Vaccine development against *Leishmania donovani*

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**INTRODUCTION**

Leishmaniasis are a wide spectrum of parasitic diseases caused by dimorphic protozoan flagellates of the genus *Leishmania*. These obligatory parasites of host macrophages are transmitted by ∼30 different species of phlebotomine sandflies (Pearson and Sousa, 1996). Leishmaniasis accounts for global morbidity of 2.4 millions (946,000 for males and 1,410,000 for females) and a mortality rate of approximately 60,000 per year1. Among the three clinical patterns of leishmaniasis (cutaneous, mucocutaneous, and visceral), visceral leishmaniasis (VL) is the most severe in terms of symptoms and clinical complications. Of the 500,000 new cases of VL every year, 90% occur in Bangladesh, Brazil, India, Ethiopia, Kenya and Sudan, countries that are strongly related to poverty (Hotez et al., 2004; Alvar et al., 2006). India alone accounts for more than half of the annual global VL infections, which is rapidly emerging as an important opportunistic HIV-coinfection.

Based on transmission characteristics, there are two types of VL: zoonotic VL caused by *Leishmania infantum*, and anthropoponic VL or kala-azar caused by *Leishmania donovani*. Kala-azar is a systemic infection clinically characterized by prolonged fever, profound cachexia, hepatosplenomegaly, hypergammaglobulinaemia, and pancytopenia, with an incubation period varying between 2 and 6 months (Bray, 1976; Chappuis et al., 2007). Almost all clinically symptomatic patients die within months if left untreated. Moreover, 5–10% of cured individuals from VL in India and 50% in Sudan may also develop cutaneous manifestations in the form of macular, maculo-papular, and nodular skin lesions containing parasites, termed as post-kala-azar dermal leishmaniasis (PKDL). PKDL represents an important yet unclear issue of parasite persistence and the putative reservoir for kala-azar between epidemic cycles that need special attention for VL eradication (Addy and Nandy, 1992; Dereure et al., 2003).

In India the few therapeutic options registered for kala-azar include pentavalent antimonials (sodium stibogluconate), liposomal/free amphotericin B, miltefosine, and paromomycin. However, all have one or more limitations or insufficiencies in the long run. Growing resistance to pentavalent antimonials, prohibitive cost of the available lipid formulations of amphotericin B, toxicity, need for hospitalization, HIV-coinfection, and increasing reports of relapse, have made the global demand for VL vaccine a major public health priority. Life-long immunity against 1

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1. http://www.who.int/tdr/disease/leish/direction.htm
reinfection indicates feasibility of successful vaccination against VL. However, no anti-leishmanial vaccine is still available for human use. Considerable inter-specific diversity of lipophosphoglycan (LPG) structure, extraordinary host evasive mechanism of the parasite and heterogeneity of human population (Beverley and Turco, 1998; Kaye and Scott, 2011) represent unprecedented challenges for VL vaccinology. Absence of an in vivo animal model accurately reflecting the clinical features of human VL adds to limitations of development of immunoprophylaxis against VL. However, current advances in genome sequencing and biotechnology have created a thrust for VL vaccinology an area which has been neglected for years. Development of a cross-protective anti-Leishmania vaccine is thus a desirable public health goal to combat this lethal infection.

Till date vaccine research against VL can be divided into three main groups: vaccination with live virulent or killed parasites (first generation vaccines), vaccination with whole-cell lysates/purified fractions, subunit vaccination with native/recombinant parasite proteins (second-generation vaccines), and plasmid DNA and viral vector based vaccines encoding parasite virulence factors (third generation vaccines). Recent concepts include use of genetically modified live-attenuated Leishmania parasites, and proteomics approach for the search of a cross-protective leishmanial vaccine that would ideally protect against both cutaneous and visceral forms of the disease.

**IMMUNOPATHOLOGY OF HUMAN VL: IMPLICATIONS FOR VACCINE DESIGN**

The clinical manifestations of leishmaniasis are determined not only by the parasite species but also by the general health and genetic constitution of the host (Bogdan et al., 1996; D’Oliveira Júnior et al., 1997). Understanding the immunological changes that are associated with resistance and susceptibility to the disease are fundamental to both designing as well as evaluating outcome of vaccines. Although mouse models have been used for study of both cutaneous and VL, very little is known about human VL.

Clinical studies reveal that human VL has a complex immunology characterized by mixed T-helper-1 (Th1)/T-helper-2 (Th2) cell milieu, where a suppressed Th1 response along with an elevated Th2 is the hallmark of the active disease. Conversely, protective immunity is achieved by upregulation of Th1 response after a successful chemotherapy (Hailu et al., 2001; Kubbar and Fragaki, 2005). The crucial step in generating anti-leishmanial immunity is maintenance of the proportion of CD4+ and CD8+ T cells that results in secretion of different cytokines required for immunomodulation in VL. Patients with kala-azar demonstrate negative delayed type hypersensitivity and non-proliferative response to parasite antigens (Sacks et al., 1987; Dominguez et al., 2002). Most of the T cell clones from the PBMCs of the active VL patients and asymptomatic subjects (seropositive and DTH positive) are CD8+ with negligible CD4+, while the situation is reversed in the cured individuals with more numbers of CD4+ than CD8+ cells (Saha et al., 2006). Probably, the CD4+ T cells are lost prior to CD8+ cells in VL, thus a depressed CD4:CD8 ratio has been observed in patients compared with controls (Rohtagi et al., 1996; Clarência et al., 2009). Associated upregulation of immunosuppressive CD4+CD25+ regulatory T cells (Treg) in active VL patients probably leads to parasite persistence and chronic infection (Saha et al., 2007).

