medicine. Biology and Epidemiology*. (Vol. 2), London, UK: Academic Press (1987). p. 1–120.
5. Rougeron V, Bañuls AL, Carme B, Simon S, Couppié P, Nacher M, et al. Reproductive strategies and population structure in *Leishmania*: substantial amount of sex in *Leishmania Viannia guyanensis*. *Mol Ecol* (2011) **20**(15):3116–27. doi:10.1111/j.1365-294X.2011.05162.x
6. Dye C. The logic of visceral leishmaniasis control. *Am J Trop Med Hyg* (1996) **55**(2):125–30.
7. Palatnik-de-Sousa CB. Vaccines for leishmaniasis in the fore coming 25 years. *Vaccine* (2008) **26**(14):1709–24. doi:10.1016/j.vaccine.2008.01.023
8. Duthie MS, Raman VS, Piazza FM, Reed SG. The development and clinical evaluation of second-generation leishmaniasis vaccines. *Vaccine* (2012) **30**(2):134–41. doi:10.1016/j.vaccine.2011.11.005
9. Antunes CM, Mayrink W, Magalhaes PA, Costa CA, Melo MN, Dias M, et al. Controlled field trials of a vaccine against new world cutaneous leishmaniasis. *Int J Epidemiol* (1986) **15**(4):572–80. doi:10.1093/ije/15.4.572
10. Vélez ID, Gilchrist K, Arbelaez MP, Rojas CA, Puerta JA, Antunes CM, et al. Failure of a killed *Leishmania amazonensis* vaccine against American cutaneous leishmaniasis in Colombia. *Trans R Soc Trop Med Hyg* (2005) **99**(8):593–8. doi:10.1016/j.trstmh.2005.04.002
11. Gradoni L, Foglia Manzillo V, Pagano A, Piantedosi D, De Luna R, Gramiccia M, et al. Failure of a multi-subunit recombinant leishmanial vaccine (MML) to protect dogs from *Leishmania infantum* infection and to prevent disease progression in infected animals. *Vaccine* (2005) **23**(45):5245–51. doi:10.1016/j.vaccine.2005.07.001
12. Trigo J, Abbehusen M, Netto EM, Nakatani M, Pedral-Sampaio G, de Jesus RS, et al. Treatment of canine visceral leishmaniasis by the vaccine Leish-111f + MPL-SE. *Vaccine* (2010) **28**(19):3333–40. doi:10.1016/j.vaccine.2010.02.089
13. da Silva VO, Borja-Cabrera GP, Correia Pontes NN, de Souza EP, Luz KG, Palatnik M, et al. A phase III trial of Efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (São Gonçalo do Amarante, RN). *Vaccine* (2000) **19**(9–10):1082–92.
14. Borja-Cabrera GP, Santos FN, Bauer FS, Parra LE, Menz I, Morgado AA, et al. Immunogenicity assay of the Leishmune® vaccine against canine visceral leishmaniasis in Brazil. *Vaccine* (2008) **26**(39):4991–7. doi:10.1016/j.vaccine.2008.07.029
15. Palatnik-de-Sousa CB, Barbosa AF, Oliveira SM, Nico D, Bernardo RR, Santos WR, et al. The FML-vaccine against canine visceral leishmaniasis: from the second generation to the synthetic vaccine. *Expert Rev Vaccines* (2008) **7**(6):833–51. doi:10.1586/14760584.7.6.833
16. Saraiva EM, de Figueiredo Barbosa A, Sanots FN, Borja-Cabrera GP, Nico D, Souza LP, et al. The FML-vaccine (Leishmune®) against canine visceral leishmaniasis: a transmission blocking vaccine. *Vaccine* (2006) **24**(13):2423–31. doi:10.1016/j.vaccine.2005.11.061
17. Palatnik-de-Sousa CB, Silva-Antunes I, Morgado Ade A, Menz I, Palatnik M, Lavor C. Decrease of the incidence of human and canine visceral leishmaniasis after dog vaccination with Leishmune® in Brazilian endemic areas. *Vaccine* (2009) **27**(27):3505–12. doi:10.1016/j.vaccine.2009.03.045
18. Palatnik-de-Sousa CB, Gomes EM, de Souza EP, dos Santos WR, de Macedo SR, de Medeiros LV, et al. The FML (fucose mannose ligand) of *Leishmania donovani*. A new tool in diagnosis, prognosis, transfusional control and vaccination against human kala-azar. *Rev Soc Bras Med Trop* (1996) **29**(2):153–63. doi:10.1590/S0037-86821996000200008
19. Palatnik-de-Sousa CB, Dutra HS, Borojevic R. *Leishmania donovani* surface glycoproteins GP36, is the major immunogen component of the fucose mannose ligand (FML). *Acta Trop* (1993) **53**(1):59–72. doi:10.1016/0001-706X(93)90006-W
20. Iovane E, Giabbai B, Muzzolini L, Matafora V, Fornili A, Minici C, et al. Structural basis for substrate specificity in group I nucleoside hydrolases. *Biochemistry* (2008) **47**(15):4418–26. doi:10.1021/bi702448s
21. Versées W, Goeminne A, Berg M, Vandemeulebroucke A, Haemers A, Augustyn K, et al. Crystal structures of *T. vivax* nucleoside hydrolase in complex with new potent and specific inhibitors. *Biochim Biophys Acta* (2009) **1794**(6):953–60. doi:10.1016/j.bbapap.2009.02.011
22. Santana DM, Borja-Cabrera GP, Paraguai de Souza E, Sturm NR, Palatnik-de-Sousa CB, Campbell DA. Nucleoside hydrolase from *Leishmania (L.) donovani* is an antigen diagnostic for visceral leishmaniasis. *Mol Biochem Parasitol* (2002) **120**(2):315–9. doi:10.1016/S0166-6851(02)00010-5
23. Mauricio IL, Yeo M, Baghaei M, Doto D, Pratlong F, Zemanova E, et al. Towards multilocus sequence typing of the *Leishmania donovani* complex: resolving genotypes and haplotypes for five polymorphic metabolic enzymes (ASAT, GPI, NH1, NH2, PGD). *Int J Parasitol* (2006) **36**(7):757–69. doi:10.1016/j.ijpara.2006.03.006
24. Lukes J, Mauricio IL, Schönian G, Dujardin JC, Soteriadou K, Dedet JP, et al. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *Proc Nat Acad Sci U S A* (2007) **104**(22):9375–80. doi:10.1073/pnas.0703678104
25. Shi W, Schramm VL, Almo SC. Nucleoside hydrolase from *Leishmania major*. Cloning, expression, catalytic properties, transition state inhibitors, and the 2.5-å crystal structure. *J Biol Chem* (1999) **274**(30):21114–20. doi:10.1074/jbc.274.30.21114
26. *Blast-Basic Local Alignment Search Tool*. National Institute of Heath (NIH) (2014). Available from: http://blast.ncbi.nlm.nih.gov/Blast.cgi
27. Paraguai de Souza E, Bernardo RR, Palatnik M, Palatnik-de-Sousa CB. Vaccination of Balb/c mice against experimental visceral leishmaniasis with the GP36 glycoprotein antigen of *Leishmania donovani*. *Vaccine* (2001) **19**(23–24):3104–15. doi:10.1016/S0264-410X(01)00031-7
28. Al-Wabel MA, Tonui WK, Cui L, Martin SK, Titus RG. Protection of susceptible BALB/c mice from challenge with *Leishmania major* by nucleoside hydrolase, a soluble exo-antigen of *Leishmania*. *Am J Trop Med Hyg* (2007) **77**(6):1060–5.

29. Aguilar-Be I, Zardo RS, Paraguai de Souza E, Borja-Cabrera GP, Rosado-Vallado M, Mut-Martin M, et al. Cross-protective efficacy of a prophylactic *Leishmania donovani* DNA vaccine against visceral and cutaneous murine leishmaniasis. *Infect Immun* (2005) **73**(2):812–9. doi:10.1128/IAI.73.2.812-819.2005
30. Chalé-Balboa WG, Mut-Martin M, Ramirez-Sierra MJ, Garcia-Miss MR, Dumonteil E. A combination DNA vaccine encoding nucleoside hydrolase 36 and glycoprotein 63 protects female but not male hamsters against *Leishmania mexicana*. *Parasite* (2009) **16**(3):227–30. doi:10.1051/parasite/2009163227
31. Souza LOP, Palatnik-de-Sousa CB. The nucleoside hydrolase DNA vaccine VR1012NH36 in prophylactic vaccination against mice tegumentar leishmaniasis. *Procedia Vaccinol* (2009) **1**(1):120–3. doi:10.1016/j.provac.2009.07.022
32. Gamboa-León R, Paraguai de Souza E, Borja-Cabrera GP, Santos FN, Miyashiro LM, Pinheiro RO, et al. Immunotherapy against visceral leishmaniasis with the nucleoside hydrolase-DNA vaccine of *L. donovani*. *Vaccine* (2007) **24**(22):4863–73. doi:10.1016/j.vaccine.2006.03.005
33. Borja-Cabrera GP, Santos FB, Nico D, Gravino AE, Manna L, Palatnik M, et al. The Leishmune®'s nucleoside hydrolase DNA vaccine as an aid in immunotherapy of canine visceral leishmaniasis. *Procedia Vaccinol* (2012) **6**:64–73. doi:10.1016/j.provac.2012.04.009
34. Nico D, Claser C, Travassos LR, Palatnik M, Soares IS, Rodrigues MM, et al. Adaptive immunity against *Leishmania* nucleoside hydrolase maps its C-terminal domain as the target of the CD4+ T cell-driven protective response. *Plos Negl Trop Dis* (2010) **4**(11):e866. doi:10.1371/journal.pntd.0000866
35. Real F, Vidal RO, Carazzolle MF, Mondego JM, Costa GG, Herai RH, et al. The genome sequence of *Leishmania* (*Leishmania*) *amazonensis*: functional annotation and extended analysis of gene models. *DNA Res* (2013) **20**(6):567–81. doi:10.1093/dnares/dst031
36. de Oliveira Cardoso FO, Souza CSF, Mendes VG, Abreu-Silva AL, Costa SCG, Calabrese KS, et al. Immunopathological studies of *Leishmania amazonensis* infection in resistant and in susceptible mice. *J Infect Dis* (2010) **201**(12):1933–40. doi:10.1086/652870
37. Costa MM, Penido M, dos Santos MS, Doro D, de Freitas E, Michalik MS, et al. Improved canine and human visceral leishmaniasis immunodiagnosis using combinations of synthetic peptides in enzyme-linked immunosorbent assay. *PLoS Negl Trop Dis* (2012) **6**(5):e1622. doi:10.1371/journal.pntd.0001622
38. Parra-López C, Calvo-Calle M, Cameron TO, Vargas LE, Salazar LM, Patarroyo ME, et al. Major histocompatibility complex and T cell interactions of a universal T-cell epitope from *Plasmodium falciparum* circumsporozoite protein. *J Biol Chem* (2006) **281**(21):14907–17. doi:10.1074/jbc.M511571200
39. Hernández-Ruiz J, Salaiza-Suazo N, Carrada G, Escoto S, Ruiz-Remigio A, Rosenstein Y, et al. CD8 cells of patients with diffuse cutaneous leishmaniasis display functional exhaustion: the latter is reversed, in vitro, by TLR2 agonists. *PLoS Negl Trop Dis* (2010) **4**(11):e871. doi:10.1371/journal.pntd.0000871
40. Basu R, Roy S, Walden P. HLA class I-restricted T cell epitopes of the kinetoplastid membrane protein-11 presented by *Leishmania donovani*-infected human macrophages. *J Infect Dis* (2007) **195**(9):1373–80. doi:10.1086/513439
41. Bertholet S, Goto Y, Carter L, Bhatia A, Howard RF, Carter D, et al. Optimized subunit vaccine protects against experimental leishmaniasis. *Vaccine* (2009) **27**(50):7036–45. doi:10.1016/j.vaccine.2009.09.066
42. Kedzierska K, Curtis JM, Valkenburg SA, Hatton LA, Kiu H, Doherty PC, et al. Induction of protective CD4+ T cell-mediated immunity by a *Leishmania* peptide delivered in recombinant influenza viruses. *PLoS One* (2012) **7**(3):e33161. doi:10.1371/journal.pone.0033161
43. Fernandes AP, Coelho EA, Machado-Coelho GL, Grimaldi G Jr, Gazzinelli RT. Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives. *Curr Opin Microbiol* (2012) **15**(4):476–85. doi:10.1016/j.mib.2012.05.002
44. Darrah PA, Patel DT, De Luca PM, Lindsay RWB, Davey DF, Flynn BG, et al. Multifunctional T_H1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat Med* (2007) **13**(7):843–50. doi:10.1038/nm1592
45. Todd SJ, Moir AJ, Johnson MJ, Moir A. Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. *J Bacteriol* (2003) **185**(11):3373–8. doi:10.1128/JB.185.11.3373-3378.2003
46. Mitterbauer R, Karl T, Adam G. *Saccharomyces cerevisiae* URH1 (encoding uridine-cytidine N-ribohydrolase): functional complementation by a nucleoside hydrolase from a protozoan parasite and by a mammalian uridine phosphorylase. *Appl Environ Microbiol* (2002) **68**(3):1336–43. doi:10.1128/AEM.68.3.1336-1343.2002
47. Ribeiro JM, Valenzuela JG. The salivary purine nucleosidase of the mosquito, *Aedes aegypti*. *Insect Biochem* (2003) **33**(1):13–22. doi:10.1016/S0965-1748(02)00078-4

Conflict of Interest Statement: The authors have declared that there is no competing interest. This work is related to the pendent Patent PI1015788-3, INPI, Brazil.

Received: 01 March 2014; accepted: 10 April 2014; published online: 01 May 2014.
*Citation: Nico D, Gomes DC, Alves-Silva MV, Freitas EO, Morrot A, Bahia D, Palatnik M, Rodrigues MM and Palatnik-de-Sousa CB (2014) Cross-protective immunity to Leishmania amazonensis is mediated by CD4+ and CD8+ epitopes of Leishmania donovani nucleoside hydrolase terminal domains. Front. Immunol. **5**:189. doi: 10.3389/fimmu.2014.00189*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 10 June 2014
doi: 10.3389/fimmu.2014.00268

Experimental validation of multi-epitope peptides including promising MHC class I- and II-restricted epitopes of four known *Leishmania infantum* proteins

Maria Agallou, Evita Athanasiou†, Olga Koutsoni†, Eleni Dotsika and Evdokia Karagouni*

Laboratory of Cellular Immunology, Department of Microbiology, Hellenic Pasteur Institute, Athens, Greece

Edited by:
Hira Nakhasi, US Food and Drug Administration, USA

Reviewed by:
Paola Massari, Boston University, USA
Syamal Roy, CSIR-Indian Institute of Chemical Biology, India

***Correspondence:**
Evdokia Karagouni, Laboratory of Cellular Immunology, Department of Microbiology, Hellenic Pasteur Institute, 127 Vas. Sofias Avenue, Athens 115 21, Greece
e-mail: ekaragouni@pasteur.gr

† Evita Athanasiou and Olga Koutsoni have contributed equally to this work.

Leishmaniasis is a significant worldwide health problem for which no vaccine exists. Activation of $CD4^+$ and $CD8^+$ T cells is crucial for the generation of protective immunity against parasite. Recent trend in vaccine design has been shifted to epitope-based vaccines that are more specific, safe, and easy to produce. In the present study, four known antigenic *Leishmania infantum* proteins, cysteine peptidase A (CPA), histone H1, KMP-11, and *Leishmania* eukaryotic initiation factor (LeIF) were analyzed for the prediction of binding epitopes to $H2^d$ MHC class I and II molecules, using online available algorithms. Based on *in silico* analysis, eight peptides including highly scored MHC class I- and II-restricted epitopes were synthesized. Peptide immunogenicity was validated in MHC compatible BALB/c mice immunized with each synthetic peptide emulsified in complete Freund's adjuvant/incomplete Freund's adjuvant. CPA_p2, CPA_p3, H1_p1, and LeIF_p6 induced strong spleen cell proliferation upon *in vitro* peptide re-stimulation. In addition, the majority of the peptides, except of LeIF_p1 and KMP-11_p1, induced IFN-γ secretion, while KMP-11_p1 indicated a suppressive effect on IL-10 production. CPA_p2, CPA_p3, LeIF_p3, and LeIF_p6 induced IFN-γ-producing $CD4^+$ T cells indicating a T_{H1}-type response. In addition, CPA_p2, CPA_p3, and H1_p1 induced also the induction of $CD8^+$ T cells. The induction of peptide-specific IgG in immunized mice designated also the existence of B cell epitopes in peptide sequences. Combining immunoinformatic tools and experimental validation, we demonstrated that CPA_p2, CPA_p3, H1_p1, H1_p3, CPA_p2, LeIF_p3, and LeIF_p6 are likely to include potential epitopes for the induction of protective cytotoxic and/or T_{H1}-type immune responses supporting the feasibility of peptide-based vaccine development for leishmaniasis.

Keywords: *in silico* analysis, cysteine peptidase A, histone H1, kinetoplastid membrane protein 11, *Leishmania* eukaryotic initiation factor, lymphocyte proliferation, $CD8^+IFN\text{-}\gamma^+$ T cells, $CD4^+IFN\text{-}\gamma^+$ T cells

INTRODUCTION

Leishmaniasis, a vector-borne parasitic disease, is caused by dimorphic protozoan flagellates of the genus *Leishmania* with a worldwide distribution. The disease is characterized by diversity and complexity, presenting a wide spectrum of clinical forms in humans ranging from self-healing cutaneous leishmaniasis (CL) to fatal visceral leishmaniasis (VL). In VL, parasites colonize internal organs, primarily the spleen, liver, and bone marrow. With an estimated 0.5 million cases per year, VL has emerged as an important public-health concern with major clinical and socioeconomic impacts[1]. In South Europe, VL is caused almost exclusively by *Leishmania (L.) infantum*, which is transmitted as a zoonosis with the domestic dog serving as the main reservoir of the parasite (1). Current attempts against leishmaniasis are based on chemotherapy to alleviate disease (2, 3) and on vector control to reduce transmission (4). Toxic side effects and growing resistance to available therapeutic drugs against VL has made the global demand for an effective vaccine capable to elicit a protective immune response, a major public-health priority.

Despite the substantial knowledge regarding the various life stages of the parasite, the considerable inter-specific diversity, the extraordinary host evasive mechanisms of parasite and the heterogeneity of population (5, 6), effective vaccine development against human VL represents an unprecedented challenge. Although a great number of potent vaccine candidates has shown promising results in mice (7, 8) and dogs (9–11), none of them has entered human trials except for LeishF1 with reported phase I and II clinical trials (12, 13).

Antigen identification is considered as a significant barrier in vaccine design, as this is usually achieved through time consuming and labor-intensive *in vitro* and *in vivo* experiments. Efforts have thus focused on developing novel strategies for more rational and faster antigen identification among large numbers of pathogen proteins. Furthermore, recent reports support that epitope-based vaccines appear to be capable of inducing more potent responses than whole protein vaccines (14). Until recently, the search of immunodominant peptides relied on the direct

[1] http://www.who.int/en/

testing of overlapping peptides or peptide libraries. T cell epitope prediction by bioinformatic analysis of protein sequences has been proposed as a promising strategy for vaccine development and an increasing number of tools have been developed, based on different algorithms and methods (15, 16). There is great possibility of missing the emergence of the sequence mutants that would potentially escape the vaccine's protective effect. Moreover, the fact that T cells from genetically distinct populations would recognize and respond to a single peptide epitope, underline the need of identifying one or more epitope(s) that bind to multiple HLA alleles and cover close to 100% of the genetically diverse human population (17). Multi-peptide-based vaccines are designed to generate a diverse immune response to incorporate antigens and to reduce limitations due to MHC restriction into a single entity.

The effectiveness of a vaccine depends on its capacity to ensure long-lasting cell-mediated immunity. In VL, there is evidence that an interplay of T helper cytokines (T_{H1}/T_{H2}) is observed, while resistance or resolution of infection is associated with dominant T_{H1} response and $CD8^+$ T cells (18–20). Furthermore, successful treatment of VL with sodium stibogluconate requires the presence of both $CD4^+$ and $CD8^+$ T cells (21) accompanied with IL-12 and IFN-γ production (22). In contrast, T_{H2} response with IL-4 and IL-10 production results in susceptibility to infection and development of severe disease. Murine models of leishmaniasis have been extensively used to study the pathogenesis of the disease and to test novel therapeutic agents or potent vaccine candidates in preclinical studies. One of the most widely studied and commonly used model of VL is the BALB/c strain of mice infected intravenously with *L. infantum*. Although this strain is considered to be susceptible and the infection progresses during the first month, the infection is then controlled by the host immune response. This mouse model is comparable to self-controlled oligosymptomatic cases and therefore is useful for the study of the protective immune response (23).

Several reports demonstrate that different leishmanial antigens elicit desired T_{H1} and CTL responses capable to sustain protection against experimental challenges (24). Among these antigens, cysteine peptidase A (CPA), histone H1, kinetoplastid membrane protein 11 (KMP-11), and *Leishmania* eukaryotic initiation factor (LeIF) are considered important immunogens, as supported by numerous studies. Specifically, CPA induces protection against *L. major* in the experimental model of CL through development of specific T_{H1} immune responses (25–27). Histone H1 and KMP-11, structural highly conserved proteins, are able to trigger specific immune responses (28–33), and immunization with these proteins confer protection against *L. major* or *L. infantum* infections in experimental animal models (34–38). Moreover, LeIF, originally described as a T_{H1}-type natural adjuvant, is capable of stimulating IL-12 mediated T_{H1} responses in PBMCs of patients (39). Furthermore, recombinant forms of CPA, histone H1, and KMP-11 act as potent B cell immunogens since they are recognized by sera from either recovered or active cases of CL and VL, as well as by sera from asymptomatic or symptomatic dogs with leishmaniasis (40–45).

In the present study, we applied immunoinformatics using currently available online algorithms in order to identify potentially immunogenic T cell epitopes from the above mentioned *L. infantum* proteins, and design multi-epitope peptides containing both MHC class I and II-restricted epitopes as possible candidate peptide vaccines for VL. Immunogenicity of the synthetic multi-epitope peptides in terms of T cell activation was validated in immunized BALB/c mice by analyzing peptide-specific proliferative responses and cytokine production by $CD8^+$ and $CD4^+$ T cells.

MATERIALS AND METHODS

PROTEIN SEQUENCE RETRIEVAL AND PREDICTION OF MHC CLASS I AND II BINDING EPITOPES

Full protein sequences of selected proteins, CPA, Histone H1, KMP-11, and LeIF, were retrieved from GenBank data[2] on JPCM5 strain (MCAN/ES/98/LLm-887) and analyzed by SignalP[3] for the prediction of signal peptides and transmembrane domains (**Table 1**). Potential MHC class I and II binding epitopes derived from the four *L. infantum* proteins, were predicted by *in silico* analysis, using three online available, binding algorithms named SYFPEITHI[4], BIMAS[5], and NetMHCII[6]. The cut-off score was adjusted to ≥18 for SYFPEITHI, ≥100 for BIMAS, and a default prediction threshold (binding affinity <500 nM) depicting accuracy >85% was used for NetMHCII (**Tables 2** and **3**).

SYNTHETIC MULTI-EPITOPE PEPTIDES

Based on the prediction results of the algorithms used, 9-mer epitopes MHC class I-restricted and 15-mer epitopes MHC class II-restricted giving high score against $H2^d$ alleles were extracted and combined in order to generate multi-epitope peptides for each *L. infantum* protein. Thus, 8 peptides, 20–30 amino acid (aa) length, were designed in a way that each peptide included at least one MHC class I-restricted epitope scored very high, as well as adjacent or overlapping MHC class II-restricted epitopes scored also high. Sequence homology between each multi-epitope peptide and mouse proteome were analyzed on BLAST database[7] and peptides with 100% identity were excluded or re-designed to

[2]http://www.ncbi.nlm.nih.gov/genbank
[3]http://www.cbs.dtu.dk/services/SignalP
[4]http://www.syfpeithi.de
[5]http://www-bimas.cit.nih.gov/molbio/hla_bind
[6]http://www.cbs.dtu.dk/services/NetMHCII
[7]blast.ncbi.nlm.nih.gov/Blast.cgi

Table 1 | *L. infantum* proteins selected as candidate antigens for epitope mapping.

Protein abbreviation	Protein name	Protein length (aa)/ mass (kDa)	GenBank accession number
CPA	Cysteine peptidase A	354/39.0	CAM67356
Histone H1	Histone H1	111/11.1	ABN54817
KMP-11	Kinetoplastid membrane protein 11	92/11.2	CAA64883
LeIF	*Leishmania* eukaryotic initiation factor	403/45.3	CAM65231

Table 2 | *In silico* predicted MHC class I-restricted 9-mer epitopes of *L. infantum* proteins.

Protein	No. of epitope	Epitope sequence	Score[a]				
			SYFPEITHI		BIMAS		
			H2-K^d	H2-L^d	H2-K^d	H2-L^d	H2-D^d
CPA	1	8-FFAIVVTIL-18	23	–	1152	–	–
	2	84-HYDVSGKFA-92	23	–	–	–	–
	3	273-LYFGGVVTL-281	23	–	2400	–	–
	4	254-AYVGKNGPV-262	22	–	1200	–	–
	5	23-SALIAQTPL-31	20	–	–	–	–
	6	102-LYLNPNYYA-110	20	–	120	–	–
	7	334-NYVVTATID-343	20	–	–	–	–
	8	7-FFFAIVVTI-15	–	–	1152	–	–
	9	165-QWALKNHSL-173	–	–	240	–	–
	10	62-RFNAFKQNM-70	–	–	144	–	–
	11	319-GYIRLAMGS-327	–	–	120	–	–
	12	179-QVLVSCDNI-187	–	–	115.20	–	–
	13	68-QNMQTAYFL-76	–	–	115.20	–	–
	14	160-GNIEGQWAL-168	–	–	115.20	–	–
Histone H1	15	46-KKAGAKKAV-54	18	–	–	–	–
	16	2-SSDSAVAAL-10	17	19	–	–	–
KMP-11	17	4-TYEEFSAKL-12	20	–	2400	–	–
	18	47-HYEKFERMI-55	19	–	2000	–	–
	19	72-HFKQFAEL-79	18	–	960	–	–
	20	69-HSEHFKQKF-77	–	18	–	–	–
	21	7-EFSAKLDRL-15	–	–	960	–	–
LeIF	22	385-HYHTQIDEL-393	24	–	2000	–	–
	23	360-RYGRKGVAI-368	23	–	2000	–	–
	24	8-APQDQDSFL-16	–	22	–	225	–
	25	25-IPSFDDMPL-33	–	22	–	150	–
	26	199-LPKDIQVAL-207	–	22	–		–
	27	344-LPTNKENYL-352	–	22	–	150	–
	28	393-LPVDFAAYL-401	–	22	–	150	–
	29	14-SFLDDQPGV-22	21	–	576	–	–
	30	197-RFLPKDIQV-205	21	–	480	–	–
	31	100-LSPTRELAL-108	–	21	–		–
	32	187-GFADQIYEI-195	20	–	960	–	–
	33	318-SRVLVTTDL-326	20	–	–	–	–
	34	23-RPIPSFDDM-31	–	20	–	150	–
	35	123-NSSKFCETF-131	–	20	–	–	–
	36	265-VSIAQSVIF-273	–	20	–	–	–
	37	222-KFMRDPVRI-230	–	–	2304	–	–
	38	195-IFRFLPKDI-203	–	–	960	–	–
	39	272-IFANTRRKV-280	–	–	288	–	–
	40	312-TFRSGSSRV-320	–	–	288	–	–
	41	164-RGALRTESL-172	–	–	–	–	120

[a]*The cut-off score was adjusted to ≥18 for SYFPEITHI, ≥100 for BIMAS, and <500 for NetMHCII.*

avoid potential autoimmunity (**Table 4**). Two multi-epitope peptides of CPA (160–189 and 273–302 aa) and Histone H1 (1–20 and 43–61 aa), one peptide of KMP-11 (4–23 aa), and three peptides of LeIF (6–35, 181–210, and 371–400 aa) were synthesized by GeneCust (Labbx, Dudelange, Luxembourg) with purity ≥95%. Synthetic peptides were dissolved in DMSO, acetic acid (10% in dH_2O), or dH_2O according to their hydrophobicity, by vigorous pipetting and stored in aliquots, in −80°C until use. Peptides solutions were found endotoxin free, since LPS concentration was <5 EU/mg as determined by LAL Test Cartridges Portable Test

Table 3 | *In silico* predicted MHC class II-restricted 15-mer epitopes of *L. infantum* proteins.

Protein	No. of epitope	Epitope sequence	Score[a]		
			SYFPEITHI		NetMHCII
			H2-IA[d]	H2-IE[d]	H2-IA[d]
CPA	1	149-MCGSCWAFATTGNIE-163	28	–	–
	2	246-PHDEEEIAAYVGKNG-260	27	–	–
	3	114-KDYKEHVHVDDSVRS-128	26	–	–
	4	257-GKNGPVAVAVDATTW-271	26	–	–
	5	32-GVDDFIASAHYGRFK-46	25	–	–
	6	312-GSSWGEKGYIRLAMG-326	–	24	–
	7	4-RNPFFFAIVVTILFV-18	23	–	–
	8	5-NPFFFAIVVTILFVV-19	23	–	–
	9	13-VTILFVVCYGSALIA-27	22	–	–
	10	172-SLVSLSEQVLVSCDN-186	22	–	–
	11	174-VSLSEQVLVSCDNID-188	22	–	–
	12	12-VVTILFVVCYGSALI-26	21	–	–
	13	260-GPVAVAVDATTWQLY-274	21	–	–
	14	279-VTLCFGLSLNHGVLV-293	21	–	–
	15	67-KQNMQTAYFLNAHNP-81	20	–	–
	16	273-LYFGGVVTLCFGLSL-287	20	–	–
	17	301-KPPYWIVKNSWGSSW-315	20	–	–
	18	328-NQCLLKNYVVTATID-342	20	–	–
	19	216-SYPYTSAGGTRPPCH-230	–	20	–
	20	308-KNSWGSSWGEKGYIR-322	–	20	–
Histone H1	21	1-MSSDSAVAALSAAMT-15	31	–	88.3
	22	27-KTAAKKAAAKKAAAK-41	29	–	182.6
	23	32-KAAAKKAAAKKAGAK-46	29	–	239.9
	24	37-KAAAKKAGAKKAGAK-51	29	–	–
	25	42-KAGAKKAGAKKAVRK-56	29	–	–
	26	2-SSDSAVAALSAAMTS-16	28	–	123.7
	27	56-KVATPKKPAKKAAKK-70	24	–	–
	28	36-KKAAAKKAGAKKAGA-50	–	24	–
	29	41-KKAGAKKAGAKKAVR-55	–	24	–
	30	71-AAKKPAKKVAKKPAK-85	–	24	–
	31	16-SPQKSPRSSPKKTAA-30	–	22	–
	32	21-PRSSPKKTAAKKAAA-35	–	22	–
	33	26-KKTAAKKAAAKKAAA-40	–	22	163.5
	34	31-KKAAAKKAAAKKAGA-45	–	22	190.3
	35	45-AKKAGAKKAVRKVAT-59	–	22	–
	36	51-KKAVRKVATPKKPAK-65	–	22	–
	37	55-RKVATPKKPAKKAAK-69	–	22	–
	38	59-TPKKPAKKAAKKAAK-73	–	22	–
	39	63-PAKKAAKKAAKKPAK-77	–	22	–
	40	67-AAKKAAKKPAKKVAK-81	–	22	–
	41	75-PAKKVAKKPAKKAAK-89	–	22	–
	42	79-VAKKPAKKAAKKPAK-93	–	22	–
	43	83-PAKKAAKKPAKKPAK-97	–	22	–
	44	87-AAKKPAKKAAKKAAK-101	–	22	–
	45	91-PAKKPAKKAAKKAAK-105	–	22	–

(Continued)

Table 3 | Continued

Protein	No. of epitope	Epitope sequence	Score[a]		
			SYFPEITHI		NetMHCII
			H2-IA[d]	H2-IE[d]	H2-IA[d]
	46	95-PAKKAAKKAAKKAAA-109	–	22	–
	47	5-SAVAALSAAMTSPQK-19	21	–	382.8
	48	76-AKKVAKKPAKKAAKK-90	21	20	–
	49	96-AKKAAKKAAKKAAAK-110	21	–	–
	50	29-AAKKAAAKKAAAKKA-43	–	–	107.9
	51	30-AKKAAAKKAAAKKAG-44	–	–	119.1
	52	25-PKKTAAKKAAAKKAA-39	–	–	119.6
	53	24-SPKKTAAKKAAAKKA-38	–	–	138.5
	54	3-SDSAVAALSAAMTSP-17	–	–	166.2
	55	28-TAAKKAAAKKAAAKK-42	–	–	183.3
	56	35-AKKAAAKKAGAKKAG-49	–	–	208.6
	57	34-AAKKAAAKKAGAKKA-48	–	–	232.0
	58	4-DSAVAALSAAMTSPQ-18	–	–	272.1
	59	48-AGAKKAVRKVATPKK-62	–	–	295.2
	60	33-AAAKKAAAKKAGAKK-47	–	–	317.8
	61	49-GAKKAVRKVATPKKP-63	–	–	457.6
KMP-11	62	4-TYEEFSAKLDRLDEE-18	23	–	–
	63	75-QKFAELLEQQKAAQN-89	19	–	–
	64	45-KEHYEKFERMIKEHT-59	–	18	–
	65	74-KQKFAELLEQQKAAQ-88	–	18	–
LeIF	66	100-LSPTRELALQTAEVI-114	28	–	264.2
	67	320-VLVTTDLVARGICVH-334	28	–	–
	68	199-LPKDIQVALFSATMP-213	27	–	–
	69	387-HTQIDELPVDFAAYL-401	27	–	–
	70	138-QDDLRKLQAGVIVAV-152	26	–	205.3
	71	166-ALRTESLRVLVLDEA-180	26	–	–
	72	169-TESLRVLVLDEADEM-183	26	–	–
	73	62-RGGDIIAQAQSGTGK-76	25	–	–
	74	140-DLRKLQAGVIVAVGT-154	24	–	256.8
	75	142-RKLQAGVIVAVGTPG-156	24	–	–
	76	259-MDLYETVSIAQSVIF-273	24	–	–
	77	223-FMRDPVRILVKRESL-237	–	24	–
	78	325-DLVARGIDVHHVNIV-339	23	–	–
	79	168-RTESLRVLVLDEADE-182	22	–	–
	80	263-ETVSIAQSVIFANTR-277	22	–	–
	81	293-TVSSMHAEMPKSDRE-307	22	–	–
	82	314-RSGSSRVLVTTDLVA-328	22	–	–
	83	268-AQSVIFANTRRKVDW-282	–	22	–
	84	16-LDDQPGVRPIPSFDD-30	20	–	–
	85	71-QSGTGKTGAFSIGLL-85	20	–	–
	86	107-ALQTAEVISRIGEFL-121	20	–	–
	87	174-VLVLDEADEMLSQGF-188	20	–	–
	88	255-LDTLMDLYETVSIAQ-269	20	–	–
	89	376-VELLHEIEAHYHTQI-390	20	–	–
	90	77-TGAFSIGLLQRLDFR-91	–	20	–

(Continued)

Table 3 | Continued

Protein	No. of epitope	Epitope sequence	Score[a]		
			SYFPEITHI		NetMHCII
			H2-IA^d	H2-IE^d	H2-IA^d
	91	81-SIGLLQRLDFRHNLI-95	–	20	–
	92	158-VSDVIKRGALRTESL-172	–	20	–
	93	102-PTRELALQTAEVISR-116	–	–	95.3
	94	103-TRELALQTAEVISRI-117	–	–	95.4
	95	101-SPTRELALQTAEVIS-115	–	–	163.8
	96	139-DDLRKLQAGVIVAVG-153	–	–	249.0
	97	99-VLSPTRELALQTAEV-113	–	–	263.1
	98	98-LVLSPTRELALQTAE-112	–	–	287.6
	99	141-LRKLQAGVIVAVGTP-155	–	–	296.7
	100	49-PSSIQQRAIAPFTRG-63	–	–	320.9
	101	48-KPSSIQQRAIAPFTR-62	–	–	378.0
	102	50-SSIQQRAIAPFTRGG-64	–	–	434.6
	103	97-GLVLSPTRELALQTA-111	–	–	452.0
	104	290-SNHTVSSMHAEMPKS-304	–	–	489.1

[a]*The cut-off score was adjusted to ≥18 for SYFPEITHI, ≥100 for BIMAS, and <500 for NetMHCII.*

System (Endosafe, Charles River Laboratories, USA). Synthetic multi-epitope peptides were also checked for the presence of 9-mer or/and 15-mer epitopes able to bind to HLA alleles (A2, A3, A24, B7, B15, DP, DQ, DR supertypes) using the above mentioned algorithms (**Table 4**). In addition, data on the crystal structure of HLA-A2 and HLA-DRB1 molecules were obtained from Protein Data Bank (PDB, codes 1HHG and 2SEB, respectively) and multi-epitope peptides of length 30 aa were transformed into PDB files using SWISS-MODEL, and each of them was docked with HLA-A2 or HLA-DRB1 molecule using the ClusPro program[8] for structure-based analysis (46–49).

IMMUNIZATION OF BALB/C MICE

Eight groups of female BALB/c mice ($n = 8$/group), 6–8 weeks old, were immunized subcutaneously at upper and lower dorsal region, with 100 μl emulsion consisting of 50 μg of each synthetic multi-epitope peptide in complete Freund's adjuvant (CFA). Mice were also received a second immunization with 100 μl emulsion of 50 μg of the same peptide in incomplete Freund's adjuvant (IFA), as well as a third immunization with 50 μg of peptide alone in PBS at 2 weeks intervals. Two sex and age matched groups of mice ($n = 8$/group) immunized similarly either with the adjuvant or with PBS alone, were served as control groups.

Animals were obtained from the breeding unit of the Hellenic Pasteur Institute (Athens, Greece) and reared in institutional facilities under specific pathogen-free conditions, receiving a diet of commercial food pellets and water *ad libitum*. All experimental procedures had been approved by the institutional Animal Bioethics Committee regulating according to the EU Directive 2010/63 and the National Law 2013/56.

[8]http://cluspro.bu.edu

CULTURE OF LYMPHOCYTES AND PROLIFERATION ASSAYS

Fifteen days post the third immunization, spleens from immunized and control mice ($n = 3$/group) were collected in aseptic conditions and used for the preparation of single cell suspensions in RPMI-1640 medium (Biochrom AG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM Hepes, 24 mM $NaHCO_3$, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) heat inactivated fetal bovine serum (FBS; Gibco, Paisley, UK) at a density of 1×10^6 cells/ml. Cell viability was >95% as determined by trypan blue exclusion. A volume of 200 μl/well were placed in triplicate into 96-well U-bottomed plates in the presence of various concentrations of each synthetic multi-epitope peptide ranging from 5 to 40 μg/ml and incubated for 96 h in 5% CO_2 at 37°C in a humidified atmosphere. The optimal concentration for each peptide was determined at 10 μg/ml and used thereafter for recall stimulation. Cells cultured in medium alone or in the presence of Concanavalin A (6 μg/ml) were served as negative or positive control, respectively. Cells were pulsed with 1 μCi/ml of ^{3}H-TdR (GE Healthcare, Buckinghamshire, UK) for the final 18 h of the culture period. Cells were harvested and ^{3}H-TdR incorporation was determined on a microplate scintillation counter (Microbeta Trilux, Wallac, Turku, Finland). The results were expressed as Δcpm (cpm of cells from immunized mice stimulated with peptide – cpm of immunized mice cultured in medium alone). Proliferative response against to each synthetic peptide giving Δcpm > 2000 was considered as positive.

CYTOKINE DETECTION AND FLOW CYTOMETRY

Spleen cells from immunized and control mice ($n = 5$/group) were also used for cytokine detection and flow cytometry. Briefly, 1 ml/well of lymphocytes in complete RPMI-1640 medium at a density of 2×10^6 cells/ml were placed in triplicate into 24-well

Table 4 | Synthetic multi-epitope peptides including MHC class I and II-restricted epitopes.

Peptide name	Synthetic multi-epitope peptide sequence	Included epitopes	HLA supertype
CPA_p2	160-GNIEGQWALKNHSLVSLSEQVLVSCDNIDD-189	165-QWALKNHSL-173 179-QVLVSCDNI-187 160-GNIEGQWAL-168 172-SLVSLSEQVLVSCDN-186 174-VSLSEQVLVSCDNID-188	HLA-A2 (A*0201), HLA-A3 (A*03), HLA-DRB1, HLA-DPA1, HLA-DQA1
CPA_p3	273-LYFGGVVTLCFGLSLNHGVLVVGFNRQAKP-302	273-LYFGGVVTL-281 279-VTLCFGLSLNHGVLV-293 273-LYFGGVVTLCFGLSL-287	HLA-A2 (A*0201), HLA-A3 (A*03), HLA-A24 (A*2402), HLA-DRB1, HLA-DPA1, HLA-DQA1
H1_p1	1-MSSDSAVAALSAAMTSPQKS-20	2-SSDSAVAAL-10 1-MSSDSAVAALSAAMT-15 2-SSDSAVAALSAAMTS-16 5-SAVAALSAAMTSPQK-19	HLA-A2 (A*0201), HLA-A3 (A*03), HLA-DRB1, HLA-DQA1
H1_p3	43-AGAKKAGAKKAVRKVATPKK-61	46-KKAGAKKAV-54 42-KAGAKKAGAKKAVRK-56 45-AKKAGAKKAVRKVAT-59	HLA-A2 (A*0201), HLA-A3 (A*03), HLA-DRB1
KMP-11_p1	4-TYEEFSAKLDRLDEEFNRKM-23	4-TYEEFSAKL-12 7-EFSAKLDRL-15 4-TYEEFSAKLDRLDEE-18	HLA-A3 (A*03), HLA-A24, HLA-DRB1, HLA-DPA1, HLA-DQA1
LeIF_p1	6-KIAPQDQDSFLDDQPGVRPIPSFDDMPLHQ-35	8-APQDQDSFL-16 25-IPSFDDMPL-33 14-SFLDDQPGV-22 23-RPIPSFDDM-31 16-LDDQPGVRPIPSFDD-30	HLA-B7 (B*5101), HLA-B15 (B62), HLA-DRB1, HLA-DPA1, HLA-DQA1
LeIF_p3	181-DEMLSQGFADQIYEIFRFLPKDIQVALFSA-210	199-LPKDIQVAL-207 197-RFLPKDIQV-205 187-GFADQIYEI-195 195-IFRFLPKDI-203 199-LPKDIQVALFSATMP-213	HLA-B7 (B*3501, B*5101), HLA-DRB1, HLA-DPA1, HLA-DQA1
LeIF_p6	371-VTEKDVELLHEIEAHYHTQIDELPVDFAAY-400	385-HYHTQIDEL-393 393-LPVDFAAYL-401 320-VLVTTDLVARGICVH-334 387-HTQIDELPVDFAAYL-401 376-VELLHEIEAHYHTQI-390	HLA-A3 (A*0301), HLA-A24, HLA-DRB1, HLA-DPA1, HLA-DQA1

plates and stimulated with 10 μg/ml of each synthetic multi-epitope peptide. Cells were cultured for 72 h in 5% CO_2 at 37°C in a humidified atmosphere. At the end of the incubation period, culture supernatants were collected and stored at −80°C until analyzed for their cytokine content. The concentrations of IFN-γ and IL-10 in the supernatants were determined by sandwich ELISA kits (900-K98, 900-K53; PeproTech, Rocky Hill, NJ, USA) according to the manufacturer's instructions. The cytokine concentrations were calculated by reference to standard curves; detection threshold for IFN-γ and IL-10 was 23 and 47 pg/ml, respectively.

In parallel, at 48 h of culture period, similarly cultured cells were exposed for 4 h to 2.5 μg/ml brefeldin A (Fluka, Buchs, Germany), washed in FACS buffer (PBS-2% FBS) and stained with anti-CD4 and anti-CD8 monoclonal antibodies (mAbs) conjugated either with FITC (anti-CD4-FITC, clone RM4-5) or PE (anti-CD4-PE, clone H129.19; anti-CD8-PE, clone 53-6.7) for 30 min. For the identification of intracellular cytokine production, cells were permeabilized using FACS buffer supplemented with 0.1% (v/v) saponin (Sigma) and stained for 30 min on ice with anti-IFN-γ conjugated with FITC (clone XMG1.2) or anti-IL-4 conjugated with PE (clone BVD4-1D11) mAbs. In all cases, control cells were processed similarly using matched isotype control. All mAbs used in the study, were purchased from BD Biosciences (Erembodegem, Belgium). For each sample, 20,000 cells were analyzed on a FACSCalibur (Becton-Dickinson, San Jose, CA, USA) and the data were processed with Cell Quest Software (Becton-Dickinson). The percentage of specific cytokine-producing $CD4^+$ or $CD8^+$ T cells relative to total numbers of $CD4^+$ or $CD8^+$ T cells was determined by analysis of FACS data using the FlowJO software package (Tree Star, Inc., Ashland, OR, USA). The percentage

of peptide-specific cytokine-producing cells was normalized to their respective proportion in unstimulated cells from mice immunized with CFA/IFA alone, in order to allow for comparison among all the synthetic multi-epitope peptides.

ENZYME LINKED IMMUNOSORBENT ASSAYS

Blood collected from each group of mice ($n = 8$/group) at fifteenth day post the third immunization, were centrifuged at $4000 \times g$ for 5 min and separated sera were aliquoted for the detection of specific antibodies against each synthetic multi-epitope peptide by specific ELISAs as previously described (50). In brief, 96-well microtiter plates were coated with 5 μg/ml of each individual peptide in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$), pH 9.6 and left overnight at 4°C. For the detection of total IgG antibodies, 10-fold dilutions of each serum sample in 1% BSA in PBS-T were added and incubated with HRP-labeled goat anti-mouse IgG (1/1000 dilution; GE Healthcare, Buckinghamshire, UK). For the detection of IgG1 and IgG2a isotypes, serum samples (1/100 dilution) were added and plates were similarly incubated either with biotin-labeled rat anti-mouse IgG1 (500 ng/ml; AbD Serotec, Oxford, UK) or IgG2a (250 ng/ml; AbD Serotec) followed by the addition of streptavidin–HRP (1/5000 dilution; AbD Serotec) and incubation for 1 h at 37°C. The cut-off value was determined as the mean OD value of normal mouse serum in a 1/100 dilution + 2SD.