Cellular immunity is considered to be the key mediator of resistance by means of IFN-γ that induces microbicidal activity against both the promastigotes and amastigote forms of *L. donovani*, in an H2O2-dependent (Murray and Cartelli, 1983; Murray et al., 1983) and NO-mediated pathway (Vouldoukis et al., 1997) for parasite clearance (Badaro et al., 1996; Badaro and Johnson, 1993; Harms et al., 1993; Squires et al., 1993; Sundar et al., 1994, 1995, 1997a,b; Sundar and Murray, 1995). Probably, impaired macrophage functions due to lack of detectable inducible NO synthase mRNA activity in liver and spleen fastens the disease progression in *L. donovani* infected hamsters, closely resembling the situation in human VL (Melby et al., 2001a). IL-12 is an immunoregulatory 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 (Kobayashi et al., 1989; Stern et al., 1990; Scharton and Scott, 1993; Trinchieri, 1993). In VL patients, IL-12 enhances Th1 responses and restores lymphocyte proliferative responses, IFN-γ production and cytotoxic responses (Ghalib et al., 1995; Bacellar et al., 1996). TNF-α is a proinflammatory chemokine associated with both protection and susceptibility to leishmaniasis. When produced at very high levels, TNF-α might have a disease-enhancing effect (Karplus et al., 2002). IL-4, considered to be a marker of Th2 response, is present in the sera, PBMC supernatant (Zwingerberger et al., 1990; Sundar et al., 1997a) and as mRNA in active VL (Carvalho et al., 1994; Kenney et al., 1998). Although largely implicated in disease exacerbation (Zwingerberger et al., 1990), beneficial role of IL-4 in driving Th1 immunity for early protection against VL has also been suggested (Kamogawa et al., 1993; Alexander et al., 2000). IL-10 primarily down-modulates innate as well as acquired immune response. Moreover, IL-10 induction by host IgG-opsonized amastigotes leads to immune suppression of Th1 response with downregulation of IL-12 for disease progression (Caldas et al., 2005). Patients with VL have increased expression of mRNA for IL-10 in bone marrow and lymph nodes with enhanced levels of IL-10 in sera and leishmanial antigen-stimulated PBMC supernatants (Saha et al., 2007). Imbalanced IL-10 production may be associated with disease progression to PKDL (Ghalib et al., 1993; Saha et al., 2007). Transforming growth factor-β (TGF-β) having immunosuppressive properties, is also documented in leishmaniasis disease progression (Nyłén and Akuffo, 2009; Donaghy et al., 2010). A high level of total TGF-β affects NO production by deactivating macrophages both in vitro and in vivo (Barral-Netto et al., 1992; Li et al., 1999). Recent studies have linked IL-13 (Babaloow et al., 2001), IL-15 (Carson et al., 1994, 1995; Armitage et al., 1995; Jullien et al., 1997; Milano et al., 2002), IL-17 and IL-22 (Pitta et al., 2009) with protection against *L. donovani*. IL-27, produced mainly by macrophages and DC, regulates Th17 cells thereby maintaining a balance between immunity and pathology in human VL (Anderson et al., 2009; Murugaiyan et al., 2009). Enhanced IL-27 in VL patients is shown to induce IL-21 which in turn promotes expansion of IL-10 secreting T cells, leading to parasite multiplication in spleen (Ansari et al., 2011).

Together with phagocytes, NK cells represent the first line of defense against pathogens by two principal mechanisms, cytolytic...
destruction of infected cells, and secretion of proinflammatory cytokines (e.g., IFNγ, TNFα; Nylen et al., 2003). Activated NK cells promote DC maturation along with clearance of autologous immature DCs (Akuffo et al., 1993; Piccioli et al., 2002). Comparatively lower frequency of NK cells in active VL than controls further confirms their role in healing (Nylen et al., 2007). Conversely, DCs are pivotal in initiating and orchestrating the immune response by phagocytosis, antigen presentation, and cytokine production (Banchereau and Steinman, 1998; Mellman and Steinman, 2001). DCs essentially guide resistance or susceptibility to Leishmania by driving the differentiation and proliferation of CD4+ T cells toward one of the two polarized pathogen-specific Th1 or Th2 phenotype (Moser and Murphy, 2000). Differential roles of DC–NK cell cross-talk at various stages of Leishmania infection present new insight on the interactions between innate immune components during infection with this parasite (Sanabria et al., 2008). Exploitation of DC biology may thus be a rationale of VL vaccine design for better antigen-delivery and presentation.

Although elevated levels of anti-leishmanial antibodies IgG, IgM, IgE, and IgG subclasses are well documented in active VL (Atta et al., 1998; Anam et al., 1999a,b; da Matta et al., 2000; Ryan et al., 2002; Ravindran et al., 2004), their precise role in protection against Leishmania is controversial and needs to be reappraised. IFN-γ probably upregulates IgG1 and IgG3, while IL-4 and IL-5 stimulate the production of high levels of IgG, IgM, IgE, and IgG subclasses like IgG4 in humans (Ozbilge et al., 2006). However, antibodies are not relevant for vaccine-induced protection in the absence of an appropriate cellular response. Overall, both CD4+ and CD8+ T cells are required to mediate parasite control and successful vaccination requires IL-12 driven IFN-γ-dependent Th1/Th2 mixed response against VL. However, high IL-4 and IL-10 production in immunized animals, despite very high IFN-γ, may lead to vaccine failure and disease exacerbation in VL (Bhowmick et al., unpublished data).

COMPLEXITY OF HOST–PARASITE INTERACTION
Leishmania spp. had been widely regarded as fastidious, obligate intracellular pathogens primarily inhabiting macrophages. But recent studies have confirmed their presence in hematopoietic cells having a common myeloid precursor (i.e., neutrophils, resident macrophages, dermal DCs, inflammatory monocyte-derived DCs) (Gueirard et al., 2008) and non-hematopoietic cells such as fibroblasts. Survival of Leishmania within the mammalian host depends on its ability to evade and manipulate the anti-leishmanial immune response to establish infection (Bogdan and Rollinghoff, 1998). Despite the presentation of leishmanial antigens and the initiation of inflammatory responses, Leishmania can reside within the host tissues for long periods of time by exploiting several evasive mechanisms (Zambrano-Villa et al., 2002). Opsonization of Leishmania promastigotes by parasite-specific IgG facilitates their uptake by macrophages and DCs which leads to establishment of infection (Prina et al., 2004). IL-12 produced by parasite-engulfed DCs, as a signature of anti-Leishmania protective response, is counteracted by immunosuppressive IL-10 produced by parasitized macrophages that activates host T_{reg} cells leading to disease progression (Woelbing et al., 2006). Notably LPG, the most abundant molecule constituting the glyocalyx of Leishmania promastigotes, plays an important role in the interactions between the parasite and the sandfly promoting the infectivity of the parasites. Considerable diversity in LPG structure among different Leishmania spp. explains their vector specificity (Sacks, 2001) and may also affect their immunological properties. Thus, LPG which is a virulence factor allowing safe passage and survival of metacyclic promastigotes in macrophages in L. major (Spith et al., 2000) and L. donovani (Desjardins and Descoteaux, 1997; Lodge et al., 2006; Vinet et al., 2009), has no such role in L. mexicana (Ilg, 2000). Profound differences in the virulence factors of L. major and L. donovani also affect their consequent pathologies as well as underlying immune evasive mechanisms, making development of a cross-protective vaccine difficult. Sandfly saliva itself can also act as a virulence factor by enhancing invasion of macrophages by promastigotes, even with low-dose inoculations (Gillespie et al., 2000). In fact, Leishmania has evolved numerous strategies to interfere with a broad range of macrophage signaling that includes protein kinase C, the JAK2/STAT1 cascade, and the MAP kinase pathway that favor its survival and persistence in host cells (Shadab and Ali, 2011). Recruitment of inhibitory CD4+CD25+FoxP3+ regulatory T cells (T_{reg}) (Engwerda et al., 2004; Peters and Sacks, 2006; Nylen et al., 2007), inhibition of macrophage phagosomal maturation (interfering with fusion of phagosomes and lysosomes) (Olivier et al., 2005), and inhibition of DC maturation and chemotaxis (Brandenfio et al., 2004) are some of the strategies employed by Leishmania to evade effector mechanisms of the host immune system. Maturation of human monocyte-derived DCs is inhibited by both L. donovani and L. amazonensis (Favalli et al., 2007; Teje et al., 2008). Further, deple- tion of CD8+ T-cell following infection with L. donovani may contribute to chronic infection (Joshi et al., 2009). Thus, the main goal of Leishmania vaccinology is establishment of a Th1-dominated cell-mediated response along with CD8+ T cells for sustained protective immunity (Scott, 2003).