STATISTICAL ANALYSIS

Data were expressed as the mean value with the standard deviation (SD) indicated. Statistical significant differences of the mean values between groups of mice immunized with synthetic multi-epitope peptide emulsified in CFA/IFA and mice immunized with CFA/IFA alone were assessed by unpaired Student's t-test. The probability (p) of <0.05 was considered to indicate statistical significance.

RESULTS

IN SILICO PREDICTION OF PROMISING EPITOPES OF *L. INFANTUM* PROTEINS BIND TO MHC CLASS I AND II MOLECULES

CPA, Histone H1, KMP-11, and LeIF have already been defined as candidate antigens. CPA, a protein significantly up-regulated in mature amastigotes (40), is predicted as a secretory protein by SignalP (cleavage site between position 24 and 25 residue), while Histone H1, an also highly expressed protein in mature amastigotes (51), KMP-11, a cytoskeleton-associated protein, and LeIF constitutively expressed in both promastigotes and amastigotes (45, 52), are predicted as non-secretory. *In silico* analysis of proteins for the prediction of binding epitopes to $H2^d$ MHC class I and II molecules revealed, in total, 41 9-mer and 104 15-mer peptides, respectively, which scored above the cut-off value of each algorithm used for the prediction (**Tables 2** and **3**).

In particular, 14 and 20 highly scored binding peptides to H2-K^d and H2-IA^d/IE^d alleles, respectively, were predicted by BIMAS and SYFPEITHI for CPA. These peptides were spanning throughout the protein amino-acid sequence and they covered the 69.2% of its length. In addition, 2 and 41 binding peptides to H2-K^d/L^d and H2-IA^d/IE^d alleles, respectively, were predicted by SYFPEITHI and NetMHCII for Histone H1. The peptides were also spanning throughout Histone H1 amino-acid sequence, covering the entire length of the protein. Five and four binding peptides to H2-K^d/L^d and H2-IA^d/IE^d alleles, respectively, were predicted by BIMAS and SYFPEITHI for KMP-11. Peptides were gathered in the middle, as well as in the amino- and carboxy-terminal region of KMP-11 sequence, and covered the 55.4% of its length. In regards to LeIF, 20 and 39 binding peptides to H2-K^d/L^d/D^d and H2-IA^d/IE^d alleles, respectively, were predicted by BIMAS, SYFPEITHI, and NetMHCII. Peptides were spanning throughout LeIF amino-acid sequence and covered the 79.4% of the entire protein.

SYNTHETIC MULTI-EPITOPE PEPTIDES CONTAINING BOTH MHC CLASS I- AND II-RESTRICTED EPITOPES

Based on the above data, eight peptides, 20–30 amino-acid length, were designed and synthesized. At least, one MHC class I-restricted epitope scored very high, as well as adjacent or overlapping MHC class II-restricted epitopes scored also high were nested in each synthetic peptide (**Table 4**). These multi-epitope peptides included CPA_p2 (160-GNIEGQWALKNHSLVSLSEQVLVSCDNIDD-189) and CPA_p3 (273-LYFGGVVTLCFGLSLNHGVLVVGFNRQAKP-302) from CPA, H1_p1 (1-MSSDSAVAALSAAMTSPQKS-20) and H1_p3 (43-AGAKKAGAKKAVRKVATPKK-61) from Histone H1, KMP-11_p1 (4-TYEEFSAKLDRLDEEFNRKM-23) from KMP-11, LeIF_p1 (6-KIAPQDQDSFLDDQPGVRPIPSFDDMPLHQ-35), LeIF_p3 (181-DEMLSQGFADQIYEIFRFL PKDIQVALFSA-210), and LeIF_p6 (371-VTEKDVELLHEIEAHYHTQIDELPVDFAAY-400) from LeIF. Synthetic peptide sequences showed to be retrieved from highly conserved regions of *L. infantum* proteins, since protein BLAST analysis revealed up to 95% residue identity to homologous sequences of corresponding proteins of strains belonging to *L. major* and *L. donovani* complexes. In addition, promiscuous 9-mer and 15-mer epitopes bound to HLA alleles (A2, A3, A24, B7, B15, DP, DQ, DR supertypes) were also nested in synthetic multi-epitope peptides as predicted by *in silico* analysis using the above mentioned algorithms.

VALIDATION OF SYNTHETIC MULTI-EPITOPE PEPTIDES IMMUNOGENICITY IN MICE

Immunogenicity of the eight synthetic multi-epitope peptides was validated in BALB/c mice ($H2^d$ haplotype) immunized with each synthetic peptide in combination with CFA/IFA, 15 days post third immunization. Specific proliferative T cell responses induced by synthetic multi-epitope peptides were firstly assessed. As shown in **Figure 1A**, CPA_p2, CPA_p3, H1_p1, and LeIF_p6 induced strong proliferation of spleen cells upon *in vitro* re-stimulation ($\Delta cpm > 1128 \pm 165$) at the optimal dose of 10 μg/ml. Of these, CPA_p2 induced the strongest proliferation, followed by LeIF_p6, CPA_p3, and H1_p1. The results indicated that four of the eight candidate peptides could effectively induce spleen cell proliferation.

To validate the profile of cytokines secreted in response to the eight synthetic multi-epitope peptides, spleen cell culture supernatants from immunized mice were analyzed for their content in IFN-γ and IL-10 at 72 h post respective peptide *in vitro* re-stimulation. Quantitation by ELISA revealed that all peptides, except from LeIF_p1 and KMP-11_p1, induced the secretion of

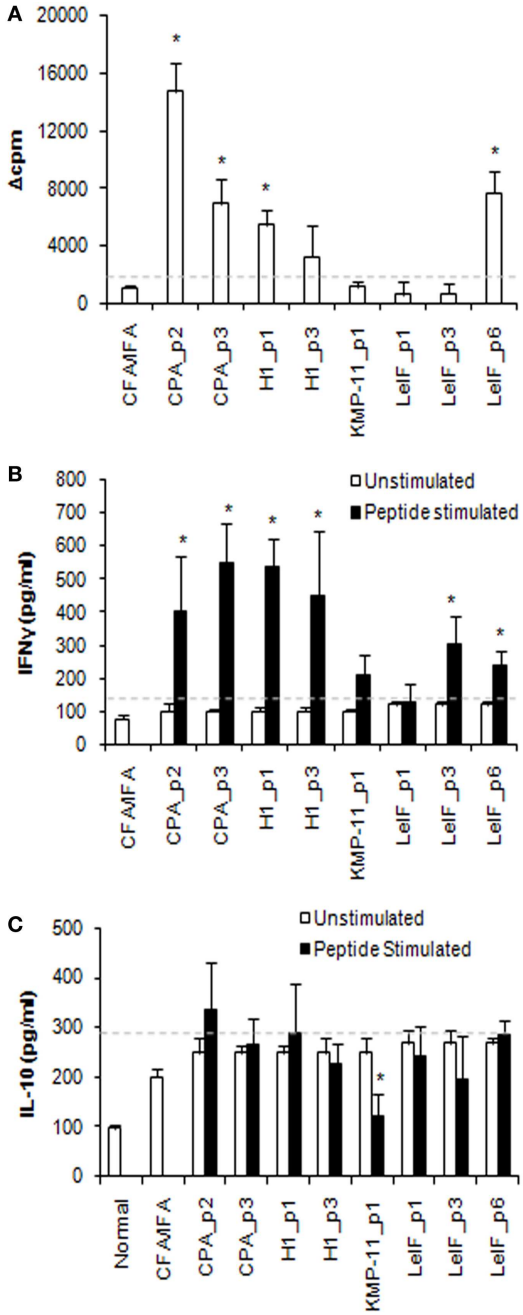


FIGURE 1 | Multi-epitope peptide-specific proliferative responses and cytokine secretion. **(A)** Proliferative responses. Spleen cells from BALB/c mice ($n = 3$/group) immunized either with individual peptide emulsified in CFA/IFA or PBS alone, were re-stimulated *in vitro* with the respective peptide (10 μg/ml) for 72 h. Cultures were pulsed for the final 18 h with 1 μCi of [^{3}H]-TdR and results are depicted as Δcpm ± SD as described in Section "Materials and Methods." Spleen cells derived from mice immunized with PBS alone, stimulated *in vitro* with ConA (Δcpm: 39743 ± 843) were used for comparison purposes. **(B)** IFN-γ and **(C)** IL-10 secretion. Cytokines were detected in culture supernatants of spleen cells from immunized BALB/c mice ($n = 5$/group), re-stimulated *in vitro* with the respective peptide (10 μg/ml) for 72 h, by ELISA. The results are expressed as pg/ml ± SD. Significant differences between groups of mice immunized with each synthetic peptide emulsified in CFA/IFA and the group of mice immunized with CFA/IFA alone are indicated by * ($P < 0.05$).

high amounts of IFN-γ in comparison to mice immunized with CFA/IFA alone (**Figure 1B**). CPA_p3, H1_p1, CPA_p2, and H1_p3 were able to induce the highest secretion of IFN-γ, followed by LeIF_p3 and LeIF_p6. In contrast, unstimulated spleen cells from immunized mice produced low levels of IFN-γ spontaneously, similar to those measured in the culture supernatants of spleen cells from mice immunized with CFA/IFA alone. The results suggest that the majority of the candidate peptides could induce IFN-γ secretion.

In addition, low levels of IL-10 were detected in the supernatants of spleen cells stimulated *in vitro* with each synthetic multi-epitope peptide (**Figure 1C**). These levels were comparable to those detected in the supernatants of unstimulated spleen cells, as well as in the supernatants of spleen cells from mice immunized with CFA/IFA alone. In particular, KMP-11_p1 indicated a rather suppressive effect on IL-10 production.

To further confirm the pattern of cytokines induced by each synthetic multi-epitope peptide, intracellular cytokine production was determined in spleen cells from immunized mice at 48 h post peptide *in vitro* re-stimulation using flow cytometry. As shown in **Figure 2A**, none of the peptides tested were able to stimulate important peptide-specific IL-4 production by $CD4^+$ T cells, although a certain predisposition in BALB/c mice has been documented by previous studies (53). In contrast, CPA_p2, CPA_p3, H1_p3, LeIF_p3, and LeIF_p6 were able to stimulate important peptide-specific IFN-γ production by $CD4^+$ T cells, indicating a T_H cell driven toward the T_{H1} type.

Regarding the ability of the synthetic multi-epitope peptides to induce the production of IFN-γ by $CD8^+$ T cells, flow cytometry revealed that one of them, H1_p1 strongly induced the production of IFN-γ by splenic $CD8^+$ T cells of immunized mice. H1_p3, CPA_p2, and CPA_p3 were also able to stimulate peptide-specific IFN-γ production by $CD8^+$ T cells in a lower level than that detected in H1_p1 (**Figure 2C**). Flow cytometry overall results indicated that most of the peptides tested induced IFN-γ production from $CD4^+$ and/or $CD8^+$ T cells confirming the results obtained with *in silico* analysis (**Figures 2B,D**).

Furthermore, specific antibodies of IgG class, as well as of IgG1 and IgG2a isotypes, were detected in the serum of mice immunized with each synthetic peptide emulsified in CFA/IFA, 15 days post third immunization, in order to evaluate peptide effect on humoral response. According to the results, all the synthetic multi-epitope peptides were able to induce the secretion of specific IgG antibodies (**Figures 3A,B**). Of these, CPA_p2, CPA_p3, LeIF_p3, and LeIF_p6 induced the highest secretion, followed by KMP-11_p1, LeIF_p1, H1_p1, and H1_p3. Analysis of isotype pattern showed that CPA_p2 strongly induced the production of both IgG2a and IgG1 isotypes, followed by CPA_p3, while LeIF_p3 induced the production of IgG2a > IgG1 (**Figure 3C**). In contrast, LeIF_p6 strongly induced the production of IgG1 isotype and weakly the production of IgG2a isotype. The other four peptides, KMP-11_p1, LeIF_p1, H1_p1, and H1_p3, had insignificant effect on the production of these two IgG isotypes.

Next, we employed a structure-based method for further analysis of the tertiary structure of the most promising synthetic peptides (CPA_p2, CPA_p3, H1_p3, LeIF_p3) that bound to HLA-A2 or HLA-DRB1 molecule, since HLA-restricted epitopes were also nested in peptide sequences according to algorithms prediction

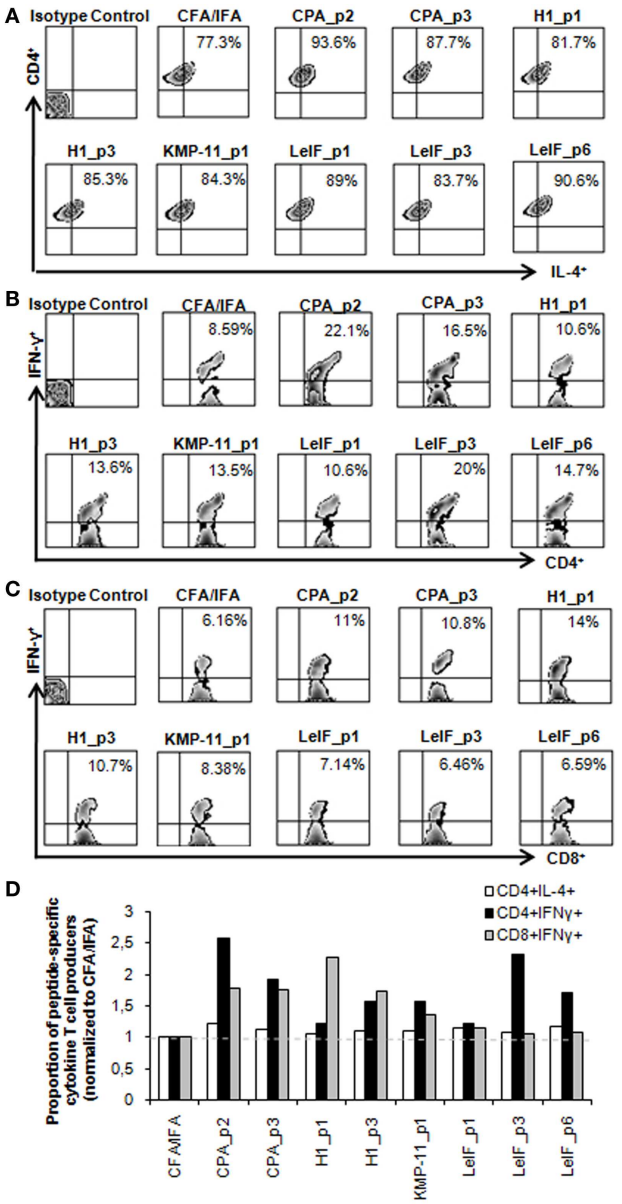


FIGURE 2 | Multi-epitope peptide-specific cytokine production by CD4$^+$ and CD8$^+$ T cells. Spleen cells from BALB/c mice ($n = 5$/group) immunized either with individual peptide emulsified in CFA/IFA or PBS alone, were re-stimulated *in vitro* with the respective peptide (10 μg/ml) for 48 h, and analyzed for CD4$^+$ and CD8$^+$ T cells producing IL-4 and IFN-γ. **(A–C)** Representative FACS plots of intracellular staining used to define IL-4- and IFN-γ-expressing CD4$^+$ and CD8$^+$ T cells in spleens derived from immunized mice. Values represent the percentages of **(A)** IL-4$^+$ cells among CD4$^+$ T cells and **(B,C)** IFN-γ$^+$ cells among CD4$^+$ and CD8$^+$ T cell populations. **(D)** Quantification of IL-4 and IFN-γ producing T cells in immunized mice. All data were normalized to CFA/IFA control group. Each bar represents the mean proportion of IL-4- and IFN-γ-producing CD4$^+$ and CD8$^+$ T cells induced by the respective peptide.

(**Table 4**). Selection of HLA-A2 and HLA-DRB1 molecules was based on published data demonstrating high frequency of these supertypes in human population (54, 55). The ClusPro program was run to predict docked conformations presenting good surface complementarity with the two MHC molecules mentioned above. The most probable 3D models according to algorithm analysis indicating peptides located onto the peptide-binding cleft of the MHC molecules with good surface complementarity are presented in **Figure 4**.

DISCUSSION

In the perspective of second generation vaccines, a variety of different parasite molecules, such as secretory or transmembrane proteins, including enzymes and receptors, has been tested to date as candidate antigens for anti-*Leishmania* vaccine development (56). Among them, CPA, Histone H1, KMP-11, and LeIF were found to be highly immunogenic as described in murine experimental models, cured VL patients and *L. infantum* infected dogs and have been considered as potential vaccine candidates (39–41, 44, 45, 57). The induction of an effective T cell response against vaccine antigens requires antigen processing and peptide presentation by antigen-presenting cells (APCs), and it is well-established that T cells recognize the peptide sequence in association to appropriate MHC molecules. The discovery of MHC-binding motifs in proteins has led to the development of several algorithms predicting MHC class I- and II-restricted epitopes for presentation to CD8$^+$ or CD4$^+$ T cells, respectively, accelerating research related to peptide-based vaccine approach (15).

In the present study, we investigated the use of three algorithms, SYFPEITHI, BIMAS, and NetMHCII to predict sequences in CPA, Histone H1, KMP-11, and LeIF able to bind to MHC class I and II molecules of the H2^d haplotype. Furthermore, combining this approach with experimental validation in MHC compatible BALB/c mice, we determined epitopes in each protein and designed multi-epitope peptides capable to induce peptide-specific T cell proliferation and cytokine production by CD4$^+$ and/or CD8$^+$ T cells.

The analysis of protein sequences yielded a significant number of possible epitopes from all four proteins, but only few of them were predicted by all algorithms used with binding efficiency to more than one supertypes or alleles. Interestingly, comparison of predicted peptides for each MHC class I and II alleles showed a low overlapping level between the results obtained from different algorithms used in the study, indicating the significant differences existing in the database source of building matrix motifs and different forms of scoring function of each algorithm. Also, it was observed an antigenic region clustering. Based on these findings and to the fact that prediction of MHC class I-restricted epitopes is considered more reliable (>85%) than that of MHC class II-restricted epitopes, we designed eight multi-epitope peptides for all proteins, based predominantly on highly scored MHC class I-restricted epitopes. Adjacent or overlapping MHC class II-restricted epitopes scored high were also nested in each synthetic peptide. These multi-epitope peptides contained epitopes recognized also by HLA class I and II molecules as defined by *in silico* analysis. Until now, very few vaccine antigens against different pathogens such as viruses, bacteria, and parasites, contain promiscuous T cell epitopes that have the ability to induce T cell-mediated protective immune responses both in mice and human by binding

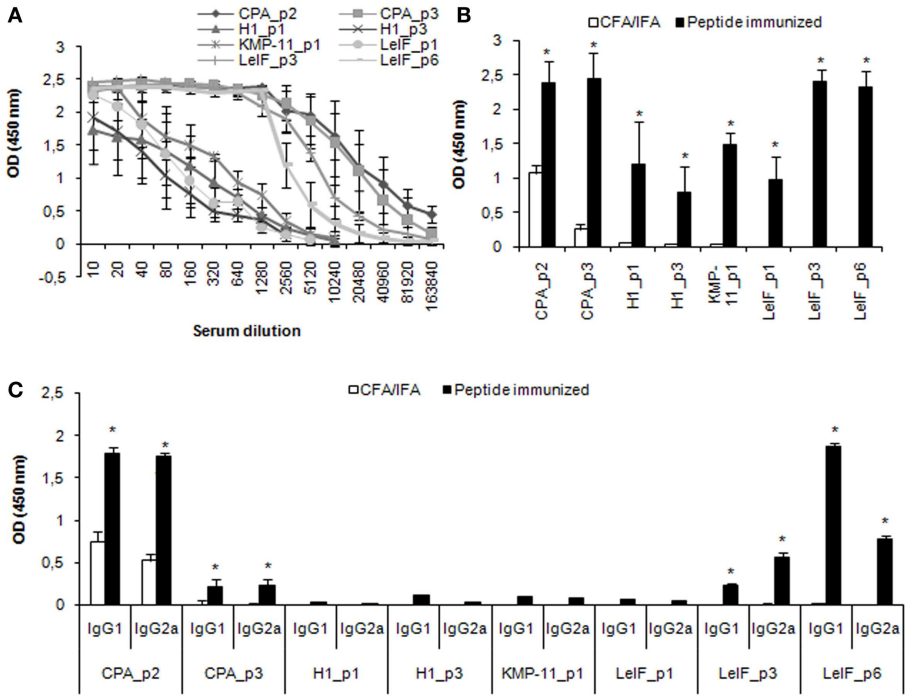


FIGURE 3 | Multi-epitope peptide-specific antibody production. BALB/c mice ($n = 9$/group) immunized either with individual peptide emulsified in CFA/IFA or PBS alone, were bled 15 days post third immunization and sera were separated. **(A,B)** total IgG Abs, and **(C)** IgG1 and IgG2a Abs against each peptide were assessed by ELISA. The results are expressed as $OD_{450} \pm SD$. Significant differences between groups of mice immunized with each synthetic peptide emulsified in CFA/IFA and the group of mice immunized with CFA/IFA alone are indicated by * ($P < 0.05$).

to several alleles of a supertype or between different supertypes (58, 59). Thus, these promiscuous epitope-driven vaccines could have the capacity of increasing the frequency of responders in genetically variable species, such as human populations (60).

The success of many vaccines is dependent on IFNγ-secreting $CD4^+$ T cells recruitment for long term protection. This accounts for better immunologic memory leading to sustained immunity after healing of live infections (61, 62). $CD4^+$ T cells are activated in terms of recognition of peptides-MHC class II complexes in the surface of APCs after protein processing in cells' endocytic compartment. Activation of IFN-γ-producing $CD4^+$ T cells plays a pivotal role in protective immune responses against leishmaniasis. Specifically, IFN-γ mediates macrophage activation against both the promastigote and amastigote forms in H_2O_2-dependent manner (63, 64) and nitric oxide production for parasite killing (65). According to our results, CPA_p2, CPA_p3, LeIF_p3, and LeIF_p6 induced peptide-specific IFN-γ production from $CD4^+$ T cells in immunized mice indicating the processing and recognition of MHC class II-specific epitopes by $CD4^+$ T cells. CPA and LeIF are considered significant candidate proteins for vaccine design against leishmaniasis. In the case of CPA, it has been shown that administration of plasmid encoding CPA induced specific T_{H1} immune responses resulting to partial protection against *L. major* in the experimental model of CL. However, protection was significantly enhanced when co-administered with CPB or as CPA/B hybrid protein (25–27), indicating the need of CPA co-administration with another protein or adjuvant. On the other hand, LeIF was originally described as a T_{H1}-type natural adjuvant and as an antigen inducing an IL-12 mediated T_{H1} response in the PBMCs of leishmaniasis patients (39). LeIF is also capable of inducing the secretion of cytokines IL-12, IL-10, and TNF-α by APCs from healthy individuals (52, 66, 67). Furthermore, recombinant trifusion vaccines (leish111; leish110f) were developed by incorporating the amino-terminal region of LeIF antigen. These vaccines were shown to be efficient in experimental or clinical trials for vaccination or immunotherapy (68).

Existing data suggest that secretory and surface exposed proteins strongly induce specific $CD8^+$ T cell responses (69, 70). A previous study applying *in silico* analysis revealed that a high number of peptides derived from *L. major* secretome could bind to H2 BALB/c molecules (71). Several studies have shown the great role played by $CD8^+$ T cells in protective immune responses against parasite in the susceptible BALB/c strain (72–74). Specifically, $CD8^+$ T cells either contributed in the destruction of *Leishmania*-infected cells by activating macrophages to oxidative burst via cytokines produced upon antigen stimulation (75, 76), or regulating $CD4^+$ T cell-mediated immune responses (77, 78). In our study, both synthetic multi-epitope peptides of CPA, CPA_p2, and CPA_p3, except from $CD4^+$IFN-γ^+ T cells activation, induced significant IFN-γ production by $CD8^+$ T cells. CPA_p2 and CPA_p3 belong to the secreted region of CPA as SignalP analysis showed (30, 31). Interestingly, another study applying *in silico* analysis in CPA sequence with MULTIPRED algorithm indicated the existence of four highly immunogenic regions recognized by the

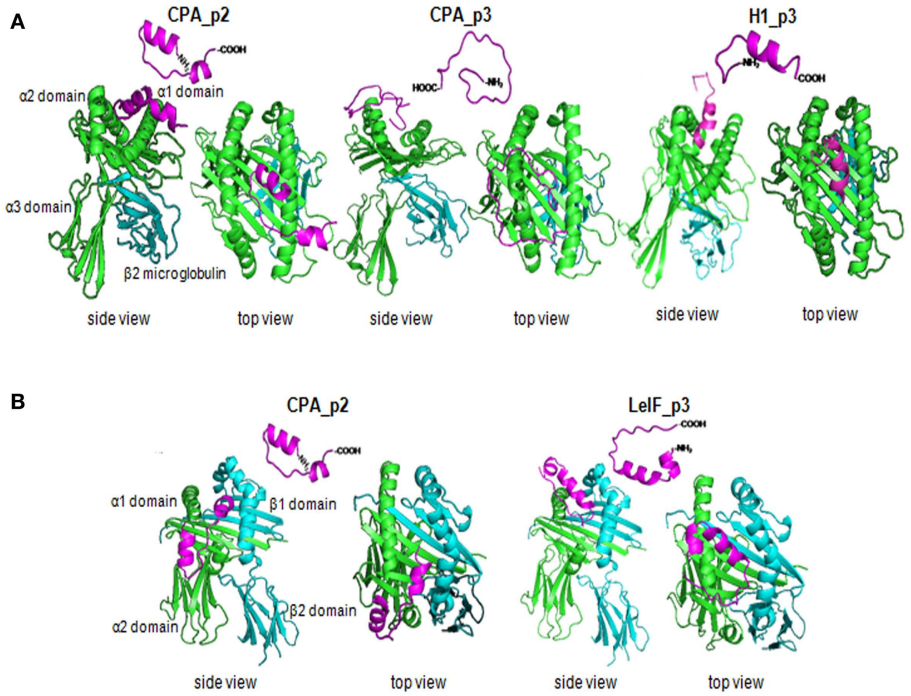


FIGURE 4 | Synthetic multi-epitope peptides docking. Ribbon diagram of 3D structural analysis of interactions between **(A)** HLA-A2 molecule (PDB-code: 1HHG) and the synthetic peptides CPA_p2, CPA_p3, H1_p3, and LeIF_p3, and **(B)** HLA-DRB1 molecule (PDB-code: 2SEB) and the synthetic peptides CPA_p2 and LeIF_p3. Candidate peptides were predicted to locate onto the peptide-binding cleft of the HLA molecules by using ClusPro program. The side and top view are shown, the α strands were shown in green, the β strands in blue, and the multi-epitope peptides in magenta.

HLA-A2 supertype (79), which harbored parts from our CPA multi-epitope peptides.

As for KMP-11, KMP-11_p1 belonged to the amino-terminal region of the protein and in contrast to previous observations this synthetic peptide was proved to be poorly immunogenic, indicated by the absence of peptide-specific proliferative response and cytokine secretion in immunized mice. Previous studies concerning the identification of T cell epitopes using infected macrophages or DCs as APCs, revealed the existence of potential HLA class I- and II-restricted T cell epitopes in the amino-terminal region, characterizing a dominant cluster between position 1 and 33 of KMP-11 sequence that could trigger specific cellular immune responses in *L. donovani*- or *L. panamensis*-infected volunteers (80, 81). Furthermore, hybrid-cell, DNA-based or heterologous KMP-11-DNA/rVV based vaccination exhibited immunoprotective capacity in susceptible VL murine models. Protection was accompanied with generation of antigen specific $CD4^+$ and $CD8^+$ T cells that produced effector cytokines such as IFN-γ, IL-2, and TNF-α (36–38, 82, 83). Also in a previous work, we demonstrated that vaccination with *ex vivo* pulsed bone marrow-derived dendritic cells with $KMP\text{-}11_{12–31aa}$ peptide and CpG as adjuvant induced strong Th1 and Th17 protective immune responses in murine model of VL (50). However, in the present study it is noteworthy that secretion of IL-10 was also abrogated. These results together suggest that KMP-11_p1 may be consisted from natural epitopes contributing in parasite host immunomodulation, allowing parasite dissemination rather than stimulate protective immune responses.

However, not only external or secreted *Leishmania* antigens are able to be presented in the context of MHC class I molecules but also intracellular proteins (84, 85). As such, in our study H1_p1 and H1_p3 induced a T cell response characterized mainly by $CD8^+$ T cell priming and production of IFN-γ in immunized mice. Although, this way of cell activation in leishmaniasis remains controversial and it is not clear how non-secretory parasite antigens such as histone H1 can be presented endogenously in the context of MHC class I molecules, a number of studies supports the induction of specific $CD8^+$ T cell responses against structural parasite proteins in animal models and VL patients (82, 84, 86, 87). Previous results from our group supported that *ex vivo* pulsed bone marrow-derived dendritic cells with the *Leishmania* histone H1 elicited significant protection in the experimental model of VL, with a pronounced enhancement of parasite-specific IFNγ-producing $CD8^+$ T cells (88). The protective effect of *Leishmania* histone H1 against *L. major* or *L. infantum* infections was also shown in different experimental animal models (34, 35) suggesting that it is also a promising vaccine candidate against leishmaniasis. In contrast, none of the LeIF peptides tested could evoke specific $CD8^+$ T cell responses. This finding was in agreement with the study of Rafati et al. showing that PBMCs from patients recovered from *L. major* failed to elicit HLA-A2-restricted $CD8^+$ T cell responses against three synthetic nonamer peptides

of LeIF, suggesting that these peptides are not able to induce a $CD8^+$ T cell-induced protective immunity (86).

The relative low concentrations of IL-10 detected in the supernatants of immune lymphocytes compared to IFN-γ after peptide re-stimulation were consistent with the suggestion of a dynamic reciprocal relationship between these two cytokines. IL-10 primarily down-modulates innate as well as acquired immunity leading to parasite establishment or disease progression. In experimental model of VL, IL-10 prevents DCs migration in spleen to activate T cells (89, 90) and suppresses both T_{H1} and T_{H2} cells (91). Also, the $CD4^+IL\text{-}4^+$ T lymphocytes detected in the presence of all peptides may be attributed to BALB/c intrinsic feature to induce the production of type-2 cytokines, such as IL-4 (53), since there was not any significant difference of IL-4 levels between peptide-immunized mice and control mice receiving the adjuvant alone. Furthermore, IFA adjuvant has a propensity to induce preferentially T_{H2} cytokines (92, 93). Similar study for the evaluation of immunoreactivity of *in silico* predicted T_{H1} epitopes of *Schistosoma japonicum* showed that high levels of IL-4 were attributed to Freund's adjuvant and BALB/c strain used (94). Therefore, in terms of proportion of intracellular cytokine production of recall $CD8^+$ and $CD4^+$ T cells, it is concluded that CPA_p3, H1_p1, H1_p3, CPA_p2, LeIF_p3, and LeIF_p6 synthetic multi-epitope peptides are likely to include potential epitopes for the induction of protective cytotoxic (CTL) and T_{H1}-type immune responses. Taken into account that the sequences of these synthetic peptides are highly conserved and bind in a promiscuous manner to murine or human MHC molecules according to *in silico* analysis and structure-based techniques, make them candidate vaccines against leishmaniasis. Based on these results, it would be worthwhile conducting future investigations for the verification of peptides' possible ability to induce protection in common or humanized mouse models of leishmaniasis. The incorporation of alternative and/or additional epitopes, the use of modern adjuvants and new antigen delivery systems should be combined. Conclusively, these findings give complementary data on epitope mapping for *Leishmania* proteins and demonstrate that combination of immunoinformatic approaches with experimental validation enables peptide identification with greater accuracy contributing to rational epitope-based vaccine development.

AUTHOR CONTRIBUTIONS

Evita Athanasiou and Olga Koutsoni contributed equally to this work. Conceived and designed the experiments: Evdokia Karagouni. Performed computational analysis: Maria Agallou, Evita Athanasiou, Olga Koutsoni, Evdokia Karagouni. Performed the experiments: Maria Agallou, Olga Koutsoni, Evita Athanasiou. Analyzed the data: Maria Agallou, Evita Athanasiou, Olga Koutsoni, Evdokia Karagouni, Eleni Dotsika. Wrote the paper: Evdokia Karagouni, Maria Agallou.

ACKNOWLEDGMENTS

This work was supported by a grant (09SYN-14-643) from EU and the National Ministry of Education and Religion Affairs under the Operational Strategic Reference Framework (NSFR 2007–2013) awarded to Evdokia Karagouni. The authors also thank Dr. Petros Giastas (Department of Neurobiology, Hellenic Pasteur Institute) for their assistance in structure-based analysis.

REFERENCES

1. Ready PD. Leishmaniasis emergence in Europe. *Euro Surveill* (2010) **15**(10):19505.
2. Singh N, Kumar M, Singh RK. Leishmaniasis: current status of available drugs and new potential drug targets. *Asian Pac J Trop Med* (2012) **5**(6):485–97. doi:10.1016/S1995-7645(12)60084-4
3. Kobets T, Grekov I, Lipoldova M. Leishmaniasis: prevention, parasite detection and treatment. *Curr Med Chem* (2012) **19**(10):1443–74. doi:10.2174/092986712799828300
4. Picado A, Dash AP, Bhattacharya S, Boelaert M. Vector control interventions for visceral leishmaniasis elimination initiative in South Asia, 2005–2010. *Indian J Med Res* (2012) **136**(1):22–31.
5. Beverley SM, Turco SJ. Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite *Leishmania*. *Trends Microbiol* (1998) **6**(1):35–40. doi:10.1016/S0966-842X(97)01180-3
6. Kaye PM, Aebischer T. Visceral leishmaniasis: immunology and prospects for a vaccine. *Clin Microbiol Infect* (2011) **17**(10):1462–70. doi:10.1111/j.1469-0691.2011.03610.x
7. Khamesipour A, Rafati S, Davoudi N, Maboudi F, Modabber F. Leishmaniasis vaccine candidates for development: a global overview. *Indian J Med Res* (2006) **123**(3):423–38.
8. Kedzierski L. Leishmaniasis. *Hum Vaccin* (2011) **7**(11):1204–14. doi:10.4161/hv.7.11.17752
9. Molano I, Alonso MG, Miron C, Redondo E, Requena JM, Soto M, et al. A *Leishmania infantum* multi-component antigenic protein mixed with live BCG confers protection to dogs experimentally infected with *L. infantum*. *Vet Immunol Immunopathol* (2003) **92**(1–2):1–13. doi:10.1016/S0165-2427(02)00315-X
10. Rafati S, Nakhaee A, Taheri T, Taslimi Y, Darabi H, Eravani D, et al. Protective vaccination against experimental canine visceral leishmaniasis using a combination of DNA and protein immunization with cysteine proteinases type I and II of *L. infantum*. *Vaccine* (2005) **23**(28):3716–25. doi:10.1016/j.vaccine.2005.02.009
11. Carson C, Antoniou M, Ruiz-Arguello MB, Alcami A, Christodoulou V, Messaritakis I, et al. A prime/boost DNA/modified vaccinia virus Ankara vaccine expressing recombinant *Leishmania* DNA encoding TRYP is safe and immunogenic in outbred dogs, the reservoir of zoonotic visceral leishmaniasis. *Vaccine* (2009) **27**(7):1080–6. doi:10.1016/j.vaccine.2008.11.094
12. Llanos-Cuentas A, Calderon W, Cruz M, Ashman JA, Alves FP, Coler RN, et al. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine when used in combination with sodium stibogluconate for the treatment of mucosal leishmaniasis. *Vaccine* (2010) **28**(46):7427–35. doi:10.1016/j.vaccine.2010.08.092
13. Chakravarty J, Kumar S, Trivedi S, Rai VK, Singh A, Ashman JA, et al. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine for use in the prevention of visceral leishmaniasis. *Vaccine* (2011) **29**(19):3531–7. doi:10.1016/j.vaccine.2011.02.096
14. De Groot AS, McMurry J, Marcon L, Franco J, Rivera D, Kutzler M, et al. Developing an epitope-driven tuberculosis (TB) vaccine. *Vaccine* (2005) **23**(17–18):2121–31. doi:10.1016/j.vaccine.2005.01.059
15. Davies MN, Flower DR. Harnessing bioinformatics to discover new vaccines. *Drug Discov Today* (2007) **12**(9–10):389–95. doi:10.1016/j.drudis.2007.03.010
16. Lundegaard C, Lund O, Kesmir C, Brunak S, Nielsen M. Modeling the adaptive immune system: predictions and simulations. *Bioinformatics* (2007) **23**(24):3265–75. doi:10.1093/bioinformatics/btm471
17. Cunha-Neto E. MHC-restricted antigen presentation and recognition: constraints on gene, recombinant and peptide vaccines in humans. *Braz J Med Biol Res* (1999) **32**(2):199–205. doi:10.1590/S0100-879X1999000200008
18. Stager S, Rafati S. CD8(+) T cells in *Leishmania* infections: friends or foes? *Front Immunol* (2012) **3**:5. doi:10.3389/fimmu.2012.00005
19. Murray HW. Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis. *J Infect Dis* (1997) **175**(6):1477–9. doi:10.1086/516482
20. Engwerda CR, Murphy ML, Cotterell SE, Smelt SC, Kaye PM. Neutralization of IL-12 demonstrates the existence of discrete organ-specific phases in the control of *Leishmania donovani*. *Eur J Immunol* (1998) **28**(2):669–80. doi:10.1002/(SICI)1521-4141(199802)28:02<669::AID-IMMU669>3.0.CO;2-N

21. Murray HW, Oca MJ, Granger AM, Schreiber RD. Requirement for T cells and effect of lymphokines in successful chemotherapy for an intracellular infection. Experimental visceral leishmaniasis. *J Clin Invest* (1989) **83**(4):1253–7. doi:10.1172/JCI114009
22. Murray HW, Montelibano C, Peterson R, Sypek JP. Interleukin-12 regulates the response to chemotherapy in experimental visceral Leishmaniasis. *J Infect Dis* (2000) **182**(5):1497–502. doi:10.1086/315890
23. Goto H, Lindoso JA. Immunity and immunosuppression in experimental visceral leishmaniasis. *Braz J Med Biol Res* (2004) **37**(4):615–23. doi:10.1590/S0100-879X2004000400020
24. Singh B, Sundar S. Leishmaniasis: vaccine candidates and perspectives. *Vaccine* (2012) **30**(26):3834–42. doi:10.1016/j.vaccine.2012.03.068
25. Ahmed SB, Touihri L, Chtourou Y, Dellagi K, Bahloul C. DNA based vaccination with a cocktail of plasmids encoding immunodominant *Leishmania (Leishmania) major* antigens confers full protection in BALB/c mice. *Vaccine* (2009) **27**(1):99–106. doi:10.1016/j.vaccine.2008.10.013
26. Rafati S, Salmanian AH, Taheri T, Vafa M, Fasel N. A protective cocktail vaccine against murine cutaneous leishmaniasis with DNA encoding cysteine proteinases of *Leishmania major*. *Vaccine* (2001) **19**(25–26):3369–75. doi:10.1016/S0264-410X(01)00081-0
27. Zadeh-Vakili A, Taheri T, Taslimi Y, Doustdari F, Salmanian AH, Rafati S. Immunization with the hybrid protein vaccine, consisting of *Leishmania major* cysteine proteinases Type I (CPB) and Type II (CPA), partially protects against leishmaniasis. *Vaccine* (2004) **22**(15–16):1930–40. doi:10.1016/j.vaccine.2003.11.014
28. Requena JM, Alonso C, Soto M. Evolutionarily conserved proteins as prominent immunogens during *Leishmania* infections. *Parasitol Today* (2000) **16**(6):246–50. doi:10.1016/S0169-4758(00)01651-3
29. Galanti N, Galindo M, Sabaj V, Espinoza I, Toro GC. Histone genes in trypanosomatids. *Parasitol Today* (1998) **14**(2):64–70. doi:10.1016/S0169-4758(97)01162-9
30. Stebeck CE, Beecroft RP, Singh BN, Jardim A, Olafson RW, Tuckey C, et al. Kinetoplastid membrane protein-11 (KMP-11) is differentially expressed during the life cycle of African trypanosomes and is found in a wide variety of kinetoplastid parasites. *Mol Biochem Parasitol* (1995) **71**(1):1–13. doi:10.1016/0166-6851(95)00022-S
31. Matos DC, Faccioli LA, Cysne-Finkelstein L, Luca PM, Corte-Real S, Armoa GR, et al. Kinetoplastid membrane protein-11 is present in promastigotes and amastigotes of *Leishmania amazonensis* and its surface expression increases during metacyclogenesis. *Mem Inst Oswaldo Cruz* (2010) **105**(3):341–7. doi:10.1590/S0074-02762010000300018
32. Tolson DL, Jardim A, Schnur LF, Stebeck C, Tuckey C, Beecroft RP, et al. The kinetoplastid membrane protein 11 of *Leishmania donovani* and African trypanosomes is a potent stimulator of T-lymphocyte proliferation. *Infect Immun* (1994) **62**(11):4893–9.
33. Jardim A, Tolson DL, Turco SJ, Pearson TW, Olafson RW. The *Leishmania donovani* lipophosphoglycan T lymphocyte-reactive component is a tightly associated protein complex. *J Immunol* (1991) **147**(10):3538–44.
34. Solioz N, Blum-Tirouvanziam U, Jacquet R, Rafati S, Corradin G, Mauel J, et al. The protective capacities of histone H1 against experimental murine cutaneous leishmaniasis. *Vaccine* (1999) **18**(9–10):850–9. doi:10.1016/S0264-410X(99)00340-0
35. Masina S, M Gicheru M, Demotz SO, Fasel NJ. Protection against cutaneous leishmaniasis in outbred vervet monkeys using a recombinant histone H1 antigen. *J Infect Dis* (2003) **188**(8):1250–7. doi:10.1086/378677
36. Basu R, Bhaumik S, Basu JM, Naskar K, De T, Roy S. Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-sensitive and -resistant strains of *Leishmania donovani* that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. *J Immunol* (2005) **174**(11):7160–71. doi:10.4049/jimmunol.174.11.7160
37. Guha R, Das S, Ghosh J, Naskar K, Mandala A, Sundar S, et al. Heterologous priming-boosting with DNA and vaccinia virus expressing kinetoplastid membrane protein-11 induces potent cellular immune response and confers protection against infection with antimony resistant and sensitive strains of *Leishmania (Leishmania) donovani*. *Vaccine* (2013) **31**(15):1905–15. doi:10.1016/j.vaccine.2013.02.025
38. Bhaumik S, Basu R, Sen S, Naskar K, Roy S. KMP-11 DNA immunization significantly protects against *L. donovani* infection but requires exogenous IL-12 as an adjuvant for comparable protection against *L. major*. *Vaccine* (2009) **27**(9):1306–16. doi:10.1016/j.vaccine.2008.12.053
39. Skeiky YA, Guderian JA, Benson DR, Bacelar O, Carvalho EM, Kubin M, et al. A recombinant *Leishmania* antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin 12. *J Exp Med* (1995) **181**(4):1527–37. doi:10.1084/jem.181.4.1527
40. Rafati S, Salmanian AH, Hashemi K, Schaff C, Belli S, Fasel N. Identification of *Leishmania major* cysteine proteinases as targets of the immune response in humans. *Mol Biochem Parasitol* (2001) **113**(1):35–43. doi:10.1016/S0166-6851(00)00377-7
41. Rafati S, Nakhaee A, Taheri T, Ghashghaii A, Salmanian AH, Jimenez M, et al. Expression of cysteine proteinase type I and II of *Leishmania infantum* and their recognition by sera during canine and human visceral leishmaniasis. *Exp Parasitol* (2003) **103**(3–4):143–51. doi:10.1016/S0014-4894(03)00097-3
42. Nakhaee A, Taheri T, Taghikhani M, Mohebali M, Salmanian AH, Fasel N, et al. Humoral and cellular immune responses against Type I cysteine proteinase of *Leishmania infantum* are higher in asymptomatic than symptomatic dogs selected from a naturally infected population. *Vet Parasitol* (2004) **119**(2–3):107–23. doi:10.1016/j.vetpar.2003.11.013
43. Pascalis H, Lavergne A, Bourreau E, Prevot-Linguet G, Kariminia A, Pradinaud R, et al. Th1 cell development induced by cysteine proteinases A and B in localized cutaneous leishmaniasis due to *Leishmania guyanensis*. *Infect Immun* (2003) **71**(5):2924–6. doi:10.1128/IAI.71.5.2924-2926.2003
44. Carmelo E, Martinez E, Gonzalez AC, Pinero JE, Patarroyo ME, Del Castillo A, et al. Antigenicity of *Leishmania braziliensis* histone H1 during cutaneous leishmaniasis: localization of antigenic determinants. *Clin Vaccine Immunol* (2002) **9**(4):808–11. doi:10.1128/CDLI.9.4.808-811.2002
45. Berberich C, Requena JM, Alonso C. Cloning of genes and expression and antigenicity analysis of the *Leishmania infantum* KMP-11 protein. *Exp Parasitol* (1997) **85**(1):105–8. doi:10.1006/expr.1996.4120
46. Kozakov D, Brenke R, Comeau SR, Vajda S. PIPER: an FFT-based protein docking program with pairwise potentials. *Proteins* (2006) **65**(2):392–406. doi:10.1002/prot.21117
47. Kozakov D, Beglov D, Bohnuud T, Mottarella SE, Xia B, Hall DR, et al. How good is automated protein docking? *Proteins* (2013) **81**(12):2159–66. doi:10.1002/prot.24403
48. Comeau SR, Gatchell DW, Vajda S, Camacho CJ. ClusPro: a fully automated algorithm for protein-protein docking. *Nucleic Acids Res* (2004) **32**:W96–9. doi:10.1093/nar/gkh354
49. Comeau SR, Gatchell DW, Vajda S, Camacho CJ. ClusPro: an automated docking and discrimination method for the prediction of protein complexes. *Bioinformatics* (2004) **20**(1):45–50. doi:10.1093/bioinformatics/btg371
50. Agallou M, Margaroni M, Karagouni E. Cellular vaccination with bone marrow-derived dendritic cells pulsed with a peptide of *Leishmania infantum* KMP-11 and CpG oligonucleotides induces protection in a murine model of visceral leishmaniasis. *Vaccine* (2011) **29**(31):5053–64. doi:10.1016/j.vaccine.2011.04.089
51. Noll TM, Desponds C, Belli SI, Glaser TA, Fasel NJ. Histone H1 expression varies during the *Leishmania major* life cycle. *Mol Biochem Parasitol* (1997) **84**(2):215–27. doi:10.1016/S0166-6851(96)02801-0
52. Probst P, Skeiky YA, Steeves M, Gervassi A, Grabstein KH, Reed SG. A *Leishmania* protein that modulates interleukin (IL)-12, IL-10 and tumor necrosis factor-alpha production and expression of B7-1 in human monocyte-derived antigen-presenting cells. *Eur J Immunol* (1997) **27**(10):2634–42. doi:10.1002/eji.1830271024
53. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* (2000) **164**(12):6166–73. doi:10.4049/jimmunol.164.12.6166
54. Cao K, Hollenbach J, Shi X, Shi W, Chopek M, Fernandez-Vina MA. Analysis of the frequencies of HLA-A, B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. *Hum Immunol* (2001) **62**(9):1009–30. doi:10.1016/S0198-8859(01)00298-1
55. Mack SJ, Tu B, Yang R, Masaberg C, Ng J, Hurley CK. Human leukocyte antigen-A, -B, -C, -DRB1 allele and haplotype frequencies in Americans originating from southern Europe: contrasting patterns of population differentiation between Italian and Spanish Americans. *Hum Immunol* (2011) **72**(2):144–9. doi:10.1016/j.humimm.2010.10.017