Though it is often assumed that the type of disease depends solely on Leishmania species, susceptibility or resistance to leishmaniasis markedly varies within and between genetically diverse human populations (Walton and Valverde, 1979; Momeni and Aminjavaheri, 1994; Weigle and Saravia, 1996; Sharma et al., 2000; Bucheton et al., 2002). For example, PKDL is influenced by genetic polymorphism in IFN-γ receptor (Mohamed et al., 2003), and susceptibility is linked to polymorphism in IL-4 gene in Sudanese patients (Blackwell et al., 2005). Natural resistance-associated macrophage protein-1 (NRAMP1) has been shown to increase the risk of acquiring infection. In mice, Nramp1/Slc11a1 Lsh locus on chromosome 1 is responsible for acute susceptibility to L. donovani (Demant et al., 1996). However, its role in human is not clear. This difference is probably due to the fact that the Lsh/Nramp1/Slc11a1 allele in susceptible mice practically abolishes the gene function (equivalent to a traditional knockout), whereas polymorphisms in the promoter, exon 3 and the intron of human SLC11A1 are expected to have less influence on susceptibility to L. donovani (Bucheton et al., 2003; Mohamed et al., 2004). Polymorphisms in genes coding HLA class I and class II molecules have been related with susceptibility to L. guyanensis (Barbier et al., 1987) and L. braziliensis (Lara et al., 1991; Petzl-Erler et al., 1991) although impact of these polymorphisms on VL species
seems ambiguous (Bucheton et al., 2003). Recent genome-wide linkage study from Sudan and Brazil has identified a putative susceptibility locus DLL1 (Delta-like 1 for Notch3) which is strongly implicated as the chromosome 6q27 VL susceptibility gene (Fakiola et al., 2011). In brief, further understanding of the complex yet unique species-specific virulence mechanisms, immunogenetics, and clinical correlates is needed for early diagnosis, customized chemotherapeutic strategies, and prophylaxis against VL.

PROGRAMMING OF INNATE IMMUNITY

The role of innate immune system comprising of macrophages, DCs, NK cells, and granulocytes forming the primary defense mechanism against pathogen, is gaining increasing attention in curing VL. Innate immune system is activated via pattern recognition receptors (PRRs) on host macrophages and DCs that recognize conserved pathogen-associated molecular patterns (PAMPs) of microbes. Activation of PRRs like Toll-like receptors (TLRs), nucleotide-binding domain (NOD)-like receptors and scavenger receptors by PAMPs leads to maturation and trafficking of the DCs to local lymph nodes for antigen presentation to naïve T cells raising the adaptive immunity against the invading pathogen (Kaisho and Akira, 2000, 2001; Bocarsly et al., 2002). Again the pattern of TLR expression varies between the APC subsets and their anatomical locations (Banchereau and Steinman, 1998). Secretion of proinflammatory cytokines and chemokines following TLR activation contribute to Th1-independent immediate defense mechanisms (Hayashi et al., 2003). Bypassing these natural innate mechanisms by Leishmania promastigotes probably marks the beginning of successful parasitism. Leishmania adopts apparently quiescent mechanisms of macrophage entry with slow or negligible stimulation of NF-kB pathway which appears to be true for all Leishmania spp., thus delaying adaptive response. This has been experimentally evidenced in mice receiving low-dose parasite challenge in the ear (Belkaid et al., 2000). Plethora of evidence suggests a crucial role of TLR-PAMP interaction for bridging innate and adaptive immunity. Past experience with vaccine success of leishmanization (consisting of live virulent parasites) as against reduced protection by killed vaccines can possibly be assumed due to loss of parasite PAMPs during inactivation process which is required for establishing future adaptive immunity. To alleviate this problem, subunit or genetic vaccines are often adjuvanted with TLR-agons for innate triggering of adaptive immunity to elicit robust cellular and humoral response. TLR ligands that mimic PAMPs to activate immune cells via Toll-like receptors (TLRs) are being developed for clinical use and immunotherapy against a variety of diseases. Adjuvant success of several TLR-agons like CpG-DNA (immunostimulatory oligodeoxynucleotides):TLR9 agonist, MPL (Monophosphoryl Lipid) A and GLA (Glucopyranosyl Lipid Adjuvant): TLR4 agonists, and imiquimod: TLR7 agonist, and imiquimod: TLR7 agonist, have been well documented against Leishmania (Buates and Matlashewski, 1999; Walker et al., 1999; Reed et al., 2003; Coler et al., 2007). Synthetic imidazoquinoline compounds (i.e., imiquimod and resiquimod) have been shown to activate human TLR7 and 8 (Gibson et al., 2002; Hemmi et al., 2002), and TLR7 in mice. TLR3 and TLR4 in synergy with TLR7, TLR8, and TLR9 in human and mouse DCs boost up Th1 response through increased production of IL-12 and IL-23. TLR synergy further establish DCs unidirectional intracellular cross-talk to acquire T cell response to a given vaccine in MyD88-dependent manner (Kang et al., 2002; Martinon and Tschopp, 2005; Napolitani et al., 2005; Zhu et al., 2008). Combinatorial synergy of MPL with CpG in L110f vaccine has shown to improve immunotherapy against murine CL probably resulting from chemokine RANTES (regulated upon activation normal T cell expressed and secreted) expression thereby recruiting activated T cells to the site of infection with enhanced IL-12 production (Raman et al., 2010). Future vaccination strategies using live non-virulent or genetically altered parasites with TLR 7/8 or TLR 4 agonists can be appropriate combination for priming innate immunity for durable, broad spectrum cross-protection against Leishmania. Designing of such novel multicomponent adjuvants combining TLRs and/or non-TLRs with vaccines for better amplification of T-cell and B-cell response merits further study.