56. Kedzierski L. Leishmaniasis vaccine: where are we today? *J Glob Infect Dis* (2010) **2**(2):177–85. doi:10.4103/0974-777X.62881
57. Jensen AT, Gasim S, Ismail A, Gaafar A, Kurtzhals JA, Kemp M, et al. Humoral and cellular immune responses to synthetic peptides of the *Leishmania donovani* kinetoplastid membrane protein-11. *Scand J Immunol* (1998) **48**(1):103–9. doi:10.1046/j.1365-3083.1998.00370.x
58. Khan AM, Miotto O, Heiny AT, Salmon J, Srinivasan KN, Nascimento EJ, et al. A systematic bioinformatics approach for selection of epitope-based vaccine targets. *Cell Immunol* (2006) **244**(2):141–7. doi:10.1016/j.cellimm.2007.02.005
59. Reche PA, Reinherz EL. Definition of MHC supertypes through clustering of MHC peptide-binding repertoires. *Methods Mol Biol* (2007) **409**:163–73. doi:10.1007/978-1-60327-118-9_11
60. Lima-Junior JC, Banic DM, Tran TM, Meyer VS, De-Simone SG, Santos F, et al. Promiscuous T-cell epitopes of Plasmodium merozoite surface protein 9 (PvMSP9) induces IFN-gamma and IL-4 responses in individuals naturally exposed to malaria in the Brazilian Amazon. *Vaccine* (2010) **28**(18):3185–91. doi:10.1016/j.vaccine.2010.02.046
61. Okwor I, Uzonna J. Persistent parasites and immunologic memory in cutaneous leishmaniasis: implications for vaccine designs and vaccination strategies. *Immunol Res* (2008) **41**(2):123–36. doi:10.1007/s12026-008-8016-2
62. Nateghi Rostami M, Keshavarz H, Edalat R, Sarrafnejad A, Shahrestani T, Mahboudi F, et al. CD8+ T cells as a source of IFN-gamma production in human cutaneous leishmaniasis. *PLoS Negl Trop Dis* (2010) **4**(10):e845. doi:10.1371/journal.pntd.0000845
63. Murray HW, Byrne GI, Rothermel CD, Cartelli DM. Lymphokine enhances oxygen-independent activity against intracellular pathogens. *J Exp Med* (1983) **158**(1):234–9. doi:10.1084/jem.158.1.234
64. Murray HW, Cartelli DM. Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes. Evidence for oxygen-dependent and -independent leishmanicidal activity. *J Clin Invest* (1983) **72**(1):32–44. doi:10.1172/JCI110972
65. Cunningham AC. Parasitic adaptive mechanisms in infection by *Leishmania*. *Exp Mol Pathol* (2002) **72**(2):132–41. doi:10.1006/exmp.2002.2418
66. Barhoumi M, Meddeb-Garnaoui A, Tanner NK, Banroques J, Kaabi B, Guizani I. DEAD-box proteins, like *Leishmania* eIF4A, modulate interleukin (IL)-12, IL-10 and tumour necrosis factor-alpha production by human monocytes. *Parasite Immunol* (2013) **35**(5–6):194–9. doi:10.1111/pim.12026
67. Barhoumi M, Garnaoui A, Kaabi B, Tanner NK, Guizani I. *Leishmania infantum* LeIF and its recombinant polypeptides modulate interleukin IL-12p70, IL-10 and tumour necrosis factor-alpha production by human monocytes. *Parasite Immunol* (2011) **33**(10):583–8. doi:10.1111/j.1365-3024.2011.01320.x
68. Fujiwara RT, Vale AM, Franca da Silva JC, da Costa RT, Quetz Jda S, Martins Filho OA, et al. Immunogenicity in dogs of three recombinant antigens (TSA, LeIF and LmSTI1) potential vaccine candidates for canine visceral leishmaniasis. *Vet Res* (2005) **36**(5–6):827–38. doi:10.1051/vetres:2005033
69. Bertholet S, Goldszmid R, Morrot A, Debrabant A, Afrin F, Collazo-Custodio C, et al. *Leishmania* antigens are presented to CD8+ T cells by a transporter associated with antigen processing-independent pathway in vitro and in vivo. *J Immunol* (2006) **177**(6):3525–33. doi:10.4049/jimmunol.177.6.3525
70. Kima PE, Ruddle NH, McMahon-Pratt D. Presentation via the class I pathway by *Leishmania amazonensis*-infected macrophages of an endogenous leishmanial antigen to CD8+ T cells. *J Immunol* (1997) **159**(4):1828–34.
71. Guerfali FZ, Ben-Abdallah H, Sghaier RM, Ben-Aissa K, Mkannez G, Attia H, et al. An in silico immunological approach for prediction of CD8+ T cell epitopes of *Leishmania major* proteins in susceptible BALB/c and resistant C57BL/6 murine models of infection. *Infect Genet Evol* (2009) **9**(3):344–50. doi:10.1016/j.meegid.2008.02.011
72. Huber M, Timms E, Mak TW, Rollinghoff M, Lohoff M. Effective and long-lasting immunity against the parasite *Leishmania major* in CD8-deficient mice. *Infect Immun* (1998) **66**(8):3968–70.
73. Hill JO, Awwad M, North RJ. Elimination of CD4+ suppressor T cells from susceptible BALB/c mice releases CD8+ T lymphocytes to mediate protective immunity against *Leishmania*. *J Exp Med* (1989) **169**(5):1819–27. doi:10.1084/jem.169.5.1819
74. Stefani MM, Muller I, Louis JA. *Leishmania major*-specific CD8+ T cells are inducers and targets of nitric oxide produced by parasitized macrophages. *Eur J Immunol* (1994) **24**(3):746–52. doi:10.1002/eji.1830240338
75. Diez H, Lopez MC, Del Carmen Thomas M, Guzman F, Rosas F, Velazco V, et al. Evaluation of IFN-gamma production by CD8 T lymphocytes in response to the K1 peptide from KMP-11 protein in patients infected with *Trypanosoma cruzi*. *Parasite Immunol* (2006) **28**(3):101–5. doi:10.1111/j.1365-3024.2005.00815.x
76. Tsagozis P, Karagouni E, Dotsika E. CD8(+) T cells with parasite-specific cytotoxic activity and a Tc1 profile of cytokine and chemokine secretion develop in experimental visceral leishmaniasis. *Parasite Immunol* (2003) **25**(11–12):569–79. doi:10.1111/j.0141-9838.2004.00672.x
77. Uzonna JE, Joyce KL, Scott P. Low dose *Leishmania major* promotes a transient T helper cell type 2 response that is down-regulated by interferon gamma-producing CD8+ T cells. *J Exp Med* (2004) **199**(11):1559–66. doi:10.1084/jem.20040172
78. Mary C, Auriault V, Faugere B, Dessein AJ. Control of *Leishmania infantum* infection is associated with CD8(+) and gamma interferon- and interleukin-5-producing CD4(+) antigen-specific T cells. *Infect Immun* (1999) **67**(11):5559–66.
79. Saffari B, Mohabatkar H. Computational analysis of cysteine proteases (Clan CA, Family Cl) of *Leishmania major* to find potential epitopic regions. *Genomics Proteomics Bioinformatics* (2009) **7**(3):87–95. doi:10.1016/S1672-0229(08)60037-6
80. Basu R, Roy S, Walden P. HLA class I-restricted T cell epitopes of the kinetoplastid membrane protein-11 presented by *Leishmania donovani*-infected human macrophages. *J Infect Dis* (2007) **195**(9):1373–80. doi:10.1086/513439
81. Delgado G, Parra-Lopez CA, Vargas LE, Hoya R, Estupinan M, Guzman F, et al. Characterizing cellular immune response to kinetoplastid membrane protein-11 (KMP-11) during *Leishmania (Viannia) panamensis* infection using dendritic cells (DCs) as antigen presenting cells (APCs). *Parasite Immunol* (2003) **25**(4):199–209. doi:10.1046/j.1365-3024.2003.00626.x
82. Basu R, Bhaumik S, Haldar AK, Naskar K, De T, Dana SK, et al. Hybrid cell vaccination resolves *Leishmania donovani* infection by eliciting a strong CD8+ cytotoxic T-lymphocyte response with concomitant suppression of interleukin-10 (IL-10) but not IL-4 or IL-13. *Infect Immun* (2007) **75**(12):5956–66. doi:10.1128/IAI.00944-07
83. Mukhopadhyay S, Sen P, Bhattacharyya S, Majumdar S, Roy S. Immunoprophylaxis and immunotherapy against experimental visceral leishmaniasis. *Vaccine* (1999) **17**(3):291–300. doi:10.1016/S0264-410X(98)90017-2
84. Iborra S, Soto M, Carrion J, Alonso C, Requena JM. Vaccination with a plasmid DNA cocktail encoding the nucleosomal histones of *Leishmania* confers protection against murine cutaneous leishmaniosis. *Vaccine* (2004) **22**(29–30):3865–76. doi:10.1016/j.vaccine.2004.04.015
85. Carneiro MW, Santos DM, Fukutani KF, Clarencio J, Miranda JC, Brodskyn C, et al. Vaccination with *L. infantum* chagasi nucleosomal histones confers protection against new world cutaneous leishmaniasis caused by *Leishmania braziliensis*. *PLoS One* (2012) **7**(12):e52296. doi:10.1371/journal.pone.0052296
86. Seyed N, Zahedifard F, Safaiyan S, Gholami E, Doustdari F, Azadmanesh K, et al. In silico analysis of six known *Leishmania major* antigens and in vitro evaluation of specific epitopes eliciting HLA-A2 restricted CD8 T cell response. *PLoS Negl Trop Dis* (2011) **5**(9):e1295. doi:10.1371/journal.pntd.0001295
87. Resende DM, Caetano BC, Dutra MS, Penido ML, Abrantes CF, Verly RM, et al. Epitope mapping and protective immunity elicited by adenovirus expressing the *Leishmania* amastigote specific A2 antigen: correlation with IFN-gamma and cytolytic activity by CD8+ T cells. *Vaccine* (2008) **26**(35):4585–93. doi:10.1016/j.vaccine.2008.05.091
88. Agallou M, Smirlis D, Soteriadou KP, Karagouni E. Vaccination with *Leishmania* histone H1-pulsed dendritic cells confers protection in murine visceral leishmaniasis. *Vaccine* (2012) **30**(34):5086–93. doi:10.1016/j.vaccine.2012.05.075
89. Basu A, Chakrabarti G, Saha A, Bandyopadhyay S. Modulation of CD11C+ splenic dendritic cell functions in murine visceral leishmaniasis: correlation with parasite replication in the spleen. *Immunology* (2000) **99**(2):305–13. doi:10.1046/j.1365-2567.2000.00939.x
90. Engwerda CR, Kaye PM. Organ-specific immune responses associated with infectious disease. *Immunol Today* (2000) **21**(2):73–8. doi:10.1016/S0167-5699(99)01549-2
91. Bodas M, Jain N, Awasthi A, Martin S, Penke Loka RK, Dandekar D, et al. Inhibition of IL-2 induced IL-10 production as a principle of phase-specific immunotherapy. *J Immunol* (2006) **177**(7):4636–43. doi:10.4049/jimmunol.177.7.4636
92. Yip HC, Karulin AY, Tary-Lehmann M, Hesse MD, Radeke H, Heeger PS, et al. Adjuvant-guided type-1 and type-2 immunity: infectious/noninfectious dichotomy defines the class of response. *J Immunol* (1999) **162**(7):3942–9.

93. Shibaki A, Katz SI. Induction of skewed Th1/Th2 T-cell differentiation via subcutaneous immunization with Freund's adjuvant. *Exp Dermatol* (2002) **11**(2):126–34. doi:10.1034/j.1600-0625.2002.110204.x
94. Zhang YL, Jia K, Zhao BP, Li Y, Yuan CX, Yang JM, et al. Identification of Th1 epitopes within molecules from the lung-stage schistosomulum of *Schistosoma japonicum* by combining prediction analysis of the transcriptome with experimental validation. *Parasitol Int* (2012) **61**(4):586–93. doi:10.1016/j.parint.2012.05.010

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 March 2014; accepted: 25 May 2014; published online: 10 June 2014.
*Citation: Agallou M, Athanasiou E, Koutsoni O, Dotsika E and Karagouni E (2014) Experimental validation of multi-epitope peptides including promising MHC class I- and II-restricted epitopes of four known Leishmania infantum proteins. Front. Immunol. **5**:268. doi: 10.3389/fimmu.2014.00268*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 04 June 2014
doi: 10.3389/fimmu.2014.00261

Antigen-pulsed CpG-ODN-activated dendritic cells induce host-protective immune response by regulating the T regulatory cell functioning in *Leishmania donovani*-infected mice: critical role of CXCL10

Saikat Majumder, Amrita Bhattacharjee, Bidisha Paul Chowdhury, Suchandra Bhattacharyya Majumdar and Subrata Majumdar*

Division of Molecular Medicine, Bose Institute, Kolkata, India

Edited by:
Hira Nakhasi, Food and Drug Administration, USA

Reviewed by:
Sreenivas Gannavaram, Food and Drug Administration, USA
Ranadhir Dey, Food and Drug Administration, USA

****Correspondence:***
Subrata Majumdar, Division of Molecular Medicine, Bose Institute, P1/12 CIT Scheme VIIM, Kolkata 700 054, India
e-mail: subrata@bic.boseinst.ernet.in

Visceral leishmaniasis (VL), caused by *Leishmania donovani*, is a systemic infection of reticulo-endothelial system. There is currently no protective vaccine against VL and chemotherapy is increasingly limited due to appearance of drug resistance to first line drugs such as antimonials and amphotericin B. In the present study, by using a murine model of leishmaniasis we evaluated the function played by soluble leishmanial antigen (SLA)-pulsed CpG-ODN-stimulated dendritic cells (SLA–CpG–DCs) in restricting the intracellular parasitic growth. We establish that a single dose of SLA–CpG–DC vaccination is sufficient in rendering complete protection against *L. donovani* infection. In probing the possible mechanism, we observe that SLA–CpG–DCs vaccination results in the significant decrease in $Foxp3^+GITR^+CTLA4^+CD4^+CD25^+$ regulatory T cells (Treg) cell population in *Leishmania*-infected mice. Vaccination with these antigen-stimulated dendritic cells results in the decrease in the secretion of TGF-β by these Treg cells by possible regulation of the SMAD signaling. Moreover, we demonstrate that a CXC chemokine, IFN-γ-inducible protein 10 (IP-10; CXCL10), has a direct role in the regulation of $CD4^+CD25^+$ Treg cells in SLA–CpG–DC-vaccinated parasitized mice as Treg cells isolated from IP-10-depleted vaccinated mice showed significantly increased TGF-β production and suppressive activity.

Keywords: ***Leishmania*, T regulatory cells, vaccine, dendritic cell, CXCL10**

INTRODUCTION

Visceral leishmaniasis (VL), a neglected tropical disease is caused by *Leishmania donovani*, a protozoan parasite (1, 2). *Leishmania* promastigotes infect the cells of the reticulo-endothelial system where they multiply (2). Emergence of severe drug resistance against the first line drugs prompts new therapeutic approach (3, 4).

We have previously reported that mice vaccinated with a single dose of soluble leishmanial antigen (SLA)-pulsed DC stimulated with CpG oligodeoxynucleotides or CpG-ODN (SLA–CpG–DCs) were protected against a subsequent leishmanial challenge (5). Stimulation with CpG-ODN, a TLR9 ligand along with SLA activates the dendritic cells and results in the development of *Leishmania* antigen-specific cytotoxic T lymphocytes, which destroys the parasite *in vivo* (5). CpG–ODN also causes the DCs to produce CXCL10, a CXC chemokine, which has previously reported anti-leishmanial properties (6). Besides dendritic cells, the major producers of CXCL10 are monocytes and macrophages (7). Moreover, a strong induction of CXCL10 is observed in *Leishmania* resistant B6 mice, thus linking it with a strong pro-inflammatory Th1 immune response (8). Earlier from our lab, we have also demonstrated that exogenously administered CXCL10 besides regulating the intracellular parasitic load can also regulate the $CD4^+CD25^+$ regulatory T cells (Treg) cells in *Leishmania*-infected mice (9). In the present study, we have investigated the potential role of SLA–CpG–DCs vaccination in the regulation of immunosuppressive $CD4^+CD25^+$ Treg cells in *Leishmania*-infected mice.

Regulatory T cells are a subpopulation of T cells, which suppress immune responses of other cell types (10). Characteristic markers of Tregs are CD25 (10), glucocorticoid-induced tumor necrosis factor receptor (GITR) (11), cytotoxic T lymphocyte antigen 4 (CTLA4) (12) and Foxp3 (13). Tregs have been reported to play a fundamental role in the progression of leishmanial disease predominantly by suppressing Th1 immune responses (14, 15). Tregs secrete high levels of immunosuppressive Th2 cytokines such as IL-10 and TGF-β (10). IL-10, a classical Th2 cytokine is produced by many different cells such as $CD4^+$ T cells, Tregs, macrophages, dendritic cells, and even NK cells (16). However, the major source of IL-10 during human VL is $CD4^+CD25^-$ T cells but not $CD4^+CD25^+$ Treg cells (17). $CD4^+CD25^+$ Tregs on the other hand are the major producers of TGF-β during active VL (18). TGF-β, a regulatory cytokine has several down regulatory effects on the host immune system; it inhibits TNF-α and IFN-γ production from activated T cells (19), decreases the nitric oxide production (20) and also abrogates the antigen presenting function of host macrophages (21). Treatment with neutralizing antibodies to TGF-β to susceptible mice results in resistance to *Leishmania* infection (22). Besides, TGF-β is also important for

the *in vivo* expansion of $CD4^+CD25^+$ Treg cells (23). Tregs isolated from TGF-β-deficient mice are defective in their suppressive property (24). Effective TGF-β signaling in Tregs also requires phosphorylation and subsequent nuclear translocation of SMAD proteins specifically SMAD4 (25–27).

Our results show that SLA–CpG–DCs vaccination inhibits the generation of $CD4^+CD25^+$ Treg cells in *Leishmania*-infected mice along with the TGF-β production by these Tregs by possible regulation of SMAD signaling. In addition, we demonstrate that the SLA–CpG–DCs-mediated reduction of Tregs can be entirely attributable to CXC chemokine IP-10, as significant increase in $CD4^+CD25^+$ Treg cells is observed in SLA–CpG–DCs-vaccinated parasitized mice depleted of IP-10.

MATERIALS AND METHODS

ETHICS STATEMENT

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental animal protocols received prior approval from the Institutional Animal Ethical Committee (Bose Institute, Registration Number: 95/99/CPCSEA).

ANIMALS, PARASITES, AND REAGENTS

BALB/c mice were purchased from the National Center for Laboratory Animal Sciences, India. For each experiment, 8–10 mice (4–6 weeks old) were used, regardless of sex. *L. donovani* strain AG-83 (MHOM/IN/1983/AG-83) was maintained *in vitro* in Medium 199 (Sigma) containing 10% fetal calf serum (FCS; Gibco BRL). Experiments were performed with stationary phase promastigotes. The CpG-ODN 1826 (5′-TCCATGACGTTCCTGACGTT-3′) and the control-ODN (non-CpG-ODN, 5'-TCCATGAGCTTCCTGAGCTT-3') was obtained from InvivoGen. CXCL10-depleting antibody was obtained from R&D Systems.

PREPARATION OF DENDRITIC CELLS

Bone marrow-derived DCs from BALB/c mice were generated as described previously (28). Non-adherent cells were collected, and 1×10^6 cells were placed in plates containing 1 ml of complete medium with GM-CSF (150 U/ml; R&D Systems) and IL-4 (75 U/ml; R&D Systems) as originally described earlier (5). Half of the medium was replaced on day 3, 5, and 7 and fresh medium containing GM-CSF and IL-4 was added. On day 8 of culture, most cells had acquired typical dendritic morphology. These cells were used as the source of DCs in subsequent experiments.

DC VACCINATION

For DC-based vaccination, DCs were pulsed with both SLA and CpG-ODNs (29) as originally described earlier (5). In case of dual stimulation, CpG-ODN (10 μg/ml) or control-ODN (10 μg/ml) was added to the media for last 6 h after 12 h of SLA stimulation. DCs were then washed with PBS thrice and injected i.v. (10^6 cells in 100 μl of PBS/mouse) into mice through the tail vein. One week later, mice were infected intravenously with 1×10^7 stationary phase *L. donovani* promastigotes. Mice were sacrificed on day 56 post-infections. Spleen and liver parasitic loads were determined from Giemsa-stained impression smears, calculated as the number of parasites per 1000 nucleated cells × organ weight (in milligrams) and expressed in Leishman Donovan Units (LDU) (30). After 28 days of infection, spleens from infected BALB/c mice were removed aseptically, and a single-cell suspension was prepared. Briefly, spleen homogenate was subjected to centrifugation on a Histopaque-1077 (Sigma) gradient and splenocytes were collected, washed, and resuspended in RPMI-1640 complete medium supplemented with 10% FCS.

IN VIVO DEPLETION OF CXCL10

For *in vivo* depletion of CXCL10, anti-mouse CXCL10 mAb (R&D Systems) were injected intraperitoneal (i.p.) on day 0 (250 mg), day 2 (100 mg), and day 4 (100 mg) after SLA–CpG–DCs vaccination as originally described earlier (5). These mice were subsequently infected with 1×10^7 stationary phase *L. donovani* promastigotes after 7 days of initial vaccination. Two hundred fifty milligrams of anti-CXCL10 mAb was again injected i.p on days 10, 15, and 24 of initial vaccination. Depletion efficiencies were assessed at regular intervals.

PURIFICATION OF $CD4^+$ T CELLS

$CD4^+$ T cells were purified from splenocytes from differently treated mice by positive selection using magnetic beads as originally described earlier (9). $CD4^+$ T cells were purified by anti-mouse CD4 (L3T4)-magnetic particles (BD Biosciences). To further separate $CD4^+$ T cells into $CD25^+$ and $CD25^-$ populations, total $CD4^+$ T cells were isolated by negative selection using magnetic beads followed by positive selection using anti-CD25 magnetic beads on a magnetic separator column into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations as per manufacturer's suggested protocol (MagCellect Treg isolation kit, R&D Systems). The cells were stained with anti-CD25 mAb, and the purity of cell preparations was determined by using FACS analysis (FACSCalibur; BD Labware). The purities of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were routinely >90 and 99%, respectively.

Additional analyses of T cell phenotypes were also performed using FACS where splenocytes were stained using 1 μg Ab/1×10^6 cells and either run immediately or fixed (3% paraformaldehyde in PBS). In some cases, the splenocytes were first stained with CD4 FITC followed by permeabilization using FACS permeabilizing solution (BD Pharmingen) and FOXP3-PE staining. The panel of Abs used for T cell phenotyping included the following: CD4, CD25, GITR, Foxp3, and CTLA4 (BD Biosciences). The dot plots were derived from the gated events based on the region encircling lymphocytes, which was set using a forward versus side scatter display, and all fluorescent parameters were gated on this population and analyzed on a Flow cytometer (FACSCalibur), using the Cell Quest program on at least 10,000 events.

PROLIFERATION ASSAY AND CYTOKINE ELISA

Splenic responder $CD4^+CD25^-$ T cells (5×10^5) and T-depleted, mitomycin C-treated syngeneic APCs (5×10^5) were cultured in the absence or presence of increasing numbers of splenic $CD4^+CD25^+$ Treg cells for 4 days in 96-well, round-bottom plates as originally described earlier (9). Soluble leishmanial antigen (SLA) (10 μg/ml) was added to the culture for stimulation. One microcurie of [3H] thymidine was added 18 h before

harvesting, and incorporated radioactivity was determined on a beta emission reader. Supernatants were collected from the co-culture of responder $CD4^+CD25^-$ and $CD4^+CD25^+$ Treg cells (1:1) at 24 h (for IL-2) or 72 h (for other cytokines). In some cases, splenocytes (2×10^6 cells/ml per well), $CD4^+CD25^-$ T cells (1×10^6 cells/ml per well), or $CD4^+CD25^+$ Treg cells (1×10^6 cells/ml per well) from different sets of treatment were stimulated with SLA (10 μg/ml) for 24 h (IL-2) or 72 h (for other cytokines). The levels of cytokines in supernatants were determined by specific ELISAs using paired mAbs for IL-2, IL-10, IFN-γ, and TGF-β along with the appropriate mouse cytokine controls (BD Biosciences and R&D Systems).

FLOW CYTOMETRY

For intracellular cytokine analysis, flow cytometry was performed for the determination of IFN-γ, IL-12, TGF-β, and IL-10 produced by CD4+ T cells in differently treated mice at the single-cell level as originally described earlier (9). In another experiment, $CD4^+CD25^+$ Treg cells were analyzed for Foxp3-positive Treg cells producing TGF-β. Splenocytes or MACS-purified $CD4^+CD25^+$ Treg cells from different groups of experimental mice were stimulated for 20–24 h with SLA (10 μg/ml). Brefeldin A (10 μg/ml) was added to the cultures 2 h before harvesting. The cells were washed in PBS containing 0.1% NaN_3/1% FCS at 4°C, and some sets were stained with FITC-conjugated anti-CD4. Cells were then permeabilized by treatment with FACS permeabilizing solution (BD Pharmingen) and stained with PE-conjugated IFN-γ, FITC-conjugated IL-12, FITC or PE-conjugated anti-mouse TGF-β mAb, PE-conjugated anti-mouse IL-10 mAb, PE-conjugated Foxp3 anti-hamster mAb, or isotype-matched control mAb and analyzed on a Flow cytometer (FACSCalibur) using the Cell Quest program on at least 10,000 events.

REAL-TIME PCR QUANTIFICATION

Total RNA was extracted from 2×10^6 $CD4^+CD25^+$ T cells or $CD4^+CD25^-$ T cells with use of TRI Reagent (Sigma), according to the manufacturer's protocol as originally described previously (9). Isolated RNA (1 μg) was then reverse transcribed using Revert Aid M-MuLV Reverse Transcriptase (Fermentas). The resulting complementary DNA was used for real-time (RT) PCR to detect different Treg cell-specific markers with use of the ABI 7500 RT-PCR system with the DNA-binding SYBR green dye (Applied Biosystems). Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as a reference. The forward- and reverse-specific primer sequences used were as follows: CTLA4 Forward, 5′-GGACGCAGATTTATGTCATTGATC-3′, CTLA4 Reverse, 5′-CCAAGCTAACTGCGAC AAGGA-3′; Foxp3 Forward, 5′-CGTACACCCAGGAAAGACAG-3′, Foxp3 Reverse, 5′-ATCCAGGAGATGATCTGCTTG-3′; GITR Forward, 5′-GACGGTCACTGCAGACTTTG-3′; GITR Reverse, 5′-GCCAT GACCAGGAAGATGAC-3′. The reaction conditions involved an initial activation step (5 min at 95°C) and a cycling step (denaturation for 30 s at 94°C, annealing for 30 s at 58.5°C, and extension for 1 min at 72°C for 40 cycles), followed by melting curve analysis. Detection of dequenched probe, calculation of threshold cycles, and further analysis of these data were performed using the Sequence Detector software (Applied Biosystems). Relative changes in CTLA4, Foxp3, and GITR messenger RNA (mRNA) expression were compared with unstimulated control, normalized to GAPDH, and quantified by the 2^{-ddCt} method.

PREPARATION OF CELL LYSATE AND IMMUNOBLOT ANALYSIS

Cell lysates were prepared as described earlier (31). Equal amounts of protein (30 μg) in each lane were subjected to SDS-10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked overnight with 3% BSA in Tris–saline buffer (pH7.5), and immunoblotting was carried out for detecting phosphorylated or dephosphorylated forms of SMAD4 as described previously (32).

STATISTICAL ANALYSIS

A minimum of five mice were used per group for *in vivo* experiments. The data, represented as mean ± standard deviation (SD), is from one experiment, which was performed at least three times. Student's *t*-test was employed to assess the significance of the differences between the mean values of control and experimental groups. A *P* value of 0.05 was considered significant and <0.001 was considered highly significant.

RESULTS

SLA–CpG–DC VACCINATION MEDIATES EFFECTIVE PROTECTION AGAINST VISCERAL LEISHMANIASIS THROUGH A POTENT PRO-INFLAMMATORY RESPONSE

Our results demonstrated that SLA-pulsed DCs in the presence of TLR9 agonist, CpG-ODN (SLA–CpG–DCs), acquired the ability to induce complete protection against *L. donovani.* SLA–CpG–DCs-vaccinated BALB/c mice on day 56 of infection, showed marked decrease in parasitic burden; 96 ± 2.6 and 97 ± 1.9% reduction in hepatic and splenic parasite burden, respectively, compared with PBS-treated infected controls (**Figure 1A**). Mice vaccinated with SLA and control-ODN-stimulated dendritic cells (SLA-Cont. ODN–DCs) were unable to give any protection against the leishmanial challenge (**Figure 1A**). Moreover, the protection conferred by SLA–CpG–DC vaccination against *L. donovani* infection is significantly dependent on a Th1 polarized anti-parasitic immune response (**Figures 1B,C**). Splenocytes from differently vaccinated mice at 28 days post-treatment were re-stimulated with SLA to evaluate the percentages of T cells secreting various pro-inflammatory or anti-inflammatory cytokines. There was about sixfold increase in IFN-γ secreting $CD4^+$ T cells along with nearly fivefold increase in IL-12 secreting $CD4^+$ T cells in splenocytes from SLA–CpG–DC-vaccinated parasitized mice compared with only infected mice (**Figures 1B,C**). Moreover, there was nearly fourfold decrease in TGF-β secreting $CD4^+$ T cells along with nearly threefold decrease in IL-10-secreting $CD4^+$ T cells in splenocytes from SLA–CpG–DC-vaccinated parasitized mice compared with only infected mice (**Figures 1D,E**).

SLA–CpG–DCs VACCINATION LEADS TO DECREASE IN SPLENIC $CD25^+CTLA4^+GITR^+Foxp3^+CD4^+$ T CELLS IN *L. DONOVANI*-INFECTED MICE

To check whether SLA–CpG–DCs vaccination of *L. donovani*-infected mice can modulate the Treg cells, which have been previously reported to play a crucial role in disease progression (33),

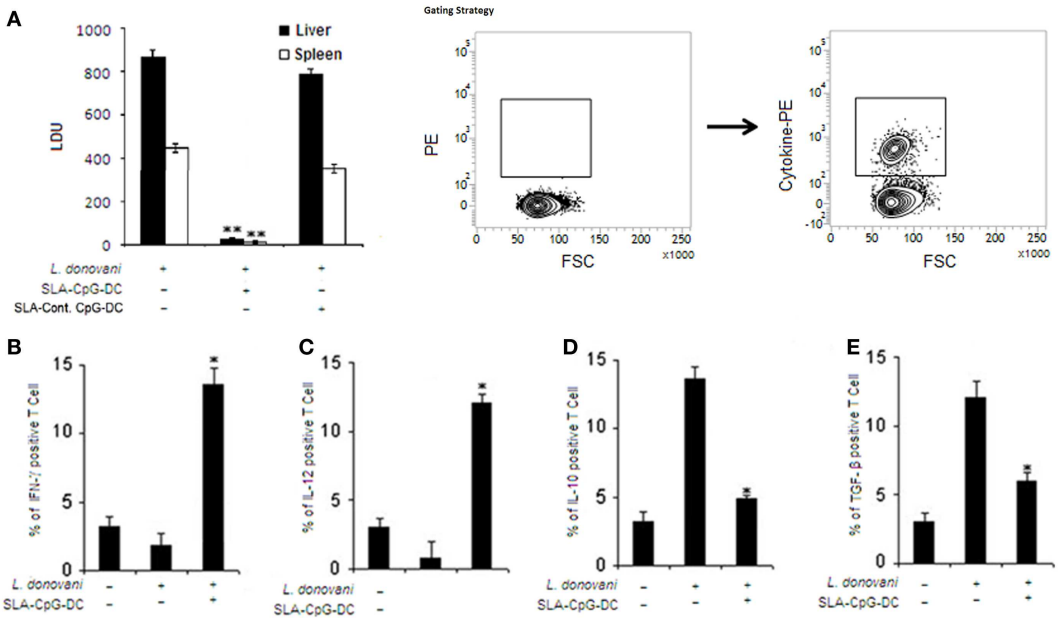


FIGURE 1 | Soluble leishmanial antigen–CpG–DC vaccination mediates effective protection against visceral leishmaniasis through a potent pro-inflammatory response. **(A)** Mice were vaccinated with SLA and CpG-ODN-pulsed DCs, SLA and control-ODN-pulsed DCs, or phosphate buffered saline (PBS; control) followed by intravenous infection with 1×10^7 stationary phase *Leishmania donovani* promastigotes after 7 days. Mice were sacrificed on day 56 after infection. Levels of parasite burden in liver and spleen samples were determined by stamp–smear method and expressed in Leishman Donovan Units (LDU). Results are from three independent experiments and represent the mean values ± standard errors of the means for five animals per group per time point. $^{**}P < 0.001$, compared to PBS-treated infected mice. In another set of experiments, splenocytes (2×10^6) from control, *L. donovani*-infected (28 days), and SLA–CpG–DC-vaccinated infected mice (28 days) were assessed for intracellular **(B)** IFN-γ, **(C)** IL-12, **(D)** IL-10, or **(E)** TGF-β staining, which was performed as mentioned in Section "Materials and Methods" and analyzed by flow cytometry. Magnetically purified $CD4^+$ T cells were analyzed for IL-12-PE, IFN-γ-PE, IL-10-PE, or TGF-β-PE staining to detect $CD4^+IL\text{-}12^+$, $CD4^+$ $IFN\text{-}\gamma^+$, $CD4^+IL\text{-}10^+$, or $CD4^+$ $TGF\text{-}\beta^+$ T cells. The bar graphs represent the mean dot plot values based on the region encircling positive cells from three independent experiments $^*P < 0.05$, compared with infected sets. The error bars represent mean ± SD of three mice per group.

FACS analysis was performed to evaluate the frequency and phenotype of splenic Treg cell population. We observed a significant decrease from 23% to 11% in $CD4^+CD25^+Foxp3^+$ Treg cells in splenocytes isolated from SLA–CpG–DCs-vaccinated infected mice compared to only infected mice (**Figures 2A,B**). Other Treg cell-specific markers like CTLA4 and GITR showed a significant decrease in splenocytes isolated from SLA–CpG–DC-vaccinated infected mice compared to infected sets (**Figures 2C,D**). We observed 2.5-fold decrease in GITR and threefold decrease in CTLA4 mRNA levels in SLA–CpG–DC-vaccinated infected sets in comparison with only infected sets.

Moreover, $CD4^+CD25^+$ Treg cells from SLA–CpG–DC-vaccinated mice failed to suppress the release of IL-2 when co-cultured with $CD4^+CD25^-$ responder T cells (1:1) compared to $CD4^+CD25^+$ Treg cells isolated from infected mice (**Figure 3B**). Additionally, $CD4^+CD25^+$ Treg cells from infected mice significantly abrogated the IFN-γ secretion by responder $CD4^+CD25^-$ T cells, while $CD4^+CD25^+$ Treg cells from SLA–CpG–DC-vaccinated parasitized mice, could not suppress the IFN-γ production from responder $CD4^+CD25^-$ T cells demonstrating parasite-specific T cell responses (**Figure 3C**).

SLA–CpG–DC VACCINATION REDUCES THE SUPPRESSIVE PROPERTIES OF THE $CD4^+CD25^+Foxp3^+$ T CELLS IN *L. DONOVANI*-INFECTED MICE

To study whether the Treg cells isolated from the *Leishmania*-infected BALB/c mice can suppress the function of effector T cells, we performed co-culture experiments using $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells purified from spleen of differently treated mice. We observed that $CD4^+CD25^+$ Treg cells isolated from untreated infected mice can efficiently suppress the proliferation of responder $CD4^+CD25^-$ T cells in a dose-dependent manner. Whereas, $CD4^+CD25^+$ Treg cells, isolated from SLA–CpG–DC-vaccinated infected mice, could not suppress the proliferation of responder $CD4^+CD25^-$ T cells (**Figure 3A**).

SLA–CpG–DC VACCINATION LEADS TO REDUCED TGF-β SECRETION FROM T REGULATORY CELLS IN *L. DONOVANI*-INFECTED MICE

SLA–CpG–DC vaccination of *L. donovani*-infected mice results in a significant decrease in Th2 cytokine secreting $CD4^+$ T cells, i.e., IL-10 and TGF-β (**Figures 4B,C**). As expected, FoxP3 levels were higher in $CD4^+CD25^+$ Treg cells isolated from infected group of mice. On the contrary, $CD4^+CD25^-$ T cells showed lower expression of Foxp3 mRNA during infection (**Figure 4A**). Now to delineate which of these two cells are the major producer of IL-10 and TGF-β, we evaluated the anti-inflammatory cytokines secreted by $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ T cells from different groups of mice. $CD4^+CD25^-$ and $CD4^+CD25^+$

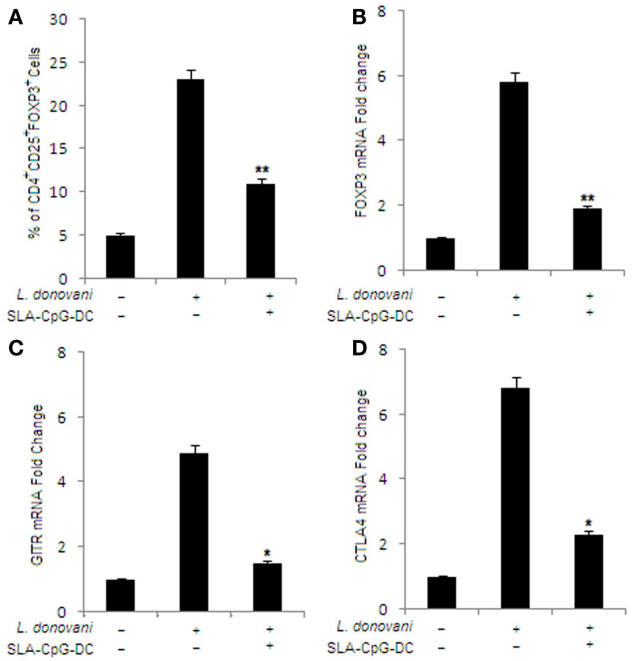


FIGURE 2 | Effect of SLA–CpG–DCs vaccination on the frequency and phenotype of T regulatory cells. **(A)** $CD4^+$ T cells (1×10^6) were purified from infected and indicated treatment groups of mice 28 days after infection, plated aseptically followed by fixation and staining for T regulatory cell-specific markers like FITC-conjugated CD25 and PE-conjugated FoxP3 mentioned in Section "Materials and Methods." The data was analyzed by flow cytometry in each group of untreated and differently treated BALB/c mice have been presented. Data represent the mean ± SD for three animals per group. In a separate experiment, $CD4^+CD25^+$ Treg cells (2×10^6) were purified from spleen of differently treated mice by MACS as described in Section "Materials and Methods." These magnetically purified Treg cells were collected in TRIZOL for mRNA extraction and real-time PCR to study mRNA expression of T regulatory cell markers. Quantitative RT-PCR showing the expression of Foxp3 **(B)**, GITR **(C)**, and CTLA4 mRNA **(D)**, where the data were presented as changes (*n*-fold) from uninfected control cells. $*P < 0.05$ and $**P < 0.001$, compared with T regulatory cells isolated from infected mice. The data represent the mean ± SD of data from three independent experiments, which yielded similar results.

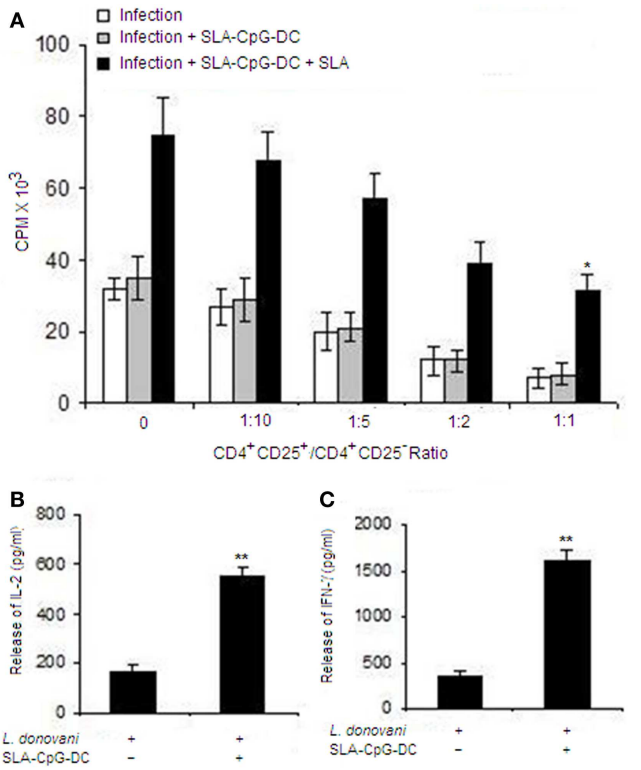


FIGURE 3 | Soluble leishmanial antigen–CpG–DCs vaccination reduces the suppressive properties of the $CD4^+CD25^+Foxp3^+$ T cells in *L. donovani*-infected mice. **(A)** $CD4^+CD25^+$ T regulatory cells and $CD4^+CD25^-$ T cells were purified from spleen of differently treated mice (Materials and Methods) after 4 weeks post-infection. $CD4^+CD25^-$ T cells (5×10^5) and T-depleted, mitomycin C-treated syngeneic APCs (5×10^5) were stimulated with SLA (10 μg/ml) and co-cultured in the presence of splenic $CD4^+CD25^+$ T cells(1:1 ratio) from differently treated sets for 4 days. Proliferation was determined by an 18 h (^3H) thymidine incorporation assay. Data are presented as counts per minute (CPM) $\times 10^3$. The supernatants were collected at 24 h for IL-2 **(B)** or at 72 h for IFN-γ **(C)** following stimulation with SLA (10 μg/ml) and levels of cytokines were determined by ELISA. The error bars represent mean ± SD of triplicate cultures. The experiments were performed three times, of which one set of representative data is shown ($**P < 0.001$ and $*P < 0.05$).

T cells from control mice showed lower levels of IL-10 and TGF-β (**Figures 4B,C**).

In infected mice, $CD4^+CD25^-$ T cells are the major producers of IL-10. On the other hand, $CD4^+CD25^+$ Treg cells are the major source of TGF-β release. Interestingly, significant decrease in TGF-β release from $CD4^+CD25^+$ Treg cells was observed in $CD4^+CD25^+$ Treg cells isolated from SLA–CpG–DC-vaccinated infected sets compared with infected mice, while $CD4^+CD25^-$ T cells showed a significant reduction in IL-10 release in vaccinated group of mice compared with infected sets (**Figures 4B,C**).

In another experiment, $CD4^+CD25^+$ Treg cells, isolated from different groups of animals, were analyzed for TGF-β secreting $Foxp3^+$ Treg cells. We observed higher levels (69.24%) of TGF-β-secreting $Foxp3^+$ Treg cells in infected mice, which were significantly abridged (24.56%) in SLA–CpG–DC-vaccinated infected mice (**Figure 4D**), indicating decrease of TGF-β secretion from $CD4^+CD25^+Foxp3^+$ Treg cells following SLA–CpG–DC vaccination during VL.

IP-10 DEPLETION ABROGATES THE TGF-β SIGNALING IN $CD4^+CD25^+$ Treg CELLS IN SLA–CpG–DC-VACCINATED PARASITIZED MICE

We have previously demonstrated that the induction of anti-leishmanial protective immunity by SLA–CpG–DCs is entirely dependent on IP-10 (5). Moreover, our lab has also shown that exogenously administered IP-10 can decrease the TGF-β secretion in *Leishmania*-infected mice (9). So to examine possible involvement of IP-10 in the secretion of TGF-β from $CD4^+CD25^+$ Treg cells following SLA–CpG–DCs vaccination, we vaccinated the mice, infected it with *Leishmania* and monitored the course of infection in the presence or absence of IP-10 (**Figure 5A**).