ANIMAL MODELS FOR VL

Among several models tried for L. donovani, BALB/c mice and Syrian golden hamsters (Mesocricetus auratus) are the most extensively studied animal models for primary screening of vaccines and drugs. Absence of functional Sc11 1a1 locus in BALB/c and C57BL6 mice renders them susceptible to L. donovani infection compared to resistant CBA mice possessing functional Sc11 1a1 gene that confers innate anti-leishmanial immunity (Bradley, 1974; Blackwell, 1996). Unlike L. major infection, Th1/Th2 dichotomy in cytokine milieu is not evident during murine VL caused by L. donovani/chagasi. The protective immunity in murine VL primarily depends on IL-12 driven Th1 response leading to increased IL-2 and IFN-γ production for parasite killing. Substantial upregulation of inducible NO synthase (iNOS, NOS2) by IFN-γ generates NO from splenic and liver macrophages thereby controlling parasite multiplication in these organs (Green et al., 1990; Blackwell, 1996). TGF-β, IL-10, and IL-13 are the major disease promoting cytokines leading to suppression of Th1 response (Wilson et al., 2005). Low levels of IL-4 usually enhance vaccine-induced protection by increasing IFN-γ production from CD8+ T cells (Stäger et al., 2003) and subsequently play no role in disease exacerbation in L. donovani infected mice. Despite the wide usage of the mouse model for vaccine studies in VL, this does not resemble the clinical outcomes seen in fatal human VL. Murine VL is largely subclinical and self-resolving disease compared to disseminated visceral infection which seems to be a major limitation in extrapolating the results to human VL. In mice, clearance of hepatic parasite burden occurs after 2 weeks post infection due to efficient granuloma formation resulting from interaction of Leishmania-specific T cells with parasitized dendritic cells and resident macrophages. Parasite killing in mature granulomas leads to resolution of hepatic infection in about 56 days in experimental VL. Hence, vaccine efficacy and disease progression in mice can be predicted from the degree of maturation of hepatic granulomas in challenged animals correlating with cell-mediated immunity (Murray et al., 1992; Carrión et al., 2006). In contrast to liver, spleen, and bone marrow act as the site of parasite persistence resulting in chronic infection in mice. The failure of parasitized marginal zone macrophages of spleen to produce IL-12, breakdown of splenic architecture and lack of granulomatous reaction leads to high parasite burden in spleen (Nieto
et al., 2011). Infecting mice with large inoculums can thus be more suitable to resemble pathological changes similar to active human VL (Carrion et al., 2006). Interestingly, inoculating BALB/c mice with Indian L. donovani strains like AG83 leads to progressive VL with clinical manifestations of hepatosplenomegaly, at least up to 6 months post infection (Mazumder et al., 2004; Bhowmick et al., 2009). The disease progression in this model is associated with suppressed Th1 response due reduced IFN-γ and IL-12 production from spleen cells along with enhanced IL-4 and IL-10, which get reversed with vaccine-induced protection. Further, infected mice also demonstrate high TGF-β and low TNF-α 4 months post infection resulting in impaired NO-mediated parasite clearance by macrophages (Bhowmick et al., 2009). However, precise correlates of pathology and protection are yet to be defined for this model for development of successful intervention against human VL.

Compared to mice, hamsters seem to a better model for progressive VL expressing clinical features like hepatosplenomegaly, immunosuppression, anemia, cachexia, and death, closely simulating human VL. Impaired macrophage activation in liver and spleen due to lack of NOS2 expression throughout disease progression seems to be the major cause of uncontrolled parasite multiplication and death in hamster (Melby et al., 2001a). High levels of TGF-β have also been reported in tissues and cultured immune cells from mice and hamsters infected with L. donovani and L. chagasi, suggesting its definitive role in disease exacerbation (Muller et al., 1992; Gomes et al., 2000; Gantt et al., 2003). Increased secretion of TNF-α during later stage of disease probably results in profound loss of infected hamsters (Tracey et al., 1988). However, inadequacy of immunological reagents limits the use of hamster model for prophylactic and therapeutic analysis of VL. High innate susceptibility of hamsters to L. donovani makes them a more suitable model for studying immunopathology of VL than evaluation of protective immunity. Among several higher non-human primate models of VL, Indian langur monkey (Presbytis entellus) has shown consistent and progressive VL similar to active human disease. Immune protection achieved by autoclaved L. donovani (ALD) along with BCG in this model correlates with high IFN-γ, IL-2, and lymphoproliferation (Dube et al., 1998; Misra et al., 2001). Difficulties in laboratory handling, non-availability and expense are some of the drawbacks of primate models that need to be solved for their routine use as VL models.

VL VACCINE DEVELOPMENT: CONVENTIONAL APPROACHES

First Generation Vaccines

“Leishmanization,” the traditional practice of inoculating live, virulent Leishmania parasites from the exudates of a cutaneous lesion is the only successful intervention against leishmaniasis and has been practiced for centuries in Middle East and Russia with efficacy levels up to 100% (Nadim et al., 1983; Khamesipour et al., 2005). Based on persistence of parasites in the primary inoculated site for cellular response, leishmanization could confer virtually life-long and complete protection which is of utmost importance for developing anti-leishmanial vaccine. Unfortunately, features of adaptive immunity elicited for successful leishmanization have not been studied in human. It is known that previous exposure to L. major can confer protection against subsequent VL (Zijlstra et al., 1994). However, leishmanization trials have never been done either against New World tegumentary leishmaniasis or human VL. Although still practiced in Uzbekistan, severe side effects and complications have eventually replaced it by first generation killed Leishmania vaccines, with or without adjuvants. In order to improve safety of leishmanization, live L. major have been co-injected with CpG-DNA without compromising the vaccine potency in murine model (Mendez et al., 2003). Success of CpG adjuvanted live vaccine in murine CL apparently depended on CD4 Th17 cells and early recruitment of IFN-γ secreting CD4 cells at inoculation site for long-term protection (Wu et al., 2010). This vaccine also resulted in activation of dermal DCs and NK cells for enhanced protection almost in without any side effect unlike leishmanization. Although effective against VL in several experimental models, recent human clinical trials of killed vaccines have been found to be safe but not as effective as leishmanization, having low efficacies (54%) when tested in humans (Sharifi et al., 1998; Momeni et al., 1999; Armijos et al., 2004; Velez et al., 2005) and dogs (Genaro et al., 1996; Mohebali et al., 2004). The success of leishmanization over killed vaccines in CL can possibly be explained by an in vitro study evaluating human T cell function when simulated by live vs. killed parasites (Nateghi Rostami et al., 2010). In addition to increased IFN-γ from CD4+ T cells, live parasites could also stimulate CD8+ T cells to secrete IFN-γ. Killed parasites, however, could stimulate CD4+ T cells only. This probably accounts for better immunologic memory leading to sustained immunity after healing of live infections (Okwor and Uzonna, 2008; Nateghi Rostami et al., 2010). Promising enough, a very recent approach have generated a killed but metabolically active L. infantum chagasi promastigotes after treating them with amotosalen and low-dose UV radiation that resulted in replication-deficient parasites that could protect mice against experimental VL (Bruhn et al., 2012). These parasite could enter host macrophages for initial transformation for stimulating robust adaptive immunity for protection lasting up to 6 weeks post infection. However, most clinical trials have been conducted for CL with very few for VL (Khalil et al., 2000, 2006). The anticipated success with CL clinical trials may lead to a few human VL trials due to the highly conserved genomes of the Leishmania spp.

Ease of production, economy, and safety of first generation leishmanial vaccines have made them attractive for immunotherapeutic strategy against VL. Combination therapy comprising of alum-precipitated autoclaved L. major (ALM) with BCG has been found to be protective in PKDL patients significantly reducing cases of relapse (Musa et al., 2008). Killed Leishmania vaccines probably hold more prospects for immunotherapy combined with drugs than their single use against VL which needs further human trials.

Second-Generation Vaccines

Vaccination with Leishmania fractions

Partially or fully purified Leishmania subfractions have been widely used in experimental models (Gradoni, 2001; Handman, 2001) owing to their excellent immunoprotective properties. Previous studies showed that vaccination with fucose-mannose ligand (FML) protects against experimental VL in several animal models such as mice (Swiss Albino and BALB/c) and dog (Palatnik de
Subunit protein vaccines
Following hundreds of native and recombinant antigens tested inducing protection against CL, several molecularly defined vaccine candidates have been evaluated against VL over the past two decades (Table 1). LPG was the first molecularly defined vaccine candidate against leishmaniasis that conferred protection against CL (Handman and Mitchell, 1985; McConville et al., 1987; Karanja et al., 2011). Concurrent studies with L. donovani-derived LPG suggested it as a promising candidate vaccine for transmission blocking (Tonui et al., 2001). The first recombinant antigen against leishmaniasis was gp63 or leishmanolysin, conserved in all Leishmania species. Although highly protective in its native form, both gp63 and Leishmania donovani (L. donovani) challenge in murine model (Tewary et al., 2009). Considerable leishmanicidal properties (dose-dependent killing promastigotes of L. amazonensis, L. major, L. braziliensis, and L. mexicana) have also been demonstrated for histones H2A and H2B (but not histone H3) (Wang et al., 2011) which show a new possibility of using these histone proteins for therapeutic vaccinations against leishmaniasis.

Open reading frame (ORF), is a single copy gene, located on chromosome 35 as a part of the multigene LD1 locus, preferentially expressed in L. donovani amastigotes (Iain and Madhubala, 2008). Purified recombinant ORF protein has been partially protective against L. donovani challenge in murine model (Tewary et al., 2004b). More recently, ORF expressed as a chimeric ubiquitin conjugate was tested against antimony-sensitive and antimony-resistant strains of L. donovani as a vaccine candidate. This study demonstrated better protective efficacy of ubiquitin-conjugated ORF than unconjugated one against experimental VL (Sharma and Madhubala, 2009). Several other subunit protein vaccines have been tested against different forms of leishmaniasis with varying success. These include dp72 (Jaffe et al., 1990; Aguilar-Be et al., 2005), ribosomal P0 protein (Iborra et al., 2005), Leishmania elongation factor 2 (LeEF-2) (Kushawaha et al., 2011), amastigote cysteine proteases (Zadeh-Vakili et al., 2004), F1 (Bhardwaj et al., 2009), hydrophilic acylated surface protein B1 (HASPB1) (Stager et al., 2000), etc. The observation that some individuals exposed to uninfected sandfly mount a protective response to Leishmania (Davies and Mazloumi Gavgani, 1999) has lead to vaccine attempts designed against sandfly salivary proteins or insect gut antigens that can protect from infection (Morris et al., 2001). Recently, hamsters immunized with LMJ 19, a novel 11kDa protein from Lutzomyia longipalpis saliva, were reported to be protected against VL (Gomes et al., 2008).

The use of synthetic peptide vaccines, particularly the T-cell epitopes also opened an interesting avenue in Leishmania vaccine since 1980s (Lamb et al., 1984; Jardim et al., 1990; Russo et al., 1993; Spitzer et al., 1999). However, this enthusiasm has waned in recent times due to inadequate memory response and inability of all individuals to respond to the peptide, which needs to be resolved.

Contrary to defined single antigen, multicomponent or polyprotein vaccines have been demonstrated to afford better protection against experimental VL. LEISH-F1 (formerly Leish111f), consisting of three recombinant antigens: Leishmania elongation initiation factor (LeIF), thiol-specific antioxidant (TSA), and L. major stress-inducible protein-1 (LmSTI1) is such a polyprotein vaccine that was able to confer protection against L. major (Skeiky et al., 2002) and L. infantum (Coler et al., 2007). A crucial component of LEISH-F1 vaccination is the selection of the adjuvant. The vaccine remained most efficacious when co-administered with IL-12, as well as detoxified derivative of lipid A from lipopolysaccharide (LPS) of Salmonella Minnesota, lipid A formulated with squalene in a stable emulsion (MPL-SE). LEISH-F1 + MPL-SE is the only second-generation vaccine that successfully cleared the clinical trial for safety and immunogenicity against VL (Vélez et al., 2009; Chakravarty et al., 2011).

Subunit vaccines based on recombinant proteins and synthetic peptides are safe. However, they are poor generators of cytotoxic T-cell (CTL) responses (Lima et al., 2004) and no single antigen could confer complete protection. To overcome this
| Antigen | Vaccine form/adjuvant used | Animal model | Challenge infection | Result | Reference |
|---------|---------------------------|--------------|---------------------|--------|-----------|
| FML     | Native antigen + Saponin  | Hamster      | L. donovani         | Protection | Palatnik de Sousa et al. (1994a) |
| FML     | Native antigen + Saponin  | Mouse        | L. donovani         | Protection | Palatnik de Sousa et al. (1994b) |
| FML     | Native antigen + Saponin/Aluminum hydroxide | Mouse | L. donovani | Protection | Santos et al. (1999) |
| FML     | Native antigen + IL-12/BCG/saponin | Mouse | L. donovani | Protection | Santos et al. (2002) |
| FML     | Native antigen + Quil A    | Dog          | L. donovani         | Protection | Borja-Cabrera et al. (2002) |
| FML     | Native antigen + Saponin   | Mouse        | L. donovani         | Protection | Santos et al. (2002) |
| FML     | Native antigen + Saponin   | Mouse        | L. donovani         | Protection | Palatnik de Sousa et al. (2004) |
| LAg     | Native antigen entrapped in cationic liposome | Mouse/Hamster | L. donovani | Protection | Afrin and Ali (1997), Ali and Afrin (1997) |
| LAg     | Native antigen entrapped in negatively charged liposome | Mouse | L. donovani | Partial protection | Mazumder et al. (2004) |
| LAg     | Native antigen entrapped in cationic liposome | Mouse | L. donovani | Protection | Mazumder et al. (2007) |
| LAg     | Native antigen entrapped in cationic liposome + BCG/MPL | Mouse | L. donovani | Protection | Ravindran et al. (2010) |
| SLA     | Native antigen entrapped in cationic liposome | Mouse | L. donovani | Protection | Bhowmick et al. (2007) |
| SLA     | Native antigen + non-coding pDNA co-entrapped in cationic liposome | Mouse | L. donovani | Protection | Mazumder et al. (2007) |
| SLA     | Native antigen entrapped in cationic liposome | Mouse | L. donovani | Protection | Ravindran et al. (2012) |
| gp36    | Native antigen + Saponin  | Mouse        | L. donovani         | Protection | Paraguai de Souza et al. (2001) |
| SA      | Native antigen + CpG-ODN  | Mouse        | L. donovani         | Protection | Tewary et al. (2004a) |
| LD 31, 51, 72, 91 | Native antigen entrapped in cationic liposome | Mouse | L. donovani | Protection | Bhowmick and Ali (2009) |
| gp70-2/dp72 | Native antigen + Corynebacterium parvum | Mouse | L. donovani | Partial protection | Jaffe et al. (1990) |
| Hsp70 + Hsp83 | Native antigen + MPL/ALD | Mouse | L. donovani | Protection | Kaur et al. (2011b) |
| gp63 + Hsp70 | Native antigen | Mouse | L. donovani | Protection | Kaur et al. (2011a) |
| LPG     | Native antigen + BCG      | Mouse/Hamster | L. donovani | No protection | Tonui et al. (2001) |
| 78kDa   | Native antigen + MPL/ALD/liposome/FCA | Mouse | L. donovani | Protection | Mukherjee et al. (2002) |
| dp72    | Native antigen + Saponin  | Mouse        | L. donovani/L. major | Cross-protection | Rachamim and Jaffe (1993) |
| gp63    | Native antigen + Saponin  | Mouse        | L. donovani         | Protection | Bhowmick et al. (2008) |
| A2      | Recombinant antigen + Propionibacterium acnes | Mouse | L. donovani | Protection | Ghosh et al. (2001b) |
| HASP81  | Recombinant antigen + IL-12 | Mouse | L. donovani | Protection | Stager et al. (2000) |
| gp63    | Recombinant antigen + S. typhimurium | Mouse | L. donovani/L. major | Cross-protection | McSorley et al. (1997) |
| gp63    | Recombinant antigen entrapped in cationic liposome + MPL/TDM | Mouse | L. donovani | Protection | Mazumder et al. (2011a) |
| F14     | Recombinant antigen + MPL | Hamster | L. donovani | Protection | Bhardwaj et al. (2009) |
| Leishmune | Recombinant antigen | Dog | L. donovani | Protection | Nogueira et al. (2005) |
| Leishmune | Recombinant antigen | Dog | L. donovani | Partial protection | Saraiva et al. (2006) |
| Antigen | Vaccine form/adjuvant used | Animal model | Challenge infection | Result | Reference |
|---------|---------------------------|--------------|---------------------|--------|-----------|
| ORFF + BT1 | Recombinant antigen | Mouse | L. donovani | Partial protection | Dole et al. (2000) |
| ORFF | Recombinant antigen + CpG-ODN | Mouse | L. donovani | Partial protection | Tewary et al. (2004b) |
| ORFF | Recombinant antigen | Mouse | L. donovani | Partial protection | Tewary et al. (2005) |
| ORFF | Recombinant antigen + IL-12 DNA | Mouse | L. donovani | Protection | Tewary et al. (2005) |
| LdyGCS | Recombinant antigen entrapped in NIV | Mouse | L. donovani | Protection | Henriquez et al. (2010) |
| LeIF2 | Recombinant antigen | Hamster | L. donovani | Protection | Kushawaha et al. (2011) |