$CD4^+CD25^+$ Treg cells from IP-10 depleted SLA–CpG–DC-vaccinated mice produced significantly higher amount of TGF-β

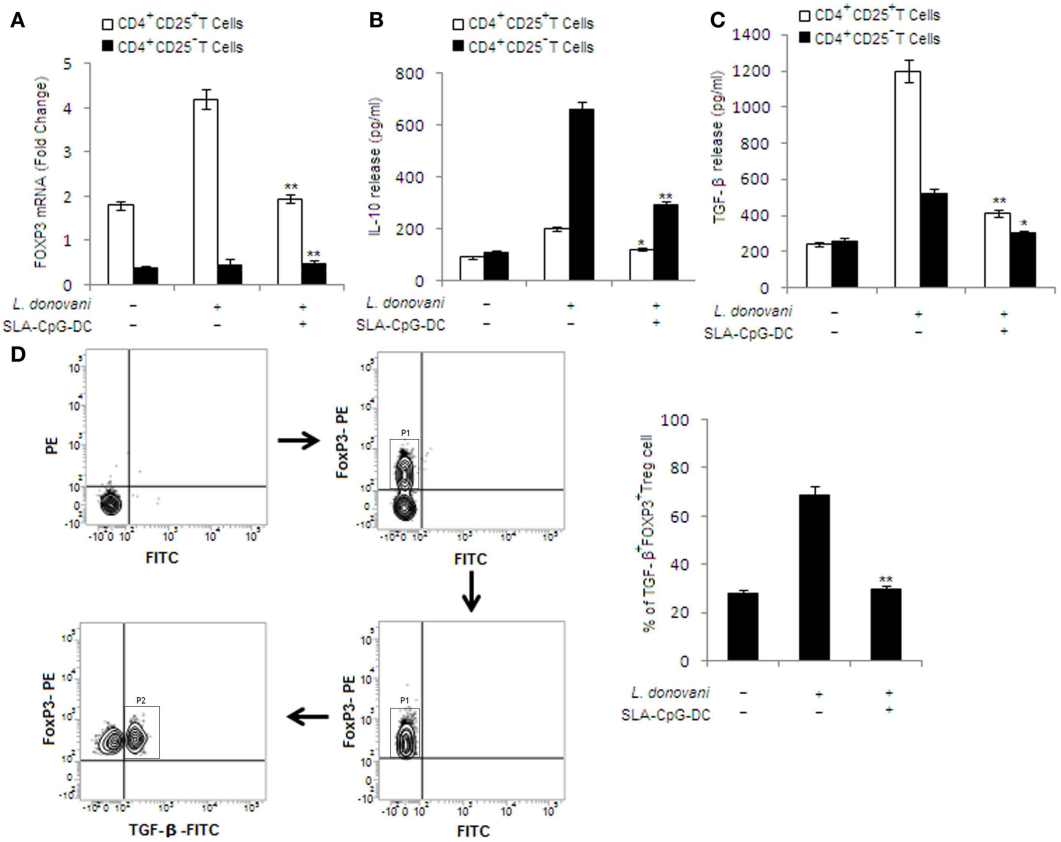


FIGURE 4 | Effect of SLA–CpG–DC vaccination on IL-10 and TGF-β release from T regulatory cells. **(A)** $CD4^+CD25^+$ Treg cells or $CD4^+CD25^-$ T cells (2×10^5), purified from spleen of differently treated mice (see Materials and Methods) 4 weeks post-infection, were collected in TRIZOL for mRNA extraction and real-time PCR to study Foxp3 mRNA expression or were stimulated with SLA for 72 h, after which the cell supernatants were collected for estimation of IL-10 **(B)** and TGF-β **(C)** by ELISA. All data were presented as mean ± SD of three triplicate wells. One of the three independent experiments is shown. $*P < 0.001$ indicates statistically significant differences compared with infected sets. In a separate experiment, $CD4^+CD25^+$ T regulatory cells (1×10^6), isolated from spleen of differently treated mice (4 weeks post-infection), were assessed for TGF-β and Foxp3 **(D)**, which was performed as mentioned in Section "Materials and Methods," and analyzed by Flow cytometry. Magnetically purified $CD4^+CD25^+$ cells were analyzed for FoxP3-PE staining to detect $FoxP3^+CD4^+CD25^+$ cells (P1 gated cell population in the sorting scheme). These $FoxP3^+CD4^+CD25^+$ cells were further analyzed for TGF-β-FITC staining to detect TGF-β^+ $FoxP3^+CD4^+CD25^+$T cells (P2 gated cell population in the sorting scheme). The bar graphs represent the mean dot plot values based on the region encircling positive cells from three independent experiments. $**P < 0.001$ and $*P < 0.05$, compared with T regulatory cells isolated from infected mice. The data represent the mean ± SD of data from three independent experiments, which yielded similar results.

together with fourfold increase in their suppressive property compared with IP-10 non-depleted vaccinated parasitized mice, which showed significant reduction in TGF-β secretion along with clear decrease in the suppressive property of the $CD4^+CD25^+$ Treg cells (**Figures 5B,C**). These findings clearly demonstrate the involvement of IP-10 in the regulation of $CD4^+CD25^+$ Treg cell functioning in SLA–CpG–DC-vaccinated parasitized mice.

Efficient TGF-β signaling requires proper activation of SMAD4 (27). To establish whether SMAD4 is involved in the SLA–CpG–DC-mediated regulation of TGF-β signaling in $CD4^+CD25^+$ Treg cells, we studied the phosphorylation of SMAD4 in $CD4^+CD25^+$ Treg cells during SLA–CpG–DC vaccination. Increased levels of phospho-SMAD4 was observed in $CD4^+CD25^+$ Treg cells isolated from infected mice, however $CD4^+CD25^+$ Treg cells isolated from SLA–CpG–DC-vaccinated mice showed lower levels of phospho-SMAD4 indicating SLA–CpG–DC-mediated regulation of TGF-β signaling through SMAD4 modulation (**Figure 5D**). Though, increased levels of phospho-SMAD4 in $CD4^+CD25^+$ Treg cells in IP-10-depleted vaccinated parasitized mice suggest the involvement of IP-10 in the SLA–CpG–DCs-mediated suppression of TGF-β.

DISCUSSION

Our results describe a novel strategy using a DC-based vaccination to confer significant protection against *Leishmania* pathogen. SLA–CpG–DC vaccination of *L. donovani*-infected mice showed significant protection as marked reduction in the hepatic and splenic parasitic burden is observed (**Figure 1A**). Besides this, significantly higher numbers of IL-12 and IFN-γ secreting T cells are observed in SLA–CpG–DC-vaccinated parasitized mice compared to unvaccinated parasitized mice. At the same time lower numbers of TGF-β and IL-10-producing $CD4^+$ T cells in vaccinated mice

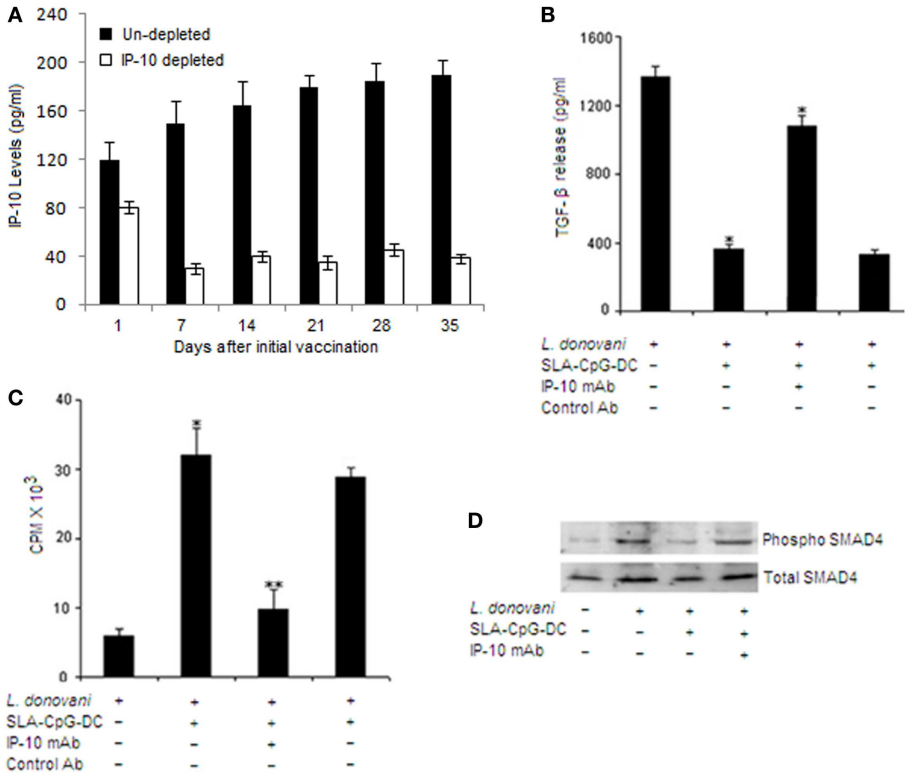


FIGURE 5 | IFN-γ-inducible protein 10 depletion abrogates the TGF-β signaling in $CD4^+CD25^+$ Treg cells in SLA–CpG–DC-vaccinated parasitized mice. **(A)** Assessment of depletion efficiency. Levels of IP-10 were measured at indicated days from IP-10 depleted and non-depleted SLA–CpG–DC-vaccinated parasitized mice. Splenic cells were isolated at indicated time points and levels of IP-10 were measured by sandwich ELISA. The experiments were carried out twice. **(B)** $CD4^+CD25^+$ Treg cells (1×10^6) purified from spleen of indicated groups of *L. donovani*-infected (28 days) mice were stimulated with SLA for 72 h. Level of TGF-β in cell culture supernatants of indicated treatment groups was determined by ELISA. Asterisks indicate statistically significant induction ($^*P < 0.05$, $^{**}P < 0.001$) of TGF-β production compared with infected sets. **(C)** $CD4^+CD25^+$ T regulatory cells and $CD4^+CD25^-$ T cells were purified from spleen of differently treated mice (see Materials and Methods) after 28 days post-infection. $CD4^+CD25^-$ T cells (5×10^5) and T-depleted, mitomycin C-treated syngeneic APCs (5×10^5) were stimulated with SLA (10 μg/ml) in the presence of splenic $CD4^+CD25^+$ T cells (1:1) for 4 days. Proliferation was determined by an 18-h (3H) thymidine incorporation assay. Data were presented as cpm $\times 10^3$. $^{**}P < 0.001$ compared with infected sets. **(D)** In a separate experiment, $CD4^+CD25^+$ Treg cells (2×10^6), purified from spleen of indicated groups of mice, were stimulated with SLA for 30 and 60 min. The cells were lysed and subjected to Western blotting with anti-pSMAD4 and anti-SMAD4 as described in Section "Materials and Methods". The experiments were carried out three times, of which one set of representative data is shown.

indicate efficient suppression of Th2 cytokines (**Figures 1B–E**). Besides $CD4^+$ T cells, SLA–CpG–DC vaccination also reduces the number of $CD25^+Foxp3^+CD4^+$ T regulatory cells in the parasitized mice along with their immunosuppressive molecules GITR and CTLA4 (**Figures 2A–D**).

Recently it has been shown that $CD4^+CD25^-$ T cells derived, but not $CD4^+CD25^+$ derived IL-10 plays a major role in the murine model of VL (34, 35). Our results also indicate that SLA–CpG–DC vaccination abrogates the secretion of IL-10 from $CD4^+CD25^-$ T cells, which can be crucial for the parasite clearance (**Figure 4B**). $CD4^+CD25^+$ Treg cells from infected mice, on the other hand secrete higher amounts of TGF-β (**Figure 4C**). TGF-β, while inhibiting pro-inflammatory cytokine responses aids in the multiplication of the parasite inside the host (36). Additionally, TGF-β is also critical for the effective functioning of Treg cells and has been implicated to play a critical role in the persistence of *Leishmania* infection. Vaccination with SLA–CpG–DC inhibits TGF-β production from $CD4^+CD25^+Foxp3^+$ Treg cells (**Figure 4D**) and also reduces their suppressive activity (**Figure 3A**), which is critical for the resistance against *Leishmania* (37).

Besides, TGF-β plays a decisive role in the generation and expansion of Treg cells and also in the induction of FoxP3 and CTLA4 in Treg cells (27, 38). SMAD proteins play a critical role here for the efficient TGF-β signaling (27). Our results indicate that SLA–CpG–DC vaccination restricted the parasite-induced enhanced phosphorylation of SMAD4 (**Figure 5D**) and thus can efficiently regulate the TGF-β signaling in Treg cells. The use of neutralizing IP-10 antibody during SLA–CpG–DC vaccination provided evidence that IP-10 besides regulating TGF-β signaling, also regulates the suppressive activity of these Treg cells (**Figures 5B,C**). Our previous finding revealed that SLA–CpG–DCs vaccination results in significant enhancement of IFN-γ secreting $CD8^+$ cytotoxic T lymphocytes along with significant increase in granzyme and perforin secreting $CD8^+$ T cells, which most likely contributes to the protection (5). The evidence

presented here indicates that the protection was also dependent on the reduction of $CD4^+CD25^+Foxp3^+$ Treg cells. Collectively, these findings illustrate that SLA–CpG–DCs vaccination induces a strong Th1 response by effective modulation of Treg cell functioning and activation of cytotoxic $CD8^+$ T cells, representing an antigen-specific immune response against *Leishmania*-induced pathogenesis.

ACKNOWLEDGMENTS

We thank Mr. Prabal Gupta for his technical assistance. Funding: The Council of Scientific and Industrial Research, Government of India, New Delhi provided fellowships to Saikat Majumder.

REFERENCES

1. Stanley AC, Engwerda CR. Balancing immunity and pathology in visceral leishmaniasis. *Immunol Cell Biol* (2007) **85**:138–47. doi:10.1038/sj.icb7100011
2. Kaye PM, Svensson M, Ato M, Maroof A, Polley R, Stager S, et al. The immunopathology of experimental visceral leishmaniasis. *Immunol Rev* (2004) **201**:239–53. doi:10.1111/j.0105-2896.2004.00188.x
3. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. *Clin Microbiol Rev* (2006) **19**:111–26. doi:10.1128/CMR.19.1.111-126.2006
4. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* (2007) **5**:873–82. doi:10.1038/nrmicro1748
5. Majumder S, Bhattacharjee S, Paul Chowdhury B, Majumdar S. CXCL10 is critical for the generation of protective CD8 T cell response induced by antigen pulsed CpG-ODN activated dendritic cells. *PLoS One* (2012) **7**:e48727. doi:10.1371/journal.pone.0048727
6. Gupta G, Bhattacharjee S, Bhattacharyya S, Bhattacharya P, Adhikari A, Mukherjee A, et al. CXC chemokine-mediated protection against visceral leishmaniasis: involvement of the proinflammatory response. *J Infect Dis* (2009) **200**:1300–10. doi:10.1086/605895
7. Luster AD, Unkeless JC, Ravetch JV. Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. *Nature* (1985) **315**:672–6. doi:10.1038/315672a0
8. Müller K, Zandbergen GV, Hansen B, Laufs H, Jahnke N, Solbach W, et al. Chemokines, natural killer cells and granulocytes in the early course of *Leishmania major* infection in mice. *Med Microbiol Immunol* (2001) **190**:73–6. doi:10.1007/s004300100084
9. Gupta G, Majumdar S, Adhikari A, Bhattacharya P, Mukherjee AK, Majumdar SB, et al. Treatment with IP-10 induces host-protective immune response by regulating the T regulatory cell functioning in *Leishmania donovani*-infected mice. *Med Microbiol Immunol* (2011) **200**:241–53. doi:10.1007/s00430-011-0197-y
10. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* (1995) **155**:1151–64.
11. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* (2002) **3**:135–42. doi:10.1038/ni759
12. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* (2000) **192**:295–302. doi:10.1084/jem.192.2.295
13. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* (2003) **4**:337–42. doi:10.1038/ni909
14. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* (2002) **420**:502–7. doi:10.1038/nature01152
15. Suffia I, Reckling SK, Salay G, Belkaid Y. A role for CD103 in the retention of CD4+CD25+ Treg and control of *Leishmania major* infection. *J Immunol* (2005) **174**:5444–55. doi:10.4049/jimmunol.174.9.5444
16. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* (2001) **19**:683–765. doi:10.1146/annurev.immunol.19.1.683
17. Nylén S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D. Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. *J Exp Med* (2007) **204**:805–17. doi:10.1084/jem.20061141
18. Rodrigues OR, Marques C, Soares-Clemente M, Ferronha MH, Santos-Gomes GM. Identification of regulatory T cells during experimental *Leishmania infantum* infection. *Immunobiology* (2009) **214**:101–11. doi:10.1016/j.imbio.2008.07.001
19. Gorelik L, Constant S, Flavell RA. Mechanism of transforming growth factor β-induced inhibition of T helper type 1 differentiation. *J Exp Med* (2002) **195**:1499–505. doi:10.1084/jem.20012076
20. Vodovotz Y, Bogdan C, Paik J, Xie QW, Nathan C. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. *J Exp Med* (1993) **178**:605–13. doi:10.1084/jem.178.2.605
21. Lee YJ, Han Y, Lu H-T, Nguyen V, Qin H, Howe PH, et al. TGF-β suppresses IFN-γ induction of class II MHC gene expression by inhibition class II transactivator messenger RNA expression. *J Immunol* (1997) **158**:2065–75.
22. Barral-Netto M, Barral A. Transforming growth factor-beta in tegumentary leishmaniasis. *Braz J Med Biol Res* (1994) **27**:1–9.
23. Peng Y, Laouar Y, Li MO, Green EA, Flavell RA. TGF-beta regulates in vivo expansion of Foxp3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci U S A* (2004) **101**:4572–7. doi:10.1073/pnas.0400810101
24. Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* (2007) **26**:579–91. doi:10.1016/j.immuni.2007.03.014
25. Bommireddy R, Doetschman T. TGFbeta1 and Treg cells: alliance for tolerance. *Trends Mol Med* (2007) **13**:492–501. doi:10.1016/j.molmed.2007.08.005
26. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* (2004) **172**:5149–53. doi:10.4049/jimmunol.172.9.5149
27. Roelen BA, Cohen OS, Raychowdhury MK, Chadee DN, Zhang Y, Kyriakis JM, et al. Phosphorylation of threonine 276 in Smad4 is involved in transforming growth factor-beta-induced nuclear accumulation. *Am J Physiol Cell Physiol* (2003) **285**:C823–30. doi:10.1152/ajpcell.00053.2003
28. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* (1992) **176**:1693–702. doi:10.1084/jem.176.6.1693
29. Ahuja SS, Reddick RL, Sato N, Montalbo E, Kostecki V, Zhao W, et al. Dendritic cell (DC)-based anti-infective strategies: DCs engineered to secrete IL-12 are a potent vaccine in a murine model of an intracellular infection. *J Immunol* (1999) **163**:3890–7.
30. Murray HW, Miralles GD, Stoeckle MY, Mcdermott DF. Role and effect of IL-2 in experimental visceral leishmaniasis. *J Immunol* (1993) **151**:929–38.
31. Majumdar S, Kane LH, Rossi MW, Volpp BD, Nauseef WM, Korchak HM. Protein kinase C isotypes and signal-transduction in human neutrophils: selective substrate specificity of calcium-dependent beta-PKC and novel calcium-independent nPKC. *Biochim Biophys Acta* (1993) **1176**:276–86. doi:10.1016/0167-4889(93)90056-U
32. Ghosh S, Bhattacharyya S, Sirkar M, Sa GS, Das T, Majumdar D, et al. *Leishmania donovani* suppresses activated protein 1 and NF-kappaB activation in host macrophages via ceramide generation: involvement of extracellular signal-regulated kinase. *Infect Immun* (2002) **70**:6828–38. doi:10.1128/IAI.70.12.6828-6838.2002
33. Campanelli AP, Roselino AM, Cavassani KA, Pereira MS, Mortara RA, Brodskyn CI, et al. CD4+CD25+ T cells in skin lesions of patients with cutaneous leishmaniasis exhibit phenotypic and functional characteristics of natural regulatory T cells. *J Infect Dis* (2006) **193**:1313–22. doi:10.1086/502980
34. Levings MK, Sangregorio R, Sartirana C, Moschin AL, Battaglia M, Orban PC, et al. Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J Exp Med* (2002) **196**:1335–46. doi:10.1084/jem.20021139
35. Gomes-Pereira S, Rodrigues OR, Rolao N, Almeida PD, Santos-Gomes GM. Hepatic cellular immune responses in mice with "cure" and "non-cure" phenotype to *Leishmania infantum* infection: importance of CD8+ T cells and TGF-beta production. *FEMS Immunol Med Microbiol* (2004) **41**:59–68. doi:10.1016/j.femsim.2004.01.003

36. Akiyama M, Ichikawa T, Miyaaki H, Motoyoshi Y, Takeshita S, Ozawa E, et al. Relationship between regulatory T cells and the combination of pegylated interferon and ribavirin for the treatment of chronic hepatitis type C. *Intervirology* (2010) **53**:154–60. doi:10.1159/000274976
37. Suffia IJ, Reckling SK, Piccirillo CA, Goldszmid RS, Belkaid Y. Infected site-restricted Foxp3+ natural regulatory T cells are specific for microbial antigens. *J Exp Med* (2006) **203**:777–88. doi:10.1084/jem.20052056
38. Zheng SG, Wang JH, Stohl W, Kim KS, Gray JD, Horwitz DA. TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells. *J Immunol* (2006) **176**:3321–9. doi:10.4049/jimmunol.176.6.3321

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 December 2013; accepted: 19 May 2014; published online: 04 June 2014.
*Citation: Majumder S, Bhattacharjee A, Paul Chowdhury B, Bhattacharyya Majumdar S and Majumdar S (2014) Antigen-pulsed CpG-ODN-activated dendritic cells induce host-protective immune response by regulating the T regulatory cell functioning in Leishmania donovani-infected mice: critical role of CXCL10. Front. Immunol. **5**:261. doi: 10.3389/fimmu.2014.00261*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
PUBLIC HEALTH

PERSPECTIVE ARTICLE
published: 08 August 2014
doi: 10.3389/fpubh.2014.00099

Vector saliva in vaccines for visceral leishmaniasis: a brief encounter of high consequence?

Shaden Kamhawi*, Hamide Aslan and Jesus G. Valenzuela*

Vector Molecular Biology Section, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA

Edited by:
Nahid Ali, Indian Institute of Chemical Biology, India

Reviewed by:
Mauricio Martins Rodrigues, Federal University of São Paulo, Brazil
Alexandre Barbosa Reis, Universidade Federal de Ouro Preto, Brazil

****Correspondence:***
Shaden Kamhawi and Jesus G. Valenzuela, Vector Molecular Biology Section, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12735 Twinbrook Parkway, Room 2E22, Rockville, MD 20878, USA
e-mail: skamhawi@niaid.nih.gov; jvalenzuela@niaid.nih.gov

Visceral leishmaniasis (VL) is a vector-borne disease transmitted by phlebotomine sand flies and remains the most serious form of the disease with no available human vaccine. Repeatedly, studies have demonstrated the immunogenicity and protective efficacy of a number of sand fly salivary proteins against cutaneous and visceral leishmaniasis. All *Leishmania* species including agents of VL are co-deposited into the skin together with vector saliva. Generally, the immune response to a protective salivary protein in vaccinated animals is rapid and possibly acts on the parasites soon after delivery into the skin by the bite of an infective sand fly. This is followed by the development of a stronger *Leishmania*-specific immunity in saliva-vaccinated animals compared to controls. Considering that several of the most efficacious protective molecules were identified from a proven vector of VL, we put forward the notion that a combination vaccine that includes a *Leishmania* antigen and a vector salivary protein has the potential to improve vaccine efficacy by targeting the parasite at it most vulnerable stage just after transmission.

Keywords: visceral leishmaniasis, sand fly vectors, vector-transmission, salivary proteins as vaccines, Th1 immune response, delayed-type hypersensitivity response

BACKGROUND

Visceral leishmaniasis (VL), also known as kala-azar, is a systemic vector-borne neglected disease that is fatal if left untreated. There are an estimated 300,000 cases of VL globally with over 20,000 deaths per year, a statistic second only to malaria among parasitic diseases (1). Over 90% of VL cases occur in six countries (Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan) where about 300 million people are at risk of infection (1, 2). From 2009 to 2012, an epidemic in South Sudan caused over 28,300 cases and nearly 900 deaths[1]. Other areas have also been affected by recent persistent epidemics of VL in Ethiopia and Kenya[1].

Visceral leishmaniasis is caused either by *Leishmania donovani* or *L. infantum*. VL caused by *L. donovani* is prevalent in East Africa and the Indian sub-continent and is considered an anthroponosis, while VL caused by *L. infantum* is prevalent in South Europe, North Africa, parts of the Middle East and Latin America (3–6). Phlebotomine sand flies are still considered the primary and stable mode of VL transmission. Different species of sand flies have been incriminated as vectors of VL. *Phlebotomus argentipes* is the only known vector of *L. donovani* in the Indian sub-continent (7–9) and *P. orientalis* represents the main sand fly species transmitting *L. donovani* within countries of East Africa, Saudi Arabia, and Yemen (10, 11). On the other hand, there are several proven vectors of VL in the Eastern Mediterranean among which *P. ariasi* and *P. perniciosus* represent the primary species transmitting *L. infantum* (12, 13), while *Lutzomyia longipalpis* is considered the primary vector of *L. infantum* across Latin America (14, 15).

Despite, its wide distribution and high mortality rate, there are no available human vaccines against VL. Even with recent improvement in treatment (16–19) and the gates initiative for the elimination of VL from the Indian sub-continent[2], there remains a need to develop a vaccine, particularly when considering the prevalence of infected individuals with subclinical infections that potentially present an uncontrolled source of parasites for the sand fly vector (20). Though the primary function of vector saliva is to facilitate blood feeding (21), a good body of evidence has shown that it modulates host immunity altering the outcome of infection with *Leishmania* and under certain circumstances, protecting from disease (22–24). Here, we give our perspective on the relevance of vector saliva in the transmission of and for vaccines against VL.

VECTOR SALIVA AND PROTECTION FROM LEISHMANIASIS

Vaccination with certain immunogenic proteins in saliva of vector sand flies confers protection from leishmaniasis (25–35). Protective molecules have mostly shared a similar property, the induction of a delayed-type hypersensitivity (DTH) response biased toward a Th1 profile. Importantly, animals previously exposed to saliva or vaccinated with a Th1-biased DTH-inducing salivary protein were protected against challenge by infected vector bites (28, 30, 35). This is significant since Peters et al. (36) showed that the innate immune response following sand fly transmission varied significantly from the response induced by needle challenge primarily related to a persistence of a neutrophilic infiltrate at the site of bite enhancing parasite virulence. Additionally, the enhanced virulence of vector-transmission was shown to abrogate protection

[1] www.who.int/leishmaniasis

[2] www.gatesfoundation.org

by *Leishmania* vaccines tested against needle challenge with parasites largely due to the need for a rapid effector immune response (37). Thus, saliva-mediated protection from vector-transmitted leishmaniasis suggests that the immune response to salivary proteins is rapid enough to restrict the establishment of *Leishmania* parasites following vector-challenge. Furthermore, the protection against vector-challenge displayed by animals vaccinated with a defined recombinant salivary protein indicates that the native protein despite its presence among others in saliva of the vector initiated an efficient recall response upon its co-deposition in skin with the parasites (28).

Recently, a study investigating the value of combining a protective salivary vaccine with promising *Leishmania* antigens tested several combinations of PpSP15, a protective salivary protein from *P. papatasi* (31, 33), with live recombinant *L. tarentolae* stably expressing the cysteine proteinases CPA and CPB (38). In both BALB/c and C57BL/6 mice, the animals primed with PpSP15 DNA and boosted with PpSP15 DNA and live recombinant CPA/CPB-expressing *L. tarentolae* exhibited the strongest protection against *L. major* infection followed by the group immunized with both PpSP15 and CPA/CPB-expressing *L. tarentolae* injected in independent sites (38). This study is the first to demonstrate the enhanced protection from leishmaniasis resulting from the inclusion of a vector salivary component to the vaccine.

The significance of vector salivary proteins in *Leishmania* vaccines is made more credible by the observed immunogenicity of saliva in exposed humans (39–41). Gomes et al. (39) first reported on the association between the appearance of antibodies to *L. longipalpis* saliva and the development of a protective cell-mediated immunity to *L. chagasi*. In another study, volunteers experimentally exposed to *L. longipalpis* produced distinct skin reactions at the bite site and displayed an increased frequency of IFN-γ- and IL-10-producing T cells (40). Additionally, the authors demonstrated that PBMC from volunteers maintained an efficient recall response 1 year after their first exposure and produced IFN-γ upon *in vitro* stimulation with saliva that was associated to a significant reduction in macrophage infection rates with *L. chagasi*. More recently, we demonstrated that the DTH response in individuals naturally exposed to bites of *P. duboscqi*, another vector sand fly, persists to mid life (41). Importantly, though PBMC from volunteers showed a Th1, Th2, or a mixed response upon *in vitro* stimulation with saliva, dermal biopsies from bite sites with a DTH response were dominated by macrophages and lymphocytes and exhibited an abundance of IFN-γ indicative of a Th1 milieu (41). Though more studies in humans are needed, the above results demonstrate that repeated exposure to sand fly saliva alters the immune response of humans to the parasites co-deposited into the wound at the site of an infected bite.

TRANSMISSION OF VISCERAL LEISHMANIASIS AND VECTOR SALIVA

Despite reports of vertical transmission of *L. infantum* (42), it is still accepted that VL, caused by *L. donovani* or *L. infantum*, is mostly transmitted by bite of infected phlebotomine sand flies. At the site of bite, the sand fly deposits few parasites (43–45) alongside saliva in the skin. Therefore, though pathology of VL is ultimately the result of failure of internal organs, mainly the spleen and liver, there is a vital phase early after transmission where the few parasites deposited in the skin are at their most vulnerable. We believe it is at this stage that immunity to a salivary protein can potentially exert a profound effect on the survival and ability of the parasites to visceralize. Studies have identified immunogenic salivary proteins from important VL vectors that induce a distinct Th1–DTH response predictive of protection from leishmaniasis (27, 29, 31, 46). In the only study investigating the potential of salivary proteins to protect against VL, LJM19, a Th1–DTH-inducing salivary protein from *L. longipalpis*, a VL vector, conferred powerful protection against progressive VL in vaccinated hamsters (29). LJM19-vaccinated animals displayed a high IFN-γ/TGF-β ratio and inducible NOS expression in the spleen and liver associated to a controlled parasite burden and survival up to 5 months post-infection. In contrast, controls and hamsters vaccinated with other salivary molecules developed progressive fatal VL within the same time frame (29). The long-term systemic protection from *L. chagasi* (*L. infantum*) conferred by immunity to LJM19 was likely driven by the initial immune response to LJM19 in the skin where a distinct DTH response with high expression of IFN-γ was observed 48 h after challenge with uninfected sand flies (29). Due to a shorter course of infection and the ease of assessing disease burden most studies of the protective capacity of immunogenic salivary proteins from saliva of *L. longipalpis* were tested using CL infection models producing promising results. Mice vaccinated with maxadilan, the vasodilator from *L. longipalpis* saliva protected mice against *L. major* infection (34), while vaccination with LJM19, protected hamsters against infections with *L. braziliensis* co-injected with saliva of the natural vector *L. intermedia* (32). LJM11, another Th1–DTH-inducing salivary protein from *L. longipalpis*, conferred partial protection against *L. infantum* in hamsters (29) and a strong protection against infections initiated by needle or vector-challenge with *L. major* in mice (28, 47). **Table 1** provides a summary of potential salivary vaccines identified from VL vectors to date.

Studies carried out using CL models of infection have demonstrated that the initial immune response directed against sand fly saliva or one of its proteins gives rise to an accelerated and potent immune response specific to the *Leishmania* parasite (28, 31). The initial saliva-specific immune response is observed as early as 2–6 h after bite up to 1 week post-challenge (29–31, 35). By 2-weeks post-infection, animals vaccinated with a salivary protein mount a stronger *Leishmania*-specific immunity with minimized pathology (28, 31). This supports our hypothesis that the initial immune response to a salivary protein in the skin can potentially alter the nature of the immune response to the parasites long-term and is therefore relevant for protection against both CL and VL.

VECTOR SALIVA IN A VACCINE FOR VISCERAL LEISHMANIASIS

RATIONALE

From the above, immunity to a vector salivary protein can potentially have an enormous impact on progression of VL. Visceralizing parasites are initially inoculated into the skin then navigate their way to the viscera in a poorly understood manner. Assuming that

Table 1 | Vaccine candidates identified from saliva of visceral leishmaniasis vectors.

Sand fly species	Salivary molecule	Immunogenicity	Protection	Animal model	Reference
L. longipalpis	Maxadilan	Th1, IgG	*L. major*	Mouse	(31)
L. longipalpis	LJM19	Th1/DTH	*L. infantum, L. braziliensis*	Hamster	(29, 32)
L. longipalpis	LJM11	DTH, IgG	*L. infantum*	Hamster (partial)	(29)
L. longipalpis	LJM11	Th1/DTH, IgG2a	*L. major*	Mouse	(28, 47)
L. longipalpis	LJM17	Th1/DTH, IgG2a	*L. infantum*	Dog	(27)
L. longipalpis	LJL143	Th1/DTH, IgG2a	*L. infantum*	Dog	(27)
P. ariasi	ParSP01	DTH		Mouse	(46)
P. ariasi	ParSP03	DTH, IgG2a		Mouse	(46)
P. ariasi	ParSP25	DTH, IgG1		Mouse	(46)

for a brief period of time these parasites are in the skin, low in number, and in close proximity to co-inoculated salivary proteins, a vaccine strategy involving immunization with a Th1-inducing salivary protein that would initiate a rapid immune response to itself at the site of bite will adversely impact the vulnerable *Leishmania* parasites while still in the skin. Such a vaccine could potentially enhance the efficacy of a VL vaccine by introducing an additional stage in which the parasites are attacked.

DIVERSITY OF VL FOCI

The complexity of VL transmission would clearly have an impact on the design and practicality of a salivary vaccine. *L. donovani*, considered an anthroponosis, is transmitted by only one species of sand flies in the Indian sub-continent but has multiple vectors in East Africa (7–11, 48). A similar situation exists for zoonotic VL due to *L. infantum* where across Latin America transmission is mostly by a single primary vector while along the Eastern Mediterranean, over six species of sand flies have been incriminated as major VL vectors (12–15). Foci where transmission involves multiple vectors would be more challenging compared to those where a vaccine needs to target a single vector species. Under these conditions, the future for salivary antigens is most likely in vaccines tailored for specific regions. Nonetheless, in several of the most important foci of VL including India, Sudan, and Latin America there is but one primary vector sand fly species, *P. argentipes*, *P. orientalis*, and *L. longipalpis*, respectively (8–10, 14, 15, 49), a situation where a tailored vaccine may be justified.

CHALLENGES AND SOLUTIONS

As mentioned above, in foci with a primary vector, inclusion of a salivary protein in a leishmaniasis vaccine can potentially enhance its efficacy. On the other hand, certain VL foci such as those in the Eastern Mediterranean region have multiple incriminated VL vectors (12, 48). For such foci, a salivary molecule with the appropriate immunogenicity needs to have close homologs in most sympatric vector species, creating a considerable obstacle. We are now addressing whether priming with a salivary protein and boosting with both the salivary antigen and a *Leishmania* antigen will drive a *Leishmania*-specific immunity strong enough to overcome the obstacle presented by specificity of vector salivary molecules. If successful, incorporating the best of the immunogenic salivary proteins with the most promising *Leishmania* antigens may present an opportunity for a pan leishmaniasis vaccine. Here, we must underscore that though a robust immunity to *Leishmania* driven by a preceding immunity to saliva has been demonstrated (28, 31), it was always generated by a challenge with virulent live parasites. It remains to be validated whether a similar level of protective immunity can be achieved with a single antigen. Considering the payback, it is a question worthy of further exploration.

FURTHER CONSIDERATIONS

Identifying salivary molecules from VL vectors that can induce a Th1-biased immunity in humans should be prioritized. Expression libraries of the secreted salivary proteins of several VL vectors are available (46, 50–53) and high throughput expression of endotoxin-free recombinant proteins of high purity has been achieved (28, 54). Developing a rapid screening assay using PBMC of healthy exposed volunteers stimulated with recombinant salivary proteins from VL vectors could rapidly reveal immunogenic antigens appropriate for further exploration as protective vaccine candidates using animal models. Additionally, we recently developed a hamster model of vector-transmitted progressive VL (55) that can further facilitate the prioritization of salivary vaccine candidates found immunogenic in humans. Here, it is important to emphasize the need to begin the search for a vaccine candidate using human cells (56). Multiple leishmaniasis vaccine candidates protected various animal models but failed to protect humans (57). This is not surprising considering that the initiation of a Th1 cellular immunity such as that induced by salivary molecules and required for protection against leishmaniasis implies efficient recognition of specific antigenic epitopes by human leukocyte antigen I (HLA-I) and HLA-II molecules for presentation to T cells (58). However, unlike anthroponotic VL where humans are the only vaccine target, zoonotic VL needs to target dogs as the domestic reservoirs and the primary source of infection to sand flies and humans (12, 48, 59, 60). Therefore, in addition to humans, salivary molecules immunogenic in dogs such as those reported for *L. longipalpis* (27), should also be considered for a canine vaccine.

SHADES OF GRAY

Though, we tend to put *Leishmania* species in clear-cut categories, nature tells us otherwise. The unique polymorphic nature

of leishmaniasis and the plasticity of *Leishmania* parasites continue to confound efforts toward disease control. There are several reports where a single parasite strain commonly causing dermatotropic symptoms manifests as a visceral infection and vice versa (61–63). Specifically, we still do not understand why *L. infantum*, associated mainly with VL, causes only cutaneous disease in some regions (64). Similarly, *L. donovani* zymodeme MON-37, the parasite strain previously associated exclusively with VL in India and East Africa, has been identified as the causative agent in recently established foci of CL in Sri Lanka (65, 66). These unusual manifestations of leishmaniasis clearly demonstrate how little we understand the factors contributing to disease. The fact that dermotropic *L. infantum* genotypes can disseminate and cause severe VL in immunosuppressed individuals is indicative of the importance of host susceptibility in the outcome of infection with *Leishmania* parasites (67). But is the etiology of leishmaniasis mainly due to host immunity or are environmental pressures, vector-derived factors and evolution of the parasite itself equally significant? Most likely the form of leishmaniasis contracted is the consequence of all the aforementioned factors. Hence, we need to keep an open mind in our search for vaccines and perhaps entertain the option of a tailored vaccine enhanced by a salivary component of a primary vector in foci of high morbidity and mortality.

CONCLUSION

To date, a human vaccine against any form of leishmaniasis is nonexistent. There is strong evidence that certain proteins in sand fly vector saliva can: (1) induce a Th1–DTH immune response; (2) protect against both CL and VL; (3) protect against vector-initiated CL; and (4) induce a *Leishmania*-specific robust immunity after challenge with minimized pathology. Considering the above, should not salivary proteins of vector sand flies be given serious consideration as candidate components in a *Leishmania* vaccine?

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program at the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

REFERENCES

1. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* (2007) **5**(11):873–82. doi:10.1038/nrmicro1748
2. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) **7**(5):e35671. doi:10.1371/journal.pone.0035671
3. Lukes J, Mauricio IL, Schonian G, Dujardin JC, Soteriadou K, Dedet JP, et al. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *Proc Natl Acad Sci U S A* (2007) **104**(22):9375–80. doi:10.1073/pnas.0703678104
4. Alvar J, Canavate C, Molina R, Moreno J, Nieto J. Canine leishmaniasis. *Adv Parasitol* (2004) **57**:1–88. doi:10.1016/S0065-308X(04)57001-X
5. Nasereddin A, Baneth G, Schonian G, Kanaan M, Jaffe CL. Molecular fingerprinting of *Leishmania infantum* strains following an outbreak of visceral leishmaniasis in central Israel. *J Clin Microbiol* (2005) **43**(12):6054–9. doi:10.1128/JCM.43.12.6054-6059.2005
6. Bader KA, Schnur LF, Nasereddin A, Pratlong F, Dedet JP, Shaheen L, et al. Palestinian infantile visceral leishmaniasis caused by a genetic variant of *Leishmania infantum* belonging to a new zymodeme. *Trop Med Int Health* (2005) **10**(6):618–20. doi:10.1111/j.1365-3156.2005.01419.x
7. Desjeux P. Leishmaniasis. Public health aspects and control. *Clin Dermatol* (1996) **14**(5):417–23. doi:10.1016/0738-081X(96)00057-0
8. Tiwary P, Kumar D, Singh RP, Rai M, Sundar S. Prevalence of sand flies and *Leishmania donovani* infection in a natural population of female *Phlebotomus argentipes* in Bihar State, India. *Vector Borne Zoonotic Dis* (2012) **12**(6):467–72. doi:10.1089/vbz.2011.0808
9. Tiwary P, Kumar D, Mishra M, Singh RP, Rai M, Sundar S. Seasonal variation in the prevalence of sand flies infected with *Leishmania donovani*. *PLoS One* (2013) **8**(4):e61370. doi:10.1371/journal.pone.0061370
10. Elnaiem DE. Ecology and control of the sand fly vectors of *Leishmania donovani* in East Africa, with special emphasis on *Phlebotomus orientalis*. *J Vector Ecol* (2011) **36**(Suppl 1):S23–31. doi:10.1111/j.1948-7134.2011.00109.x
11. Dereure J, Rageh HA, Daoud W, Rioux JA. Eco-epidemiology of visceral and cutaneous leishmaniasis in the Arab Republic of Yemen. II. A survey using intradermal reaction to leishmanin in a zone of mixed infestation with *Leishmania tropica*, *L. donovani* and *L. infantum*. *Bull Soc Pathol Exot Filiales* (1989) **82**(5):665–8.
12. Ready PD. Leishmaniasis emergence in Europe. *Euro Surveill* (2010) **15**(10):19505.
13. Giorgobiani E, Lawyer PG, Babuadze G, Dolidze N, Jochim RC, Tskhvaradze L, et al. Incrimination of *Phlebotomus kandelakii* and *Phlebotomus balcanicus* as vectors of *Leishmania infantum* in Tbilisi, Georgia. *PLoS Negl Trop Dis* (2012) **6**(4):e1609. doi:10.1371/journal.pntd.0001609
14. Silva ES, Gontijo CM, Pacheco RS, Fiuza VO, Brazil RP. Visceral leishmaniasis in the Metropolitan Region of Belo Horizonte, State of Minas Gerais, Brazil. *Mem Inst Oswaldo Cruz* (2001) **96**(3):285–91. doi:10.1590/S0074-02762001000300002
15. Lainson R, Rangel EF. *Lutzomyia longipalpis* and the eco-epidemiology of American visceral leishmaniasis, with particular reference to Brazil: a review. *Mem Inst Oswaldo Cruz* (2005) **100**(8):811–27. doi:10.1590/S0074-02762005000800001
16. Dorlo TP, Balasegaram M, Beijnen JH, de Vries PJ. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *J Antimicrob Chemother* (2012) **67**(11):2576–97. doi:10.1093/jac/dks275
17. Sundar S, Chakravarty J. Liposomal amphotericin B and leishmaniasis: dose and response. *J Glob Infect Dis* (2010) **2**(2):159–66. doi:10.4103/0974-777X.62886
18. Chakravarty J, Sundar S. Drug resistance in leishmaniasis. *J Glob Infect Dis* (2010) **2**(2):167–76. doi:10.4103/0974-777X.62887
19. Meheus F, Balasegaram M, Olliaro P, Sundar S, Rijal S, Faiz MA, et al. Cost-effectiveness analysis of combination therapies for visceral leishmaniasis in the Indian subcontinent. *PLoS Negl Trop Dis* (2010) **4**(9):e818. doi:10.1371/journal.pntd.0000818
20. Bern C, Haque R, Chowdhury R, Ali M, Kurkjian KM, Vaz L, et al. The epidemiology of visceral leishmaniasis and asymptomatic leishmanial infection in a highly endemic Bangladeshi village. *Am J Trop Med Hyg* (2007) **76**(5):909–14.
21. Ribeiro JM, Francischetti IM. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu Rev Entomol* (2003) **48**:73–88. doi:10.1146/annurev.ento.48.060402.102812
22. Kamhawi S. The biological and immunomodulatory properties of sand fly saliva and its role in the establishment of *Leishmania* infections. *Microbes Infect* (2000) **2**(14):1765–73. doi:10.1016/S1286-4579(00)01331-9
23. Andrade BB, de Oliveira CI, Brodskyn CI, Barral A, Barral-Netto M. Role of sand fly saliva in human and experimental leishmaniasis: current insights. *Scand J Immunol* (2007) **66**(2–3):122–7. doi:10.1111/j.1365-3083.2007.01964.x
24. Oliveira F, de Carvalho AM, de Oliveira CI. Sand-fly saliva – man: the trigger trio. *Front Immunol* (2013) **4**:375. doi:10.3389/fimmu.2013.00375
25. Belkaid Y, Kamhawi S, Modi G, Valenzuela J, Noben-Trauth N, Rowton E, et al. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. *J Exp Med* (1998) **188**(10):1941–53. doi:10.1084/jem.188.10.1941
26. Carregaro V, Costa DL, Brodskyn C, Barral AM, Barral-Netto M, Cunha FQ, et al. Dual effect of *Lutzomyia longipalpis* saliva on *Leishmania braziliensis* infection is mediated by distinct saliva-induced cellular recruitment into BALB/c mice ear. *BMC Microbiol* (2013) **13**:102. doi:10.1186/1471-2180-13-102