BCG: Mycobacterium bovis Bacillus Calmette–Guerin; LPG, lipophosphoglycan; FML, fucose-mannose ligand; LdyGCS, L. donovani gamma-glutamyl cysteine synthetase; LeIF2, L. donovani elongation factor2; gp, glycoprotein; ORFF, open reading frame F; BT1, biopterin transporter; dp72, L. donovani promastigote antigen of 72 kDa; HASPB1, hydrophilic acylated surface protein B1; Hisp, heat-shock protein; LAg, leishmanial membrane antigen; NIV, non-ionic surfactant vesicle; CpG-ODN, CpG oligodeoxynucleotides; ALD, autoclaved L. donovani.

Inherent problem of subunit vaccines, several studies are being carried out with promising adjuvants like Bacillus–Calmette–Guerin (BCG), alum, saponin, liposomes, MPLA, and other TLR ligands for vaccine success against VL (Blowmick et al., 2008). Long-term immunity generated by subcutaneous immunization of liposomal soluble leishmanial antigen (SLA) with MPL-TDM (monophosphoryl lipid–trehalose dicarboxymycoclate) in BALB/c mice largely depends on high IFN-γ and CD8+ T cells, along with downregulation of IL-4 (Ravindran et al., 2012). RAPSODI, a human–compatible second-generation vaccine current is currently undergoing preclinical trial heading toward a pan-Leishmania vaccine (Rapsodi against leishmaniasis2; accessed 2 January 2012). Based on the most immunodominant antigen, promastigote surface antigen (PSA) expressed in L. tarentolae system (LEXYS, Jena Bioscience), RAPSODI holds a promising future for a cross-protective Leishmania vaccine.

**Dendritic cell (DC) – based vaccines**

DCs are a class of specialized APCs that orchestrate both innate and acquired immunity. TLR-PAMP interaction leads to maturation of DCs lying in the interface external environment and mucosal surface, inducing the expression of co-stimulatory molecules CD40, CD80, and CD86 required for T-cell activation. The secreted cytokines, namely IFNs and IL-12 drive the T-cell differentiation toward CD40, CD80, and CD86 required for T-cell activation. The mucosal surface, inducing the expression of co-stimulatory molecules on DCs lying in the interface external environment and acquired immunity. TLR:PAMP interaction leads to maturation of DCs in a murine model of VL (Agallou et al., 2011). In another study, combination therapy using SLDA-pulsed syngeneic bone marrow-derived DC and antimony-based chemotherapy could cure established murine VL via CD4+ T cell dependent Th1 response (Ghosh et al., 2003). Similar protective effects of microbial antigen-loaded DCs have also been reported for other pathogens (Bourquin et al., 1998; George-Chandy et al., 2001; McShane et al., 2002) and antitumour immunity (Kobukai et al., 2011). Another interesting approach has implemented hybrid cell vaccination therapy using L. donovani kinetoplastid membrane protein-11 (KMP-11)-transfected bone marrow-derived macrophages from BALB/c mice with allogeneic bone marrow-derived DCs from C57BL/6 mice to resolve infection by establishing strong antigen-specific CD8+ CTL response (Basu et al., 2007). DCs have been proposed to play a critical role as immunomodulators in vaccination and therapy, and are therefore an important tool for prophylactic vaccination against serious infections. Integrating DC vaccinations with standard anti-leishmanial chemotherapy could thus be highly beneficial for therapeutic vaccination against VL. Nevertheless, this strategy has its limitations. Large-scale generation of leishmanial Ag-pulsed and/or cytokine-transduced autologous DC vaccine seems laborious and expensive, and could thus possibly limit its use in developing countries. Moreover, the choice of DC subtype, antigen-loading strategy, maturational status, route and frequency of immunization, and adjuvant cytokines all affect the performance of a DC vaccine.