27. Collin N, Gomes R, Teixeira C, Cheng L, Laughinghouse A, Ward JM, et al. Sand fly salivary proteins induce strong cellular immunity in a natural reservoir of visceral leishmaniasis with adverse consequences for *Leishmania*. *PLoS Pathog* (2009) **5**(5):e1000441. doi:10.1371/journal.ppat.1000441
28. Gomes R, Oliveira F, Teixeira C, Meneses C, Gilmore DC, Elnaiem DE, et al. Immunity to sand fly salivary protein LJM11 modulates host response to vector-transmitted *Leishmania* conferring ulcer-free protection. *J Invest Dermatol* (2012) **132**(12):2735–43. doi:10.1038/jid.2012.205
29. Gomes R, Teixeira C, Teixeira MJ, Oliveira F, Menezes MJ, Silva C, et al. Immunity to a salivary protein of a sand fly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. *Proc Natl Acad Sci U S A* (2008) **105**(22):7845–50. doi:10.1073/pnas.0712153105
30. Kamhawi S, Belkaid Y, Modi G, Rowton E, Sacks D. Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies. *Science* (2000) **290**(5495):1351–4. doi:10.1126/science.290.5495.1351
31. Oliveira F, Lawyer PG, Kamhawi S, Valenzuela JG. Immunity to distinct sand fly salivary proteins primes the anti-*Leishmania* immune response towards protection or exacerbation of disease. *PLoS Negl Trop Dis* (2008) **2**(4):e226. doi:10.1371/journal.pntd.0000226
32. Tavares NM, Silva RA, Costa DJ, Pitombo MA, Fukutani KF, Miranda JC, et al. *Lutzomyia longipalpis* saliva or salivary protein LJM19 protects against *Leishmania braziliensis* and the saliva of its vector, *Lutzomyia intermedia*. PLoS Negl Trop Dis (2011) **5**(5):e1169. doi:10.1371/journal.pntd.0001169
33. Valenzuela JG, Belkaid Y, Garfield MK, Mendez S, Kamhawi S, Rowton ED, et al. Toward a defined anti-*Leishmania* vaccine targeting vector antigens: characterization of a protective salivary protein. *J Exp Med* (2001) **194**(3):331–42. doi:10.1084/jem.194.3.331
34. Morris RV, Shoemaker CB, David JR, Lanzaro GC, Titus RG. Sandfly maxadilan exacerbates infection with *Leishmania major* and vaccinating against it protects against *L. major* infection. *J Immunol* (2001) **167**(9):5226–30. doi:10.4049/jimmunol.167.9.5226
35. Teixeira C, Gomes R, Oliveira F, Meneses C, Gilmore DC, Elnaiem DE, et al. Characterization of the early inflammatory infiltrate at the feeding site of infected sand flies in mice protected from vector-transmitted *Leishmania major* by exposure to uninfected bites. *PLoS Negl Trop Dis* (2014) **8**(4):e2781. doi:10.1371/journal.pntd.0002781
36. Peters NC, Egen JG, Secundino N, Debrabant A, Kimblin N, Kamhawi S, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science* (2008) **321**(5891):970–4. doi:10.1126/science.1159194
37. Peters NC, Kimblin N, Secundino N, Kamhawi S, Lawyer P, Sacks DL. Vector transmission of *Leishmania* abrogates vaccine-induced protective immunity. *PLoS Pathog* (2009) **5**(6):e1000484. doi:10.1371/journal.ppat.1000484
38. Zahedifard F, Gholami E, Taheri T, Taslimi Y, Doustdari F, Seyed N, et al. Enhanced protective efficacy of nonpathogenic recombinant *Leishmania tarentolae* expressing cysteine proteinases combined with a sand fly salivary antigen. *PLoS Negl Trop Dis* (2014) **8**(3):e2751. doi:10.1371/journal.pntd.0002751
39. Gomes RB, Brodskyn C, de Oliveira CI, Costa J, Miranda JC, Caldas A, et al. Seroconversion against *Lutzomyia longipalpis* saliva concurrent with the development of anti-*Leishmania chagasi* delayed-type hypersensitivity. *J Infect Dis* (2002) **186**(10):1530–4. doi:10.1086/344733
40. Vinhas V, Andrade BB, Paes F, Bomura A, Clarencio J, Miranda JC, et al. Human anti-saliva immune response following experimental exposure to the visceral leishmaniasis vector, *Lutzomyia longipalpis*. *Eur J Immunol* (2007) **37**(11):3111–21. doi:10.1002/eji.200737431
41. Oliveira F, Traore B, Gomes R, Faye O, Gilmore DC, Keita S, et al. Delayed-type hypersensitivity to sand fly saliva in humans from a leishmaniasis-endemic area of Mali is Th1-mediated and persists to midlife. *J Invest Dermatol* (2013) **133**(2):452–9. doi:10.1038/jid.2012.315
42. Rosypal AC, Troy GC, Zajac AM, Frank G, Lindsay DS. Transplacental transmission of a North American isolate of *Leishmania infantum* in an experimentally infected beagle. *J Parasitol* (2005) **91**(4):970–2. doi:10.1645/GE-483R.1
43. Kimblin N, Peters N, Debrabant A, Secundino N, Egen J, Lawyer P, et al. Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies. *Proc Natl Acad Sci U S A* (2008) **105**(29):10125–30. doi:10.1073/pnas.0802331105
44. Secundino NF, de Freitas VC, Monteiro CC, Pires AC, David BA, Pimenta PF. The transmission of *Leishmania infantum chagasi* by the bite of the *Lutzomyia longipalpis* to two different vertebrates. *Parasit Vectors* (2012) **5**:20. doi:10.1186/1756-3305-5-20
45. Maia C, Seblova V, Sadlova J, Votypka J, Volf P. Experimental transmission of *Leishmania infantum* by two major vectors: a comparison between a viscerotropic and a dermotropic strain. *PLoS Negl Trop Dis* (2011) **5**(6):e1181. doi:10.1371/journal.pntd.0001181
46. Oliveira F, Kamhawi S, Seitz AE, Pham VM, Guigal PM, Fischer L, et al. From transcriptome to immunome: identification of DTH inducing proteins from a *Phlebotomus ariasi* salivary gland cDNA library. *Vaccine* (2006) **24**(3):374–90. doi:10.1016/j.vaccine.2005.07.085
47. Xu X, Oliveira F, Chang BW, Collin N, Gomes R, Teixeira C, et al. Structure and function of a "yellow" protein from saliva of the sand fly *Lutzomyia longipalpis* that confers protective immunity against *Leishmania major* infection. *J Biol Chem* (2011) **286**(37):32383–93. doi:10.1074/jbc.M111.268904
48. Ready PD. Epidemiology of visceral leishmaniasis. *Clin Epidemiol* (2014) **6**:147–54. doi:10.2147/CLEP.S44267
49. WHO. Control of the leishmaniases. Report of a WHO expert committee. *World Health Organ Tech Rep Ser* (2010) **949**:1–200.
50. Vlkova M, Sima M, Rohousova I, Kostalova T, Sumova P, Volfova V, et al. Comparative analysis of salivary gland transcriptomes of *Phlebotomus orientalis* sand flies from endemic and non-endemic foci of visceral leishmaniasis. *PLoS Negl Trop Dis* (2014) **8**(2):e2709. doi:10.1371/journal.pntd.0002709
51. Valenzuela JG, Garfield M, Rowton ED, Pham VM. Identification of the most abundant secreted proteins from the salivary glands of the sand fly *Lutzomyia longipalpis*, vector of *Leishmania chagasi*. *J Exp Biol* (2004) **207**(Pt 21):3717–29. doi:10.1242/jeb.01185
52. Anderson JM, Oliveira F, Kamhawi S, Mans BJ, Reynoso D, Seitz AE, et al. Comparative salivary gland transcriptomics of sandfly vectors of visceral leishmaniasis. *BMC Genomics* (2006) **7**:52. doi:10.1186/1471-2164-7-52
53. Rohousova I, Subrahmanyam S, Volfova V, Mu J, Volf P, Valenzuela JG, et al. Salivary gland transcriptomes and proteomes of *Phlebotomus tobbi* and *Phlebotomus sergenti*, vectors of leishmaniasis. *PLoS Negl Trop Dis* (2012) **6**(5):e1660. doi:10.1371/journal.pntd.0001660
54. Teixeira C, Gomes R, Collin N, Reynoso D, Jochim R, Oliveira F, et al. Discovery of markers of exposure specific to bites of *Lutzomyia longipalpis*, the vector of *Leishmania infantum chagasi* in Latin America. *PLoS Negl Trop Dis* (2010) **4**(3):e638. doi:10.1371/journal.pntd.0000638
55. Aslan H, Dey R, Meneses C, Castrovinci P, Jeronimo SM, Oliva G, et al. A new model of progressive visceral leishmaniasis in hamsters by natural transmission via bites of vector sand flies. *J Infect Dis* (2013) **207**(8):1328–38. doi:10.1093/infdis/jis932
56. Das S, Freier A, Boussoffara T, Das S, Oswald D, Losch FO, et al. Modular multi-antigen T cell epitope-enriched DNA vaccine against human leishmaniasis. *Sci Transl Med* (2014) **6**(234):234ra56. doi:10.1126/scitranslmed.3008222
57. Singh B, Sundar S. Leishmaniasis: vaccine candidates and perspectives. *Vaccine* (2012) **30**(26):3834–42. doi:10.1016/j.vaccine.2012.03.068
58. Kamhawi S, Oliveira F, Valenzuela JG. Using humans to make a human leishmaniasis vaccine. *Sci Transl Med* (2014) **6**(234):234fs18. doi:10.1126/scitranslmed.3009118
59. Courtenay O, Carson C, Calvo-Bado L, Garcez LM, Quinnell RJ. Heterogeneities in *Leishmania infantum* infection: using skin parasite burdens to identify highly infectious dogs. *PLoS Negl Trop Dis* (2014) **8**(1):e2583. doi:10.1371/journal.pntd.0002583
60. Reis AB, Giunchetti RC, Carrillo E, Martins-Filho OA, Moreno J. Immunity to *Leishmania* and the rational search for vaccines against canine leishmaniasis. *Trends Parasitol* (2010) **26**(7):341–9. doi:10.1016/j.pt.2010.04.005
61. Ponce C, Ponce E, Morrison A, Cruz A, Kreutzer R, McMahon-Pratt D, et al. *Leishmania donovani chagasi*: new clinical variant of cutaneous leishmaniasis in Honduras. *Lancet* (1991) **337**(8733):67–70. doi:10.1016/0140-6736(91)90734-7
62. Pratlong F, Bastien P, Perello R, Lami P, Dedet JP. Human cutaneous leishmaniasis caused by *Leishmania donovani* sensu stricto in Yemen. *Trans R Soc Trop Med Hyg* (1995) **89**(4):398–9. doi:10.1016/0035-9203(95)90025-X

63. Marty P, Pratlong F, Marcelet B, Adda A, Le Fichoux Y. *Leishmania infantum* variant MON-24 isolated from a cutaneous lesion acquired in the suburban area of Nice (France). *Parasite* (1994) **1**(2):175–6.
64. Svobodova M, Alten B, Zidkova L, Dvorak V, Hlavackova J, Myskova J, et al. Cutaneous leishmaniasis caused by *Leishmania infantum* transmitted by *Phlebotomus tobbi*. *Int J Parasitol* (2009) **39**(2):251–6. doi:10.1016/j.ijpara.2008.06.016
65. Siriwardana HV, Noyes HA, Beeching NJ, Chance ML, Karunaweera ND, Bates PA. *Leishmania donovani* and cutaneous leishmaniasis, Sri Lanka. *Emerg Infect Dis* (2007) **13**(3):476–8. doi:10.3201/eid1303.060242
66. Karunaweera ND. *Leishmania donovani* causing cutaneous leishmaniasis in Sri Lanka: a wolf in sheep's clothing? *Trends Parasitol* (2009) **25**(10):458–63. doi:10.1016/j.pt.2009.07.002
67. Gradoni L, Gramiccia M. *Leishmania infantum* tropism: strain genotype or host immune status? *Parasitol Today* (1994) **10**(7):264–7. doi:10.1016/0169-4758(94)90142-2

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 May 2014; accepted: 14 July 2014; published online: 08 August 2014.
*Citation: Kamhawi S, Aslan H and Valenzuela JG (2014) Vector saliva in vaccines for visceral leishmaniasis: a brief encounter of high consequence? Front. Public Health **2**:99. doi: 10.3389/fpubh.2014.00099*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Public Health.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 31 March 2014
doi: 10.3389/fimmu.2014.00134

Live vaccination tactics: possible approaches for controlling visceral leishmaniasis

Noushin Saljoughian, Tahareh Taheri and Sima Rafati*

Molecular Immunology and Vaccine Research Laboratory, Pasteur Institute of Iran, Tehran, Iran

Edited by:
Nahid Ali, Indian Institute of Chemical Biology, India

Reviewed by:
Urszula Krzych, Walter Reed Army Institute of Research, USA
Hira Nakhasi, Center for Biologics Evaluation Research and Review, USA

Correspondence:
Sima Rafati, Molecular Immunology and Vaccine Research Laboratory, Pasteur Institute of Iran, 69 Pasteur Avenue, Tehran 13164, Iran
e-mail: s_rafati@yahoo.com, sima-rafatisy@pasteur.ac.ir

Vaccination with durable immunity is the main goal and fundamental to control leishmaniasis. To stimulate the immune response, small numbers of parasites are necessary to be presented in the mammalian host. Similar to natural course of infection, strategy using live vaccine is more attractive when compared to other approaches. Live vaccines present the whole spectrum of antigens to the host immune system in the absence of any adjuvant. Leishmanization was the first effort for live vaccination and currently used in a few countries against cutaneous leishmaniasis, in spite of their obstacle and safety. Then, live attenuated vaccines developed with similar promotion of creating long-term immunity in the host with lower side effect. Different examples of attenuated strains are generated through long-term *in vitro* culturing, culturing under drug pressure, temperature sensitivity, and chemical mutagenesis, but none is safe enough and their revision to virulent form is possible. Attenuation through genetic manipulation and disruption of virulence factors or essential enzymes for intracellular survival are among other approaches that are intensively under study. Other designs to develop live vaccines for visceral form of leishmaniasis are utilization of live avirulent microorganisms such as *Lactococcus lactis*, *Salmonella enterica*, and *Leishmania tarentolae* called as vectored vaccine. Apparently, these vaccines are intrinsically safer and can harbor the candidate antigens in their genome through different genetic manipulation and create more potential to control *Leishmania* parasite as an intracellular pathogen.

Keywords: *Leishmania*, visceral leishmaniasis, live vaccine, live attenuated vaccines, live non-attenuated vaccines

INTRODUCTION

Several species of the protozoan genus *Leishmania* (*L*) causes a group of parasitic diseases called Leishmaniasis which generates different clinical symptoms from cutaneous (CL) to visceral leishmaniasis (VL). People living in Latin America, the Middle East, parts of Africa, Asia, and India have been affected by VL (also named Kala azar) which is a very deadly disease caused mainly by *L. (d) infantum*, *L. (d) donovani*, and *L. (d) chagasi* species. Kala azar causes a clinical syndrome identified by repetitive fever, anemia, hepatosplenomegaly, and a wasting disease accompanied with muscular atrophy and finally leads to death after all the sufferings. Sand flies that have already bitten infected dogs or humans transfer parasites to other humans through their bites. These *Leishmania* parasites have numerous survival strategies among which the intracellular replication is the most famous one and prevents the parasites from direct contact to the immune system by the surrounding host cells.

A Th1 type cytokine milieu causes the parasite load to clear while a Th2 type leads to the host's susceptibility. Th1 cytokines can trigger macrophages, which are the major cells to destroy *Leishmania* parasites. To clear intracellular parasites, Th2 cells do not suffice since they induce a humoral response which has little or no effect on the parasites. Nowadays, controlling the disease depends mainly on chemotherapy as prophylactic or therapeutic vaccines are unavailable. VL chemotherapies have certain disadvantages such as the lengthy treatment time, costly drugs, and teratogenic effects. The reason for concern about resistance emergence is the long half-life of the chemotherapeutics (1–3).

The complex life cycle of *Leishmania* parasites, which consists of stages in animal or human and the sand fly vector, makes vaccine development more challenging (**Figure 1A**). An ideal antileishmanial vaccine should be able to solve current problems and limitations of other existing vaccines. As shown in **Figure 1B**, it should be safe, stable, reproducible, less risky, easily administered, stored and delivered, not reversible to infectious state, and able to induce long-term immunological memory and humoral and cellular responses.

In CL form of disease, the life-long protection is generated against the same disease and this is the fact that promises the feasibility of a vaccine. Deliberate infection with parasites at hidden body sites where scars ensue is a method that has been exploited in the leishmanization (LZ) practices of the last century (4). Nations, particularly in the Middle East, have successfully used the strategy for mass prevention of CL, but it need to improve due to persistence of monthly adverse effects and local lesions in 2–3% of cases (5).

In the late 1930s, researchers in Brazil showed that killed parasites were efficient when used as therapeutic as well as prophylactic; afterward first generation vaccines were produced from the whole killed *Leishmania* parasites (6). For many years, these vaccines

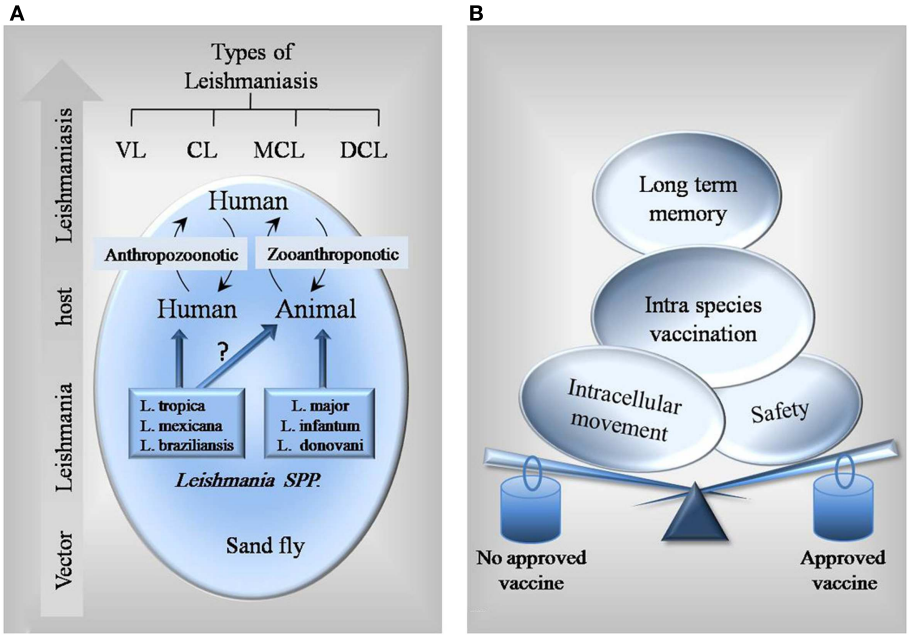


FIGURE 1 | Schematic figure of *Leishmania* interplay and different factors to consider in vaccine design. (A) Most of *Leishmania* parasites such as *L. infantum* and *L. donovani* are known to have both human and animal hosts, so preventive vaccines could be designed for both. However, there is no identified reservoir animal host for some species like *L. tropica*. For these species, specific vaccines for human are needed. **(B)** Balance between different factors leads a vaccine to get approved.

were tested either alone or combined with different adjuvants. So far, killed parasites had no enough efficacy as a potent vaccine to prevent disease, although they have demonstrated well-tolerated safety profiles (7).

First generation vaccines produced from VL *Leishmania* species have had no chance to be tested in clinical trials, since most vaccine studies have concentrated on CL. What have been included for the progression of *Leishmania* second generation vaccines are recombinant proteins, poly-proteins, DNA vaccines, and combinations thereof. In experimental infection systems, not only defined single molecules, but also multi-component vaccines have shown protection against VL. Coler et al. worked on LEISH-F1 + MPL-SE, which consisted of three recombinant *Leishmania* poly-protein (TSA–LmSTI1–LeIF), in association with monophosphoryl lipid and squalene as adjuvants (MPL-SE) (8). The synthetic RAPSODI[1] and two other DNA vaccines are in preclinical trials in Europe; one is being developed based on a viral vector by Paul Kaye (York University, UK) and another, LEISHDNAVAX[2], by Mologen (Berlin, Germany) using a new technology named minimalistic immunogenically defined gene expression (MIDGE) to deliver selected *Leishmania* antigens; the latter can be used either solely or accompaniment to a synthetic adjuvant – double stem loop immunomodulator (dSLIM).

It is believed that if a candidate vaccine could stimulate immune system more similar to the natural disease, we will have a more efficient immune response. As the success of smallpox, measles, mumps, and rubella vaccines indicate that live attenuated vaccines are the touchstone for protection against their specific causing pathogen. As shown in **Figure 2**, different approaches were used based on whole parasite vaccine ranging from live active *Leishmania* vaccine (LZ) to live non-pathogenic vaccines.

Some attenuated strains were also developed through different approaches such as physical, chemical, and genetically attenuation. Much interest has been arisen in the development of genetically attenuated parasite vaccines due to the knowledge obtained in potential parasite virulence factors and the increased understanding of the antigens participating in immunity acquisition. Targeting and deleting genes that encode virulence factor genes essential for intracellular survival is the major general approach toward genetic attenuation of *Leishmania* parasites. Recently, there are few successful reports about live attenuated *Plasmodium* through genetical modification that can elicit long-lasting memory protection by producing antibodies and cellular immune responses (9). Interestingly, in recent human clinical trial using *Plasmodium falciparum* genetically attenuated parasites (*Pf*GAP) as vaccine on volunteers showed the first in human proof of concept of this strategy that could inhibit the expansion of disease by decreasing the sporozoites (10).

Using BCG as a vaccine against *Mycobacterium tuberculosis* infection is a method which is comparable with utilizing non-pathogenic *Leishmania* species, such as a lizard parasite *L. tarentolae*, to develop live non-pathogenic parasites as VL vaccines. Although *L. tarentolae* can infect mammalian cells and change to amastigotes, it does not cause any disease or clinical symptoms

[1]http://www.fp7-rapsodi.eu/
[2]http://www.leishdnavax.org/

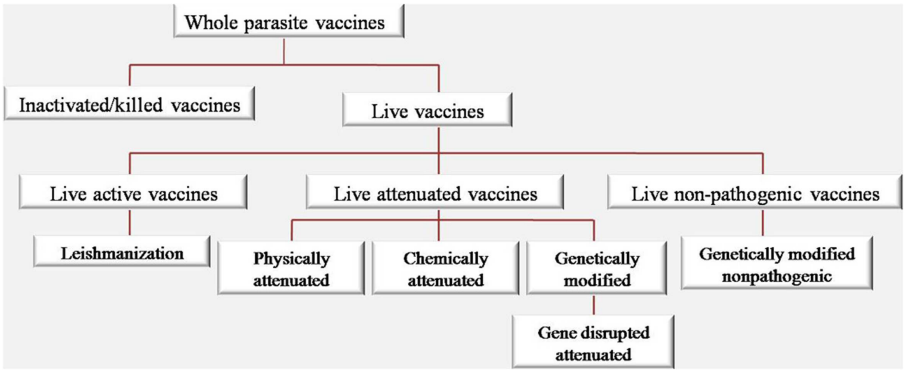


FIGURE 2 | Categorization of vaccine types based on whole *Leishmania* parasite.

in either mouse or hamster models (11, 12). Furthermore, due to general feasibility of human vaccination with live *Salmonella* and *Lactococcus* expressing exogenous antigens, they could serve another means to develop vaccine against leishmaniasis.

In this review, we have limited our scope to all types of live vaccinations against leishmaniasis and have considered them as vaccine candidates against leishmaniasis.

LEISHMANIZATION (LIVE ACTIVE VACCINES)

In the past, mothers used to expose their children's arms to be bitten by sand flies because they knew by experience that this would protect them from the severe disease in future. LZ was accepted in Israel and Russia after a method for axenic culture of the parasites was established (13). Using LZ was stopped because of HIV spreading, the use of immunosuppressive drugs, ethical reasons, uncontrolled permanent skin lesions, parasite persistence, and the inoculum quality control problems. The only usage of LZ at the present time is found in one of the endemic country, Uzbekistan, which is licensed and in Iran its efficacy is in human trials. Scientists are trying to improve the safety of this practice because it is the only way against *Leishmania* that has proved efficient in humans. The severity of primary lesions is reduced and wound healing is accelerated by including killed parasites in the inoculums and using adjuvants that improve quick immune responses (14, 15).

LIVE ATTENUATED VACCINES

Different methods such as physical attenuation: long-term vitro cultures (16), temperature sensitivity (17), γ-attenuation (18), and chemical attenuation: chemical mutagenesis (19), and parasite culture under drug pressure (20) were used to develop attenuated strains.

Instead, using a targeted gene disruption strategy can lead to a genetic alteration of the *Leishmania* genome that could help identifying essential genes for survival and/or virulence (21–27) (**Table 1**). Generally speaking, live attenuated organisms are quite acceptable for vaccination because, first, such vaccines render native antigen into cells and improve activation of antigen-presenting cells at the same time by imitating the natural course of infection, which will lead to an optimal polarization of $CD4^+$T cells (28); second, the memory repertoire of the immune system is increased since a collection of complete antigens is delivered (in comparison with subunit-defined vaccines); and third, they assure antigen persistency by generating prolonged sub-clinical infection. Then, generation of antigen-specific effector and memory cells which react soon after infection may be allowed (29). Substantial protection in murine models against challenge has been conferred by attenuated strains, but potential for reversion is possible for ever, which makes them inappropriate for use in human vaccination. Actually, risk of subsequent reactivation, especially in HIV/*Leishmania* co-infection, is raised by the persistence of asymptomatic *Leishmania* infections. In addition, a loss of effectiveness for protective immunity can be resulted from physical and chemical attenuation, either because a sub-clinical infection cannot be formed by such strains or because they do not express critical antigen epitopes anymore (30). Although the experimental results have been promising so far, there are still some safety points that need to be considered in relation to the use of genetically attenuated parasites as vaccines. Prolonged immunity after re-infection induces live attenuated vaccines through maintaining a low level asymptomatic infection. Since the persistence of antigen is essential to generate effective memory responses to *Leishmania*, the establishment of sub-clinical infection is considered quite valuable. Patients who are immunocompromised (e.g., after HIV infection) have shown reactivation of *Leishmania*. This is the reason why it is necessary that the safety of attenuated parasites that cause a sub-clinical infection should be carefully investigated.

LIVE PHYSICALLY ATTENUATED VACCINES

It was shown by Mitchell et al. that long-term cultured promastigotes of *L. major* and *L. tropica* isolates could not cause lesions after cutaneous injection to mice (16). One year later, the effect of long-term cultivation of *L. donovani* promastigotes on cultured mouse and hamster macrophages *in vitro* was evaluated by Nolan et al. In a period of 48 days, the number of amastigotes derived from long-term promastigote cultures decreased only slightly in mice but rapidly in hamsters (46). In another experiment, 8 weeks after infection, long-term cultured *L. amazonensis* promastigotes induced smaller lesions, produced higher IFN-γ, and made smaller parasite load compared to the short-term cultured counterparts. Macrophages infected by long-term cultured parasites expressed

Table 1 | Live attenuated vaccines against leishmaniasis.

Attenuated vaccine form	Species	Animal model	Result	Reference
PHYSICALLY ATTENUATED				
Long-term cultured	*L. major*	C57BL/6 and	C57BL/: completely resistant; BALB/c: partially	(16)
	L. tropica	BALB/c	protection, persistent low-grade cutaneous disease	
	L. major	BALB/c	Protection	(31)
	L. chagasi	BALB/c	No protection	(30)
	L. amazonensis	C57BL/6	Smaller lesions, ↑ IFN-γ, ↓ parasite load	(32)
Temperature sensitivity	*L. braziliensis*	BALB/c	Protection	(17)
Radio-attenuated	*L. major*	CBA	Resistance to subsequent infection with *L. mexicana*	(33)
Gamma irradiation	*L. major*	CBA and BALB/c	Protection against homologs and heterologous challenge	(18)
CHEMICALLY ATTENUATED				
With *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine	Avirulent *lpg*$^{-}$ deficient *L. major*	BALB/c	↓ Lesion size, resistance to a subsequent challenge	(19)
Culturing *in vitro* under gentamicin pressure	*L. mexicana* and *L. major*	BALB/c	No lesions, Th1-like responses ↓ Th2 responses, modulate the host immune response Significant protection	(20, 34, 35)
Culturing *in vitro* under gentamicin pressure	*L. infantum*	Dogs	No clinicopathological abnormalities ↑ IFN-γ, ↓ IL-10, ↑ IgG2 ↑ CD4+ and CD8+ T cells	(36–38)
GENETICALLY ATTENUATED				
dhfr-ts Null mutant	*L. major*	BALB/c	Protective	(21)
Cysteine proteinase-deficient mutant	*L. mexicana*	BALB/c, C57BL/6, CBA/Ca	Immune response modulation, Th1 response	(24)
dhfr-ts Null mutant	*L. major*	Monkeys	No protection	(39)
lpg2-	*L. major*	BALB/c	Protection, no strong Th1 response	(26)
Cysteine proteinase-deficient mutants	*L. mexicana*	Hamsters	Delayed disease onset ↓ Smaller lesions ↓ Parasite burden, ↓ IL-10 and TGF-beta, and protection	(40)
LiSIR2(±) mutant	*L. infantum*	BALB/c	↑ IFN-γ/IL-10 ratio, ↑ NO, protection	(27)
Phosphomannomutase-deficient mutant	*L. major*	BALB/c	↓ IL-10 and IL-13, ↑ CD44hi T cell recruitment Protection	(41)
LdCen1($^{-/-}$) mutant	*L. donovani*	BALB/c SCID hamsters	↑ IFN-γ, IL-2, TNF, ↑ IgG2a, ↑ IFN-γ/IL-10 ratio, ↑ NO, Th1 response, long-lasting protection in hamsters	(42)
HSP70-II null mutant	*L. infantum*	BALB/c	↑ NO, type 1 responses	(43)
Ldp27($^{-/-}$) mutant	*L. donovani*	BALB/c	Long-term protection	(44)
cLdCen($^{-/-}$) mutant	*L. donovani*	Dogs	↑ Type 1, ↓ Type 2 ↑ Immunogenicity	(45)

high level of chemokine CXCL10 mRNA, which might activate these cells to kill the parasites (32). Nevertheless, there are several similar trials which led to ineffectiveness, such as long-term *in vitro* culture of *L. chagasi* that did not create protective immunity (30). Using temperature-sensitive avirulent parasite clones, the immunized susceptible BALB/c mice were successfully protected against *L. braziliensis* (17). Radio-attenuation, first introduced in 1974 by Lemma et al., is another physical approach for preparation of *Leishmania* vaccine (47). The resistance of CBA mice to subsequent infection with *L. mexicana* is highly increased by administration of radio-attenuated *L. major* vaccines (33). In another experiment, gamma irradiation of *L. major* elicited a high degree of protection against homologs and heterologous challenge in CBA and BALB/c mice (18). Although most of these methods showed promising protective effects, they were not further used in research studies of vaccination against *Leishmania* species, due to safety issues regarding incomplete inactivation and reversion of infectivity (**Table 2**).

Table 2 | Properties of different types of live vaccines based on whole organisms.

Type of live vaccines	Benefits	Concerns
Leishmanization Live non-attenuated vaccines	Life-long protection Almost successful and immunity (48, 50)	No safety (48) and high risk (49) Exacerbate the disease, reversion to virulence, large persistent lesions, psoriasis, and immunosuppression Not reproducible (48), no efficacy, and no standardization and quality control (48, 50) Risk of HIV transmission
Physically attenuated	Cheaper	No safety, high risk, incomplete attenuation, no efficacy, not reproducible, non-specific attenuation (51), and reversion to virulence (51) Not acceptable for humans (50), risk of DNA damage
Chemically attenuated	Cheaper	No safety, high risk, incomplete attenuation, no efficacy, not reproducible, risk of random mutations, non-specific attenuation (51), and reversion to virulence (51)
Genetically attenuated	Safer, more stable (48) Natural course of infection (50)	Reversion to virulence Presence of antibiotic resistance genes (52); storage and delivery
Non-pathogenic organism	Safer (52), cross-reactivity between species (48), induce both humoral and cellular response (48) Lower risk of reversion to the virulent phenotype, highly immunogenic Natural course of infection For some easy administration	Not appealing prospect (48) Possible reversion to virulence or reactivation (52) Presence of antibiotic resistance genes (52) Storage and delivery

LIVE CHEMICALLY ATTENUATED VACCINES

To immunize susceptible BALB/c mice against challenge with virulent *L. major*, Kimsey et al. used an avirulent clone of *L. major* which was prepared after several *in vitro* treatments of a virulent population of *L. major* with the mutagen, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), and could control lesion size in the challenge mice model (19). It has been shown that an avirulent lipophosphoglycan-deficient *L. major* clone is able to elicit resistance to a subsequent challenge with virulent *L. major* while it is unable to produce cutaneous lesions in susceptible BALB/c mice (19). Similarly, in another experiment, avirulent lipophosphoglycan-deficient *L. donovani* parasites could not generate visceral infection in hamster model after inoculation through the intra cardiac route, contrary to virulent *L. donovani* (53). Different species of *Leishmania* have been attenuated by culturing *in vitro* under gentamicin pressure successfully such as *L. mexicana*, *L. major*, *L. infantum*, and *L. donovani*. While wild-type (WT) parasites survived and multiplied, the attenuated strains were able to invade but they neither could survive within bone marrow-derived macrophages *in vitro* nor induce cutaneous lesions in BALB/c mice after about 12 weeks. High level of protection was induced in mice against challenge with WT parasites by both attenuated lines of *L. mexicana* and *L. major* (20). This was accompanied by a $CD4^{+}$Th1-like response in BALB/c mice that was shown by the cytokine profile of their WT *L. mexicana* promastigotes-stimulated splenocytes (34). Growth of the WT parasites was excessively controlled in experiments wherein mice were simultaneously inoculated (either at the same site or on separate sites) with attenuated and WT parasites, showing that the attenuated parasites have a possible therapeutic role. Comparing dogs infected with either WT *L. infantum* or gentamicin-attenuated *L. infantum* H-line, no pathological abnormalities were observed in the latter group, which induced significantly higher IFN-γ and lower IL-10 levels with the highest levels of IgG2 subclass in their sera (37). Also, proliferation of mononuclear cells is associated with cellular immunity in immunized dogs (38). However, in addition to the difficulty of large-scale production of these physically attenuated vaccines and their delivery to the field in appropriate conditions, the major drawback is their loss of effectiveness for protective immunity due to their inability to form sub-clinical infection and express critical antigen epitopes (30) (**Table 2**).

LIVE GENETICALLY ATTENUATED VACCINES

Development of transfection technology has acted as a powerful reverse molecular genetics tool for genetic modifications in the last two decades. Gene delivery into such unicellular pathogens as *Leishmania* has created a great revolution in making genetically defined vaccines through knocking out/in certain genes. DNA delivery by physical methods is a very efficient and easy system; DNA fragments are best transferred into parasites nuclei by transfection through electroporation (54). A linearized construct containing antibiotic resistant genes should be integrated into the genome through homologous recombination (HR) to remove a gene. This allows a DNA sequence transfer into the locus of interest in the *Leishmania* genome using two flanking sequences in both sides of the gene (54).

To generate an absolute knockout, the *Leishmania* parasite needs a second construct to bear another antibiotic resistant gene to replace the second gene alleles. The cell phenotype is altered by this manipulation and new parasite features are naturally

transferred to the next generations through inheritance. Controlling the gene in its new genome location is the most crucial concern in gene targeting because it may affect the normal gene functionality in both sides of the target. Therefore, gene entrance location is very important and should be confirmed by molecular genetics methods although *Leishmania* genome is relatively easy to manipulate. Phenotypic changes (e.g., morphology, growth, infectivity) of the manipulated parasite after each transfection are other critical issues that need to be studied.

In this direction, one of the first experiments to vaccinate mice against challenge with virulent *L. major* was done by Titus et al. (21) using *dhfr-ts* null mutant of *L. major* obtained by gene targeting. Although it could not produce protective immunity in primates and needed further improvement for vaccine application (39), it could elicit considerable resistance phenotype after BALB/c mice challenge with virulent *L. major* (21). *L. mexicana* mutants lacking cysteine proteinase genes generated by targeted gene disruption were tested on murine and hamster models in another attempt and could induce delayed disease onset, smaller lesions, and lower parasite burden in mice and hamsters (24, 40). Thus, the idea of the feasibility of using genetically attenuated live *Leishmania* to achieve protective immunity was supported by such findings. Uzonna et al. showed that highly susceptible mice could be protected against virulent challenge without inducing a strong Th1 response when vaccinated with phosphoglycan-deficient *L. major* (26). A much less capacity compared to the WT parasites was shown in *L. donovani* BT1 null mutant for inducing infection in mice, and those susceptible to infection against *L. donovani* challenge attained protective immunity (25). Silvestre et al. showed that SIR2-deficient (silent information regulatory 2) *L. infantum* induced a clear IFN-γ/IL-10 pattern that is associated with protection patterns (27). In another study, susceptible BALB/c mice showed protection against infection when vaccinated with avirulent *L. major* phosphomannomutase-deficient parasites (41). Kedzierski et al. concluded that the factors that play essential parts in eliciting protection against *Leishmania* are increase in the number of T cells, their rapid recruitment to lymph nodes upon infection, and lower production of IL-13 and IL-10 (which leads to high IFN-γ/IL-10 ratio). It was shown in 2009 that live attenuated *L. donovani* parasites by gene disruption of centrin gene (LdCen1$^{-/-}$) could be live, safe, and induce protection in susceptible BALB/c mice, immunocompromised severe combined immunodeficiency (SCID) mice and hamsters. Infection with *L. braziliensis*, which causes mucocutaneous leishmaniasis, could be prevented if mice were immunized with LdCen1$^{-/-}$ (42). It was shown by Fiuza et al. that strong antibody production, Type 1 polarization, and Type 2 inhibition could be induced by LdCen$^{-/-}$ vaccine in dogs, as an important reservoir host (45). Dey et al. have shown that *L. donovani* mutant of amastigote-specific protein p27 knockout (Ldp27$^{-/-}$) as live attenuated parasites are safe, induce protective immunity, and can provide protection against homologous and heterologous *Leishmania* species (44). Carrion et al. believe that the ability of a safe genetically modified *L. infantum* mutant, which lacks both HSP70-II alleles (ΔHSP70-II), provide protection against *L. major* infection in BALB/c and can lead to the production of high levels of NO, type 1 immune responses, and IgG subclass analyses in mice (43). However, there are some limitations for their extensive use such as safety constraints due to reversion to virulent form especially in immunosuppressed individuals and manufacturing concerns.

LIVE NON-PATHOGENIC VACCINES

Utilization of non-pathogenic species as *Salmonella enteric*, *Lactococcus lactis*, and *L. tarentolae* to develop live attenuated parasites as VL vaccines is another approach. This approach has shown enhanced antigen presentation and potent Th1 response similar to BCG, a successful vaccine against *M. tuberculosis* infection (**Table 3**). These methods can be further refined through the use of their recombinants expressing antigens of virulent *Leishmania* spp. In general, the most promising strategic alternative against VL can be claimed to be the use of live, non-pathogenic/genetically engineered strains of these species.

SALMONELLA ENTERICA

Salmonella (*S*) are intracellular pathogens that upon entrance to human macrophages induce a viscerotropic immune response similar to *Leishmania*. Development of live *Salmonella* vaccines as a method for delivering heterologous antigens was discussed for the first time in 1987 (66). The important advantage of using attenuated *Salmonella* for vaccination against VL is their low production cost, storage at room temperature, and their oral, needle-free application if rehydrated. Since orally administered live attenuated *Salmonella* spp. that express heterologous antigens are safe and highly immunogenic, they are promising candidates; they can elicit prolonged, protective, systemic, and mucosal immune responses against the heterologous pathogen (67).

In vivo inducible promoters and optimized expression systems are used to construct novel attenuated *Salmonella* vaccines that deliver antigens and show a host protective effect in small rodent models of VL. Live *Salmonella* needs more studies to promote their further application.

Furthermore, for delivery and expression of vaccine antigens in the host, several attenuated lines of *S. typhimurium* have been generated. For more safety, more than one attenuating mutation can be incorporated in a vaccine. Several derived antigens (target carbohydrate, protein) or epitopes from different pathogens, viruses, bacteria, and eukaryotic parasites are expressed by combined *Salmonella* vaccines in the form of capsules, fimbria, or flagellum, either within or on the surface of the cell (68). A very significant resistance was developed against a *L. major* challenge infection by the mice that had been orally immunized with gp63-transformed *S. typhimurium* (55, 56). *S. typhimurium* derivatives (GIDMIF, GIDIL2, GIDIFN, and GIDTNF) expressed cytokines *in vitro* under anaerobic conditions. They were stably colonized in orally immunized BALB/c mice more than 14 days and showed protective effect which correlated with the induction of inducible nitric oxide synthase (57).

Lange et al. showed that production of IFN-γ could induce protection against *L. major* infection in susceptible BALB/c mice and were enhanced as a result of using LACK antigens in DNA-*Salmonella* primer-booster vaccination compared to that with the DNA alone (59). In a recent study, Schroeder et al. identified two novel candidate vaccine antigens (LinJ08.1190 and LinJ23.0410)

Table 3 | Live non-pathogenic vaccines against leishmaniasis.

Vaccine form	Species	Animal model	Result	Reference
SALMONELLA ENTERICA				
S. typhimurium aroA-+gp63 (SL3261-gp63)	*L. major*	CBA	↑ T helper 1 protection	(55)
S. typhimurium aroA- aroD-+ +gp63 (GID101)	*L. major*	BALB/c	↑ Th1 subset of CD4+ T cells protection	(56)
S. typhimurium aroA- aroD- (BRD509), +MIF, IL-2, IFN-γ, or TNF-alpha (GIDMIF, GIDIL2, GIDIFN, and GIDTNF)	*L. major*	BALB/c	Limited lesion development ↑ Nitric oxide synthase (iNOS) ↓ Parasite loads, protection	(57)
S. typhi delta aroC, delta aroD (CVD 908), ++gp63 (SL3261-gp63)	*L. m. mexicana*	F1 (BALB/ cXC57BL/6)	T cell-mediated response Protection or resolution of the infection	(58)
DNA-*Salmonella*+ +LACK antigens primer-booster	*L. major*	BALB/c	↑ Th1, ↑ IFN-γ, ↑ IgG2a Protection	(59)
S. typhimurium SL3261+ +LinJ08.1190 and LinJ23.0410	*L. major* and *L. donovani*	BALB/c	↑ Resistance against visceral leishmaniasis	(60)
LACTOCOCCUS LACTIS				
A2-expressing *Lactococcus lactis*	*L. donovani*	BALB/c	↑ Liver parasitemia ↑ Antibody titers, critical influence on the immune response	(61)
Lactococcus lactis co-expressing LACK and IL-12	*L. major*	BALB/c	↓ Parasite burden ↑ Th1 response Partially protection Delay in footpad swelling	(62) (63)
LEISHMANIA TARENTOLAE				
L. tarentolae	*L. donovani*	BALB/c	↑ *Leishmania*-specific TH1 immune response Protection	(12)
Recombinant *L. tarentolae* expressing A2 gene	*L. infantum*	BALB/c	Intraperitoneal administration: ↑ IFN-γ, ↓ IL-5, ↑ Th1, protection	(64)
Recombinant *L. tarentolae* expressing A2–CPA–CPB^{-CTE}	*L. infantum*	BALB/c	↑ IFN-γ, ↓ IL-10, ↑ NO ↑ IFN-γ/IL-10 ratio ↓ Parasite burden, protection	(65)

by reverse vaccinology and utilized them in the construction of live *Salmonella* carriers against VL, which reduced visceralization considerably and increased resistancy against *L. donovani* infection in susceptible BALB/c mice (60).

LACTOCOCCUS LACTIS

Lactococcus lactis is a Gram-positive, non-pathogenic, non-colonizing lactic acid bacterium (69), which is industrially important and is frequently used in the preparation of fermented foods and dairies; FDA has given it a generally recognized as safe (GRAS) status [(70); aminopeptidase enzyme preparation derived from *L. lactis* (21CFR184.1985)].

It has been used as a live bacterial delivery vector for more than 10 years (71) and scientists are being encouraged to use it as a live vaccine against leishmaniasis. A2-expressing *L. lactis* live vaccines have been generated and evaluated by Yam et al. against *L. donovani* in BALB/c mice. This A2 anchored to the cell wall has a critical influence on the immune response; this subcellular location of antigen expression causes the highest reduction in liver parasitemia, induces the highest level of antigen-specific antibody titers which is seen at both low- and high-dose *L. donovani* parasite challenges (61). In another study of this group it was shown, using LACK- and IL-12-expressing *L. lactis*, that subcutaneous immunization against *L. major* infection delays footpad swelling, indicating the necessity for co-administration of *L. lactis*/sec IL-12 (secreting IL-12) as a Th1-inducing adjuvant (63). Again in another study, the same group showed that if live *L. lactis* secreting both LACK and IL-12 was used, oral immunization was the only regimen that could protect BALB/c mice partially against *L. major* infection (62). The *L. lactis* line generated in these studies provides an attractive cornerstone for further research on live-based vaccines against leishmaniasis and other pathogens.

LEISHMANIA TARENTOLAE

Recently, the use of a non-pathogenic *Leishmania* vector (*L. tarentolae*) was suggested by Breton et al. (12) as a vaccine candidate against leishmaniasis which is known as non-pathogenic for human since it is not able to generate any manifestation of human leishmaniasis. Although this parasite is non-pathogenic in either

mouse or hamster models because it lacks any clinical symptoms, it can infect mammalian cells and transform into amastigotes (72). Genome sequence analyses have revealed that this parasite is syntonic to the three sequenced pathogenic *Leishmania* species (*L. major*, *L. braziliensis*, and *L. infantum*) and that more than 90% of the approximately 8200 genes are shared by all the species. Nevertheless, some of the essential genes that are relevant to pathogenicity in pathogenic strains or expressed in amastigote form are absent in *L. tarentolae* or were in variable copy number. This supports the idea that some of these genes are possible to be associated with reduction of pathogenic capacity in *L. tarentolae* and make it an intracellular parasite and its diminished pathogenic potential to humans. As an example, the amastin family, especially the delta group as just two copy number in *L. tarentolae* while high copy numbers (12–25) are found in the pathogenic species (73). Why *L. tarentolae* cannot replicate efficiently in mammalian macrophages can be explained by the absence of these proteins. It has been shown in experimental vaccine trials that a single intra peritoneal immunization of *L. tarentolae* elicited a protective immune response against *L. donovani* in susceptible BALB/c mice; it was concluded that it was a result of an enhanced antigen presentation and potent Th1 immune response (12). Since *L. tarentolae* is a safe vector for use as a vaccine, it can be more effective anti-*Leishmania* vaccine by genetic manipulation in order to induce transgenic *L. tarentolae* which expresses certain immunodominant *Leishmania* antigens.

Effort has also been made to use *L. tarentolae* as a specific deliver and expression system for *Leishmania* antigens in host. The *L. donovani* A2 antigen was expressed in *L. tarentolae*, which normally lacks this protein (74) and used as a vaccine strain in an experimental mouse model. The susceptible mice were protected against *L. infantum* infection through vaccination following high levels of IFN-γ were produced (64). In addition, *L. tarentolae* can be used as a promising live vaccine vector against intracellular pathogens. This idea was examined for the first time in an experiment using a recombinant *L. tarentolae* expressing HIV-1 Gag protein as a candidate HIV-1 vaccine. It was shown that the vaccine induces a strong cell-mediated immunity in BALB/c mice and decreases HIV-1 replication in an *ex vivo* condition (75). Also, a novel live vaccine using recombinant *L. tarentolae* expressing E7 protein for the protection of mice against HPV-associated tumors was produced and evaluated (76). It is worth mentioning that this vaccine showed the best protection and minimum tumor size among all other groups against TC-1-induced tumors (76).

Our team produced a recombinant *L. tarentolae* expressing the A2–CPA–CPB^{-CTE} tri-gene fusion that are three important vaccine candidate antigens of *L. infantum*, as a new live vaccination strategy against visceral form of leishmaniasis in two-modalities, namely DNA/live and live/live vaccination in BALB/c mice. We demonstrated how prime-boost (DNA/live) strategies using recombinant *L. tarentolae*-based vaccines elicited promising immunization against a high-dose virulent *L. infantum* challenge (65). We also tested live/live *L. tarentolae*-A2–CPA–CPB^{-CTE} prime-boost vaccination regime in hamsters and showed that it represented an appropriate animal model in the discovery of potential antigens that could be used in the control of canine VL (unpublished data). The parasite loads in both visceral organs were controlled in the vaccinated hamsters reaching a negligible level by day 56 post challenge, demonstrating its strong vaccine potential. Five weeks after infection by *L. infantum*, hamsters that had received the live vaccine produced higher levels of anti-*L. infantum* lysate antibodies than those injected with PBS control.

In another attempt, we tested the efficacy of a novel combination of established protective parasite antigens expressed by *L. tarentolae* together with saliva antigens as a vaccine strategy against *L. major* infection. Different DNA/live and live/live prime-boost vaccination modalities with live recombinant *L. tarentolae* stably expressing cysteine proteinases (type I and II, CPA/CPB) and PpSP15, an immunogenic salivary protein from *Phlebotomus papatasi*, a natural vector of *L. major*, were tested in both susceptible BALB/c and resistant C57BL/6 mice. In both strains of mice, the strongest protective effect was observed when priming with PpSP15DNA and boosting with PpSP15 DNA and live recombinant *L. tarentolae* stably expressing cysteine proteinase genes (accepted in PLoS NTD, 2014).

Regarding vaccine development in dogs, with lack of enough knowledge about canine leishmaniasis and canine immunity, it is almost impossible to predict the results obtained from the mouse and hamster models, if vaccine candidates can work in dogs. Therefore, it is essential to do more studies on dogs for both new vaccine candidates and immune response analyses. Whether or not protection will be achieved, results of such tests would be valuable for the advancement of knowledge about canine leishmaniasis and giving a guided direction to future protection strategies. It is worth to mention that our group is testing the genetically knock in *L. tarentolae* expressing the A2–CPA–CPB^{-CTE} tri-gene fusion as a live vaccination strategy with different modalities in outbreed dogs.

CONCLUSION

Unlike most other pathogens, *Leishmania* never clears fully by immune system and we do not need sterile immunity. The important issue for maintenance of immunity is believed to be the presence of small number of live parasite in the host. Live replicating parasites or just persistent antigens are believed to be important for the maintenance of effector memory like T cells but not for central memory T cells. It has been reported that the quality of memory cells in the presence and absence of live parasite are different in CL (77). In the case of VL, persistence of parasite antigen is important for generating antigen-specific effector T cells, although more depth studies are required to be analyzed in the case of non-pathogenic and/or genetically attenuated *Leishmania* parasite (44). During *Leishmania* infection, we need a methodical understanding of how the immunological memory is generated and maintained, what the sustained long-term protective immune responses are, and through what mechanisms vaccines stimulate protective immunity. An ideal anti-*Leishmania* vaccine must maintain constant turnover of *Leishmania*-specific memory cells in vaccinated host, otherwise repeated booster injections would be required (78).

Immune response to *Leishmania* is very complicated and for wisely designing vaccines we need to know which T cell determinants act as IFN-γ inducer (CD8+ or CD4+ T cell) and are

essential for long-term immunity. Long-lasting protective immunity induced by vaccination is a pragmatic goal for control of parasitic infections. In LZ, the only successful strategy that has been used to induce resistance to cutaneous leishmaniasis, after obviation of the infection, individuals are resistant to re-infection. It is now clear that in mice infected with WT parasites, heterogeneous memory CD4+ T cell pool contain two subsets, specified by their expression of the LN-homing molecule CD62L, one of them, effector memory T cells, has the characteristics of effector cells (CD62Llo) and the other one, central memory T cells, act as a repository of antigen-specific T cells (CD62Lhi) and can extend upon rechallenge, differentiate into effector T cells, and refill the effector cell population (79, 80). The latter which expressed CD62L and lodged to the lymph nodes, expand early after infection with *L. major* (81). However, the first population of cells CD62Llo effector T cells could intercede resistance faster than the CD62Lhi central memory T cells (80). In other words, at providing immunity to rechallenge in leishmaniasis central memory CD4+ T cells that could be maintained without persistent parasites were less effective. This observation indicates that for immunity maintenance and providing long-term immunologic memory, persistent parasites may well be needed (82). Therefore, on this basis the idea of using live vaccine either in attenuated or non-pathogenic form is strengthened.

It is preferred that attenuating process of *Leishmania* strains for the production of live vaccine be done selectively (i.e., only in intracellular form or amastigotes); this will allow the cultivation of promastigotes in large-scale. Attenuation needs to be optimized so that the power of live parasite vaccines can be improved, but it should be noted that reversion of these parasites to the virulent form restricts their use. In other words, returning back to virulence is also probable; hence, the need for the production of new safer live vaccine vectors such as non-pathogenic *L. tarentolae* harboring immunogenic antigens that can enhance antigen presentation and elicit potent immune responses, without any risk of disease development in humans, becomes obvious. Using *L. tarentolae* as non-pathogenic vector is promised because of its safety and easy adaptation to mammalian system. Also, it has not the ability to revert to pathogenic form due to its non-pathogenic intrinsic property (11, 12). But what is certain is that *L. tarentolae* could not long survive in the mammalian cell, so it is best to think of some strategies to prolong its life there. Finally, there are still several obstacles for utilization of live non-pathogenic *Leishmania*, such as lyophilization and storage of this organism, which need special attention and serious research.

ACKNOWLEDGMENTS

We thank Amir Mizbani for critically reading and editing the manuscript. This work was supported by Pasteur Institute of Iran, Iran Ministry of Health, and National Science Foundation of Iran (grant number 91053539).

REFERENCES

1. Sundar S, Chatterjee M. Visceral leishmaniasis – current therapeutic modalities. *Indian J Med Res* (2013) **123**(3):345–52.
2. Olliaro P, Darley S, Laxminarayan R, Sundar S. Cost-effectiveness projections of single and combination therapies for visceral leishmaniasis in Bihar, India. *Trop Med Int Health* (2009) **14**:918–25. doi:10.1111/j.1365-3156.2009.02306.x
3. Olliaro P, Sundar S. Anthropometrically derived dosing and drug costing calculations for treating visceral leishmaniasis in Bihar, India. *Trop Med Int Health* (2009) **14**:88–92. doi:10.1111/j.1365-3156.2008.02195.x
4. Nadim A, Javadian E, Tahvildar-Bidruni G, Ghorbani M. Effectiveness of leishmanization in the control of cutaneous leishmaniasis. *Bull Soc Pathol Exot Filiales* (1983) **76**:377–83.
5. Hosseini SM, Hatam GR, Ardehali S. Characterization of *Leishmania* isolated from unhealed lesions caused by leishmanization. *East Mediterr Health J* (2005) **11**:240–3.
6. Noazin S, Modabber F, Khamesipour A, Smith PG, Moulton LH, Nasseri K, et al. First generation leishmaniasis vaccines: a review of field efficacy trials. *Vaccine* (2008) **26**:6759–67. doi:10.1016/j.vaccine.2008.09.085
7. Noazin S, Khamesipour A, Moulton LH, Tanner M, Nasseri K, Modabber F, et al. Efficacy of killed whole-parasite vaccines in the prevention of leishmaniasis: a meta-analysis. *Vaccine* (2009) **27**:4747–53. doi:10.1016/j.vaccine.2009.05.084
8. Coler RN, Goto Y, Bogatzki L, Raman V, Reed SG. Leish-111f, a recombinant polyprotein vaccine that protects against visceral leishmaniasis by elicitation of CD4+ T cells. *Infect Immun* (2007) **75**:4648–54. doi:10.1128/IAI.00394-07
9. Mueller A-K, Labaied M, Kappe SH, Matuschewski K. Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine. *Nature* (2005) **433**:164–7. doi:10.1038/nature03188
10. Finney OC, Keitany GJ, Smithers H, Kaushansky A, Kappe S, Wang R. Immunization with genetically attenuated *P. falciparum* parasites induces long-lived antibodies that efficiently block hepatocyte invasion by sporozoites. *Vaccine* (2014). doi:10.1016/j.vaccine.2014.02.055
11. Wilson V, Southgate B. *Lizard Leishmania. Biology of the Kinetoplastida.* San Diego, CA: Academic Press (1978). p. 244–68.
12. Breton M, Tremblay MJ, Ouellette M, Papadopoulou B. Live nonpathogenic parasitic vector as a candidate vaccine against visceral leishmaniasis. *Infect Immun* (2005) **73**:6372–82. doi:10.1128/IAI.73.10.6372-6382.2005
13. Koufman Z, Egoz N, Greenblatt CL, Handman E, Montilio B, Even-Paz Z. Observations on immunization against cutaneous leishmaniasis in Israel. *Isr J Med Sci* (1978) **14**:218–22.
14. Khamesipour A, Dowlati Y, Asilian A, Hashemi-Fesharki R, Javadi A, Noazin S, et al. Leishmanization: use of an old method for evaluation of candidate vaccines against leishmaniasis. *Vaccine* (2005) **23**:3642–8. doi:10.1016/j.vaccine.2005.02.015
15. Tabbara KS, Peters NC, Afrin F, Mendez S, Bertholet S, Belkaid Y, et al. Conditions influencing the efficacy of vaccination with live organisms against *Leishmania major* infection. *Infect Immun* (2005) **73**:4714–22. doi:10.1128/IAI.73.8.4714-4722.2005
16. Mitchell GF, Handman E, Spithill TW. Vaccination against cutaneous leishmaniasis in mice using nonpathogenic cloned promastigotes of *Leishmania major* and importance of route of injection. *Aust J Exp Biol Med Sci* (1984) **62**(Pt 2):145–53. doi:10.1038/icb.1984.14
17. Gorczynski RM. Immunization of susceptible BALB/c mice against *Leishmania braziliensis*. II. Use of temperature-sensitive avirulent clones of parasite for vaccination purposes. *Cell Immunol* (1985) **94**:11–20. doi:10.1016/0008-8749(85)90081-4
18. Rivier D, Shah R, Bovay P, Mauel J. Vaccine development against cutaneous leishmaniasis. Subcutaneous administration of radioattenuated parasites protects CBA mice against virulent *Leishmania major* challenge. *Parasite Immunol* (1993) **15**:75–84. doi:10.1111/j.1365-3024.1993.tb00587.x
19. Kimsey PB, Theodos CM, Mitchen TK, Turco SJ, Titus RG. An avirulent lipophosphoglycan-deficient *Leishmania major* clone induces CD4+ T cells which protect susceptible BALB/c mice against infection with virulent *L. major*. *Infect Immun* (1993) **61**:5205–13.
20. Daneshvar H, Coombs GH, Hagan P, Phillips RS. *Leishmania mexicana* and *Leishmania major*: attenuation of wild-type parasites and vaccination with the attenuated lines. *J Infect Dis* (2003) **187**:1662–8. doi:10.1086/374783
21. Titus RG, Gueiros-Filho FJ, De Freitas LA, Beverley SM. Development of a safe live *Leishmania* vaccine line by gene replacement. *Proc Natl Acad Sci U S A* (1995) **92**:10267–71. doi:10.1073/pnas.92.22.10267
22. Dumas C, Ouellette M, Tovar J, Cunningham ML, Fairlamb AH, Tamar S, et al. Disruption of the trypanothione reductase gene of *Leishmania* decreases its ability to survive oxidative stress in macrophages. *EMBO J* (1997) **16**:2590–8. doi:10.1093/emboj/16.10.2590
23. Hubel A, Krobitsch S, Horauf A, Clos J. *Leishmania major* Hsp100 is required chiefly in the mammalian stage of the parasite. *Mol Cell Biol* (1997) **17**:5987–95.

24. Alexander J, Coombs GH, Mottram JC. *Leishmania mexicana* cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. *J Immunol* (1998) **161**:6794–801.
25. Papadopoulou B, Roy G, Breton M, Kundig C, Dumas C, Fillion I, et al. Reduced infectivity of a *Leishmania donovani* biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. *Infect Immun* (2002) **70**:62–8. doi:10.1128/IAI.70.1.62-68.2002
26. Uzonna JE, Spath GF, Beverley SM, Scott P. Vaccination with phosphoglycan-deficient *Leishmania major* protects highly susceptible mice from virulent challenge without inducing a strong Th1 response. *J Immunol* (2004) **172**:3793–7.
27. Silvestre R, Cordeiro-Da-Silva A, Santarem N, Vergnes B, Sereno D, Ouaissi A. SIR2-deficient *Leishmania infantum* induces a defined IFN-gamma/IL-10 pattern that correlates with protection. *J Immunol* (2007) **179**:3161–70.
28. Sporri R, Reis e Sousa C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol* (2005) **6**:163–70. doi:10.1038/ni1162
29. Foulds KE, Wu CY, Seder RA. Th1 memory: implications for vaccine development. *Immunol Rev* (2006) **211**:58–66. doi:10.1111/j.0105-2896.2006.00400.x
30. Streit JA, Recker TJ, Filho FG, Beverley SM, Wilson ME. Protective immunity against the protozoan *Leishmania chagasi* is induced by subclinical cutaneous infection with virulent but not avirulent organisms. *J Immunol* (2001) **166**:1921–9.
31. Segovia M, Artero JM, Mellado E, Chance ML. Effects of long-term in vitro cultivation on the virulence of cloned lines of *Leishmania major* promastigotes. *Ann Trop Med Parasitol* (1992) **86**:347–54.
32. de Souza MC, De Assis EA, Gomes RS, Marques Da Silva Ede A, Melo MN, Fietto JL, et al. The influence of ecto-nucleotidases on *Leishmania* amazonensis infection and immune response in C57B/6 mice. *Acta Trop* (2010) **115**:262–9. doi:10.1016/j.actatropica.2010.04.007
33. Alexander J. A radioattenuated *Leishmania major* vaccine markedly increases the resistance of CBA mice to subsequent infection with *Leishmania mexicana mexicana*. *Trans R Soc Trop Med Hyg* (1982) **76**:646–9. doi:10.1016/0035-9203(82)90232-2
34. Daneshvar H, Hagan P, Phillips RS. *Leishmania mexicana* H-line attenuated under pressure of gentamicin, potentiates a Th1 response and control of cutaneous leishmaniasis in BALB/c mice. *Parasite Immunol* (2003) **25**:589–96. doi:10.1111/j.0141-9838.2004.00671.x
35. Daneshvar H, Burchmore R, Hagan P, Phillips RS. *Leishmania major* H-line attenuated under pressure of gentamicin, induces a Th1 response which protects susceptible BALB/c mice against infection with virulent *L. major*. *Parasitology* (2009) **136**:1243–50. doi:10.1017/S0031182009990679
36. Daneshvar H, Molaei MM, Afshar RM, Kamiabi H, Burchmore R, Hagan P, et al. Gentamicin-attenuated *Leishmania infantum*: a clinicopathological study in dogs. *Vet Immunol Immunopathol* (2009) **129**:28–35. doi:10.1016/j.vetimm.2008.12.002
37. Daneshvar H, Molaei M, Kamiabi H, Burchmore R, Hagan P, Stephen Phillips R. Gentamicin-attenuated *Leishmania infantum*: cellular immunity production and protection of dogs against experimental canine leishmaniasis. *Parasite Immunol* (2010) **32**:722–30. doi:10.1111/j.1365-3024.2010.01237.x
38. Daneshvar H, Sedghy F, Dabiri S, Kamiabi H, Molaei MM, Phillips S, et al. Alteration in mononuclear cell subpopulations in dogs immunized with gentamicin-attenuated *Leishmania infantum*. *Parasitology* (2012) **139**:1689–96. doi:10.1017/S0031182012001187
39. Amaral VF, Teva A, Oliveira-Neto MP, Silva AJ, Pereira MS, Cupolillo E, et al. Study of the safety, immunogenicity and efficacy of attenuated and killed *Leishmania (Leishmania) major* vaccines in a rhesus monkey (*Macaca mulatta*) model of the human disease. *Mem Inst Oswaldo Cruz* (2002) **97**:1041–8. doi:10.1590/S0074-02762002000700019
40. Saravia NG, Escorcia B, Osorio Y, Valderrama L, Brooks D, Arteaga L, et al. Pathogenicity and protective immunogenicity of cysteine proteinase-deficient mutants of *Leishmania mexicana* in non-murine models. *Vaccine* (2006) **24**:4247–59. doi:10.1016/j.vaccine.2005.05.045
41. Kedzierski L, Curtis JM, Doherty PC, Handman E, Kedzierska K. Decreased IL-10 and IL-13 production and increased CD44hi T cell recruitment contribute to *Leishmania major* immunity induced by non-persistent parasites. *Eur J Immunol* (2008) **38**:3090–100. doi:10.1002/eji.200838423
42. Selvapandiyan A, Dey R, Nylen S, Duncan R, Sacks D, Nakhasi HL. Intracellular replication-deficient *Leishmania donovani* induces long lasting protective immunity against visceral leishmaniasis. *J Immunol* (2009) **183**:1813–20. doi:10.4049/jimmunol.0900276
43. Carrion J, Folgueira C, Soto M, Fresno M, Requena JM. *Leishmania infantum* HSP70-II null mutant as candidate vaccine against leishmaniasis: a preliminary evaluation. *Parasit Vectors* (2011) **4**:150. doi:10.1186/1756-3305-4-150
44. Dey R, Dagur PK, Selvapandiyan A, Mccoy JP, Salotra P, Duncan R, et al. Live attenuated *Leishmania donovani* p27 gene knockout parasites are nonpathogenic and elicit long-term protective immunity in BALB/c mice. *J Immunol* (2013) **190**:2138–49. doi:10.4049/jimmunol.1202801
45. Fiuza JA, Santiago Hda C, Selvapandiyan A, Gannavaram S, Ricci ND, Bueno LL, et al. Induction of immunogenicity by live attenuated *Leishmania donovani* centrin deleted parasites in dogs. *Vaccine* (2013) **31**:1785–92. doi:10.1016/j.vaccine.2013.01.048
46. Nolan TJ, Herman R. Effects of long-term in vitro cultivation on *Leishmania donovani* promastigotes. *J Protozool* (1985) **32**:70–5. doi:10.1111/j.1550-7408.1985.tb03015.x
47. Lemma A, Cole L. *Leishmania enrietti*: radiation effects and evaluation of radioattenuated organisms for vaccination. *Exp Parasitol* (1974) **35**:161–9. doi:10.1016/0014-4894(74)90019-8
48. Mutiso JM, Macharia JC, Kiio MN, Ichagichu JM, Rikoi H, Gicheru MM. Development of *Leishmania* vaccines: predicting the future from past and present experience. *J Biomed Res* (2013) **27**:85–102. doi:10.7555/JBR.27.20120064
49. Selvapandiyan A, Duncan R, Debrabant A, Lee N, Sreenivas G, Nakhasi HL. Genetically modified live attenuated parasites as vaccines for leishmaniasis. *Indian J Med Res* (2006) **123**:455–66.
50. Evans KJ, Kedzierski L. Development of vaccines against visceral leishmaniasis. *J Trop Med* (2012) **2012**:892817. doi:10.1155/2012/892817
51. Selvapandiyan A, Dey R, Gannavaram S, Lakhal-Naouar I, Duncan R, Salotra P, et al. Immunity to visceral leishmaniasis using genetically defined live-attenuated parasites. *Re Dai Yi Xue Za Zhi* (2012) **2012**:631460. doi:10.1155/2012/631460
52. Silvestre R, Cordeiro-Da-Silva A, Ouaissi A. Live attenuated *Leishmania* vaccines: a potential strategic alternative. *Arch Immunol Ther Exp (Warsz)* (2008) **56**:123–6. doi:10.1007/s00005-008-0010-9
53. Mukhopadhyay S, Sen P, Bhattacharyya S, Majumdar S, Roy S. Immunoprophylaxis and immunotherapy against experimental visceral leishmaniasis. *Vaccine* (1999) **17**:291–300. doi:10.1016/S0264-410X(98)90017-2
54. Papadopoulou B, Dumas C. Parameters controlling the rate of gene targeting frequency in the protozoan parasite *Leishmania*. *Nucleic Acids Res* (1997) **25**:4278–86. doi:10.1093/nar/25.21.4278
55. Yang DM, Fairweather N, Button LL, Mcmaster WR, Kahl LP, Liew FY. Oral *Salmonella typhimurium* (AroA-) vaccine expressing a major leishmanial surface protein (gp63) preferentially induces T helper 1 cells and protective immunity against leishmaniasis. *J Immunol* (1990) **145**:2281–5.
56. Xu D, Mcsorley SJ, Chatfield SN, Dougan G, Liew FY. Protection against *Leishmania major* infection in genetically susceptible BALB/c mice by gp63 delivered orally in attenuated *Salmonella typhimurium* (AroA- AroD-). *Immunology* (1995) **85**:1–7.
57. Xu D, Mcsorley SJ, Tetley L, Chatfield S, Dougan G, Chan WL, et al. Protective effect on *Leishmania major* infection of migration inhibitory factor, TNF-alpha, and IFN-gamma administered orally via attenuated *Salmonella typhimurium*. *J Immunol* (1998) **160**:1285–9.
58. Gonzalez CR, Noriega FR, Huerta S, Santiago A, Vega M, Paniagua J, et al. Immunogenicity of a Salmonella typhi CVD 908 candidate vaccine strain expressing the major surface protein gp63 of *Leishmania mexicana mexicana*. *Vaccine* (1998) **16**:1043–52. doi:10.1016/S0264-410X(97)00267-3
59. Lange UG, Mastroeni P, Blackwell JM, Stober CB. DNA-salmonella enterica serovar *Typhimurium* primer-booster vaccination biases towards T helper 1 responses and enhances protection against *Leishmania major* infection in mice. *Infect Immun* (2004) **72**:4924–8. doi:10.1128/IAI.72.8.4924-4928.2004
60. Schroeder J, Brown N, Kaye P, Aebischer T. Single dose novel *Salmonella* vaccine enhances resistance against visceralizing *L. major* and *L. donovani* infection in susceptible BALB/c mice. *PLoS Negl Trop Dis* (2011) **5**:e1406. doi:10.1371/journal.pntd.0001406
61. Yam KK, Hugentobler F, Pouliot P, Stern AM, Lalande JD, Matlashewski G, et al. Generation and evaluation of A2-expressing *Lactococcus lactis* live vaccines against *Leishmania donovani* in BALB/c mice. *J Med Microbiol* (2011) **60**:1248–60. doi:10.1099/jmm.0.029959-0

62. Hugentobler F, Di Roberto RB, Gillard J, Cousineau B. Oral immunization using live *Lactococcus lactis* co-expressing LACK and IL-12 protects BALB/c mice against *Leishmania major* infection. *Vaccine* (2012) **30**:5726–32. doi:10.1016/j.vaccine.2012.07.004
63. Hugentobler F, Yam KK, Gillard J, Mahbuba R, Olivier M, Cousineau B. Immunization against *Leishmania major* infection using LACK- and IL-12-expressing *Lactococcus lactis* induces delay in footpad swelling. *PLoS One* (2012) **7**:e30945. doi:10.1371/journal.pone.0030945
64. Mizbani A, Taheri T, Zahedifard F, Taslimi Y, Azizi H, Azadmanesh K, et al. Recombinant *Leishmania tarentolae* expressing the A2 virulence gene as a novel candidate vaccine against visceral leishmaniasis. *Vaccine* (2009) **28**:53–62. doi:10.1016/j.vaccine.2009.09.114
65. Saljoughian N, Taheri T, Zahedifard F, Taslimi Y, Doustdari F, Bolhassani A, et al. Development of novel prime-boost strategies based on a tri-gene fusion recombinant *L. tarentolae* vaccine against experimental murine visceral Leishmaniasis. *PLoS Negl Trop Dis* (2013) **7**:e2174. doi:10.1371/journal.pntd.0002174
66. Dougan G, Hormaeche CE, Maskell DJ. Live oral *Salmonella* vaccines: potential use of attenuated strains as carriers of heterologous antigens to the immune system. *Parasite Immunol* (1987) **9**:151–60. doi:10.1111/j.1365-3024.1987.tb00496.x
67. Bumann D, Hueck C, Aebischer T, Meyer TF. Recombinant live Salmonella spp. for human vaccination against heterologous pathogens. *FEMS Immunol Med Micro* (2000) **27**:357–64. doi:10.1111/j.1574-695X.2000.tb01450.x
68. Hormaeche CE. Live attenuated *Salmonella* vaccines and their potential as oral combined vaccines carrying heterologous antigens. *J Immunol Methods* (1991) **142**:113–20. doi:10.1016/0022-1759(91)90298-T
69. Casalta E, Montel MC. Safety assessment of dairy microorganisms: the *Lactococcus* genus. *Int J Food Microbiol* (2008) **126**:271–3. doi:10.1016/j.ijfoodmicro.2007.08.013
70. FDA. (1995). Available from: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1985
71. Bahey-El-Din M, Gahan CG, Griffin BT. Lactococcus lactis as a cell factory for delivery of therapeutic proteins. *Curr Gene Ther* (2010) **10**:34–45. doi:10.2174/156652310790945557
72. Taylor VM, Munoz DL, Cedeno DL, Velez ID, Jones MA, Robledo SM. *Leishmania tarentolae*: utility as an in vitro model for screening of antileishmanial agents. *Exp Parasitol* (2010) **126**:471–5. doi:10.1016/j.exppara.2010.05.016
73. Raymond F, Boisvert S, Roy G, Ritt JF, Legare D, Isnard A, et al. Genome sequencing of the lizard parasite *Leishmania tarentolae* reveals loss of genes associated to the intracellular stage of human pathogenic species. *Nucleic Acids Res* (2012) **40**:1131–47. doi:10.1093/nar/gkr834
74. Azizi H, Hassani K, Taslimi Y, Najafabadi HS, Papadopoulou B, Rafati S. Searching for virulence factors in the non-pathogenic parasite to humans *Leishmania tarentolae*. *Parasitology* (2009) **136**:723–35. doi:10.1017/S0031182009005873
75. Breton M, Zhao C, Ouellette M, Tremblay MJ, Papadopoulou B. A recombinant non-pathogenic *Leishmania* vaccine expressing human immunodeficiency virus 1 (HIV-1) Gag elicits cell-mediated immunity in mice and decreases HIV-1 replication in human tonsillar tissue following exposure to HIV-1 infection. *J Gen Virol* (2007) **88**:217–25. doi:10.1099/vir.0.81995-0
76. Salehi M, Taheri T, Mohit E, Zahedifard F, Seyed N, Taslimi Y, et al. Recombinant *Leishmania tarentolae* encoding the HPV type 16 E7 gene in tumor mice model. *Immunotherapy* (2012) **4**:1107–20. doi:10.2217/imt.12.110
77. Okwor I, Uzonna J. Persistent parasites and immunologic memory in cutaneous leishmaniasis: implications for vaccine designs and vaccination strategies. *Immunol Res* (2008) **41**:123–36. doi:10.1007/s12026-008-8016-2
78. Okwor I, Mou Z, Liu D, Uzonna J. Protective immunity and vaccination against cutaneous leishmaniasis. *Front Immunol* (2012) **3**:128. doi:10.3389/fimmu.2012.00128
79. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* (1999) **401**:708–12. doi:10.1038/44385
80. Zaph C, Uzonna J, Beverley SM, Scott P. Central memory T cells mediate long-term immunity to *Leishmania major* in the absence of persistent parasites. *Nat Med* (2004) **10**:1104–10. doi:10.1038/nm1108
81. Pakpour N, Zaph C, Scott P. The central memory CD4+ T cell population generated during *Leishmania major* infection requires IL-12 to produce IFN-gamma. *J Immunol* (2008) **180**:8299–305.
82. Colpitts S, Scott P. Memory T-cell subsets in parasitic infections. In: Zanetti M, Schoenberger SP, editors. *Memory T Cells*. New York: Springer (2010). p 145–54.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 January 2014; paper pending published: 05 February 2014; accepted: 17 March 2014; published online: 31 March 2014.
*Citation: Saljoughian N, Taheri T and Rafati S (2014) Live vaccination tactics: possible approaches for controlling visceral leishmaniasis. Front. Immunol. **5**:134. doi: 10.3389/fimmu.2014.00134*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 14 May 2014
doi: 10.3389/fimmu.2014.00213

Genetically modified organisms and visceral leishmaniasis

Rudra Chhajer and Nahid Ali*

Infectious Diseases and Immunology Division, Council of Scientific and Industrial Research-Indian Institute of Chemical Biology, Kolkata, India

Edited by:
Hira Nakhasi, Food and Drug Administration, USA

Reviewed by:
Sreenivas Gannavaram, Food and Drug Administration, USA
Ennio De Gregorio, Novartis Vaccines and Diagnostics, Italy
Ranadhir Dey, Food and Drug Administration, USA

***Correspondence:**
Nahid Ali, Infectious Diseases and Immunology Division, Council of Scientific and Industrial Research-Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Kolkata 700032, India
e-mail: nali@iicb.res.in

Vaccination is the most effective method of preventing infectious diseases. Since the eradication of small pox in 1976, many other potentially life compromising if not threatening diseases have been dealt with subsequently. This event was a major leap not only in the scientific world already burdened with many diseases but also in the mindset of the common man who became more receptive to novel treatment options. Among the many protozoan diseases, the leishmaniases have emerged as one of the largest parasite killers of the world, second only to malaria. There are three types of leishmaniasis namely cutaneous (CL), mucocutaneous (ML), and visceral (VL), caused by a group of more than 20 species of *Leishmania* parasites. Visceral leishmaniasis, also known as kala-azar is the most severe form and almost fatal if untreated. Since the first attempts at leishmanization, we have killed parasite vaccines, subunit protein, or DNA vaccines, and now we have live recombinant carrier vaccines and live attenuated parasite vaccines under various stages of development. Although some research has shown promising results, many more potential genes need to be evaluated as live attenuated vaccine candidates. This mini-review attempts to summarize the success and failures of genetically modified organisms used in vaccination against some of major parasitic diseases for their application in leishmaniasis.

Keywords: vaccines, immunology, *Leishmania*, genetically modified parasites, visceral leishmaniasis

INTRODUCTION

The leishmaniases comprise a group of largely neglected tropical diseases, transmitted during the blood meal of the phlebotomine sandfly (**Figure 1**). The disease outcome ranges from the mild cutaneous, more severe mucocutaneous to the almost fatal visceral leishmaniasis (followed by PKDL in a small proportion of VL patients) depending upon the transmitted species of *Leishmania* parasite. With more than 90% of the VL patients concentrated in south-east Asia and Africa, the statistics indicate that almost 200 million people are at risk worldwide, which is only a rough estimate, as a major population remains asymptomatic and hence unrecognized (1). VL ranks fourth in morbidity among all tropical diseases with an annual incidence of 2.5/1000 persons (2) and is second only to malaria in terms of mortality (3).

Despite abundant research in recent years, the available treatment options are far from satisfactory. The drugs are associated with toxicity, high cost, and/or resistance. In this context, multidrug combinatorial therapies have shown some promise (4). Prevention by vaccination is favored by the fact that healing from leishmaniasis is almost always associated with lifelong resistance to infection. A desirable vaccine would provide long term immunity; elicit a T-cell immune response that would be a balance of Th1 mediated immune activation against the pathogen and Th2 mediated suppression to avoid excess tissue damage, produce a strong memory and effector response upon subsequent challenge, be persistent, and highly immunogenic (3). However, the vaccine should not elicit an auto-immune response and be safe even in immune-compromised SCID mice and HIV patients (5).

Based on the general nature of the formulation, there are three types of anti-leishmanial vaccines (6). The first generation of vaccines is comprised of live, virulent parasites injected at hidden body parts so as to avoid lesion visibility (leishmanization) or of inactivated parasites achieved by heat, radiation, antibiotics, chemical mutagenesis, and selection for temperature sensitivity or long passages in-culture (7). The second generation includes crude whole cell lysates, purified fractions, or subunit vaccines composed of single or multiple recombinant or native antigens. The only approved vaccine for human trial is Leish111f, a multivalent vaccine, composed of a thiol-specific antioxidant, *Leishmania major* stress inducible protein 1, and *L. major* elongation initiation factor (8). The third generation of vaccines consists largely of DNA in the form of mammalian expression plasmids or viral vectors encoding virulence factors (9). Unfortunately, the efficacy of available DNA and protein subunit vaccine candidates are limited (10). Recent concepts introduce the use of sandfly salivary antigens, T-cell epitope based peptides, antigen pulsed DC's, and genetically modified live attenuated parasites (11). In contrast, vaccination using live attenuated parasites mimics natural infection and overcomes most of these limitations (12). Additionally, their persistence and display of parasites entire antigenic repertoire alleviates the need for an adjuvant. The recent success of live attenuated vaccination (LAV) in malaria, the clear genetic profile, and safety from reversion of complete knock-outs further encourages this endeavor.

GENETIC MODIFICATION IN *LEISHMANIA*: APPLICATIONS AND TYPES

Due to advances in axenic parasite culture, transfection efficiency, availability of genetic manipulation vectors (for expression, recombination, or integration), and the plethora of sequence based information available (from databases, like GeneDB, LeishCyc, LeishBase, KEGG, TriTrypDB, and TDR Targets), the ease and

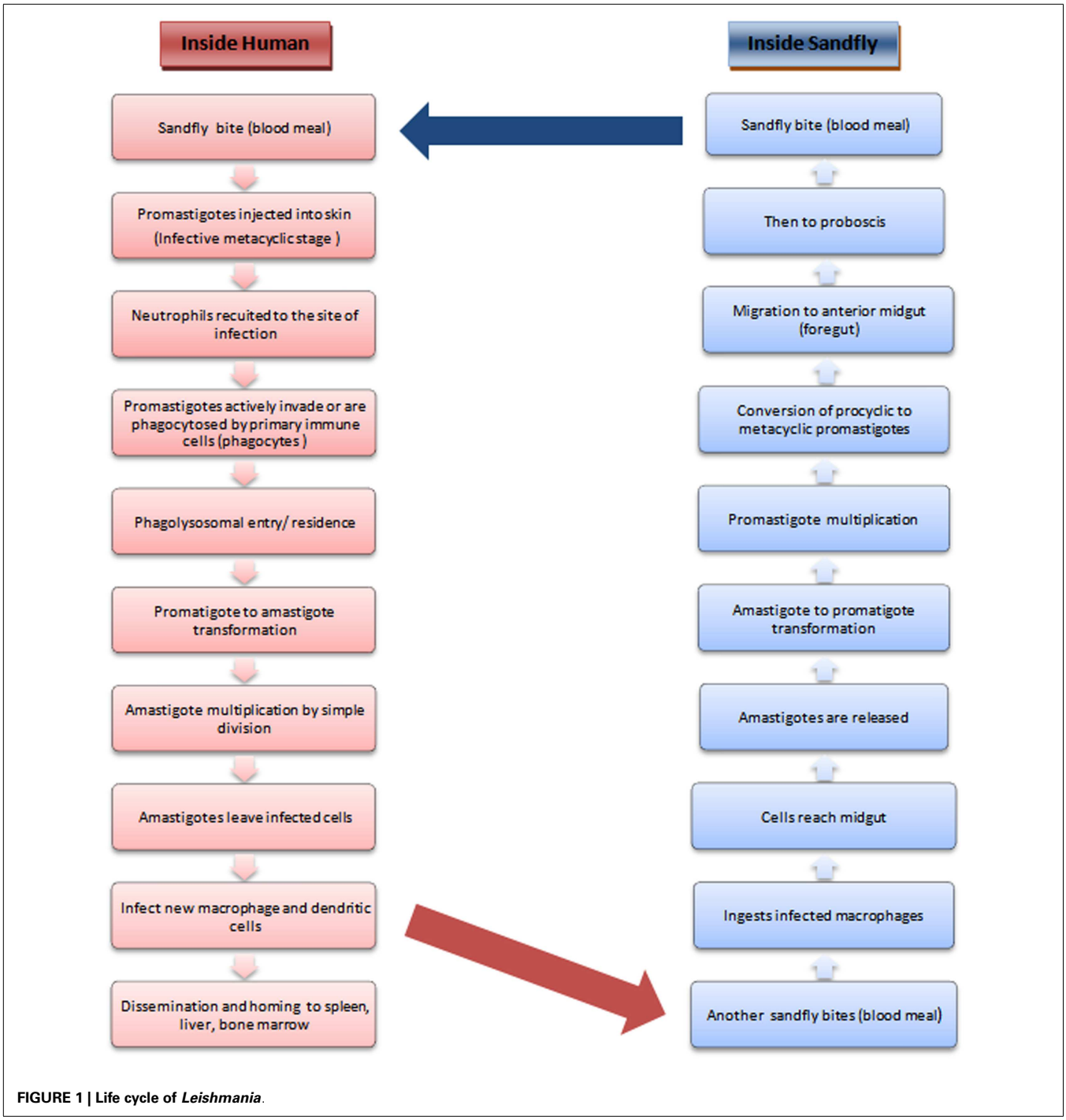


FIGURE 1 | Life cycle of *Leishmania*.

scope of creating live attenuated parasites has increased tremendously (13). Such parasites can be used to elucidate novel drug targets as well as vaccine candidates based on whether the gene under study is essential for both the promastigote and amastigote stages of the parasite or, only the amastigote stage. In addition, genetically modified organisms can also be used in metabolic pathways studies, structure-function relationship investigates (14), screening of new drugs (15), host–parasite interaction, and post-infection analysis among others, to enhance our understanding of these lower eukaryotes. Considering the success of LAV strategies against many viral, bacterial, and protozoan diseases (although to different extents), these are now considered the gold standard for protection against intra-cellular pathogens (12).

Foreign or self genes can be introduced in either episomal or integrated form, for expression of particular proteins to study their effects on various aspects of the parasites life cycle. In the episomal form, the gene's expression is under the control of the vector specific promoter, which can be inducible or not (for stage

specific expression analysis). For integration, the genes are generally targeted downstream of the ribosomal RNA locus to study the effects of constitutive expression at all stages of the life cycle. In either case, the genes can be fused to fluorescent reporter genes for ease of monitoring their expression (15). In addition, there are methods to selectively knock-out particular regions of interest heterologously or homologously using gene specific targeting constructs (16–18). During deletion, the targeted region is replaced by an antibiotic selection marker. Its expression makes the modified cells resistant to that antibiotic, thereby facilitating selection. Multiple genes can be targeted simultaneously. This exchange is generally brought about by the double strand break repair model of homologous recombination (19) whose major role has been the maintenance of its multi-gene families, conferring a selective advantage to parasites stressed by antifolate drugs (by upregulation of resistance genes) (14, 16). Alternatively, the transcripts of the genes can also be simply knocked down by anti-sense RNA interference technique, thereby blocking translation. However, with a few exceptions most leishmanial species lack the RNAi machinery (20).

SUCCESS OF LIVE ATTENUATED VACCINATION IN OTHER DISEASES

Herein, we will discuss LAV strategies in various mosquito borne, viral, protozoal, and bacterial diseases. Malaria, which exerts significant mortality, morbidity, and economic burden, is spread by intra-cellular parasitic apicomplexans of the genus *Plasmodium*. Like *Leishmania*, *Plasmodium* has multiple hosts and forms and rapid amplification is key to its survival and spread. Their pathogenic liver and transmission stages have been the most often chosen targets for attenuation because compared to the blood stages, they are low in numbers and exhibit limited antigenic variation, making it less probable that a vaccine will fail against heterologous parasite strains. The search for a live attenuated malaria vaccine provided some invaluable insights that can be applied to leishmanial as well as other infectious diseases. The failure of the inactivated sporozoites, and success of γ-irradiated ones, demonstrated the requirement of live and host cell invasive parasites to confer protection (21–23). The ability of the *UIS3*$^{-/-}$ sporozoites to confer protection against sporozoite re-infection but not blood stage transfusion, demonstrates stage specific immunity, herein, liver stage. Hence, not all stages of a parasites cycle may be equally useful for LAV approaches (24). The deletion of liver stage specific fatty acid synthesis pathway genes, however, had no effect on replication and gametogenesis, indicating that only essential metabolic pathways should be targeted for attenuation. Furthermore, multiple deletions sometimes may be more effective, as combined *p26/p52* knock-out provided better protection than either of the single knock-outs in both chimeric mouse harboring human hepatocytes as well as both low/high dose human trials (22, 25, 26). These mutants exhibited complete growth arrest during the liver stages. However, their pre-erythrocytic stages were unhampered, thereby not hindering the possibility of large-scale production. Similarly, for leishmania, an unaffected promastigote growth stage would be desirable for a strain to be used for vaccination.

Another virus that largely affects the cloven hoofed animals worldwide is the foot and mouth disease virus (FMDV). Control by limiting animal movements and herd destruction has been mostly practiced due to insufficient protection by the available inactivated vaccine against all three FMDV variants. Recently, however, a reverse genetics approach has yielded a novel vaccine candidate by substitutions in a few amino-acids showing remarkable protection. These mutants too had normal growth properties as desirable for large-scale vaccine production (27).

One of the most successful and oldest examples of live attenuated vaccines is the 17D strain of yellow fever virus. It has also served as a model for vaccination strategies against dengue, a viral disease caused by transmission of one of its four serotypes 1–4 by the *Aedes* mosquito. Sanofi Pasteur's ChimeriVax Dengue tetravalent vaccine (CVD1–4) is the most advanced product so far and a chimera in the truest sense utilizing the licensed YFV 17D vaccine as backbone, each expressing the *prM* and *E* genes of one of the four DENV serotypes. An effective dengue vaccine should consist of a tetravalent formulation, with components representing each serotype (28). A "stem-loop" genomic region implicated in its pathogenicity has been deleted to create the rDEN(1,2,4)Δ30 strains that impart adequate protection. However, the rDEN3Δ30 was not protective, indicating differences among strains. Hence, a novel chimerization led to a creation of rDEN3/4Δ30(ME) – a recombinant virus backbone of serotype 4 with Δ30 deletion, containing the *ME* region of a naturally attenuated serotype 3 strain, having manifold lower replication and transmission. This is a perfect example of successful extrapolation from sabin polio virus whose second component was also a naturally attenuated polio strain (29).

The MMR vaccine against measles, mumps, and rubella given to expecting mothers is another successful example of a multivalent vaccine that reduces the number of doses and avoids unnecessary delays and problems of spacing live attenuated vaccines (30). With pandemic capacity (31), the influenza vaccine, has been a huge challenge with its constantly varying epitopes resulting in antigenically drifted strains (32). In such cases, focusing on the most constant regions is the best strategy. However, till a strain specific vaccine is available, reasonable protection can be offered by a recombinant adenoviral vector expressing antigens from H5, H7, and H9 avian influenza virus strains (33). The success of multivalent, dengue, influenza, and MMR vaccines offers the idea for such a vaccine against CL, ML, and VL too.

Among bacteria, *Streptococcus suis*, that causes swine flu is a global health hazard to the swine industry, associated with septic shock, pneumonia, meningitis, and arthritis. The current vaccine against it is a Sly gene deletion attenuated strain undergoing some refinement by association with other surface antigens and adjuvants (34). The Bacillus Calmette Guerin vaccine for tuberculosis is created by long *in vitro* passaging of the intracellular bacteria *Mycobacterium tuberculosis*. The gradual loss of the RD loci has been reported as the major cause for this attenuation. Hence, attempts at manually creating these deletions are on. Recombinant BCG vaccines co-expressing other antigens from pathogens are also in clinical trials (35, 36). For cholera too, many endogenously produced live attenuated vaccines (Peru15 and Bengal15) are available as a traveler's vaccine in different countries (37–39).

ELUCIDATION OF NOVEL VACCINE CANDIDATES AND DRUG TARGETS: ATTEMPTS MADE IN *LEISHMANIA*

In contrast to leishmanial species causing CL, research on genetic modification in VL has been limited. However, recent years have seen a significant improvement in this scenario (**Table 1**). Though mostly focused at elucidating metabolic pathways, cellular processes, and host–parasite interactions; it has simultaneously led to the discovery of novel drug targets and vaccine candidates. The major pathways targeted were those that are unique to the parasite's life cycle or metabolism, components sufficiently different from the homolog in hosts. Today, bio-informatic databases, proteomic screens (40), and reverse vaccinology, aid in the identification of novel vaccine candidates based on their expression stage, abundance, sub-cellular localization, sequence conservation in leishmanial species, non-homology to their human counterparts, trans-membrane helix predictions, and T-cell epitopic regions (12). Using the same genetically modified strain, research collaborations between labs working on different aspects of leishmaniasis can greatly speed up and enhance this search. Some of the most important pathways and their components, that have surfaced, are briefly discussed below.

POLYAMINE METABOLISM

Polyamines are essential for proliferative processes and trypanothione synthesis. Their biosynthesis involves arginase, ornithine decarboxylase, *S*-adenosylmethionine decarboxylase, and spermidine synthase. In *Leishmania*, spermidine along with trypanothione reductase and trypanothione synthetase replace the antioxidant pathways of the host and are necessary for survival. Deletion of any of these enzymes implicates the essentiality of polyamine biosynthesis in both promastigotes and amastigotes, rendering them important drug targets.

NUCLEOTIDE METABOLISM

Purines and pyrimidines are indispensable to all life. However, *Leishmania* are purine auxotrophs. Surprisingly, deletion of any of the purine salvages enzymes, namely hypoxanthine–guanine phosphoribosyl transferase (*Hgprt*), adenine phosphoribosyl transferase (*Aprt*), and xanthine phosphoribosyl transferase (*Xprt*); guanylate nucleotide synthesis enzyme namely inosine monophosphate dehydrogenase (IMPDH) or; adenine aminohydrolase (*Aah*) does not prove their essentiality for either salvage, virulence, or viability. However, multiple knock-out strains such as Δ*hgprt*/Δ*xprt* and Δ*aah*/Δ*hgprt*/Δ*xprt* are avirulent and hence potential vaccine candidates. However, the upregulation of *Xprt* in combined mutants implicate their therapeutic potential. Similarly, although both adenylosuccinate synthetase (*Adss*) and adenylosuucinate lyase (*Asl*) null mutants show diminished virulence, only the Δ*asl* null mutants are profoundly incapacitated in their ability to infect mice and essential for purine salvage by both life cycle stages.