**THIRD GENERATION VACCINES**

Genetic vaccination has been a better and flexible strategic alternative for inducing stronger Th1 biased immune response than their recombinant protein counterparts which often suffer from poor immunogenicity due to alerted ppathoantigenic epitopes (Gurunathan et al., 1998; Rafati et al., 2006). An important feature of DNA vaccination is the induction of CD8+ cytotoxic T cells along with sufficient CD4+ T cell and humoral response. This contributes to cytolysis of parasite-infected cells by CD8+ T cells and increased IFN-γ secretion for efficient leishmanicidal activity (Aguilar-Be et al., 2005; Carter et al., 2007). However, priming and expansion of CD8+ T cells solely depends upon CD4+ T cells which is vital for initiation and maintenance of immune memory (Bourgeois et al., 2002; Jansen et al., 2003). This concept of Th cell help for CTL response has led to further improvement.
of DNA vaccine constructs and adjuvant strategies over time. In addition to codon-optimized vectors, fusion of immune modulators (like CpG motifs, GM-CSF, Flt3 ligands, and cytokine genes) and novel delivery systems like electroporation, and the concept of heterologous prime-boosting have been employed successfully against *Leishmania* and several other pathogens. gp63, *Leishmania* analog of the receptor kinase C (LACK) and ORFF are the most extensively studied DNA vaccine candidates against VL till date (Table 2). Vaccination with a plasmid encoding LACK gene with or without co-administration of IL-12 resulted in robust, long-lasting protection in mice against *L. major* (Gurunathan et al., 1997, 1998; Stobie et al., 2000). Intranasal immunization with plasmid carrying LACK has also been protective against *L. chagasi* both in mice (Gomes et al., 2007) and hamster model (DE Oliveira Gomes et al., 2011) of VL. In contrast to LACK, which failed to protect against *L. donovani* (Melby et al., 2001b), priming with gp63 DNA with protein boost afforded robust, long-term protection in susceptible BALB/c mice (Mazumder et al., 2011b). Similarly, ORFF (Tewary et al., 2005), KMP-11 (Basu et al., 2005), and cysteine proteases (Rafati et al., 2006) have been protective as DNA vaccines against different *Leishmania* species. Protection was significantly improved when vaccines were composed of a cocktail of plasmids expressing immunodominant *Leishmania* genes. Accordingly, vaccination of mice with cocktail of plasmids harboring gp63 and Hsp70 genes elicited robust protection against *L. donovani* with enhanced IFN-γ and IL-2 driving toward an excellent Th1 biased immune response (Kaur et al., 2011a). Further studies are required in higher animal models for such promising combination vaccines to ensure protection before they are finally evaluated in patients with VL. An interesting approach based on cDNA libraries has been adopted to identify protective *L. donovani* antigens through cDNA immunizations (Melby et al., 2000). Plasmid DNAs isolated from several cDNA sublibraries constructed from amastigote-derived RNA have been screened by immunization of BALB/c mice. Several groups of cDNA that conferred in vivo protection against subsequent *L. donovani* challenge have been identified by this technique.

Several lines of evidence suggest a central role of DCs in antigen presentation after DNA vaccination (Corr et al., 1996; Akbari et al., 1999) which explains the success of DC-targeted adjuvants like GM-CSF, TLRs, and Flt3 ligands when co-administered with plasmid DNA (Encke et al., 2006). LEISHDNAVAX, based on MIDGE vector, developed by Mologen AG (Berlin, Germany) is presently undergoing preclinical trials in Europe, against visceral and cutaneous forms of leishmaniasis, along with a double stem loop immunomodulator (DSLIM).

### Table 2 | Third generation vaccine candidates for *L. donovani.*

| Antigen         | Vaccine form/adjuvant used          | Animal model | Challenge infection | Result                | Reference                  |
|-----------------|-------------------------------------|--------------|---------------------|-----------------------|----------------------------|
| **L. DONOVANI-DERIVED ANTIGENS TESTED AGAINST L. DONOVANI** |
| p36 LACK        | DNA vaccine + IL-12                 | Mouse        | *L. donovani*       | No protection          | Melby et al. (2001b)       |
| A2              | DNA vaccine                        | Mouse        | *L. donovani*       | Protection             | Ghosh et al. (2001a)       |
| A2              | Expression in Lactococcus lactis   | Mouse        | *L. donovani*       | Protection             | Yam et al. (2011)          |
| ORFF            | DNA vaccine                        | Mouse        | *L. donovani*       | Partial protection     | Sukumaran et al. (2003)    |
| ORFF-HPB        | DNA vaccine + recombinant protein   | Mouse        | *L. donovani*       | Protection             | Tewary et al. (2005)       |
| ORFF            | Ubiquitin conjugated DNA vaccine    | Mouse        | *L. donovani*       | Protection             | Sharma and Madhubala (2009) |
| KMP-11          | DNA vaccine                        | Hamster      | *L. donovani*       | Protection             | Basu et al. (2005)         |
| KMP-11          | DNA vaccine + IL-12                 | Mouse        | *L. donovani*, *L. major* | Cross-protection       | Bhaumik et al. (2009)     |
| γ GCS           | DNA vaccine                        | Mouse        | *L. donovani*       | Protection             | Carter et al. (2007)       |
| H2A,H2B,H3,H4,LACK | Multiantigen DNA vaccine        | Dog          | *L. donovani*       | Partial protection     | Saldarriaga et al. (2006)  |
| PPG-N-terminal domain | DNA vaccine                     | Hamster      | *L. donovani*       | Protection             | Samant et al. (2009)       |
| gp63/polytope   | DNA vaccine/polytope/polytope fused with hsp70 | Mouse | *L. donovani* | Protection | Sachdeva et al. (2009) |
| P1-HPB          | DNA vaccine + recombinant protein   | Hamster      | *L. donovani*       | Protection             | Masih et al. (2011)        |
| gp63-HPB        | DNA vaccine + recombinant protein + CpG-ODN | Mouse | *L. donovani*, *L. major* | Cross-protection | Mazumder et al. (2011b) |

| **L. DONOVANI-DERIVED ANTIGENS TESTED AGAINST OTHER LEISHMANIA SPECIES** |
|-----------------|-------------------------------------|--------------|---------------------|-----------------------|----------------------------|
| NH36            | DNA vaccine                        | Mouse        | *L. chagasi*, *L. mexicana* | Cross-protection       | Aguilar-Be et al. (2005)  |
| NH36            | DNA vaccine                        | Mouse        | *L. chagasi*        | Protection             | Gambro-León et al. (2006) |
| A2              | Expressed in adenovirus            | Mouse        | *L. chagasi*        | Protection             | Resende et al. (2008)     |
| A2              | Expressed in L. tarentolae         | Mouse        | *L. infantum*       | Protection             | Miziani et al. (2009)     |

NH36, nucleoside hydrolase 36; HPB, heterologous prime-boost; KMP-11, kinetoplastid membrane protein-11; LACK, *Leishmania* analog of the receptors of activated C kinase; PPG, proteophosphoglycan.
Vaccination with genetically modified \( \text{Leishmania} \) parasite is based on decoupling virulence for induction of protective immunity. This generally involves targeted disruption of virulence or essential leishmanial genes resulting in mutated parasites lacking virulence. These mutants closely mimic the natural course of infection through PAMPs required for adequate stimulation of innate immunity for long-term immune memory. Such live-attenuated vaccines have been successfully used against several viral and bacterial pathogens (Nakhasi et al., 1989; Larsen et al., 2009). Despite several attempts, very limited numbers of genetically modified live-attenuated forms of \( L. \) \textit{donovani} exist as vaccine candidates. Recently, \( L. \) \textit{donovani} centrin null mutants (\( \text{Ld Cen}^{-/-} \)) have been reported to protect BALB/c mice and Syrian hamsters against homologous as well as heterologous infectious challenge (Selvapandiyan et al., 2009). Immune protection upon 10 week post-virulent challenge correlated with Th1 biased response with significant increase in IFN-\( \gamma \), IL-12, and TNF producing T cells that significantly lowered the parasite burden in spleen along with complete clearance of parasites in liver. Similar Th1 biased protection has been shown in mice immunized with \( L. \) \textit{donovani} bioterpin transporter null mutant (\( \text{BT1}^{-/-} \)) (Papadopoulou et al., 2002) and silent information regulatory 2 single-knock out mutants (\( \text{Li SIR2}^{-/-} \)) of \( L. \) \textit{infantum} against virulent challenge (Silvestre et al., 2007). Similarly, cytochrome C oxidase component p27 null mutants (\( \text{Ld p27}^{-/-} \)) of \( L. \) \textit{donovani} showed less virulence both in human macrophages and in BALB/c mice (Dey et al., 2010) which may be explored further. More recently, a preliminary evaluation using \( L. \) \textit{infantum} heat-shock protein (\( \text{HSP70-II} \)) null mutant seems promising in inducing protection in \( L. \) \textit{major}-BALB/c infection model (Carrión et al., 2011).

An interestingly different approach using non-pathogenic species like \( L. \) \textit{tarentolae} as live vaccine showed enhanced antigen presentation and potent Th1 response against \( L. \) \textit{donovani} challenge in BALB/c mice (Breton et al., 2005). Further refinement of this approach using recombinant \( L. \) \textit{tarentolae} expressing \( L. \) \textit{donovani} A2 antigen was cross-protective against both \( L. \) \textit{infantum} (Mizbani et al., 2009, 2011) and \( L. \) \textit{donovani} (Yam et al., 2011). Overall, the use of live, non-pathogenic/genetically engineered \textit{Leishmania} seems to be the most promising strategic alternative against VL. However, the underlying risk of reversion to virulence chiefly in immunocompromised individuals is an important limitation for their large-scale use. Additionally, presence of antibiotic resistant genes in attenuated parasite strains hinders their use in clinical studies. Moreover, safety blocking of vaccine strain transmissibility by sandfly vector is another important issue that needs clarification.

### PROTEOMIC APPROACHES

Proteomics, being widely applied in studying \textit{Leishmania} spp., is a powerful tool for elucidating both molecular pathology and mechanistic searching of novel vaccine targets often missed following conventional techniques. Current immunoproteomics using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) approach focuses on identification and evaluation of responses to parasite T-cell epitopes as putative vaccine targets. Recent proteomics-based methodology using 2-D Western blot analyses from clinical isolates and sera of Indian VL patients have led to detection of 330 different immunogenic antigens (Forgber et al., 2006). Another systematic study detected a total of 33 T-cell stimulating antigens from soluble proteins of \( L. \) \textit{donovani} clinical isolate, M.W. ranging from 68 to 97.4 kDa (Gupta et al., 2007) that showed significant prophylactic potential against \( L. \) \textit{donovani} challenge in hamster model of VL (Garg et al., 2006). Availability of annotated 32.8-Mb sequenced genome with 8272 protein coding genes\(^4\) of \( L. \) \textit{major} permits identification of all potential protein products precisely outlining heterogeneity of complex host responses to leishmanial antigenic variation, and will be useful in rational designing of vaccine. Additional proteomic approaches analyzing sandfly salivary components have identified a large prospective candidate repertoire for vaccine development against leishmaniasis (Titus and Ribeiro, 1988; Gillespie et al., 2000; Valenzuela et al., 2004).

However, the field of proteomics in \textit{Leishmania} mostly limits itself to cytosolic proteins thereby excluding many pathogenic extracellular membrane proteins due to difficulties in solubilizing their hydrophobic transmembrane domains. Also, the approach remains insufficient for identification of protein subsets actually being expressed in parasites. Combining biological functions of identified proteins via activity-based techniques would accelerate this process of post-genomic vaccine research in future.

### SUBTRACTIVE GENOMICS

A very recently adopted methodology is based on subtraction of sequence between host and parasite proteome. Availability of whole genome sequence for human and pathogenic microbes has been utilized to find non-human homologous vaccine and therapeutic targets against \textit{Helicobacter pylori} (Huynen et al., 1998; Dutta et al., 2006), \textit{Pseudomonas aeruginosa} (Sakhrkar et al., 2004; Perumal et al., 2007), \textit{Mycobacterium pneumonia} (Gupta et al., 2010), \textit{Borrelia burgdorferi} (Madagi et al., 2011), \textit{Chlamydia trachomatis} (Praveena et al., 2011) as well as \( L. \) \textit{donovani} (Mandage and Wadhnerkar, 2010) that are currently facing the problems of drug resistance or absence of vaccines. Thus sorted key pathogenic proteins essential for parasite survival but non-homologous to human host, are subsequently checked for their cellular localization within the pathogen, exo-membrane topology and role in metabolic pathways to be used for drug or

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\(^3\)http://ec.europa.eu/research/health/infectious-diseases/neglected-diseases/projects/006.en.html

\(^4\)http://www.genedb.org/leish/index.jsp
prophylactic targets. Usually, conserved secreted and membrane proteins from pathogens are selected to design novel vaccines (Dutta et al., 2006; Barh et al., 2010). A preliminary work based on computational subtractive genomics has identified protein kinase, adenylate kinase and MAP kinase homolog of L. donovani for regulating normal cell cycle and differentiation of the parasite (Mandage and Wadnerkar, 2010). The experimental evaluation of such vaccine targets is required to validate their prophylactic potential. Further prediction of functional sites and exploration of Leishmania proteome are required to get the full benefit of this approach for a cross-protective VL vaccine. Moreover, it is advisable to consider all the pathogenic strains undertaking in silico mutagenesis approach and pangenomics data base, for further improvement of this basic strategy.

CHALLENGES AND FUTURE PROSPECT

A major challenge in vaccination against Leishmania and several other intracellular pathogens is the requirement of strong cell-mediated immune response in mediating protection. Most of the licensed prophylactic vaccines for human use rely completely on humoral response which have limited efficacy against Leishmania. Although adjuvants like alum, AS04 (composed of MPLA adsorbed to alum), MF59 and AS03 (oil-in-water emulsion) are approved for human use; inability to trigger robust T-cell response remains a potential short-coming of killed/subunit vaccines. None of the Leishmania vaccines reaching clinical trials could show adequate efficacy due to poor CD8+ T-cell responses arising from inefficient cross-presentation. Live-attenuated vaccines are advantageous in generating durable cellular (both CD4+ and CD8+) response mostly due to temporary parasite persistence and proper antigen presentation. However, they are largely precluded due to safety reasons. Although, in the current scenario, a plasmid DNA vaccine following heterologous prime-boost strategy seems to be adequate efficacy due to poor CD8+ T-cell responses arising from inefficient cross-presentation. Live-attenuated vaccines are advantageous in generating durable cellular (both CD4+ and CD8+) response mostly due to temporary parasite persistence and proper antigen presentation. However, they are largely precluded due to safety reasons. Although, in the current scenario, a plasmid DNA vaccine following heterologous prime-boost strategy seems to be a promising prophylactic choice for generating robust cellular and humoral response, they are largely tempered by chances of chronic inflammation, autoantibody generation and systemic lupus erythematosus (SLE), or integration into host genome that may either lead to absolute prophylactic anti-leishmanial vaccine in the near future.

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