In contrast to purines, *Leishmania* are prototrophic for pyrimidines. Nevertheless, they also possess some salvage enzymes. Deletion of the uridine monophosphate synthase (*Umps*), a bifunctional enzyme for UMP biosynthesis established this enzyme as essential for pyrimidine biosynthesis. Additionally, although single deletions of either uracil phosphoribosyl transferase (*Uprt*) or carbamoyl phosphate synthetase (*Cprt*) did not affect parasite growth, their combined deletion mutants were completely attenuated exhibiting reduced survivability, hence potential live vaccine candidates.

AMASTIGOTE STAGE SPECIFIC PROTEINS

Amastigote stage specific genes are considered good targets for attenuation. Vaccination with null mutants of the biopterin transporter 1 (*Bt1*) gene, involved in biopterin transport; centrin (*Cen*), involved in the cell division cycle; *p27*, a cytochrome *c* oxidase complex component; *Lpg-2* (Golgi GDP mannose transporter), involved in phosphoglycan synthesis, which is essential for host–parasite interactions or ubiquitin fold modifier-1 (*Ufm-1*) gene involved in fatty acid metabolism produced a strong protective immunity against challenge infection. Their reduced virulence and survivability confirms their vaccine candidature and demands further investigations. However, similar attempts with *A2* (amastigote specific expression 2) genes failed due to their multiplicity and rapid compensation by amplification of the remaining genes.

PROTEASES

Proteases play key roles in the life cycle, host–parasite relationship and pathogenesis of parasitic diseases. The deletion of genes for cathepsin B cysteine protease, oligopeptidase B serine protease, or subtilisin protease resulted in avirulent strains causing proteome remodeling, upregulation of gene-transcription in macrophages, or reduced promastigote to amastigote differentiation *in vitro*, respectively. As in many other diseases, proteases form attractive drug targets.

CYTOSKELETAL ELEMENTS

Some flagellar components were also found to play important roles in the parasites life cycle. The deletion of *myosin XXI*, that encodes a novel class of myosin; the 70 kDa subunit of the outer dynein arm docking complex; a novel actin related protein (ORF LmjF.13.0950) or the over-expression of *ARL-3A* (ADP-ribosylation factor like protein), a homolog of human ARL-3, all resulted in impairment of flagellar assembly, motility, and survival. They also affected intra-cellular trafficking, virulence *in vitro* and mitochondrial membrane potential to various extents. Hence, a novel group of putatively essential components that hold promise for further studies were identified.

In addition to these, components of some other pathways have also been manipulated to assess their functional role and dispensability. Heterozygous mutants of glyoxalase I (*GLO I*), involved in methylglyoxal metabolism and *CYP5122A1*, involved in xenobiotic metabolism and sterol biosynthesis, impaired growth, mitochondrial membrane potential, and normal metabolism. Altered drug susceptibility and virulence were also observed in the latter mutants. Moreover, attempts at homozygous deletions did not permit survival. In addition, knock-outs of some chaperone proteins like *HSP70-II, HSP90*, and co-chaperones like *SGT* (small glutamine rich tetra trichopeptide) also had deleterious effects. Also, trials of *LiHSP70-II* null mutants to provide protection against *L. major* infection model demonstrated both safety and protection. In another study, the over-expression of a kinase, *CK1.4* (casein kinase 1 isoform 4), increased virulence and metacyclogenesis. As

Table 1 | Genetic deletions that led to the discovery of novel drug or vaccine candidates in VL causing organisms.

Organism	Target gene	Animal model	Immune response	Persistence	Inference		Reference
					Drug	LAV	
L. mexicana *L. major*	Arginase	NA	NA	NA	+	UC	(41–43)
L. donovani	Ornithine decarboxylase	BALB/c mice	Reduced virulence *in vitro* and *in vivo*	NA	+	+	(44–46)
L. donovani	Spermidine synthase	BALB/c mice	Decreased organ parasite burden	4 weeks	+	UC	(47)
L. donovani	*S*-adenosylmethionine decarboxylase	NA	NA	NA	+	UC	(48)
L. donovani	Trypanothione reductase	NA	Reduced virulence *in vitro*	NA	+	UC	(49–52)
L. donovani	Trypanothione synthetase	NA	NA	NA	+	UC	(42, 53)
L. donovani	Hypoxanthine–guanine phosphoribosyl transferase	NA	No effect on virulence *in vitro* and *in vivo*	NA	X	X	(54)
L. donovani	Adenine phosphoribosyl transferase	NA	No effect on virulence *in vitro* and *in vivo*	NA	X	X	(54, 55)
L. donovani	Xanthine phosphoribosyl transferase	NA	No effect on virulence *in vitro* and *in vivo*	NA	+	UC	(54, 56)
L. donovani	Inosine monophosphate dehydrogenase	BALB/c mice	No effect on virulence *in vivo*	NA	X	X	(57)
L. donovani	Adenine aminohydrolase	BALB/c mice	No significant effect on parasitemia *in vitro* or in organ parasite burden	NA	+	UC	(58)
L. donovani	Hypoxanthine–guanine phosphoribosyl transferase/xanthine phosphoribosyl transferase	NA	Highly reduced virulence *in vitro*	NA	–	+	(59)
L. donovani	Adenine aminohydrolase/hypoxanthine–guanine phosphoribosyl transferase/xanthine phosphoribosyl transferase	BALB/c mice	Avirulent *in vitro* and *in vivo*	4 weeks	–	+	(58)
L. donovani	Adenylosuccinate synthetase	BALB/c mice	Reduced virulence *in vitro* but not *in vivo*	NA	X	X	(60)
L. donovani	Adenylosuccinate lyase	BALB/c mice	Reduced virulence *in vitro* and *in vivo*	NA	+	UC	(60)
L. donovani	Uridine monophosphate synthase	NA	NA	NA	+	UC	(61)
L. donovani	Uracil phosphoribosyl transferase	BALB/c mice	No effect on virulence *in vitro* or *in vivo*	NA	+	UC	(62, 63)
L. donovani	Carbamoyl phosphate synthetase	BALB/c mice	Reduced virulence *in vitro* and decreased parasite burden	NA	+	UC	(62)
L. donovani	Uracil phosphoribosyl transferase/carbamoyl phosphate synthetase	BALB/c mice	Reduced virulence *in vivo*	4 weeks	–	+	(62)
L. donovani	Biopterin transporter 1	BALB/c mice	Reduced virulence *in vivo*. Protective against challenge infection. Increased IFN-γ production upon splenocyte stimulation	3 months	UC	+	(64)

(Continued)

Table 1 | Continued

Organism	Target gene	Animal model	Immune response	Persistence	Inference		Reference
					Drug	LAV	
L. donovani	Centrin	BALB/c mice, SCID mice, golden Syrian hamsters	Long term protection against challenge infection-early clearance. Protective Th1-type immune response. Increase of single and multiple cytokine (IFN-γ, IL-2, and TNFα) producing cells, IFN-γ/IL-10 ratio, IgG2a immunoglobulins and NO production. Reduced organ parasite burden. Cross-protective against *L. braziliensis* challenge	10 weeks	UC	+	(65, 66)
L. donovani	P27, a cytochrome *c* oxidase component	BALB/c mice	Reduced virulence *in vivo*. NO generation, Ag-specific multifunctional CD4 and CD8 T-cells, enhanced secretion of pro-inflammatory cytokines IFN-γ, TNF-α, IL-12, and anti-inflammatory cytokines IL-10, IL-4, and IL-13	20 weeks	UC	+	(67)
L. donovani	Ubiquitin fold modifier-1	NA	Reduced virulence in human macrophages	NA	+	+	(68)
L. donovani	Golgi GDP mannose transporter	BALB/c mice	Reduced virulence *in vitro* and *in vivo*	Long term	+	+	(69)
L. donovani	Amastigote specific expression protein-2	BALB/c mice	Decreased virulence *in vitro* and *in vivo*	NA	+	X	(70)
L. donovani	Cathepsin b cysteine protease	NA	Decreased virulence in U937 macrophage cells	NA	+	UC	(71)
L. donovani	Oligopeptidase b serine protease	BALB/c mice	Decreased virulence in the murine footpad infection model. Massive upregulation in gene-transcription	NA	+	UC	(72)
L. donovani	Subtilisin protease	BALB/c mice, golden Syrian hamsters	Reduced virulence *in vivo*	NA	+	UC	(73)
L. donovani	Myosin	NA	NA	NA	+	UC	(74)
L. donovani	70 kDa subunit of outer dynein arm docking complex	NA	Increased virulence *in vitro*	NA	X	X	(75)
L. donovani	Actin	NA	Reduced survival *in vitro* mice peritoneal macrophage cells	NA	+	UC	(76)
L. donovani	ADP-ribosylation factor like protein-3A	NA	NA	NA	+	UC	(77)
L. infantum	Heat shock protein 70 type II	*L. major* model of infection in BALB/c mice, SCID mice, golden Syrian hamster	Increased NO production and protection by type 1 immune response in BALB/c mice	NA	UC	+	(78)
L. donovani	Small glutamine rich tetra trichopeptide	NA	NA	NA	+	UC	(79)
L. donovani	Casein kinase 1 isoform 4	NA	Increased virulence *in vitro* mice peritoneal macrophage cells	NA	+	UC	(80)

(Continued)

Table 1 | Continued

Organism	Target gene	Animal model	Immune response	Persistence	Inference		Reference
					Drug	LAV	
L. donovani	Glyoxalase I	NA	NA	NA	+	UC	(81)
L. donovani	cyp5122A1, a cytochrome P450	Golden Syrian hamsters	Decreased virulence *in vitro* and *in vivo*	NA	+	UC	(82)

Color codes	Role	Symbols/short forms	Interpretation
Purple	Polyamine metabolism	NA	Not available
Blue	Purine metabolism	+	Positive indication
Gray	Pyrimidine metabolism	–	Not evaluated
Green	Amastigote stage	UC	Uncertain
Yellow	Protease	X	Negative indication
Peach	Cytoskeletal involvement		
Pink	Chaperones		
White	Others		

seen, majorly these studies implicate the therapeutic potential of the target genes. Simultaneous evaluation of their LAV potential would greatly fasten the search for an ideal leishmanial vaccine.

CHALLENGES AND SCOPE FOR THE FUTURE

Although a large proportion of currently licensed vaccines are based on inactivated or whole live attenuated organisms, the scope of LAV gets largely restricted due to safety issues. Foremost, is the risk of reversion to wild type or expression of compensatory genes. The *Leishmania* genome being highly plastic, this has a high probability. Additionally, critical consideration of the position of knock-outs, their effects on upstream and downstream genes, the restriction to manipulate only amastigote stage specific and single copy genes and availability of few selectable markers limits the potential targets and simultaneous multi-gene targeting, respectively (12). Furthermore, the retention of antibiotic resistance genes (20) and generation of cross resistance to anti-leishmanial drugs as in the case of neomycin to paromomycin is undesirable (83). Moreover, prior to human clinical trials, the cultivation of parasites in serum free media, their large-scale production, storage, validation of the best challenge methods-syringe or sandfly mediated, and many months of post challenge follow-up impose practical and as yet unresolved issues (84). In contrast, subunit and DNA vaccines are relatively safe and without these limitations. However, the low predictive power of available pre-clinical models to determine the human outcome of vaccination and the lack of knowledge of convincing markers to monitor their safety or efficacy remain common to all vaccination strategies (2).

The following road map may be considered a basic guideline while working with live attenuated vaccines. Preliminary phenotypic and genotypic screening of the parasites after each recombination event should be followed by vigorous *in vitro* studies on human cell lines. The parasites compartmentalization, proliferation, cellular responses, and activation markers should be closely monitored (85). After successful *in vitro* screening, the *in vivo* experiments in Golden Syrian hamsters and BALB/c mice models should be supported by those on chimeric humanized mice (25, 86). Continuous monitoring assays to test for reversion or attenuation retention by sensitive molecular biology techniques like PCR, microarrays should be done (87). Timely splenic biopsies for parasite load and multiparametric FACS analysis and ELISA for monitoring cytokine responses would help in elucidating the immune correlates of protection or disease development (6). Additionally, the comparison of these results among different groups, namely asymptomatic carriers, non-endemic healthy, endemic healthy, infected-cured, and infected individuals would greatly enhance our knowledge of disease pathogenesis. With the advent of modern imaging techniques, bioluminescent parasites can provide unsurpassable insight at each level of disease progression in real time (beginning from host cell–parasite interaction to dissemination and homing to various organs) also requiring lower number of animals to obtain statistically significant data (88). Lastly, human trials to provide proof of concept studies would strengthen our hypothesis derived from pre-clinical studies.

Parasite gene deletion mutants have helped in numerous pathway studies and elucidation of novel drug targets and vaccine candidates (**Table 1**). They also offer the possibility of co-administration with adjuvants or drugs to improve disease outcome. Moreover, vectored formulations in recombinant vaccinia (89), *Lactobacillus* (90), adenovirus, or *Salmonella* (91) carriers offer non-pathogenic and genetically modifiable alternatives for safe mucosal delivery, the major entry portal of pathogens. The concept of the flying vaccinator, genetically engineered blood-feeding insects to deliver vaccines to replace mosquito populations is a novel attempt tried in antimalarial programs and can be applied for sandfly eradication (92) too. Lastly, well-defined clinical trials with attenuated parasites will enhance the number of potential therapeutic targets, which are urgently needed to combat leishmaniasis.

ACKNOWLEDGMENTS

This work was supported by grants from the Council of Scientific and Industrial Research, Government of India. We thank Md Asad

and Anirban Bhattacharya for their help during preparation of the manuscript.

REFERENCES

1. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) **7**:e35671. doi:10.1371/journal.pone.0035671
2. Schroeder J, Aebischer T. Vaccines for leishmaniasis: from proteome to vaccine candidates. *Hum Vaccin* (2011) **7**(Suppl):10–5. doi:10.4161/hv.7.0.14556
3. Kaye PM, Aebischer T. Visceral leishmaniasis: immunology and prospects for a vaccine. *Clin Microbiol Infect* (2011) **17**:1462–70. doi:10.1111/j.1469-0691.2011.03610.x
4. Kumar N, Sinha PK, Pandey K, Verma N, Lal CS, Ranjan A, et al. A rare case of visceral leishmaniasis with multiple relapse and multi-drug unresponsive: successfully treated with combination therapy. *Int J Clin Pharm* (2011) **33**:726–9. doi:10.1007/s11096-011-9544-8
5. Evans KJ, Kedzierski L. Development of vaccines against visceral leishmaniasis. *J Trop Med* (2012) **2012**:892817. doi:10.1155/2012/892817
6. Das A, Ali N. Vaccine development against *Leishmania donovani*. *Front Immunol* (2012) **3**:99. doi:10.3389/fimmu.2012.00099
7. Noazin S, Modabber F, Khamesipour A, Smith PG, Moulton LH, Nasseri K, et al. First generation leishmaniasis vaccines: a review of field efficacy trials. *Vaccine* (2008) **26**:6759–67. doi:10.1016/j.vaccine.2008.09.085
8. Trigo J, Abbehusen M, Netto EM, Nakatani M, Pedral-Sampaio G, De Jesus RS, et al. Treatment of canine visceral leishmaniasis by the vaccine Leish-111f+MPL-SE. *Vaccine* (2010) **28**:3333–40. doi:10.1016/j.vaccine.2010.02.089
9. Tabbara KS. Progress towards a *Leishmania* vaccine. *Saudi Med J* (2006) **27**:942–50.
10. Kedzierski L. Leishmaniasis. *Hum Vaccin* (2011) **7**:1204–14. doi:10.4161/hv.7.11.17752
11. Okwor I, Uzonna J. Vaccines and vaccination strategies against human cutaneous leishmaniasis. *Hum Vaccin* (2009) **5**:291–301. doi:10.4161/hv.5.5.7607
12. Silvestre R, Cordeiro-Da-Silva A, Ouaissi A. Live attenuated *Leishmania* vaccines: a potential strategic alternative. *Arch Immunol Ther Exp (Warsz)* (2008) **56**:123–6. doi:10.1007/s00005-008-0010-9
13. Timmers LF, Pauli I, Barcellos GB, Rocha KB, Caceres RA, De Azevedo WF Jr, et al. Genomic databases and the search of protein targets for protozoan parasites. *Curr Drug Targets* (2009) **10**:240–5. doi:10.2174/138945009787581195
14. Tobin JF, Wirth DF. A sequence insertion targeting vector for *Leishmania* enrietti. *J Biol Chem* (1992) **267**:4752–8.
15. Singh N, Gupta R, Jaiswal AK, Sundar S, Dube A. Transgenic *Leishmania donovani* clinical isolates expressing green fluorescent protein constitutively for rapid and reliable ex vivo drug screening. *J Antimicrob Chemother* (2009) **64**:370–4. doi:10.1093/jac/dkp206
16. Tobin JF, Laban A, Wirth DF. Homologous recombination in *Leishmania* enrietti. *Proc Natl Acad Sci U S A* (1991) **88**:864–8. doi:10.1073/pnas.88.3.864
17. Hwang HY, Gilberts T, Jardim A, Shih S, Ullman B. Creation of homozygous mutants of *Leishmania donovani* with single targeting constructs. *J Biol Chem* (1996) **271**:30840–6. doi:10.1074/jbc.271.48.30840
18. Fulwiler AL, Soysa DR, Ullman B, Yates PA. A rapid, efficient and economical method for generating leishmanial gene targeting constructs. *Mol Biochem Parasitol* (2011) **175**:209–12. doi:10.1016/j.molbiopara.2010.10.008
19. Papadopoulou B, Dumas C. Parameters controlling the rate of gene targeting frequency in the protozoan parasite *Leishmania*. *Nucleic Acids Res* (1997) **25**:4278–86. doi:10.1093/nar/25.21.4278
20. Roberts SC. The genetic toolbox for *Leishmania* parasites. *Bioeng Bugs* (2011) **2**:320–6. doi:10.4161/bbug.2.6.18205
21. Hoffman SL, Billingsley PF, James E, Richman A, Loyevsky M, Li T, et al. Development of a metabolically active, non-replicating sporozoite vaccine to prevent *Plasmodium falciparum* malaria. *Hum Vaccin* (2010) **6**:97–106. doi:10.4161/hv.6.1.10396
22. Vaughan AM, Wang R, Kappe SH. Genetically engineered, attenuated whole-cell vaccine approaches for malaria. *Hum Vaccin* (2010) **6**:107–13. doi:10.4161/hv.6.1.9654
23. Matuschewski K, Hafalla JC, Borrmann S, Friesen J. Arrested *Plasmodium* liver stages as experimental anti-malaria vaccines. *Hum Vaccin* (2011) **7**(Suppl):16–21. doi:10.4161/hv.7.0.14557
24. Mueller AK, Labaied M, Kappe SH, Matuschewski K. Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine. *Nature* (2005) **433**:164–7. doi:10.1038/nature03188
25. Vanbuskirk KM, O'neill MT, De La Vega P, Maier AG, Krzych U, Williams J, et al. Preerythrocytic, live-attenuated *Plasmodium falciparum* vaccine candidates by design. *Proc Natl Acad Sci U S A* (2009) **106**:13004–9. doi:10.1073/pnas.0906387106
26. Spring M, Murphy J, Nielsen R, Dowler M, Bennett JW, Zarling S, et al. First-in-human evaluation of genetically attenuated *Plasmodium falciparum* sporozoites administered by bite of Anopheles mosquitoes to adult volunteers. *Vaccine* (2013) **31**:4975–83. doi:10.1016/j.vaccine.2013.08.007
27. Li P, Bai X, Sun P, Li D, Lu Z, Cao Y, et al. Evaluation of a genetically modified foot-and-mouth disease virus vaccine candidate generated by reverse genetics. *BMC Vet Res* (2012) **8**:57. doi:10.1186/1746-6148-8-57
28. Wan SW, Lin CF, Wang S, Chen YH, Yeh TM, Liu HS, et al. Current progress in dengue vaccines. *J Biomed Sci* (2013) **20**:37. doi:10.1186/1423-0127-20-37
29. Blaney JE Jr, Hanson CT, Firestone CY, Hanley KA, Murphy BR, Whitehead SS. Genetically modified, live attenuated dengue virus type 3 vaccine candidates. *Am J Trop Med Hyg* (2004) **71**:811–21.
30. White SJ, Boldt KL, Holditch SJ, Poland GA, Jacobson RM. Measles, mumps, and rubella. *Clin Obstet Gynecol* (2012) **55**:550–9. doi:10.1097/GRF.0b013e31824df256
31. Lopez-Macias C. Virus-like particle (VLP)-based vaccines for pandemic influenza: performance of a VLP vaccine during the 2009 influenza pandemic. *Hum Vaccin Immunother* (2012) **8**:411–4. doi:10.4161/hv.18757
32. Wikramaratna PS, Sandeman M, Recker M, Gupta S. The antigenic evolution of influenza: drift or thrift? *Philos Trans R Soc Lond B Biol Sci* (2013) **368**:20120200. doi:10.1098/rstb.2012.0200
33. Vemula SV, Ahi YS, Swaim AM, Katz JM, Donis R, Sambhara S, et al. Broadly protective adenovirus-based multivalent vaccines against highly pathogenic avian influenza viruses for pandemic preparedness. *PLoS One* (2013) **8**:e62496. doi:10.1371/journal.pone.0062496
34. Du H, Huang W, Xie H, Ye C, Jing H, Ren Z, et al. The genetically modified suilysin, rSLY(P353L), provides a candidate vaccine that suppresses proinflammatory response and reduces fatality following infection with *Streptococcus suis*. *Vaccine* (2013) **31**:4209–15. doi:10.1016/j.vaccine.2013.07.004
35. Kaufmann SH, Gengenbacher M. Recombinant live vaccine candidates against tuberculosis. *Curr Opin Biotechnol* (2012) **23**:900–7. doi:10.1016/j.copbio.2012.03.007
36. Romano M, Huygen K. An update on vaccines for tuberculosis – there is more to it than just waning of BCG efficacy with time. *Expert Opin Biol Ther* (2012) **12**:1601–10. doi:10.1517/14712598.2012.721768
37. Ryan ET, Calderwood SB, Qadri F. Live attenuated oral cholera vaccines. *Expert Rev Vaccines* (2006) **5**:483–94. doi:10.1586/14760584.5.4.483
38. Chowdhury MI, Sheikh A, Qadri F. Development of Peru-15 (CholeraGarde), a live-attenuated oral cholera vaccine: 1991-2009. *Expert Rev Vaccines* (2009) **8**:1643–52. doi:10.1586/erv.09.137
39. Shin S, Desai SN, Sah BK, Clemens JD. Oral vaccines against cholera. *Clin Infect Dis* (2011) **52**:1343–9. doi:10.1093/cid/cir141
40. Paape D, Aebischer T. Contribution of proteomics of *Leishmania* spp. to the understanding of differentiation, drug resistance mechanisms, vaccine and drug development. *J Proteomics* (2011) **74**:1614–24. doi:10.1016/j.jprot.2011.05.005
41. Roberts SC, Tancer MJ, Polinsky MR, Gibson KM, Heby O, Ullman B. Arginase plays a pivotal role in polyamine precursor metabolism in *Leishmania*. Characterization of gene deletion mutants. *J Biol Chem* (2004) **279**:23668–78. doi:10.1074/jbc.M402042200
42. Heby O, Persson L, Rentala M. Targeting the polyamine biosynthetic enzymes: a promising approach to therapy of African sleeping sickness, Chagas' disease, and leishmaniasis. *Amino Acids* (2007) **33**:359–66. doi:10.1007/s00726-007-0537-9
43. Reguera RM, Balana-Fouce R, Showalter M, Hickerson S, Beverley SM. *Leishmania major* lacking arginase (ARG) are auxotrophic for polyamines but retain infectivity to susceptible BALB/c mice. *Mol Biochem Parasitol* (2009) **165**:48–56. doi:10.1016/j.molbiopara.2009.01.001
44. Jiang Y, Roberts SC, Jardim A, Carter NS, Shih S, Ariyanayagam M, et al. Ornithine decarboxylase gene deletion mutants of *Leishmania donovani*. *J Biol Chem* (1999) **274**:3781–8. doi:10.1074/jbc.274.6.3781

45. Boitz JM, Yates PA, Kline C, Gaur U, Wilson ME, Ullman B, et al. *Leishmania donovani* ornithine decarboxylase is indispensable for parasite survival in the mammalian host. *Infect Immun* (2009) **77**:756–63. doi:10.1128/IAI.01236-08
46. Roberts SC, Kline C, Liu W, Ullman B. Generating knock-in parasites: integration of an ornithine decarboxylase transgene into its chromosomal locus in *Leishmania donovani*. *Exp Parasitol* (2011) **128**:166–9. doi:10.1016/j.exppara.2011.02.020
47. Gilroy C, Olenyik T, Roberts SC, Ullman B. Spermidine synthase is required for virulence of *Leishmania donovani*. *Infect Immun* (2011) **79**:2764–9. doi:10.1128/IAI.00073-11
48. Roberts SC, Scott J, Gasteier JE, Jiang Y, Brooks B, Jardim A, et al. S-adenosylmethionine decarboxylase from *Leishmania donovani*. Molecular, genetic, and biochemical characterization of null mutants and overproducers. *J Biol Chem* (2002) **277**:5902–9. doi:10.1074/jbc.M110118200
49. Kelly JM, Taylor MC, Smith K, Hunter KJ, Fairlamb AH. Phenotype of recombinant *Leishmania donovani* and *Trypanosoma cruzi* which over-express trypanothione reductase. Sensitivity towards agents that are thought to induce oxidative stress. *Eur J Biochem* (1993) **218**:29–37. doi:10.1111/j.1432-1033.1993.tb18348.x
50. Dumas C, Ouellette M, Tovar J, Cunningham ML, Fairlamb AH, Tamar S, et al. Disruption of the trypanothione reductase gene of *Leishmania* decreases its ability to survive oxidative stress in macrophages. *EMBO J* (1997) **16**:2590–8. doi:10.1093/emboj/16.10.2590
51. Tovar J, Cunningham ML, Smith AC, Croft SL, Fairlamb AH. Down-regulation of *Leishmania donovani* trypanothione reductase by heterologous expression of a trans-dominant mutant homologue: effect on parasite intracellular survival. *Proc Natl Acad Sci U S A* (1998) **95**:5311–6. doi:10.1073/pnas.95.9.5311
52. Tovar J, Wilkinson S, Mottram JC, Fairlamb AH. Evidence that trypanothione reductase is an essential enzyme in *Leishmania* by targeted replacement of the tryA gene locus. *Mol Microbiol* (1998) **29**:653–60. doi:10.1046/j.1365-2958.1998.00968.x
53. Saudagar P, Dubey VK. Cloning, expression, characterization and inhibition studies on trypanothione synthetase, a drug target enzyme, from *Leishmania donovani*. *Biol Chem* (2011) **392**:1113–22. doi:10.1515/BC.2011.222
54. Boitz JM, Ullman B. *Leishmania donovani* singly deficient in HGPRT, APRT or XPRT are viable in vitro and within mammalian macrophages. *Mol Biochem Parasitol* (2006) **148**:24–30. doi:10.1016/j.molbiopara.2006.02.015
55. Kaur K, Iovannisci DM, Ullman B. Adenine phosphoribosyltransferase-deficient *Leishmania donovani*. *Adv Exp Med Biol* (1986) **195**(Pt B):553–7. doi:10.1007/978-1-4684-1248-2_86
56. Jardim A, Bergeson SE, Shih S, Carter N, Lucas RW, Merlin G, et al. Xanthine phosphoribosyltransferase from *Leishmania donovani*. Molecular cloning, biochemical characterization, and genetic analysis. *J Biol Chem* (1999) **274**:34403–10. doi:10.1074/jbc.274.48.34403
57. Fulwiler AL, Boitz JM, Gilroy C, Yates PA, Jardim A, Ullman B. IMP dehydrogenase deficiency in *Leishmania donovani* causes a restrictive growth phenotype in promastigotes but is not essential for infection in mice. *Mol Biochem Parasitol* (2011) **180**:123–6. doi:10.1016/j.molbiopara.2011.08.006
58. Boitz JM, Strasser R, Hartman CU, Jardim A, Ullman B. Adenine aminohydrolase from *Leishmania donovani*: unique enzyme in parasite purine metabolism. *J Biol Chem* (2012) **287**:7626–39. doi:10.1074/jbc.M111.307884
59. Boitz JM, Ullman B. A conditional mutant deficient in hypoxanthine-guanine phosphoribosyltransferase and xanthine phosphoribosyltransferase validates the purine salvage pathway of *Leishmania donovani*. *J Biol Chem* (2006) **281**:16084–9. doi:10.1074/jbc.M600188200
60. Boitz JM, Strasser R, Yates PA, Jardim A, Ullman B. Adenylosuccinate synthetase and adenylosuccinate lyase deficiencies trigger growth and infectivity deficits in *Leishmania donovani*. *J Biol Chem* (2013) **288**:8977–90. doi:10.1074/jbc.M112.431486
61. French JB, Yates PA, Soysa DR, Boitz JM, Carter NS, Chang B, et al. The *Leishmania donovani* UMP synthase is essential for promastigote viability and has an unusual tetrameric structure that exhibits substrate-controlled oligomerization. *J Biol Chem* (2011) **286**:20930–41. doi:10.1074/jbc.M111.228213
62. Wilson ZN, Gilroy CA, Boitz JM, Ullman B, Yates PA. Genetic dissection of pyrimidine biosynthesis and salvage in *Leishmania donovani*. *J Biol Chem* (2012) **287**:12759–70. doi:10.1074/jbc.M112.346502
63. Soysa R, Wilson ZN, Elferich J, Forquer I, Shinde U, Riscoe MK, et al. Substrate inhibition of uracil phosphoribosyltransferase by uracil can account for the uracil growth sensitivity of *Leishmania donovani* pyrimidine auxotrophs. *J Biol Chem* (2013) **288**:29954–64. doi:10.1074/jbc.M113.478826
64. Papadopoulou B, Roy G, Breton M, Kundig C, Dumas C, Fillion I, et al. Reduced infectivity of a *Leishmania donovani* biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. *Infect Immun* (2002) **70**:62–8. doi:10.1128/IAI.70.1.62-68.2002
65. Selvapandiyan A, Dey R, Nylen S, Duncan R, Sacks D, Nakhasi HL. Intracellular replication-deficient *Leishmania donovani* induces long lasting protective immunity against visceral leishmaniasis. *J Immunol* (2009) **183**:1813–20. doi:10.4049/jimmunol.0900276
66. Fiuza JA, Santiago Hda C, Selvapandiyan A, Gannavaram S, Ricci ND, Bueno LL, et al. Induction of immunogenicity by live attenuated *Leishmania donovani* centrin deleted parasites in dogs. *Vaccine* (2013) **31**:1785–92. doi:10.1016/j.vaccine.2013.01.048
67. Dey R, Dagur PK, Selvapandiyan A, Mccoy JP, Salotra P, Duncan R, et al. Live attenuated *Leishmania donovani* p27 gene knockout parasites are nonpathogenic and elicit long-term protective immunity in BALB/c mice. *J Immunol* (2013) **190**:2138–49. doi:10.4049/jimmunol.1202801
68. Gannavaram S, Connelly PS, Daniels MP, Duncan R, Salotra P, Nakhasi HL. Deletion of mitochondrial associated ubiquitin fold modifier protein Ufm1 in *Leishmania donovani* results in loss of beta-oxidation of fatty acids and blocks cell division in the amastigote stage. *Mol Microbiol* (2012) **86**:187–98. doi:10.1111/j.1365-2958.2012.08183.x
69. Gaur U, Showalter M, Hickerson S, Dalvi R, Turco SJ, Wilson ME, et al. *Leishmania donovani* lacking the Golgi GDP-man transporter LPG2 exhibit attenuated virulence in mammalian hosts. *Exp Parasitol* (2009) **122**:182–91. doi:10.1016/j.exppara.2009.03.014
70. Zhang WW, Matlashewski G. Characterization of the A2-A2rel gene cluster in *Leishmania donovani*: involvement of A2 in visceralization during infection. *Mol Microbiol* (2001) **39**:935–48. doi:10.1046/j.1365-2958.2001.02286.x
71. Mundodi V, Kucknoor AS, Gedamu L. Role of *Leishmania (Leishmania) chagasi* amastigote cysteine protease in intracellular parasite survival: studies by gene disruption and antisense mRNA inhibition. *BMC Mol Biol* (2005) **6**:3. doi:10.1186/1471-2199-6-3
72. Swenerton RK, Zhang S, Sajid M, Medzihradszky KF, Craik CS, Kelly BL, et al. The oligopeptidase B of *Leishmania* regulates parasite enolase and immune evasion. *J Biol Chem* (2011) **286**:429–40. doi:10.1074/jbc.M110.138313
73. Swenerton RK, Knudsen GM, Sajid M, Kelly BL, Mckerrow JH. *Leishmania* subtilisin is a maturase for the trypanothione reductase system and contributes to disease pathology. *J Biol Chem* (2010) **285**:31120–9. doi:10.1074/jbc.M110.114462
74. Katta SS, Tammana TV, Sahasrabuddhe AA, Bajpai VK, Gupta CM. Trafficking activity of myosin XXI is required in assembly of Leishmania flagellum. *J Cell Sci* (2010) **123**(Pt 12):2035–44. doi:10.1242/jcs.064725
75. Harder S, Thiel M, Clos J, Bruchhaus I. Characterization of a subunit of the outer dynein arm docking complex necessary for correct flagellar assembly in *Leishmania donovani*. *PLoS Negl Trop Dis* (2010) **4**:e586. doi:10.1371/journal.pntd.0000586
76. Singh K, Veluru NK, Trivedi V, Gupta CM, Sahasrabuddhe AA. An actin-like protein is involved in regulation of mitochondrial and flagellar functions as well as in intramacrophage survival of *Leishmania donovani*. *Mol Microbiol* (2014) **91**:562–78. doi:10.1111/mmi.12477
77. Cuvillier A, Redon F, Antoine JC, Chardin P, DeVos T, Merlin G. LdARL-3A, a *Leishmania* promastigote-specific ADP-ribosylation factor-like protein, is essential for flagellum integrity. *J Cell Sci* (2000) **113**(Pt 11):2065–74.
78. Carrion J, Folgueira C, Soto M, Fresno M, Requena JM. *Leishmania infantum* HSP70-II null mutant as candidate vaccine against leishmaniasis: a preliminary evaluation. *Parasit Vectors* (2011) **4**:150. doi:10.1186/1756-3305-4-150
79. Ommen G, Chrobak M, Clos J. The co-chaperone SGT of *Leishmania donovani* is essential for the parasite's viability. *Cell Stress Chaperones* (2010) **15**:443–55. doi:10.1007/s12192-009-0160-7
80. Dan-Goor M, Nasereddin A, Jaber H, Jaffe CL. Identification of a secreted casein kinase 1 in *Leishmania donovani*: effect of protein over expression on parasite growth and virulence. *PLoS One* (2013) **8**:e79287. doi:10.1371/journal.pone.0079287
81. Chauhan SC, Madhubala R. Glyoxalase I gene deletion mutants of *Leishmania donovani* exhibit reduced methylglyoxal detoxification. *PLoS One* (2009) **4**:e6805. doi:10.1371/journal.pone.0006805

82. Verma S, Mehta A, Shaha C. CYP5122A1, a novel cytochrome P450 is essential for survival of *Leishmania donovani. PLoS One* (2011) **6**:e25273. doi:10.1371/journal.pone.0025273
83. Titus RG, Gueiros-Filho FJ, De Freitas LA, Beverley SM. Development of a safe live *Leishmania* vaccine line by gene replacement. *Proc Natl Acad Sci U S A* (1995) **92**:10267–71. doi:10.1073/pnas.92.22.10267
84. Selvapandiyan A, Duncan R, Debrabant A, Lee N, Sreenivas G, Salotra P, et al. Genetically modified live attenuated parasites as vaccines for leishmaniasis. *Indian J Med Res* (2006) **123**:455–66.
85. Guy B. The perfect mix: recent progress in adjuvant research. *Nat Rev Microbiol* (2007) **5**:505–17. doi:10.1038/nrmicro1681
86. Gupta S. Visceral leishmaniasis: experimental models for drug discovery. *Indian J Med Res* (2011) **133**:27–39.
87. Moreira N, Vitoriano-Souza J, Roatt BM, Vieira PM, Ker HG, De Oliveira Cardoso JM, et al. Parasite burden in hamsters infected with two different strains of *Leishmania (Leishmania) infantum*: "Leishman Donovan units" versus real-time PCR. *PLoS One* (2012) **7**:e47907. doi:10.1371/journal.pone.0047907
88. Beattie L, Evans KJ, Kaye PM, Smith DF. Transgenic *Leishmania* and the immune response to infection. *Parasite Immunol* (2008) **30**:255–66. doi:10.1111/j.1365-3024.2008.01020.x
89. Verheust C, Goossens M, Pauwels K, Breyer D. Biosafety aspects of modified vaccinia virus Ankara (MVA)-based vectors used for gene therapy or vaccination. *Vaccine* (2012) **30**:2623–32. doi:10.1016/j.vaccine.2012.02.016
90. Leblanc JG, Aubry C, Cortes-Perez NG, De Moreno De Leblanc A, Vergnolle N, Langella P, et al. Mucosal targeting of therapeutic molecules using genetically modified lactic acid bacteria: an update. *FEMS Microbiol Lett* (2013) **344**:1–9. doi:10.1111/1574-6968.12159
91. Chorobik P, Marcinkiewicz J. Therapeutic vaccines based on genetically modified *Salmonella*: a novel strategy in cancer immunotherapy. *Pol Arch Med Wewn* (2011) **121**:461–6.
92. Yamamoto DS, Nagumo H, Yoshida S. Flying vaccinator; a transgenic mosquito delivers a *Leishmania* vaccine via blood feeding. *Insect Mol Biol* (2010) **19**:391–8. doi:10.1111/j.1365-2583.2010.01000.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 February 2014; accepted: 28 April 2014; published online: 14 May 2014.
*Citation: Chhajer R and Ali N (2014) Genetically modified organisms and visceral leishmaniasis. Front. Immunol. **5**:213. doi: 10.3389/fimmu.2014.00213*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 23 May 2014
doi: 10.3389/fimmu.2014.00241

Biomarkers of safety and immune protection for genetically modified live attenuated *Leishmania* vaccines against visceral leishmaniasis – discovery and implications

Sreenivas Gannavaram[1]*, Ranadhir Dey[1], Kumar Avishek[2], Angamuthu Selvapandiyan[3], Poonam Salotra[2] and Hira L. Nakhasi[1]*

[1] *Division of Emerging and Transfusion Transmitted Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA*
[2] *National Institute of Pathology, Indian Council of Medical Research, New Delhi, India*
[3] *Institute of Molecular Medicine, New Delhi, India*

Edited by:
Nahid Ali, Indian Institute of Chemical Biology, India

Reviewed by:
Stephen M. Beverley, Washington University in St. Louis, USA
Abhay Satoskar, The Ohio State University, USA

****Correspondence:***
Sreenivas Gannavaram and Hira L. Nakhasi, Division of Emerging and Transfusion Transmitted Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20892, USA
e-mail: sreenivas.gannavaram@fda.hhs.gov; hira.nakhasi@fda.hhs.gov

Despite intense efforts there is no safe and efficacious vaccine against visceral leishmaniasis, which is fatal and endemic in many tropical countries. A major shortcoming in the vaccine development against blood-borne parasitic agents such as *Leishmania* is the inadequate predictive power of the early immune responses mounted in the host against the experimental vaccines. Often immune correlates derived from in-bred animal models do not yield immune markers of protection that can be readily extrapolated to humans. The limited efficacy of vaccines based on DNA, subunit, heat killed parasites has led to the realization that acquisition of durable immunity against the protozoan parasites requires a controlled infection with a live attenuated organism. Recent success of irradiated malaria parasites as a vaccine candidate further strengthens this approach to vaccination. We developed several gene deletion mutants in *Leishmania donovani* as potential live attenuated vaccines and reported extensively on the immunogenicity of LdCentrin1 deleted mutant in mice, hamsters, and dogs. Additional limited studies using genetically modified live attenuated *Leishmania* parasites as vaccine candidates have been reported. However, for the live attenuated parasite vaccines, the primary barrier against widespread use remains the absence of clear biomarkers associated with protection and safety. Recent studies in evaluation of vaccines, e.g., influenza and yellow fever vaccines, using systems biology tools demonstrated the power of such strategies in understanding the immunological mechanisms that underpin a protective phenotype. Applying similar tools in isolated human tissues such as PBMCs from healthy individuals infected with live attenuated parasites such as LdCen$^{-/-}$ *in vitro* followed by human microarray hybridization experiments will enable us to understand how early vaccine-induced gene expression profiles and the associated immune responses are coordinately regulated in normal individuals. In addition, comparative analysis of biomarkers in PBMCs from asymptomatic or healed visceral leishmaniasis individuals in response to vaccine candidates including live attenuated parasites may provide clues about determinants of protective immunity and be helpful in shaping the final *Leishmania* vaccine formulation in the clinical trials.

Keywords: *Leishmania*, vaccine, genetically modified organisms, live attenuated parasites, vaccine-induced immunity, systems vaccinology, biomarkers of protection

INTRODUCTION

An estimated 100,000 VL cases are reported annually in the endemic foci of northeastern India, Nepal, and Bangladesh alone and ~150 million people are at risk for infection (1). The current programs for elimination of VL include early diagnosis and treatment, coordinated vector control, and effective disease surveillance through passive and active case detection (2). Vector or reservoir control, toxicity of currently available drugs, and increasing parasite resistance underline the need for an effective prophylactic vaccine against leishmaniasis (3).

Estimates of potential economic value of a prophylactic vaccine indicated that even a vaccine with a relatively short duration of protection and modest efficacy could prevent a substantial number of cases at low-cost. Further, a vaccine providing ~5 years duration of protection with as little as 50% efficacy remains cost–effective compared with chemotherapy (4). Development of a prophylactic vaccine against *Leishmania* has gone through a long trajectory that includes phases of systematic selection of antigens, adjuvants, natural immune parameters to identify correlates of protection [reviewed in Ref. (5)]. Despite these advances, there is no effective vaccine even though vaccine is thought to be feasible. Protection against reinfection following a natural infection with *Leishmania major* historically has been the reason for feasibility of a vaccine (6). Similarly people successfully cured from visceral leishmaniasis

develop *Leishmania* specific Th1-type cellular-mediated responses and protection against new infections (7). The absence of an effective vaccine to a large extent is related to the absence of clear understanding of correlates of protection. Concerns also remain about the ability of current experimental models to predict protection against natural, sandfly transmitted infection (8). A recent survey of past clinical trials with killed *Leishmania* antigens in Central American countries, Iran, and Sudan showed absence of efficacy against developing CL (3). This further underlines the lack of understanding of the immune mechanisms that drive protection not only in human leishmaniasis but in other parasitic diseases as well. This lack of vaccine efficacy owing to lack of knowledge of correlates of protection is even more powerfully illustrated in the recently concluded malaria vaccine trials where only a subpopulation of the vaccines was protected (9). The parameters measured in the vaccine study did not reveal any correlations that could explain the observed differences. In contrast, tremendous progress has been made in the understanding of vaccine-induced immunity in several viral vaccines including AIDS (10), yellow fever (11), and influenza (12) that showed the power of systematic analysis of early immune responses can have immense value in providing a clear understanding of immune mechanisms of protection.

BIOMARKERS OF ASYMPTOMATIC CARRIERS OF VL-IMPLICATIONS TO VACCINE SUCCESS

In the Indian subcontinent, humans are the only reservoir of the parasite *L. donovani*. An estimated 100,000 VL cases are reported annually in the endemic foci of northeastern India, Nepal, and Bangladesh, and in addition a significant number of asymptomatic carriers also occur (13). Various epidemiologic studies have reported that asymptomatic infections outnumber clinical VL cases including in India and Nepal (9:1) and Bangladesh (4:1) (14, 15). Thus, early diagnosis and treatment, and effective disease surveillance through passive and active case detection have been identified as key components of *Leishmania* elimination (16). Importantly, the asymptomatic carriers with no overt signs of disease but could potentially develop active VL could serve as reservoirs of parasites in the endemic areas. In practice, more asymptomatic individuals than those with active VL are identified in high endemicity areas (17). A 2-year longitudinal study in the hyperendemic regions in India showed that seropositivity in direct agglutination test (DAT)- and rK39-based tests at baseline cannot predict development of VL (18). Persistence of antibodies over periods of several years against *L. donovani* further complicates the careful identification of active versus past exposure (19). In order to develop biomarkers of parasitemia DAT, ELISA based on rK39 and whole cell lysate and quantitative PCR tests were employed in a recent clinical study (20). Developing tools that can predict progression to VL disease will lead to early intervention and treatment, but also have implications for future vaccination trials. The comparative diagnostic tests revealed the limited complementarity between serology-based tests and DNA-based diagnostic assays underlining the fact that the presence of *L. donovani* DNA is transient, as was also described in a larger survey of asymptomatic children in Brazil (21). Further, the low predicted parasite burden in the asymptomatic carriers in the study (the median 0.1 *L. donovani* DNA equivalents/milliliter of blood) implied that more robust PCR methods need to be developed to detect low parasite burdens. In a whole blood-based IFN-γ release assay in high VL endemic region in India showed that whole blood from active and cured VL cases can produce antigen-specific IFN-γ (19). Secretion of IFN-γ from active VL cases in whole blood assays strongly suggested that no Th1 response deficit exists but the pathogenesis is indicative of immune suppression. The only discriminating factor between active and cured VL was IL-10 where only active VL cases secreted IL-10 (13). This establishes a pattern of biomarkers that will be helpful in a vaccine trial to identify asymptomatic carriers and also raises important considerations for testing of *in vitro* correlates in PBMCs versus whole blood cultures.

VACCINATION APPROACHES IN *LEISHMANIA*

Heat killed *Leishmania* and recombinant antigens have the longest history of clinical trials against CL in parts of South America including Brazil, Ecuador, and also in Iran and Sudan [reviewed in Ref. (3)]. A majority of these trials used *Leishmania* skin test (LST) as a biomarker for vaccine efficacy. This is due to a strong correlation between LST positivity and protection after recovery from the disease caused by several species (22). A retrospective analysis indicated that reproducible evidence of protective efficacy against CL has not emerged from these clinical trials using heat killed *Leishmania* vaccines. Absence of demonstrable efficacy in most of the randomized controlled trials is consistent with the killed whole parasite preparations being inadequate to produce long lasting, relevant immune responses required for protection. Even though vaccinated groups in some trials showed larger LST, the observed immunogenicity was not translated into protective efficacy against CL. Thus measuring LST as a correlate of vaccine-induced immunity has only limited predictive power. However, conversion from negative LST reaction to LST >5 after vaccination has been observed to be associated with significantly lower infection incidence in Brazil, Iran, and Sudan (3). It must be noted that in several of the heat killed *Leishmania* vaccines, BCG was a common adjuvant and the immune reaction caused by BCG compounded the LST-based interpretation significantly. A meta-analysis further confirmed that LST conversion may be associated with an immune response that can provide some protection by its ability to distinguish a subpopulation of responders to leishmanial antigens or BCG after vaccination even though such response had a huge variability (16–68% conversion rate) in these studies (23).

In early vaccination studies, choice of antigens in majority of vaccine formulations related to CL as well as VL was empirical. Systematic studies to identify potential vaccine antigens against VL were undertaken more recently using proteome serology (24), bioinformatics approaches, and reactivity with serum from active VL cases (25, 26). Previous efforts to identify antigens that showed protective efficacy against *L. donovani* infection in experimental VL models include K26/HASPB (27), A2 (28), kinetoplastid membrane protein-11 (29), nucleoside hydrolase (30), cysteine proteinase B (31), and sterol 24-C-methyltransferase (25, 32). Recently, these antigens were evaluated in peripheral blood obtained from a limited number of cases of healed VL and previously unexposed controls to test if the cytokines released in response to *Leishmania* antigen can reveal markers that could predict efficacy of the six candidates (7). If the selected antigens elicit

cellular immune responses correlating with protection or cure (e.g., IFN-γ production in previously exposed and cured individuals) that may indicate the potential to be good candidates for prophylactic and therapeutic vaccines. Of the cytokines tested IFN-γ, IL-2, IL-4, IL-5, IL-10, and TNF-α, only soluble *Leishmania* antigen-specific IFN-γ in healed VL cases showed significantly higher secretion compared to controls (7). Interestingly, TNF-α secretion was observed along with high IFN-γ secretion in samples that respond to SLA (7) resembling a pattern of multifunctional Th1 cells that correlate with protection observed against *L. major* infection (33). Interestingly, KMP-11 and K26/HASPB did not show Th1-related IFN-γ production in cured VL subjects even though these antigens were selected on the basis of previous studies with experimental animal models that showed a protective role for KMP-11 and K26/HASPB (27, 29). The observed discrepancy in protective efficacy of these antigens in mouse versus human pre-clinical studies could be due to several reasons including their induction of CD8+ T cells, but not CD4+ cells the latter of which have been shown to be the main source of IFN-γ in cured VL (34) and to limitations in the measurement of responses by CD8+ T cells (7). These studies have demonstrated the importance of comparing antigens for their protective efficacy and allow selection for a future vaccine and importantly revealed the dichotomy in the results obtained in the experimental mouse models and human pre-clinical studies.

Previous studies with HASPB and KMP-11 as prophylactic vaccines indicated good protection associated with the development of CD8+ T cell responses in hamsters and isolated human PBMCs with KMP11 (35, 36) and immunogenicity in dogs against HASPB1 antigen (37). When these antigens were tested in a recent study as potential therapeutic vaccines, experiments with mice revealed that route and dose influenced the breadth and magnitude of the observed CD8+ T cell responses (38). For instance, the response to the HASPB C-terminal epitope was twofold greater in the footpad compared to subcutaneous administration (38). Footpad vaccination induced clear and dose-dependent IgG1 and IgG2a responses, a proxy for CD4+ T cell response but in subcutaneous vaccination such response was undetectable suggesting that route of administration as a determinant of the host response (38). Although significant reduction in splenic parasite burdens was observed, the failure to elicit CD8+ T cell responses indicated that HASPB and KMP11 indicated that they might not be dominant antigens in terms of CD8+ T cell recognition as was seen in several antigen interference studies (39). However, the reduction in the parasite burdens in the immunized mice implied that correlation with strong CD4+ T cell responses upon treatment with *Leishmania* soluble antigen may not be a necessary prerequisite as suggested by the human pre-clinical studies with these antigens. In addition, the data suggested an interesting possibility that for an antigen to be effective as a vaccine candidate, it may not have to be the dominant antigen during natural infection (38).

GENETICALLY MODIFIED LIVE ATTENUATED *L. DONOVANI* AS VACCINE CANDIDATES

Though several multi-antigen recombinant protein and DNA vaccines have been and continue to be tested as vaccine candidates, no effective vaccine against VL has been developed so far. In contrast to subunit vaccines, live attenuated parasite vaccines have several advantages in terms of their ability to induce adaptive immune responses relevant to protection by mimicking a natural infection without causing overt disease and likely induce an immune response consistent with protection (40). Early live attenuated *Leishmania* parasites were developed by targeted deletion of dihydrofolate reductase–thymidylate synthase (DHFR-TS) in *L. major* and cysteine proteases cpa and cpb in *L. mexicana* (41, 42). These studies demonstrated the feasibility of using live attenuated parasites for vaccination against CL and provided the rationale for developing and testing several gene deletion mutants in other *Leishmania* species including *L. donovani* subsequently. Further, early progress demonstrated in the vaccination studies in CL using live attenuated parasites led to identification of specific parameters to be evaluated in a discussions sponsored by the WHO (TDR News 2005; http://www.who.int/tdr/publications/documents/tdrnews-issue-75.pdf).

Despite the early progress in CL vaccine studies based on murine models of *L. major* infections, it is well-recognized that the immune mechanisms mediating visceral disease in the liver and spleen caused by *L. donovani* differ significantly from other species of *Leishmania* causing CL and mucocutaneous disease (43, 44). Consequently, vaccine-induced immunity required for protection in CL versus VL is likely to differ in significant ways although cross protection studies indicated common mechanisms of protection (32, 45–49).

Acquisition of protective immunity following leishmanization in cutaneous leishmaniasis (3), development of *Leishmania* specific Th1-type immune response, and protection against new infection in individuals successfully cured from VL (7) and in case of VL a complete *Leishmania* cDNA expression library injected into mice was more protective than subpools of the library plasmids, emphasizing the idea that the whole parasite makes the best vaccine (50). Immunization with live attenuated parasites is likely to deliver several antigens than the limited number possible with subunit or recombinant antigens as revealed by studies in *L. major* (40, 51). Recent success with irradiated *Plasmodium falciparum* sporozoites in inducing strong protection upon intravenous administration further demonstrates the feasibility of using attenuated parasites as vaccine candidates (52). Relative ease of genetic manipulation of genes allowed to produce several gene knock out *L. donovani* parasite strains (5). The live attenuated parasites organisms have the advantage of presenting a complete antigen spectrum like in a natural infection, which may result in a robust immunity unlike subunit vaccines that are limited in antigenic repertoire. The attenuated parasites persist in the host for a limited period of time providing the immune system persistent antigens that allowing for the generation of antigen-specific memory cells that are important for providing a protective response following subsequent infection. Several targeted gene deletions have been carried out to develop *Leishmania*-attenuated vaccine strains. Similar to CL studies, protection against virulent challenge was reported in experimental mouse immunization with *L. donovani* deleted for biopterin transporter (53) A2–rel gene cluster in *L. donovani* (54) SIR2 gene in *L. infantum* (55) and Hsp70-II (56) as immunogens induced protection against virulent challenge

Table 1 | Genetically modified live attenuated vaccine candidates in *L. donovani* complex.

Target of deletion	Animal model	Results of immunization	Reference
Biopterin transporter, LdBT1$^{-/-}$	Balb/C	Protective immunity, antigen-specific IFN-γ secretion	Papadopoulou et al. (53)
Centrin 1, LdCen$^{-/-}$	Balb/C mice, hamsters, dogs	Protective immunity against *L. donovani* and *L. braziliensis* challenge. Increased IFNγ, IL-2, and TNF producing cells and IFNγ/IL-10 ratio, presence of multifunctional cells	Selvapandiyan et al. (48), Fiuza et al. (58)
Silent information regulatory two single allele deletion, LiSIR2±	Balb/C	Protective immunity, increased antigen-specific IFNγ/IL-10 ratio	Silvestre et al. (55)
Cytochrome *c* oxidase complex component p27, Ldp27$^{-/-}$	Balb/C	12-Week survival in host, protective immunity against *L. donovani*, *L. braziliensis*, and *L. major* challenge. Increased IFNγ, IL-2, and TNF producing cells and IFNγ/IL-10 ratio	Dey et al. (49)
Heat shock protein 70, LiHsp70$^{-/-}$	Balb/C	Cross protection against *L. major*, high IgG2a relative to IgG1	Carrion et al. (56)

in BALB/c mice (**Table 1**). These experiments demonstrated the potential of generating live attenuated vaccines by targeted gene disruptions. Our laboratory has developed a *L. donovani* mutant (*LdCen*$^{-/-}$) deleted for the centrin gene. Centrin is a growth regulating gene in the protozoan parasites *Leishmania*. *LdCen*$^{-/-}$ is specifically attenuated at the amastigote stage and not as the promastigote (57). The mutant *Leishmania* amastigotes showed cytokinesis arrest in the cell cycle and persisted for a short duration in animals (mice and hamsters) or *ex vivo* in human macrophages and were eventually cleared (48). Mouse immunization experiments revealed that *LdCen*$^{-/-}$ can protect mice against virulent challenge and this protection was accompanied by the induction of robust *Leishmania* specific multifunctional T cell responses (48) as was reported in previous studies that showed strong protection against an *L. major* challenge (33). Immunization experiments also revealed that intrinsic growth defect of *LdCen*$^{-/-}$ amastigotes allows for limited replication in the mouse as was revealed by their clearance after a limited period. Immunization experiments in dogs with *LdCen*$^{-/-}$ revealed that these attenuated parasites can be inducing potent immunogenicity and early indication of protection against virulent challenge (58). Similarly, we developed *L. donovani* mutant (*Ldp27*$^{-/-}$) deleted for the *p27* gene, an essential component of cytochrome *c* oxidase complex involved in oxidative phosphorylation (59). Similar to *LdCen*$^{-/-}$, *Ldp27*$^{-/-}$ parasites also induced *Leishmania*-specific multifunctional T cell responses in mice and showed strong potential as a candidate vaccine (49). We have also developed *L. donovani* mutants deleted for a ubiquitin-like protein in *Leishmania* (*LdUfm1*$^{-/-}$) and the processing enzyme Ufsp (*LdUfsp*$^{-/-}$) that converts the precursor Ufm1 into its conjugatable form (60, 61). *L. donovani* Ufm1 conjugates to and modifies the enzymes involved in β-oxidation of fatty acids. Since the Ufm1 protein is part of a pathway involving activities of several proteins, it is possible to create additional deletion mutants deficient in multiple genes without causing additive loss of virulence thus enhancing the safety of the live attenuated parasites. Together, these examples of genetically altered parasites provide opportunities for testing as live attenuated vaccine candidates in pre-clinical and clinical conditions. Despite the advantages of live attenuated parasites as vaccines, there are considerable challenges. A major concern with live attenuated vaccines is the risk of reversion to a virulent parasite. Hence, biomarkers of safety are essential to assess the genetic and physiological traits of the organism to assess stability of the attenuated parasites in addition to biomarkers of efficacy that are just as important to any kind of anti-*Leishmania* vaccine. Several issues regarding the live attenuated vaccines must be overcome including safety, genetic stability, lack of transmissibility, limited persistence, and conditions of cryopreservation in order for these vaccines to be used in human trials (62). In addition, growing the attenuated organisms in media containing serum of bovine origin constitutes a possible safety risk due to BSE-related hazard. Our recent studies have shown that the live attenuated *L. donovani* parasites can be grown in serum free media (63). The presence of antibiotic markers in the live attenuated organisms can be potential safety concern. Recent clinical trials for treating lung cancer and glioblastoma with tumor cell vaccines or retroviral vectors containing Neo^{r} and Hyg^{r} markers that confer resistance against neomycin and hygromycin suggest that these markers may be permissible in clinical trials (64, 65). However, recent developments in genome engineering methods including use of zinc finger nucleases, transcription activator-like effector nucleases might be pertinent for developing marker free live attenuated *Leishmania* parasites as was the use of Tn5 transposon or thymidine kinase-based antibiotic marker removal (66–69). The ability to create marker free attenuated mutants might also be helpful in creating *Leishmania* parasites lacking multiple genes thus limiting the probability of reversion to virulence due to mutations in secondary loci as observed in lpg2$^{-/-}$ in *L. major* (70). Similarly, infection experiments under immunosuppressive conditions to show limited persistence and sand fly infections post-immunization to show lack of transmissibility might be necessary for demonstrating the safety of live attenuated *Leishmania* parasites. Our preliminary experiments to investigate transmissibility by sand flies have shown that the live attenuated *L. donovani* parasites (*LdCen*$^{-/-}$ and *Ldp27*$^{-/-}$) do not establish an infection in sand flies (Dey et al., unpublished).

IMMUNOLOGY OF HUMAN VL

The immunopathology of VL has been extensively studied in experimental mouse model, dog model, and to a limited extent in humans. It has long been established that protection against

Leishmania is derived predominantly from T cell-mediated immunity with both Th1- and Th2-associated cytokines contributing to vaccine-induced resistance (71). Even though the correlates of immunity to human VL are not fully understood, the protection requires antigen-specific CD+4 and CD+8 T cell responses (72). PBMCs from active VL patients typically do not proliferate or produce IFN-γ in response to *Leishmania* antigen where as PBMCs from cured VL cases do proliferate [(73), Avishek et al., unpublished data]. Recent studies have clarified that human VL is not associated with Th2-biased responses dominated by IL-4 and or IL-13 but produce elevated serum IL-1, IL-6, IL-8, IL-12, IL-15, IP-10, IFN-γ, and TNFα (74, 75). The role of IL-10 in promoting pathology of VL has long been demonstrated in human studies. This is primarily accomplished by turning macrophages unresponsive to activation signals. DCs are important for initiating immunity and further modifying the immune response. Secretion of cytokines by DCs strongly influences the outcome of T cell responses. Stimulation of DCs to produce IL-12 drives creation of antigen-specific IFN-γ secreting Th1-type CD4 and CD8 cells that aid in resolution of infection (76). Several human studies indicated that IFN-γ secretion is the biomarker for protection even though both VL and CL can progress despite the presence of a Th1-type of response. IL-10 expression is the only other marker that has been consistently shown to negatively correlate with the protection. Elevated serum levels of IL-10, IL-6, and IL-8 in serum have been shown to be associated with active disease (73).

Poor understanding of the mechanism of immune protection has thus impaired development of effective vaccine against human VL so far. This is in many respects paralleled by attempts at developing a vaccine against malaria. More recently, results from the clinical trials with RTS,S/AS01 in the infants have underlined the importance of biomarkers of efficacy. Efficacy of RTS,S/AS01 was only 26% against sever malaria (9). Absence of knowledge about the nature of adaptive immune responses in certain protected population in malaria endemic areas and whether such immunological correlates of protection can be mimicked by vaccination still remain unanswered. The protection observed only in small fraction of infants suggests that it is important to determine which vaccine elicited responses are necessary for such protection (77). Since typical clinical trials can last for several years, absence of knowledge on biomarkers of protection can considerably impede the progress of vaccine development. This is reflected by the limited success rate (22%) observed in vaccine development (78). Comprehensive analysis of antibodies, cytokines, immune cells, and whole-genome transcription to identify key host responses associated with an effective protection would be necessary to reveal those answers.

SYSTEMS BIOLOGICAL APPROACHES IN VACCINE-INDUCED IMMUNITY

Majority of the currently licensed vaccines have mostly developed empirically, and protection by these vaccines is generally conferred by antigen-specific antibodies, which prevent infection. Many viral vaccines, such as the live attenuated vaccines, work by mimicking pathogens, to stimulate lasting and protective immunity in the host (79). Only recently, the immune mechanisms underlying the protection are beginning to be understood in the otherwise empirically developed viral vaccines. Recent technological advances in molecular genetics, molecular and cellular immunology, structural biology, bioinformatics, computational biology, nanotechnology, formulation technologies, and systems biology have facilitated new developments in antigen discovery/design, adjuvant discovery, and immune monitoring that offer substantial potential for discovering new biomarkers of protective immunity, and identify the limitations of animal models for screening and prioritizing human vaccines (80). Recent progress in understanding how the innate immune system recognizes microbial stimuli and regulates adaptive immunity is being applied to vaccine discovery in what is termed "systems vaccinology" (81). Systems vaccinology is an offshoot of systems biology for which tools of a number of high-throughput technologies including DNA microarrays, RNA-seq, protein arrays, deep sequencing, mass spectrometry along with sophisticated computational tools have been originally developed (81). Together, these tools enable system-wide unbiased molecular measurements, which can then be used to reconstruct the perturbations in the immune networks (82).

The tools of systems biology were first applied for the highly effective yellow fever vaccine YF-17D, which is a live attenuated virus. Yellow fever vaccine has been one of the most successful vaccines known. Past immunological studies have revealed that YF-17D induces neutralizing antibodies, cytotoxic T cells and Th1/Th2 cells, and signal via Toll-like receptors 2, 7, 8, and 9 on subsets of dendritic cells (83). Querec et al. (11) and Gaucher et al. (84) first applied systems biological approaches in evaluating immune responses to vaccination to discern new and fundamental insights about the workings of the immune system by detailed study of innate and adaptive immune responses to vaccination (Yellow Fever Vaccine) in humans. When the PBMCs from the vaccinated and control groups were tested in human microarray hybridization experiments, results revealed that genes of the antiviral type I interferon pathway, complement pathway, and inflammasome were induced 3–7 days after vaccination, concomitant with the development of the adaptive immune response. In addition, a broad spectrum of gene regulations for innate sensing, including TLR7, RIG-I, MDA5, and LGP2, and for innate signaling, including JUN, STAT1, IRF7, and RNF36, was also observed. Analysis of immune networks revealed that it is possible to predict titers of neutralizing antibodies by the early gene expression signatures.

Later studies with seasonal influenza model using systems biology approaches compared immune responses to a live attenuated virus vaccine, or a trivalent-inactivated vaccine (12). This comparison revealed important differences between the live attenuated versus trivalent-inactivated vaccines. The live attenuated vaccine induced the expression of several interferon-related genes, common to live viral vaccines where as, the inactivated vaccine, induced genes highly expressed in plasma B cells. Such comparative analysis of gene expression in human microarrays in the first week of vaccination allowed prediction of antibody responses in the vaccines based on the expression of markers such as the receptor gene for B cell growth factor BLyS (12). This clearly demonstrated that analysis of early transcriptomic profiles from the peripheral blood

can be used in identifying novel immune networks and also predict vaccine efficacy.

The gene expression profiles once obtained can also be utilized to identify if the correlates of protection are universally applicable. For instance, the metabolic changes of the aging body, including the increased presence of apoptotic cells and of oxidative stress induce the immune system to change its "quiescent" state to a different, often higher level of basal activation [reviewed in Ref. (85)]. Similarly, the role of sex hormones in affecting the vaccine-induced immunity is not often studied. A meta-analysis of the yellow fever vaccine study revealed substantially higher expression signatures in the toll-like receptor–interferon signaling in women compared to men (86). Similar studies with influenza vaccine revealed an immunosuppressive role for testosterone in influenza vaccination. Unexpectedly, this immunosuppression was linked to genes that participate in lipid metabolism including leukotriene A4 hydrolase, revealing the power of systems analysis (87). The main appeal of using systems biology approaches to examine vaccine-induced immunity is the ability to study early responses in peripheral blood by comparing genes and pathways induced before/after immunization. This paradigm can be applied to compare efficacy of different vaccine formulations including live attenuated parasite vaccines, recombinant antigen vaccines if knowledge of biomarkers of protection is available.

Since immune cells migrate through the blood stream between lymph nodes, spleen, and peripheral tissues obtaining meaningful information of an immune response from this dynamic mix of cells remains a complex challenge. Several attempts have been made at decomposing the gene expression profile from a heterogeneous mix of cells into that of respective cell types based on unique gene expression profiles independently obtained from a pure cell population such as monocytes, lymphocytes, neutrophils, eosinophils, and basophils (88).

With the development of genomic and proteomic tools along with advances in computational approaches, global profiling of cellular states in terms of gene and protein expression has been applied to study a broad range of immunological phenomena. These approaches have been used to study T cell activation signatures (89, 90), blood cell states in patients with autoimmunity (91), and the responses of host cells to HCV infection (92).

Even though the approaches outlined here can provide fundamental insights into the immune responses to complex pathogens, it must be noted that so far only viral vaccines were studied. In most such cases, protection is mediated by antibodies whereas clearance of parasitic pathogens needs predominantly cell-mediated immunity. The role of antibody responses in protection is at best inconclusive in human leishmaniasis (73). In addition, it is conceivable that parasites engage with human immunity in multiple levels as is evidenced by immune suppression in humans with active VL. Computational approaches developed for the analysis of innate immunity point to the possibility of such analysis in human VL (93).

CONCLUSION

A major shortcoming in the vaccine development is the inadequate predictive power of the early immune responses mounted in the host against the vaccines. Also, for the live attenuated parasite vaccines, the primary barrier against widespread use remains safe in terms of avirulence of the parasites in host. Therefore, understanding of the pathogenesis of live attenuated parasites such as *LdCen*$^{-/-}$ in human PBMC in different clinical groups will provide valuable information regarding avirulence of these parasites and efficacy in terms of immune- and non-immune-related responses prior to the evaluation in human trials. Recent influenza vaccine studies substantiated that the role of non-immune parameters in protective immunity is generally under appreciated and often missed in conventional vaccine studies that measure only immunological parameters. Obtaining such information via systems biology approaches, as has been applied to study viral vaccines, will enable us to understand how vaccine-induced responses are coordinately regulated in healthy, asymptomatic infected individuals, and individuals recovered from VL. This will provide information regarding correlates of protection as well as biomarkers of safety and enable identification of immune and non-immune predictors hitherto unidentified to *Leishmania* antigens or live attenuated vaccine candidates in the human cells and might be useful in shaping the final vaccine formulation in the clinical trials. Studies comparing expression profiles of PBMCs from bonafide asymptomatic carriers with those that acquired protective immunity following clinical cure will likely provide biomarkers other than the IFN-γ secretion as is currently practiced. Understanding of immune modulators that confer protection in VL can lead to new targets for immune therapy.

REFERENCES

1. Sundar S, Chakravarty J. Recent advances in the diagnosis and treatment of kala-azar. *Natl Med J India* (2012) **25**:85–9.
2. Matlashewski G, Das VN, Pandey K, Singh D, Das S, Ghosh AK, et al. Diagnosis of visceral leishmaniasis in Bihar India: comparison of the rK39 rapid diagnostic test on whole blood versus serum. *PLoS Negl Trop Dis* (2013) **7**:e2233. doi:10.1371/journal.pntd.0002233
3. Noazin S, Modabber F, Khamesipour A, Smith PG, Moulton LH, Nasseri K, et al. First generation leishmaniasis vaccines: a review of field efficacy trials. *Vaccine* (2008) **26**:6759–67. doi:10.1016/j.vaccine.2008.09.085
4. Bacon KM, Hotez PJ, Kruchten SD, Kamhawi S, Bottazzi ME, Valenzuela JG, et al. The potential economic value of a cutaneous leishmaniasis vaccine in seven endemic countries in the Americas. *Vaccine* (2013) **31**:480–6. doi:10.1016/j.vaccine.2012.11.032
5. Selvapandiyan A, Dey R, Gannavaram S, Lakhal-Naouar I, Duncan R, Salotra P, et al. Immunity to visceral leishmaniasis using genetically defined live-attenuated parasites. *J Trop Med* (2012) **2012**:631460. doi:10.1155/2012/631460
6. Khamesipour A, Dowlati Y, Asilian A, Hashemi-Fesharki R, Javadi A, Noazin S, et al. Leishmanization: use of an old method for evaluation of candidate vaccines against leishmaniasis. *Vaccine* (2005) **23**:3642–8. doi:10.1016/j.vaccine.2005.02.015
7. Kumar R, Goto Y, Gidwani K, Cowgill KD, Sundar S, Reed SG. Evaluation of ex vivo human immune response against candidate antigens for a visceral leishmaniasis vaccine. *Am J Trop Med Hyg* (2010) **82**:808–13. doi:10.4269/ajtmh.2010.09-0341
8. Costa CH, Peters NC, Maruyama SR, de Brito EC Jr, Santos IK. Vaccines for the leishmaniases: proposals for a research agenda. *PLoS Negl Trop Dis* (2011) **5**:e943. doi:10.1371/journal.pntd.0000943
9. Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BG, Kabwende AL, et al. A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. *N Engl J Med* (2012) **367**:2284–95. doi:10.1056/NEJMoa1208394
10. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med* (2012) **366**:1275–86. doi:10.1056/NEJMoa1113425

11. Querec TD, Akondy RS, Lee EK, Cao W, Nakaya HI, Teuwen D, et al. Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. *Nat Immunol* (2009) **10**:116–25. doi:10.1038/ni.1688
12. Nakaya HI, Wrammert J, Lee EK, Racioppi L, Marie-Kunze S, Haining WN, et al. Systems biology of vaccination for seasonal influenza in humans. *Nat Immunol* (2011) **12**:786–95. doi:10.1038/ni.2067
13. Singh OP, Gidwani K, Kumar R, Nylen S, Jones SL, Boelaert M, et al. Reassessment of immune correlates in human visceral leishmaniasis as defined by cytokine release in whole blood. *Clin Vaccine Immunol* (2012) **19**:961–6. doi:10.1128/CVI.00143-12
14. Bern C, Haque R, Chowdhury R, Ali M, Kurkjian KM, Vaz L, et al. The epidemiology of visceral leishmaniasis and asymptomatic leishmanial infection in a highly endemic Bangladeshi village. *Am J Trop Med Hyg* (2007) **76**:909–14.
15. Das VN, Siddiqui NA, Verma RB, Topno RK, Singh D, Das S, et al. Asymptomatic infection of visceral leishmaniasis in hyperendemic areas of Vaishali district, Bihar, India: a challenge to kala-azar elimination programmes. *Trans R Soc Trop Med Hyg* (2011) **105**:661–6. doi:10.1016/j.trstmh.2011.08.005
16. Matlashewski G, Arana B, Kroeger A, Battacharya S, Sundar S, Das P, et al. Visceral leishmaniasis: elimination with existing interventions. *Lancet Infect Dis* (2011) **11**:322–5. doi:10.1016/S1473-3099(10)70320-0
17. Ostyn B, Gidwani K, Khanal B, Picado A, Chappuis F, Singh SP, et al. Incidence of symptomatic and asymptomatic *Leishmania donovani* infections in high-endemic foci in India and Nepal: a prospective study. *PLoS Negl Trop Dis* (2011) **5**:e1284. doi:10.1371/journal.pntd.0001284
18. Gidwani K, Rai M, Chakravarty J, Boelaert M, Sundar S. Evaluation of leishmanin skin test in Indian visceral leishmaniasis. *Am J Trop Med Hyg* (2009) **80**:566–7.
19. Gidwani K, Picado A, Rijal S, Singh SP, Roy L, Volfova V, et al. Serological markers of sand fly exposure to evaluate insecticidal nets against visceral leishmaniasis in India and Nepal: a cluster-randomized trial. *PLoS Negl Trop Dis* (2011) **5**:e1296. doi:10.1371/journal.pntd.0001296
20. Vallur AC, Duthie MS, Reinhart C, Tutterrow Y, Hamano S, Bhaskar KR, et al. Biomarkers for intracellular pathogens: establishing tools as vaccine and therapeutic endpoints for visceral leishmaniasis. *Clin Microbiol Infect* (2013). doi:10.1111/1469-0691.12421
21. dos Santos Marques LH, Gomes LI, da Rocha IC, da Silva TA, Oliveira E, Morais MH, et al. Low parasite load estimated by qPCR in a cohort of children living in urban area endemic for visceral leishmaniasis in Brazil. *PLoS Negl Trop Dis* (2012) **6**:e1955. doi:10.1371/journal.pntd.0001955
22. Ben Salah A, Louzir H, Chlif S, Mokni M, Zaatour A, Raouene M, et al. The predictive validity of naturally acquired delayed-type hypersensitivity to leishmanin in resistance to *Leishmania major*-associated cutaneous leishmaniasis. *J Infect Dis* (2005) **192**:1981–7. doi:10.1086/498042
23. Noazin S, Khamesipour A, Moulton LH, Tanner M, Nasseri K, Modabber F, et al. Efficacy of killed whole-parasite vaccines in the prevention of leishmaniasis: a meta-analysis. *Vaccine* (2009) **27**:4747–53. doi:10.1016/j.vaccine.2009.05.084
24. Forgber M, Basu R, Roychoudhury K, Theinert S, Roy S, Sundar S, et al. Mapping the antigenicity of the parasites in *Leishmania donovani* infection by proteome serology. *PLoS One* (2006) **1**:e40. doi:10.1371/journal.pone.0000040
25. Goto Y, Coler RN, Reed SG. Bioinformatic identification of tandem repeat antigens of the *Leishmania donovani* complex. *Infect Immun* (2007) **75**:846–51. doi:10.1128/IAI.01205-06
26. Goto Y, Bhatia A, Raman VS, Liang H, Mohamath R, Picone AF, et al. KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. *Clin Vaccine Immunol* (2011) **18**:1118–24. doi:10.1128/CVI.05024-11
27. Stager S, Smith DF, Kaye PM. Immunization with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. *J Immunol* (2000) **165**:7064–71. doi:10.4049/jimmunol.165.12.7064
28. Ghosh A, Labrecque S, Matlashewski G. Protection against *Leishmania donovani* infection by DNA vaccination: increased DNA vaccination efficiency through inhibiting the cellular p53 response. *Vaccine* (2001) **19**:3169–78. doi:10.1016/S0264-410X(01)00023-8
29. Basu R, Bhaumik S, Basu JM, Naskar K, De T, Roy S. Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-sensitive and -resistant strains of *Leishmania donovani* that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. *J Immunol* (2005) **174**:7160–71.
30. Aguilar-Be I, da Silva Zardo R, Paraguai de Souza E, Borja-Cabrera GP, Rosado-Vallado M, et al. Cross-protective efficacy of a prophylactic *Leishmania donovani* DNA vaccine against visceral and cutaneous murine leishmaniasis. *Infect Immun* (2005) **73**:812–9. doi:10.1128/IAI.73.2.812-819.2005
31. Rafati S, Zahedifard F, Nazgouee F. Prime-boost vaccination using cysteine proteinases type I and II of *Leishmania infantum* confers protective immunity in murine visceral leishmaniasis. *Vaccine* (2006) **24**:2169–75. doi:10.1016/j.vaccine.2005.11.011
32. Goto Y, Bhatia A, Raman VS, Vidal SE, Bertholet S, Coler RN, et al. *Leishmania infantum* sterol 24-C-methyltransferase formulated with MPL-SE induces cross-protection against *L. major* infection. *Vaccine* (2009) **27**:2884–90. doi:10.1016/j.vaccine.2009.02.079
33. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat Med* (2007) **13**:843–50. doi:10.1038/nm1592
34. Kemp K, Kemp M, Kharazmi A, Ismail A, Kurtzhals JA, Hviid L, et al. *Leishmania*-specific T cells expressing interferon-gamma (IFN-gamma) and IL-10 upon activation are expanded in individuals cured of visceral leishmaniasis. *Clin Exp Immunol* (1999) **116**:500–4. doi:10.1046/j.1365-2249.1999.00918.x
35. de Carvalho LP, Soto M, Jeronimo S, Dondji B, Bacellar O, Luz V, et al. Characterization of the immune response to *Leishmania infantum* recombinant antigens. *Microbes Infect* (2003) **5**:7–12. doi:10.1016/S1286-4579(02)00051-5
36. da Silva RA, Tavares NM, Costa D, Pitombo M, Barbosa L, Fukutani K, et al. DNA vaccination with KMP11 and *Lutzomyia longipalpis* salivary protein protects hamsters against visceral leishmaniasis. *Acta Trop* (2011) **120**:185–90. doi:10.1016/j.actatropica.2011.08.007
37. Moreno J, Nieto J, Masina S, Canavate C, Cruz I, Chicharro C, et al. Immunization with H1, HASPB1 and MML *Leishmania* proteins in a vaccine trial against experimental canine leishmaniasis. *Vaccine* (2007) **25**:5290–300. doi:10.1016/j.vaccine.2007.05.010
38. Maroof A, Brown N, Smith B, Hodgkinson MR, Maxwell A, Losch FO, et al. Therapeutic vaccination with recombinant adenovirus reduces splenic parasite burden in experimental visceral leishmaniasis. *J Infect Dis* (2012) **205**:853–63. doi:10.1093/infdis/jir842
39. Kedl RM, Kappler JW, Marrack P. Epitope dominance, competition and T cell affinity maturation. *Curr Opin Immunol* (2003) **15**:120–7. doi:10.1016/S0952-7915(02)00009-2
40. Handman E. Leishmaniasis: current status of vaccine development. *Clin Microbiol Rev* (2001) **14**:229–43.
41. Titus RG, Gueiros-Filho FJ, de Freitas LA, Beverley SM. Development of a safe live Leishmania vaccine line by gene replacement. *Proc Natl Acad Sci U S A* (1995) **92**:10267–71.
42. Alexander J, Coombs GH, Mottram JC. Leishmania mexicana cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. *J Immunol* (1998) **161**:6794–801.
43. McMahon-Pratt D, Alexander J. Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? *Immunol Rev* (2004) **201**:206–24. doi:10.1111/j.0105-2896.2004.00190.x
44. Soong L, Henard CA, Melby PC. Immunopathogenesis of non-healing American cutaneous leishmaniasis and progressive visceral leishmaniasis. *Semin Immunopathol* (2012) **34**:735–51. doi:10.1007/s00281-012-0350-8
45. Porrozzi R, Teva A, Amaral VF, Santos da Costa MV, Grimaldi G Jr. Cross-immunity experiments between different species or strains of *Leishmania* in rhesus macaques (*Macaca mulatta*). *Am J Trop Med Hyg* (2004) **71**:297–305.
46. Rachamim N, Jaffe CL. Pure protein from *Leishmania donovani* protects mice against both cutaneous and visceral leishmaniasis. *J Immunol* (1993) **150**:2322–31.
47. Tonui WK, Titus RG. Cross-protection against Leishmania donovani but not L. Braziliensis caused by vaccination with L. Major soluble promastigote exogenous antigens in BALB/c mice. *Am J Trop Med Hyg* (2007) **76**:579–84.
48. Selvapandiyan A, Dey R, Nylen S, Duncan R, Sacks D, Nakhasi HL. Intracellular replication-deficient *Leishmania donovani* induces long lasting protective immunity against visceral leishmaniasis. *J Immunol* (2009) **183**:1813–20. doi:10.4049/jimmunol.0900276
49. Dey R, Dagur PK, Selvapandiyan A, McCoy JP, Salotra P, Duncan R, et al. Live attenuated *Leishmania donovani* p27 gene knockout parasites are nonpathogenic

and elicit long-term protective immunity in BALB/c mice. *J Immunol* (2013) **190**:2138–49. doi:10.4049/jimmunol.1202801
50. Melby PC, Ogden GB, Flores HA, Zhao W, Geldmacher C, Biediger NM, et al. Identification of vaccine candidates for experimental visceral leishmaniasis by immunization with sequential fractions of a cDNA expression library. *Infect Immun* (2000) **68**:5595–602. doi:10.1128/IAI.68.10.5595-5602.2000
51. Rivier D, Bovay P, Shah R, Didisheim S, Mauel J. Vaccination against *Leishmania major* in a CBA mouse model of infection: role of adjuvants and mechanism of protection. *Parasite Immunol* (1999) **21**:461–73. doi:10.1046/j.1365-3024.1999.00244.x
52. Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, et al. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. *Science* (2013) **341**:1359–65. doi:10.1126/science.1241800
53. Papadopoulou B, Roy G, Breton M, Kundig C, Dumas C, Fillion I, et al. Reduced infectivity of a *Leishmania donovani* biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. *Infect Immun* (2002) **70**:62–8. doi:10.1128/IAI.70.1.62-68.2002
54. Zhang WW, Matlashewski G. Characterization of the A2-A2rel gene cluster in *Leishmania donovani*: involvement of A2 in visceralization during infection. *Mol Microbiol* (2001) **39**:935–48. doi:10.1046/j.1365-2958.2001.02286.x
55. Silvestre R, Cordeiro-Da-Silva A, Santarem N, Vergnes B, Sereno D, Ouaissi A. SIR2-deficient *Leishmania infantum* induces a defined IFN-gamma/IL-10 pattern that correlates with protection. *J Immunol* (2007) **179**:3161–70. doi:10.4049/jimmunol.179.5.3161
56. Carrion J, Folgueira C, Soto M, Fresno M, Requena JM. *Leishmania infantum* HSP70-II null mutant as candidate vaccine against leishmaniasis: a preliminary evaluation. *Parasit Vectors* (2011) **4**:150. doi:10.1186/1756-3305-4-150
57. Selvapandiyan A, Debrabant A, Duncan R, Muller J, Salotra P, Sreenivas G, et al. Centrin gene disruption impairs stage-specific basal body duplication and cell cycle progression in *Leishmania*. *J Biol Chem* (2004) **279**:25703–10. doi:10.1074/jbc.M402794200
58. Fiuza JA, Santiago Hda C, Selvapandiyan A, Gannavaram S, Ricci ND, Bueno LL, et al. Induction of immunogenicity by live attenuated *Leishmania donovani* centrin deleted parasites in dogs. *Vaccine* (2013) **31**:1785–92. doi:10.1016/j.vaccine.2013.01.048
59. Dey R, Meneses C, Salotra P, Kamhawi S, Nakhasi HL, Duncan R. Characterization of a *Leishmania* stage-specific mitochondrial membrane protein that enhances the activity of cytochrome *c* oxidase and its role in virulence. *Mol Microbiol* (2010) **77**:399–414. doi:10.1111/j.1365-2958.2010.07214.x
60. Gannavaram S, Connelly PS, Daniels MP, Duncan R, Salotra P, Nakhasi HL. Deletion of mitochondrial associated ubiquitin fold modifier protein Ufm1 in *Leishmania donovani* results in loss of beta-oxidation of fatty acids and blocks cell division in the amastigote stage. *Mol Microbiol* (2012) **86**:187–98. doi:10.1111/j.1365-2958.2012.08183.x
61. Gannavaram S, Davey S, Lakhal-Naouar I, Duncan R, Nakhasi HL. Deletion of ubiquitin fold modifier protein Ufm1 processing peptidase Ufsp in *L. donovani* abolishes Ufm1 processing and alters pathogenesis. *PLoS Negl Trop Dis* (2014) **8**(2):e2707. doi:10.1371/journal.pntd.0002707
62. TDR News. Live attenuated *Leishmania* vaccines. (2005) **75**:6.
63. Dey R, Gannavaram S, Selvapandiyan A, Fiuza J, Duncan R, Nakhasi HL. Current status and future challenges for the development of genetically altered live attenuated Leishmania vaccines. In: Satoskar A, Durvasula R, editors. *Pathogenesis of Leishmaniasis: New Developments in Research*. New York: Springer Science+Business Media (2014). p. 45–66. doi:10.1007/978-1-4614-9108-8
64. Ram Z, Culver KW, Oshiro EM, Viola JJ, DeVroom HL, Otto E, et al. Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* (1997) **3**:1354–61. doi:10.1038/nm1297-1354
65. Nemunaitis J, Dillman RO, Schwarzenberger PO, Senzer N, Cunningham C, Cutler J, et al. Phase II study of belagenpumatucel-L, a transforming growth factor beta-2 antisense gene-modified allogeneic tumor cell vaccine in non-small-cell lung cancer. *J Clin Oncol* (2006) **24**:4721–30. doi:10.1200/JCO.2005.05.5335
66. Gaj T, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* (2013) **31**:397–405. doi:10.1016/j.tibtech.2013.04.004
67. Denise H, Coombs GH, Mottram JC. Generation of *Leishmania* mutants lacking antibiotic resistance genes using a versatile hit-and-run targeting strategy. *FEMS Microbiol Lett* (2004) **235**:89–94. doi:10.1111/j.1574-6968.2004.tb09571.x
68. Laurentino EC, Ruiz JC, Brito LO, Fiandt M, Nicoletti LM, Jamur MC, et al. The use of Tn5 transposable elements in a gene trapping strategy for the protozoan *Leishmania*. *Int J Parasitol* (2007) **37**:735–42. doi:10.1016/j.ijpara.2006.12.021
69. Mureev S, Kushnir S, Kolesnikov AA, Breitling R, Alexandrov K. Construction and analysis of *Leishmania* tarentolae transgenic strains free of selection markers. *Mol Biochem Parasitol* (2007) **155**:71–83. doi:10.1016/j.molbiopara.2007.05.007
70. Späth GF, Lye LF, Segawa H, Turco SJ, Beverley SM. Identification of a compensatory mutant (lpg2-REV) of *Leishmania major* able to survive as amastigotes within macrophages without LPG2-dependent glycoconjugates and its significance to virulence and immunization strategies. *Infect Immun* (2004) **72**:3622–7. doi:10.1128/IAI.72.6.3622-3627.2004
71. Kaye PM, Curry AJ, Blackwell JM. Differential production of Th1- and Th2-derived cytokines does not determine the genetically controlled or vaccine-induced rate of cure in murine visceral leishmaniasis. *J Immunol* (1991) **146**:2763–70.
72. Kaye P, Scott P. Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol* (2011) **9**:604–15. doi:10.1038/nrmicro2608
73. Kumar R, Nylen S. Immunobiology of visceral leishmaniasis. *Front Immunol* (2012) **3**:251. doi:10.3389/fimmu.2012.00251
74. Ansari NA, Saluja S, Salotra P. Elevated levels of interferon-gamma, interleukin-10, and interleukin-6 during active disease in Indian kala azar. *Clin Immunol* (2006) **119**:339–45. doi:10.1016/j.clim.2006.01.017
75. Nylen S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D. Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. *J Exp Med* (2007) **204**:805–17. doi:10.1084/jem.20061141
76. Nylen S, Akuffo H. Tracing immunity to human leishmaniasis. *Future Microbiol* (2009) **4**:241–54. doi:10.2217/17460913.4.2.241
77. Daily JP. Malaria vaccine trials – beyond efficacy end points. *N Engl J Med* (2012) **367**:2349–51. doi:10.1056/NEJMe1213392
78. Struck MM. Vaccine R&D success rates and development times. *Nat Biotechnol* (1996) **14**:591–3. doi:10.1038/nbt0596-591
79. Pulendran B. Learning immunology from the yellow fever vaccine: innate immunity to systems vaccinology. *Nat Rev Immunol* (2009) **9**:741–7. doi:10.1038/nri2629
80. Koff WC, Burton DR, Johnson PR, Walker BD, King CR, Nabel GJ, et al. Accelerating next-generation vaccine development for global disease prevention. *Science* (2013) **340**:1232910. doi:10.1126/science.1232910
81. Pulendran B, Li S, Nakaya HI. Systems vaccinology. *Immunity* (2010) **33**:516–29. doi:10.1016/j.immuni.2010.10.006
82. Germain RN, Meier-Schellersheim M, Nita-Lazar A, Fraser ID. Systems biology in immunology: a computational modeling perspective. *Annu Rev Immunol* (2011) **29**:527–85. doi:10.1146/annurev-immunol-030409-101317
83. Li S, Nakaya HI, Kazmin DA, Oh JZ, Pulendran B. Systems biological approaches to measure and understand vaccine immunity in humans. *Semin Immunol* (2013) **25**:209–18. doi:10.1016/j.smim.2013.05.003
84. Gaucher D, Therrien R, Kettaf N, Angermann BR, Boucher G, Filali-Mouhim A, et al. Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. *J Exp Med* (2008) **205**:3119–31. doi:10.1084/jem.20082292
85. Boraschi D, Aguado MT, Dutel C, Goronzy J, Louis J, Grubeck-Loebenstein B, et al. The gracefully aging immune system. *Sci Transl Med* (2013) **5**:185ps8.
86. Klein SL, Jedlicka A, Pekosz A. The Xs and Y of immune responses to viral vaccines. *Lancet Infect Dis* (2010) **10**:338–49. doi:10.1016/S1473-3099(10)70049-9
87. Furman D, Hejblum BP, Simon N, Jojic V, Dekker CL, Thiebaut R, et al. Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination. *Proc Natl Acad Sci U S A* (2014) **111**:869–74. doi:10.1073/pnas.1321060111
88. Shen-Orr SS, Tibshirani R, Khatri P, Bodian DL, Staedtler F, Perry NM, et al. Cell type-specific gene expression differences in complex tissues. *Nat Methods* (2010) **7**:287–9. doi:10.1038/nmeth.1439
89. Haining WN, Wherry EJ. Integrating genomic signatures for immunologic discovery. *Immunity* (2010) **32**:152–61. doi:10.1016/j.immuni.2010.02.001
90. Haining WN, Pulendran B. Identifying gnostic predictors of the vaccine response. *Curr Opin Immunol* (2012) **24**:332–6. doi:10.1016/j.coi.2012.03.010

91. Pascual V, Chaussabel D, Banchereau J. A genomic approach to human autoimmune diseases. *Annu Rev Immunol* (2010) **28**:535–71. doi:10.1146/annurev-immunol-030409-101221
92. Diamond DL, Syder AJ, Jacobs JM, Sorensen CM, Walters KA, Proll SC, et al. Temporal proteome and lipidome profiles reveal hepatitis C virus-associated reprogramming of hepatocellular metabolism and bioenergetics. *PLoS Pathog* (2010) **6**:e1000719. doi:10.1371/journal.ppat.1000719
93. Amit I, Regev A, Hacohen N. Strategies to discover regulatory circuits of the mammalian immune system. *Nat Rev Immunol* (2011) **11**:873–80. doi:10.1038/nri3109

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 February 2014; accepted: 09 May 2014; published online: 23 May 2014.
*Citation: Gannavaram S, Dey R, Avishek K, Selvapandiyan A, Salotra P and Nakhasi HL (2014) Biomarkers of safety and immune protection for genetically modified live attenuated Leishmania vaccines against visceral leishmaniasis – discovery and implications. Front. Immunol. **5**:241. doi: 10.3389/fimmu.2014.00241*
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology.