Abhay Satoskar · Ravi Durvasula
Editors

Pathogenesis of Leishmaniasis

New Developments in Research

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Mechanisms of Immunopathology of Leishmaniasis

Gayathri Natarajan*, Steve Oghumu*, Sanjay Varikuti, Alexandria Thomas, and Abhay Satoskar

Abstract While cutaneous leishmaniasis is characterized by lesions proximal to the site of the sandfly bite, visceral leishmaniasis is associated with immunopathology in the liver and spleen of the infected host. This chapter offers a brief overview of the immune responses generated during infections with cutaneous and visceral leishmaniasis. Insights on how the immune responses generated during cutaneous and visceral leishmaniasis coupled with other host- and pathogen-derived factors determine the clinical pathology and outcome of the disease are also discussed.

Keywords Cytokines • Chemokines • Visceral • Splenomegaly • Immunopathology • Cutaneous

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* Gayathri Natarajan and Steve Oghumu are equal contributors of this chapter.

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Introduction

Immunity to intracellular pathogens requires the involvement of cell-mediated immune responses coupled with multiple cellular signaling pathways targeted at activating macrophages and dendritic cells (DCs) as well as killing infected cells. These processes require tight regulation to avoid uncontrolled amplification of the immune response, which may lead to immunopathology. *Leishmania* parasites have devised various mechanisms to circumvent the hosts' attempts at restricting parasite growth and disease establishment (Soong et al. 2012). In some cases, this involves inducing immunosuppressive conditions that favor parasite proliferation, or interfering with the migration and cellular interactions of critical immune cells involved in immunity against the parasite (Oghumu et al. 2010; Gupta et al. 2013). Host genetic and environmental factors also affect immunity to *Leishmania*. How these processes contribute to pathology in human disease as well as in animal models of leishmaniasis will be the focus of this review.

Cutaneous Leishmaniasis

Pathology of Cutaneous Leishmaniasis

Cutaneous leishmaniasis (CL) is caused by several species of *Leishmania*. Patients generally present with ulcers or nodules, usually on exposed parts of the body, such as the arms, legs, and face (Chappuis et al. 2007). Local cutaneous leishmaniasis (LCL) is characterized by the development of a lesion typically at the site of the sandfly bite. LCL lesions start with the development of erythema, eventually evolving into a small papule, which progressively ulcerates over a period of weeks to months, when the parasite spreads to lymph nodes and triggers an immunological response. The lesion development, progression, and resolution timeline varies depending upon the species of infection as well as the immunological status of the patient (Reithinger et al. 2007). CL lesions can develop anywhere from a few weeks to months after the initial infection (David and Craft 2009). LCL is caused by *Leishmania major*, *Leishmania tropica*, and *Leishmania aethiopica* in the regions of North and East Africa, Central Asia, and the Middle East. *Leishmania mexicana* and *Leishmania amazonensis* cause LCL in parts of Central and South America, the USA, and Mexico (Reithinger et al. 2007).

Under certain conditions, *L. aethiopica*, *L. mexicana*, and *L. amazonensis* also cause a diffuse form of the disease which is characterized by the development of non-ulcerative lesions away from the initial site of infection. Known as diffuse cutaneous leishmaniasis (DCL), these lesions do not have a tendency to self-cure. LCL and DCL are not life threatening but in the event of a late diagnosis, increase in lesion sizes could lead to scaring and development of secondary bacterial infections (David and Craft 2009). Migration of parasites to oropharyngeal mucosal sites leads to disfiguring mucosal lesions, called mucocutaneous leishmaniasis (MCL) which

is typically caused by *Leishmania braziliensis* in South America, parts of Central America, and Mexico (Reithinger et al. 2007). Patients with MCL present with severe mucosal inflammation and stuffiness (Chappuis et al. 2007; Reithinger et al. 2007), and suffer from progressively destructive mucosal ulcerations, extending from the nose and mouth to internal oropharyngeal organs, with possible perforation of the septum (Reithinger et al. 2007; Chappuis et al. 2007). MCL like DCL does not heal spontaneously and can be fatal and lesions are usually seen months or years after the first CL symptom (Chappuis et al. 2007).

Immune Responses to Cutaneous Leishmaniasis

Immunological mechanisms of CL have been elucidated mostly by studies on inbred mouse strains which have helped in the characterization of immune factors that are responsible for disease resolution or susceptibility. The most relevant mouse model that mimics human LCL is the *L. major* infection of C57BL/6 mouse strain which leads to a spontaneously healing lesion formed at the site of parasite injection (Belkaid et al. 2000).

Early Phase of Infection and Innate Immune Responses

It is well established that the sandfly bite injects *Leishmania* metacyclic parasites into the dermis of the skin (Mougneau et al. 2011). The skin is an important immune organ which consists of a variety of immune cells such as Langerhans cells, dermal dendritic cells, macrophages, and T cells. Keratinocytes are also involved in innate immune responses by releasing cytokines and chemokines as well as by participating in antigen presentation (Ilkovitch 2011). *Leishmania* parasites are phagocytosed by resident dermal dendritic cells and infiltrating neutrophils within 30 min of injection at the cutaneous site. However, infiltrating monocytes and macrophages make up the majority of cells that are infected with the parasite after several days (Ribeiro-Gomes et al. 2012b; Ng et al. 2008). The initial entry of parasites into neutrophils leads to changes in neutrophil physiology. Infected neutrophils are stimulated by the parasite to produce high levels of monocyte attracting chemokine, MIP-1β. Ultimately, parasite-infected neutrophils become apoptotic and the uptake of dying neutrophils by macrophages results in the secretion of anti-inflammatory cytokines such as TGF-β and reduced production of pro-inflammatory cytokines such as TNF-α. This works to create an anti-inflammatory milieu which promotes parasite survival in macrophages (van Zandbergen et al. 2004). Dermal dendritic cells also participate in the phagocytosis of dying infected neutrophils which results in an attenuation of *Leishmania* antigen presentation, expression of surface activation markers, and the ability to prime $CD4^+$ T cells (Ribeiro-Gomes et al. 2012b). Thus, *Leishmania* employ neutrophils as “Trojan Horses” to infect macrophages and dendritic cells and establish the initial phase of cutaneous infection. Interestingly,

at 1 week post-infection, a second wave of neutrophil recruitment has been documented in experimental models in addition to macrophage recruitment (Ribeiro-Gomes et al. 2012b; Ribeiro-Gomes and Sacks 2012). It is likely that these neutrophils are recruited in an IL-17-dependent manner due to the observation of poorer neutrophil recruitment in *IL-17*$^{-/-}$ mice (Lopez et al. 2009). The first 4–5 weeks of infection is a quiescent phase which is typified by rapid parasite multiplication without overt development of cutaneous pathology (Belkaid et al. 2000).

Nonimmune cells also play a vital role in the CL disease progression. Keratinocytes in the epidermal skin layer participate in modulating the immune response by the secretion of chemokines such as CCL2 and CCL5 which induces the recruitment of macrophages to the site of infection. Keratinocytes are also involved in the production of IL-4 and IL-6 in a temporally regulated manner within the first 8 h of infection thereby mediating a protective Th1 immune response (Ehrchen et al. 2010).

The second stage of infection that follows the silent phase ensues at 5 weeks post-infection (Belkaid et al. 2000). In experimental murine models using low dose parasite infection at a dermal infection site which mimics natural transmission, this second phase is concomitant with lesion evolution. During this phase, there is an influx of neutrophils, dendritic cells, and macrophages to the site of infection (Belkaid et al. 2000). Ultimately, macrophages are the preferred cell type that is targeted by the parasite for survival and replication. Clearance of the parasite is determined by the ability of macrophages to produce nitric oxide (NO) by the activation of the enzyme inducible nitric oxide synthase (iNOS). In addition to NO, the production of other oxidative intermediates by activated macrophages complements its leishmanicidal activity. Macrophages are activated to produce nitric oxide by cytokines such as IFN-γ and TNF-α while cytokines like IL-4, IL-10, IL-13, and TGF-β dampen NO production, thereby attenuating the leishmanicidal capability of the cell (Liu and Uzonna 2012).

Dendritic Cell Recruitment and Engagement of the Adaptive Immune Response

As mentioned above, coincident with lesion development is the increased presence of CD11c^{+} dendritic cells in the skin lesion (von Stebut 2007b). Dendritic cells are vital antigen presenting cells that participate in the initiation of an adaptive immune response. The fact that dendritic cell recruitment in the lesion coincides with increased IFN-γ production indicates that these cells contribute to the development of a protective immune response at the infection site and the draining lymph node (Belkaid et al. 2000). Dendritic cells preferentially uptake amastigotes versus promastigote forms of the parasite and present antigen via major histocompatibility complex (MHC) class I and II as well as accessory co-stimulatory molecules to T cells (Woelbing et al. 2006; von Stebut 2007a). Further, the uptake of *L. major* amastigotes leads to IL-12 release which directs the differentiation of naïve CD4^{+} T

helper cell to IFN-γ producing Th1 cells (von Stebut 2007a). *L. major*-specific CD8$^+$ T cells are also activated and contribute to IFN-γ production at the draining lymph nodes and lesion site (von Stebut 2007a). The role of B cells and the humoral response during *L. major* infection is unclear with differences in susceptibility being observed depending on the parasite strain (McMahon-Pratt and Alexander 2004).

Lesion Resolution, Wound Healing, and Immunity to Reinfection

Parasite elimination in macrophages is promoted by IFN-γ from activated CD4$^+$ and CD8$^+$ T cells (von Stebut 2007a). This leads to rapid decrease in parasite numbers in the lesion (Belkaid et al. 2000). However, complete elimination of parasites is not achieved, as there are few persistent parasites in the infection site and draining lymph node (Belkaid et al. 2001). IL-10 is required for parasite persistence as well as the maintenance of protective immunity to reinfection since sterile cure observed in *IL-10*$^{-/-}$ mice is also concomitant with the loss of protective memory responses (Belkaid et al. 2002). Two groups of CD4$^+$ T cells mediate immunity to reinfection and develop during the course of lesion healing: parasite-independent central memory T cells and parasite-dependent effector T cells (von Stebut 2007a). Thus, immunological memory in *Leishmania* infection is a case of concomitant immunity where, in addition to the central memory T cell population, there is a chronic stimulation of the immune system by persistent parasites which maintains an effector T cell population (Okwor and Uzonna 2008). Further, CD8$^+$ T cells are also important in secondary immune responses (Okwor and Uzonna 2008). Regulatory CD4$^+$ CD25$^+$ T cells (Tregs) modulate the immune response by curtailing excessive Th1 and Th2 immunity. In an IL-10-dependent and -independent manner, Tregs also allow for parasite persistence and, thus, in the maintenance of immunologic memory (Belkaid et al. 2002). Immunity to reinfection is a complex phenomenon which involves the presence and interactions of various immune cells and the residual parasites present in the host, mechanisms of which are still being explored. Pathology during infection is principally dependent on host genetics and infecting parasite species. Intact wound healing responses which determine the ability to rapidly induce tissue repair and skin healing are also vital besides T cell immune responses in resistance to CL (Mougneau et al. 2011).

Diverse Immunopathology Among Cutaneous **Leishmania** *Causing Species*

Phylogenetic studies indicate significant genetic diversity among various *Leishmania* complexes. It is therefore not surprising that the cutaneous disease causing species *L. major* and *L. mexicana* are as distinct from each other as visceralizing species are

different from those that cause CL. This is apparent from the variation in virulence factors that are required for pathogenesis by each of the species. For example, LPG is a virulence factor in *L. major* but not in *L. mexicana* (McMahon-Pratt and Alexander 2004).

The immunological mechanisms that regulate resistance also vary widely between species. Although an IFN-γ-dependent Th1 immune response with $CD4^+$ and $CD8^+$ T cell involvement is required for protection against CL, *L. mexicana* and *L. amazonensis* (unlike *L. major*) have developed mechanisms to survive in conditions of limited Th1 immune responses in the host. *L. amazonensis* parasites show enhanced replication in IFN-γ-activated macrophages (Qi et al. 2004). Paradoxically, progressive lesion development in *L. amazonensis* infection has been attributed to $CD4^+$ Th1 cells which promote the recruitment of cells important for parasite persistence to the infection site. It is therefore evident that compared to *L. major*, New World CL species require a more robust Th1 immune response for their clearance (McMahon-Pratt and Alexander 2004). There are also species-specific differences in the immune responses that govern disease exacerbation. While the role for B cells and antibodies is unclear in *L. major* infection, humoral immune responses have been shown to lead to susceptibility in infections of *L. mexicana* and *L. amazonensis* (McMahon-Pratt and Alexander 2004).

Interestingly, differences in immune mechanisms also exist within a given species depending upon the nature of CL infection. LCL caused by *L. aethiopica* is characterized by ulcerative lesions and a strong T cell response compared to the non-ulcerative pathology of DCL which is typified by T cell unresponsiveness (Nylen and Eidsmo 2012). Clinical isolates of LCL species induce higher IFN-γ, IL-4, and IL-6 production upon infection with human blood-derived monocytes compared to DCL species which induce more IL-10, a cytokine that is generally associated with chronic infections (Akuffo et al. 1997). Patients infected with strains of *L. mexicana* which cause DCL lesions contain fewer $CD8^+$ T cells that are deficient in IFN-γ production, proliferative capability, and cytotoxicity compared to patients infected with LCL strains. These immunological responses are characteristic of T cell exhaustion which may correlate with parasite persistence and chronic infection in DCL. MCL, on the other hand, is associated with mixed Th1 and Th2 immune responses (Castes and Tapia 1998). Higher IL-2, IL-4, and IL-5 production has been observed in MCL causing *L. braziliensis* compared to LCL causing *L. braziliensis* (Nogueira et al. 2008). Tissue destruction in MCL has been attributed to higher concentrations of TNF-α and the cytolytic action of $CD8^+$ T cells, NK cells, and neutrophils (Nylen and Eidsmo 2012; Blackwell 1999).

The extent of tissue damage and ulcerative lesion development has been linked to factors such as Fas ligand (FasL) and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) which mediate apoptosis in Fas and TRAIL-receptor expressing cells, respectively. In LCL, keratinocytes upregulate Fas, TRAIL, and TRAIL-receptor 2 which deliver apoptotic signals. Similarly, FasL upregulation has been observed in MCL (Nylen and Eidsmo 2012).

Depending on the species of *Leishmania* causing infection, host genetics, immunological status, and environmental and other factors, CL displays a variety of

pathological and clinical manifestations. Although differences in virulence factors have been defined between the various CL species, additional research will be required to understand how various *Leishmania* species manipulate host immunological factors to induce tissue damage in certain cases like LCL and MCL infections or minimize ulcerative lesion development in case of DCL. In addition to drugs that target the parasite, agents that modulate host immune mechanisms and wound healing responses could potentially limit pathology, particularly during CL infections.

Visceral Leishmaniasis

Unlike cutaneous and mucosal leishmaniasis, visceral leishmaniasis (VL) is a systemic disease which is fatal if left untreated. It is caused by *L. donovani* in East Africa and the Indian subcontinent and *L. infantum/chagasi* in North Africa, Europe, and Latin America (Chappuis et al. 2007). Clinical presentation of the infection usually occurs 2–6 months after initial contact with an infected sandfly. The parasites are introduced intradermally, travel through blood vessels to blood-filtering organs, and invade the reticulo-endothelial system, including the lymph nodes, spleen, and liver, causing them to become enlarged (Chappuis et al. 2007; McCall et al. 2013). Patients infected with VL present several symptoms that indicate systemic infection such as weakness, fatigue, fever, weight loss, and loss of appetite (Chappuis et al. 2007). A complication of VL commonly observed in Sudan and other East African countries as well as in immunocompromised VL patients is post-kala-azar dermal leishmaniasis (PKDL) characterized by nodular lesions which contain numerous parasites (Chappuis et al. 2007). However, many who become infected with visceral *Leishmania* species do not manifest any clinical symptoms (Chappuis et al. 2007; McCall et al. 2013).

Immunopathology of Visceral Leishmaniasis

As noted earlier, the pathogenesis of visceralizing species of *Leishmania* is markedly different from those that cause cutaneous disease. Prevailing immune responses generated in the spleen, liver, and bone marrow ultimately determine the disease outcome of patients infected with VL. However, mechanisms of resistance to VL share a number of similarities with CL. As demonstrated by animal models, Th1 responses characterized by IFN-γ production are required for resistance, while production of the immunosuppressive cytokine IL-10 is associated with susceptibility to VL. Cytokine-mediated macrophage activation as well as production of reactive oxygen and nitrogen intermediates is critical for killing of intracellular amastigotes. There are, however, unique parasite- and host-specific factors that affect immunopathology to *L. donovani* and *L. infantum/chagasi* as demonstrated by a number of studies.

In humans, VL presents as an asymptomatic, subclinical infection or as an active disease. Very few infected patients develop active disease (Soong et al. 2012; Badaro et al. 1986). This suggests that host genetic and environmental factors play a major role in immunity to VL. Indeed, the mechanisms of immunopathology to VL in humans are complex and are still incompletely understood. Much of what is known about immunity to VL is based on murine and hamster infection models. Infection of hamsters with *L. donovani* or *L. infantum/chagasi* results in progressive disease and parasite replication in the spleen, liver, and bone marrow eventually resulting in death (Melby et al. 2001), which resembles active disease in humans. On the other hand, infection of mice with visceralizing *Leishmania* is a better model of subclinical infection in humans (Wilson et al. 2005).

As with CL, neutrophils are among the first cells recruited to the area of the bite of a visceralizing *Leishmania*-infected sandfly (Thalhofer et al. 2011). Eventually, parasites disseminate to visceral organs through mechanisms that are still incompletely understood, although macrophage and dendritic cell populations seem to play a role in this process (Ribeiro-Gomes et al. 2012a; Zhang et al. 2003). Parasite virulence factors no doubt also contribute to organ dissemination and a number of virulence genes in *L. donovani* and *L. infantum/chagasi* have been identified as required for visceralization (Zhang and Matlashewski 2001; McCall and Matlashewski 2012). Host factors affecting immunity to VL in humans as suggested by population genetic studies in endemic areas include a number of cytokines such as TNF-α (Karplus et al. 2002), IL-4 (Mohamed et al. 2003) and TGF-β (Frade et al. 2011), chemokine and cytokine receptors CXCR2 (Mehrotra et al. 2011) and IL-2 receptor (Bucheton et al. 2007), as well as other immune-related genes. These studies suggest that Th1 immune responses are involved in control of VL in humans, although this response alone is not sufficient to protect against disease (McCall et al. 2013). Additionally, the prevalence of HIV coinfection as well as malnutrition in developing countries negatively affects immunity to VL (Soong et al. 2012).

Using animal models, much has been learned about immunity to *L. donovani*. Elements of the innate and adaptive immune system have been shown to play a role in resistance to VL. Mice genetically resistant to VL, such as CBA mice, control early parasite replication due to the presence of a functional phagosomal ion transporter-related gene, slc11a1 (Blackwell et al. 2001; Gruenheid et al. 1997). Mice with a genetic mutation in slc11a1 such as BALB/c and C57BL/6 mice display enhanced parasite growth at early time points (Gruenheid et al. 1997; Kaye et al. 2004). Studies using SCID mice or *rag*$^{-/-}$ mice show that adaptive T cell-mediated responses are vital for resistance. The importance of other immune-related genes has also been demonstrated using mouse models, such as reactive oxygen and nitrogen intermediates (Murray and Nathan 1999), Th1 inducing cytokines IFN-γ and IL-12 (Murray et al. 1997; Engwerda et al. 1998; Squires et al. 1989), as well as transcription factor T-bet (Rosas et al. 2006b). Th1 suppressing cytokines such as TGF-β and IL-10 mediate susceptibility to VL (Murphy et al. 2001; Goto and Lindoso 2004). However, unlike *L. major*, immunity to VL extends beyond the generation of a Th1 response. Studies using *IL4*$^{-/-}$ and *IL4Rα*$^{-/-}$ mice show that IL-4 signaling is important for parasite clearance in the spleen and liver (Alexander et al. 2000; Stager et al. 2003). Also surprising is the fact that mice deficient in

STAT1, a mediator of IFN-γ signaling, are highly resistant to *L. donovani* infection and develop minimal immunopathology (Rosas et al. 2006b). This shows that other immunologic factors in addition to Th1 immune responses play a role in determining immunity and pathology of VL.

Organ-Specific Immunopathology in Visceral Leishmaniasis

Immunity to *L. donovani* is primarily organ specific. As demonstrated by experimental murine models of infection, the liver is the site of acute infection which usually resolves, resulting in minimal tissue damage (Engwerda et al. 1998). However, in the spleen and bone marrow, the parasite persists accompanied by significant pathology (Stanley and Engwerda 2007). The hallmark of parasite control in the liver of infected mice is the formation of mature granulomas around infected macrophages. Granuloma formation is dependent on the production of chemokines CCL2 (MCP-1), CCL3 (MIP-1α), and CXCL10 (IP-10) by infected Kupffer cells and subsequent recruitment of neutrophils and monocytes within the first few days of infection (Soong et al. 2012; Murray 2001; Oghumu et al. 2010). Eventually, $CD4^+$ and $CD8^+$ T cells are recruited to the granuloma and produce IFN-γ, TNF-α, and other pro-inflammatory cytokines which lead to classical macrophage activation and generation of reactive oxygen species (ROS) and NO. NO production by IFN-γ-activated macrophages is critical for parasite killing in the liver. Interestingly, despite increased expression of IFN-γ and TNF-α in the liver of hamsters, progressive infection still develops. In this model, it has been shown that IFN-γ-mediated NO production by infected macrophages is severely attenuated in the liver, leading to progressive disease similar to what is found in active human VL.

Although required for parasite clearance, uncontrolled Th1 hyperimmune responses could contribute to immunopathology in the liver. The production of immunosuppressive cytokines such as IL-10, TGF-β, and IL-27 could help to control pro-inflammatory immune responses (Stanley and Engwerda 2007). IL-27 has been shown to prevent severe immunopathology in this organ (Rosas et al. 2006a).

In mice the spleen is generally the site of progressive infection and severe pathology. It is not completely known why control of VL is different in the spleens and livers of infected mice. Research which provides a better understanding of these differences could better explain mechanisms of immunopathology in active human VL and possible therapeutic interventions. Early during infection, marginal zone (MZ) macrophages are infected by *Leishmania*. Migration of dendritic cells from the MZ to the periarteriolar lymphoid sheath (PALS) is critical for the generation of effector T cell responses early during infection, a process mediated by production of chemokines CCL19 and CCL21 by stromal cells in the PALS and expression of chemokine receptor CCR7 by migrating DCs (Ato et al. 2006; Oghumu et al. 2010). During the course of infection, this migration is lost due to a reduction of CCR7 expression as well as CCL19 and CCL21 production (Wilson et al. 2005; Stanley and Engwerda 2007; Soong et al. 2012). A major immune factor implicated in causing cellular mis-localization in the spleen is excessive production of TNF-α

(Ato et al. 2002; Engwerda et al. 2002). In contrast with the liver where regulated production of TNF-α is vital for effective parasite control, excessive TNF-α production in the spleen has been shown to be responsible for parasite growth and severe immunopathology. IL-10 induction in the spleen, caused by TNF-α production, also contributes to parasite persistence and defective immune responses (Ato et al. 2002; Wilson et al. 2005; Murphy et al. 2001). Eventually the splenic architecture is severely damaged, with extensive remodeling of the MZ and selective loss of MZ macrophages (Engwerda et al. 2002). Follicular dendritic cell networks and germinal centers are destroyed, although the precise immune mechanisms are still yet to be fully defined (Smelt et al. 1997). Similar immunopathology is observed in humans with progressive VL (Zijlstra and el-Hassan 2001).

It is obvious that mechanisms of immunopathology in VL are dependent on host genetic and immunologic factors as well as parasite virulence determinants. The success of visceralizing species of *Leishmania* in the establishment of chronic disease and pathology in mammalian hosts is based on their ability to subvert host signaling pathways and induce immunosuppressive and/or hyper-responsive immunologic states in visceral organs.

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References

Akuffo H, Maasho K, Blostedt M, Hojeberg B, Britton S, Bakhiet M (1997) Leishmania aethiopica derived from diffuse leishmaniasis patients preferentially induce mRNA for interleukin-10 while those from localized leishmaniasis patients induce interferon-gamma. J Infect Dis 175(3):737–741

Alexander J, Carter KC, Al-Fasi N, Satoskar A, Brombacher F (2000) Endogenous IL-4 is necessary for effective drug therapy against visceral leishmaniasis. Eur J Immunol 30(10):2935–2943. doi:10.1002/1521-4141(200010)30:10<2935::AID-IMMU2935>3.0.CO;2-Q

Ato M, Stager S, Engwerda CR, Kaye PM (2002) Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. Nat Immunol 3(12):1185–1191. doi:10.1038/ni861

Ato M, Maroof A, Zubairi S, Nakano H, Kakiuchi T, Kaye PM (2006) Loss of dendritic cell migration and impaired resistance to Leishmania donovani infection in mice deficient in CCL19 and CCL21. J Immunol 176(9):5486–5493

Badaro R, Jones TC, Lorenco R, Cerf BJ, Sampaio D, Carvalho EM, Rocha H, Teixeira R, Johnson WD Jr (1986) A prospective study of visceral leishmaniasis in an endemic area of Brazil. J Infect Dis 154(4):639–649

Belkaid Y, Mendez S, Lira R, Kadambi N, Milon G, Sacks D (2000) A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. J Immunol 165(2):969–977. doi:ji_v165n2p969 [pii]

Belkaid Y, Hoffmann KF, Mendez S, Kamhawi S, Udey MC, Wynn TA, Sacks DL (2001) The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. J Exp Med 194(10):1497–1506

Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL (2002) $CD4^{+}CD25^{+}$ regulatory T cells control Leishmania major persistence and immunity. Nature 420(6915):502–507. doi:10.1038/nature01152 [doi]; nature01152 [pii]

Blackwell JM (1999) Tumour necrosis factor alpha and mucocutaneous leishmaniasis. Parasitol Today 15(2):73–75. doi:S0169-4758(98)01355-6 [pii]

Blackwell JM, Goswami T, Evans CA, Sibthorpe D, Papo N, White JK, Searle S, Miller EN, Peacock CS, Mohammed H, Ibrahim M (2001) SLC11A1 (formerly NRAMP1) and disease resistance. Cell Microbiol 3(12):773–784

Bucheton B, Argiro L, Chevillard C, Marquet S, Kheir MM, Mergani A, El-Safi SH, Dessein AJ (2007) Identification of a novel G245R polymorphism in the IL-2 receptor beta membrane proximal domain associated with human visceral leishmaniasis. Genes Immun 8(1):79–83. doi:10.1038/sj.gene.6364355

Castes M, Tapia FJ (1998) Immunopathology of American tegumentary leishmaniasis. Acta Cient Venez 49(1):42–56

Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, Boelaert M (2007) Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol 5(11):873–882. doi:10.1038/nrmicro1748

David CV, Craft N (2009) Cutaneous and mucocutaneous leishmaniasis. Dermatol Ther 22(6):491–502. doi:10.1111/j.1529-8019.2009.01272.x

Ehrchen JM, Roebrock K, Foell D, Nippe N, von Stebut E, Weiss JM, Munck NA, Viemann D, Varga G, Muller-Tidow C, Schuberth HJ, Roth J, Sunderkotter C (2010) Keratinocytes determine Th1 immunity during early experimental leishmaniasis. PLoS Pathog 6(4):e1000871. doi:10.1371/journal.ppat.1000871 [doi]

Engwerda CR, Murphy ML, Cotterell SE, Smelt SC, Kaye PM (1998) Neutralization of IL-12 demonstrates the existence of discrete organ-specific phases in the control of Leishmania donovani. Eur J Immunol 28(2):669–680. doi:10.1002/(SICI)1521-4141(199802)28:02<669::AID-IMMU669>3.0.CO;2-N

Engwerda CR, Ato M, Cotterell SE, Mynott TL, Tschannerl A, Gorak-Stolinska PM, Kaye PM (2002) A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during Leishmania donovani infection. Am J Pathol 161(2):429–437

Frade AF, Oliveira LC, Costa DL, Costa CH, Aquino D, Van Weyenbergh J, Barral-Netto M, Barral A, Kalil J, Goldberg AC (2011) TGFB1 and IL8 gene polymorphisms and susceptibility to visceral leishmaniasis. Infect Genet Evol 11(5):912–916. doi:10.1016/j.meegid.2011.02.014

Goto H, Lindoso JA (2004) Immunity and immunosuppression in experimental visceral leishmaniasis. Braz J Med Biol Res 37(4):615–623

Gruenheid S, Pinner E, Desjardins M, Gros P (1997) Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. J Exp Med 185(4):717–730

Gupta G, Oghumu S, Satoskar AR (2013) Mechanisms of immune evasion in leishmaniasis. Adv Appl Microbiol 82:155–184. doi:10.1016/B978-0-12-407679-2.00005-3

Ilkovitch D (2011) Role of immune-regulatory cells in skin pathology. J Leukoc Biol 89(1):41–49. doi:jlb.0410229 [pii]; 10.1189/jlb.0410229 [doi]

Karplus TM, Jeronimo SM, Chang H, Helms BK, Burns TL, Murray JC, Mitchell AA, Pugh EW, Braz RF, Bezerra FL, Wilson ME (2002) Association between the tumor necrosis factor locus and the clinical outcome of Leishmania chagasi infection. Infect Immun 70(12):6919–6925

Kaye PM, Svensson M, Ato M, Maroof A, Polley R, Stager S, Zubairi S, Engwerda CR (2004) The immunopathology of experimental visceral leishmaniasis. Immunol Rev 201:239–253. doi:10.1111/j.0105-2896.2004.00188.x

Liu D, Uzonna JE (2012) The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. Front Cell Infect Microbiol 2:83. doi:10.3389/fcimb.2012.00083 [doi]

Lopez KS, Dinges S, Griewank K, Iwakura Y, Udey MC, von Stebut E (2009) IL-17 promotes progression of cutaneous leishmaniasis in susceptible mice. J Immunol 182(5):3039–3046. doi:182/5/3039 [pii]; 10.4049/jimmunol.0713598 [doi]

McCall LI, Matlashewski G (2012) Involvement of the Leishmania donovani virulence factor A2 in protection against heat and oxidative stress. Exp Parasitol 132(2):109–115. doi:10.1016/j.exppara.2012.06.001

McCall LI, Zhang WW, Matlashewski G (2013) Determinants for the development of visceral leishmaniasis disease. PLoS Pathog 9(1):e1003053. doi:10.1371/journal.ppat.1003053

McMahon-Pratt D, Alexander J (2004) Does the Leishmania major paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? Immunol Rev 201:206–224. doi:10.1111/j.0105-2896.2004.00190.x [doi]; IMR190 [pii]

Mehrotra S, Fakiola M, Oommen J, Jamieson SE, Mishra A, Sudarshan M, Tiwary P, Rani DS, Thangaraj K, Rai M, Sundar S, Blackwell JM (2011) Genetic and functional evaluation of the role of CXCR1 and CXCR2 in susceptibility to visceral leishmaniasis in north-east India. BMC Med Genet 12:162. doi:10.1186/1471-2350-12-162

Melby PC, Chandrasekar B, Zhao W, Coe JE (2001) The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like cytokine response. J Immunol 166(3):1912–1920

Mohamed HS, Ibrahim ME, Miller EN, Peacock CS, Khalil EA, Cordell HJ, Howson JM, El Hassan AM, Bereir RE, Blackwell JM (2003) Genetic susceptibility to visceral leishmaniasis in The Sudan: linkage and association with IL4 and IFNGR1. Genes Immun 4(5):351–355. doi:10.1038/sj.gene.6363977

Mougneau E, Bihl F, Glaichenhaus N (2011) Cell biology and immunology of Leishmania. Immunol Rev 240(1):286–296. doi:10.1111/j.1600-065X.2010.00983.x [doi]

Murphy ML, Wille U, Villegas EN, Hunter CA, Farrell JP (2001) IL-10 mediates susceptibility to Leishmania donovani infection. Eur J Immunol 31(10):2848–2856. doi:10.1002/1521-4141(2001010)31:10<2848::AID-IMMU2848>3.0.CO;2-T

Murray HW (2001) Tissue granuloma structure-function in experimental visceral leishmaniasis. Int J Exp Pathol 82(5):249–267

Murray HW, Nathan CF (1999) Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral Leishmania donovani. J Exp Med 189(4):741–746

Murray HW, Hariprashad J, Coffman RL (1997) Behavior of visceral Leishmania donovani in an experimentally induced T helper cell 2 (Th2)-associated response model. J Exp Med 185(5):867–874

Ng LG, Hsu A, Mandell MA, Roediger B, Hoeller C, Mrass P, Iparraguirre A, Cavanagh LL, Triccas JA, Beverley SM, Scott P, Weninger W (2008) Migratory dermal dendritic cells act as rapid sensors of protozoan parasites. PLoS Pathog 4(11):e1000222. doi:10.1371/journal.ppat.1000222 [doi]

Nogueira MF, Goto H, Sotto MN, Cuce LC (2008) Cytokine profile in Montenegro skin test of patients with localized cutaneous and mucocutaneous leishmaniasis. Rev Inst Med Trop Sao Paulo 50(6):333–337. doi:S0036-46652008000600004 [pii]

Nylen S, Eidsmo L (2012) Tissue damage and immunity in cutaneous leishmaniasis. Parasite Immunol 34(12):551–561. doi:10.1111/pim.12007 [doi]

Oghumu S, Lezama-Davila CM, Isaac-Marquez AP, Satoskar AR (2010) Role of chemokines in regulation of immunity against leishmaniasis. Exp Parasitol 126(3):389–396. doi:10.1016/j.exppara.2010.02.010

Okwor I, Uzonna J (2008) Persistent parasites and immunologic memory in cutaneous leishmaniasis: implications for vaccine designs and vaccination strategies. Immunol Res 41(2):123–136. doi:10.1007/s12026-008-8016-2 [doi]

Qi H, Ji J, Wanasen N, Soong L (2004) Enhanced replication of Leishmania amazonensis amastigotes in gamma interferon-stimulated murine macrophages: implications for the pathogenesis of cutaneous leishmaniasis. Infect Immun 72(2):988–995

Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S (2007) Cutaneous leishmaniasis. Lancet Infect Dis 7(9):581–596. doi:S1473-3099(07)70209-8 [pii]; 10.1016/S1473-3099(07)70209-8 [doi]

Ribeiro-Gomes FL, Sacks D (2012) The influence of early neutrophil-Leishmania interactions on the host immune response to infection. Front Cell Infect Microbiol 2:59. doi:10.3389/fcimb.2012.00059 [doi]

Ribeiro-Gomes FL, Peters NC, Debrabant A, Sacks DL (2012a) Efficient capture of infected neutrophils by dendritic cells in the skin inhibits the early anti-leishmania response. PLoS Pathog 8(2):e1002536. doi:10.1371/journal.ppat.1002536

Ribeiro-Gomes FL, Peters NC, Debrabant A, Sacks DL (2012b) Efficient capture of infected neutrophils by dendritic cells in the skin inhibits the early anti-leishmania response. PLoS Pathog 8(2):e1002536. doi:10.1371/journal.ppat.1002536 [doi]; PPATHOGENS-D-11-01509 [pii]

Rosas LE, Satoskar AA, Roth KM, Keiser TL, Barbi J, Hunter C, de Sauvage FJ, Satoskar AR (2006a) Interleukin-27R (WSX-1/T-cell cytokine receptor) gene-deficient mice display enhanced resistance to leishmania donovani infection but develop severe liver immunopathology. Am J Pathol 168(1):158–169. doi:10.2353/ajpath.2006.050013

Rosas LE, Snider HM, Barbi J, Satoskar AA, Lugo-Villarino G, Keiser T, Papenfuss T, Durbin JE, Radzioch D, Glimcher LH, Satoskar AR (2006b) Cutting edge: STAT1 and T-bet play distinct roles in determining outcome of visceral leishmaniasis caused by Leishmania donovani. J Immunol 177(1):22–25

Smelt SC, Engwerda CR, McCrossen M, Kaye PM (1997) Destruction of follicular dendritic cells during chronic visceral leishmaniasis. J Immunol 158(8):3813–3821

Soong L, Henard CA, Melby PC (2012) Immunopathogenesis of non-healing American cutaneous leishmaniasis and progressive visceral leishmaniasis. Semin Immunopathol 34(6):735–751. doi:10.1007/s00281-012-0350-8

Squires KE, Schreiber RD, McElrath MJ, Rubin BY, Anderson SL, Murray HW (1989) Experimental visceral leishmaniasis: role of endogenous IFN-gamma in host defense and tissue granulomatous response. J Immunol 143(12):4244–4249

Stager S, Alexander J, Carter KC, Brombacher F, Kaye PM (2003) Both interleukin-4 (IL-4) and IL-4 receptor alpha signaling contribute to the development of hepatic granulomas with optimal antileishmanial activity. Infect Immun 71(8):4804–4807

Stanley AC, Engwerda CR (2007) Balancing immunity and pathology in visceral leishmaniasis. Immunol Cell Biol 85(2):138–147. doi:10.1038/sj.icb7100011

Thalhofer CJ, Chen Y, Sudan B, Love-Homan L, Wilson ME (2011) Leukocytes infiltrate the skin and draining lymph nodes in response to the protozoan Leishmania infantum chagasi. Infect Immun 79(1):108–117. doi:10.1128/IAI.00338-10

van Zandbergen G, Klinger M, Mueller A, Dannenberg S, Gebert A, Solbach W, Laskay T (2004) Cutting edge: neutrophil granulocyte serves as a vector for Leishmania entry into macrophages. J Immunol 173(11):6521–6525. doi:173/11/6521 [pii]

von Stebut E (2007a) Cutaneous Leishmania infection: progress in pathogenesis research and experimental therapy. Exp Dermatol 16(4):340–346. doi:EXD554 [pii]; 10.1111/j.1600-0625.2007.00554.x [doi]

von Stebut E (2007b) Immunology of cutaneous leishmaniasis: the role of mast cells, phagocytes and dendritic cells for protective immunity. Eur J Dermatol 17(2):115–122. doi:ejd.2007.0122 [pii]; 10.1684/ejd.2007.0122 [doi]

Wilson ME, Jeronimo SM, Pearson RD (2005) Immunopathogenesis of infection with the visceralizing Leishmania species. Microb Pathog 38(4):147–160. doi:10.1016/j.micpath.2004.11.002

Woelbing F, Kostka SL, Moelle K, Belkaid Y, Sunderkoetter C, Verbeek S, Waisman A, Nigg AP, Knop J, Udey MC, von Stebut E (2006) Uptake of Leishmania major by dendritic cells is mediated by Fcgamma receptors and facilitates acquisition of protective immunity. J Exp Med 203(1):177–188. doi:jem.20052288 [pii]; 10.1084/jem.20052288 [doi]

Zhang WW, Matlashewski G (2001) Characterization of the A2-A2rel gene cluster in Leishmania donovani: involvement of A2 in visceralization during infection. Mol Microbiol 39(4):935–948

Zhang WW, Mendez S, Ghosh A, Myler P, Ivens A, Clos J, Sacks DL, Matlashewski G (2003) Comparison of the A2 gene locus in Leishmania donovani and Leishmania major and its control over cutaneous infection. J Biol Chem 278(37):35508–35515. doi:10.1074/jbc.M305030200

Zijlstra EE, el-Hassan AM (2001) Leishmaniasis in Sudan. Visceral leishmaniasis. Trans R Soc Trop Med Hyg 95(suppl 1):S27–S58

Update on *Leishmania* Mediators That Promote Immune Evasion

Peter E. Kima

Abstract Parasites of the *Leishmania* genus are the causative agents of the complex disease called leishmaniasis. Clinical manifestations in this disease range from cutaneous lesions, disseminated cutaneous lesions, mucocutaneous lesions, and visceral disease. Even though immune responses are elicited in response to infection, infection with several *Leishmania* species is not self-limited. The mechanism(s) by which parasites avoid the lethal effects of the immune response is the subject of intense research. This research includes studies on the identity and targets of the parasite mediators that are deployed to limit effectors of the immune response. This review will profile some of the parasite mediators for which there has been recent progress and the processes that they target.

Keywords *Leishmania* • Immune evasion • Tryparedoxin peroxidase • ROS • RNS • Type 1 IFN

Introduction

The survival of *Leishmania* in the mammalian host is dependent on the outcome of multiple host–parasite interactions throughout the duration of the infection. *Leishmania* lesions are a milieu that is rich in immunologic activity: chemokines produced by infected or bystander cells induce cell recruitment to that site; cytokines released there activate cells in the lesion. In light of the fact that *Leishmania* persist in lesions, it can be inferred that either the immune effectors in lesions are not sufficiently antiparasitic or parasites deploy many strategies to limit the effect of those responses.

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Both anti-inflammatory and proinflammatory cytokines and chemokines are produced during *Leishmania* infections. There is active research aimed at determining how *Leishmania* parasites contribute to the development of those immune responses. Several recent reviews have considered the impact of elicitation of cross regulatory mediators on *Leishmania* infections (Nylén and Sacks 2007; Alexander and Brombacher 2012; Kima and Soong 2013). Going forward, it is of interest to determine whether elicitation of responses with counter-regulatory effects occurs fortuitously or whether it is a *Leishmania* strategy for survival.

There is convincing evidence that intracellular signaling pathways that transmit responses to cytokines or to stimuli that result in cytokine production are modulated by the infection. The reader is referred to several excellent reviews that have provided detailed summaries of those studies (Ben-Othman et al. 2009; Gregory and Olivier 2005; Gupta et al. 2013; Kima 2007). Examples of the signaling mediators that are modulated by infection include the MAP kinases, signal transducers and activators of transcription (STATs), and nuclear factor kappa b (NF-κB). The effect on the infection process is that infected cells are refractory to most immunologic effectors in their vicinity. Our understanding of the parasite mediators that target many of these host processes is still incomplete. However, several recent studies have provided valuable insight into parasite mediators that are elaborated during infection. The identity of those parasite molecules and their targets in the host will be the focus of this review. An issue that is raised, for which there is limited evidence, is the schemes by which molecules that are synthesized by *Leishmania* parasites within vacuolar compartments gain access to their host targets. For parasite molecules with targets in the infected cell cytosol, they have to be exported across the parasitophorous vacuole (PV) membrane; for parasite molecules whose targets are in the host cell nucleus, an additional membrane, the nuclear membrane, has to be overcome; some parasite molecules have targets outside the infected cell.

Parasite mediators that detoxify the respiratory burst. Macrophages are the primary hosts of *Leishmania* parasites. The respiratory burst in macrophages is composed of several oxygen and nitrogen reactive compounds. Generation of these compounds occurs in response to distinct stimuli. However, once the response is initiated, oxygen and nitrogen radicals can synergize to form more potent species. Reactive oxygen species (ROS) including superoxide radical anion and H_2O_2 are primarily produced by the NADPH oxidase (NOX) and dual-oxidase (DUOX) family of enzymes (Nauseef 2008). They are produced in response to innate stimuli such as phagocytosis. NOX-2 (out of five variants) is the most important in macrophages. This enzyme is composed of five subunits that assemble at the site of phagocytosis and subsequently on the limiting membrane of phagosomes that harbor internalized particles. In contrast to ROS, nitric oxide (NO), the initial reactive nitrogen species (RNS), is produced in response to immune stimuli, specifically cytokines. NO production in macrophages is the product of the inducible nitric oxide synthetase (iNOS) that acts on arginine in the presence of oxygen to catalyze the release of NO. NO can be subsequently converted to nitrogen oxide or peroxynitrite, which are potent oxidative species. ROS and RNS can cause oxidative

damage to biomolecules such as lipids, proteins, and DNA, which leads to loss of membrane integrity, defective replication, and eventually cell death.

Both the promastigote and amastigote forms of *Leishmania* are internalized by phagocytosis. It should be expected therefore that ROS is produced during parasite internalization. However, several studies with the amastigote forms of *Leishmania* have shown that uptake of amastigote forms is silent (Channon et al. 1984; Pham et al. 2005; Lodge and Descoteaux 2006). The situation with promastigote forms is more controversial as species differences in superoxide production have been reported (Lodge et al. 2006). These observations imply that *Leishmania* amastigotes elaborate a mechanism to prevent superoxide production during parasite uptake. Details on the proposed mechanisms by which *Leishmania* parasites limit superoxide production have been discussed in several reviews (Kima 2007; Moradin and Descoteaux 2012).

Thioredoxins and peroxiredoxins: These are members of a family of proteins that are characterized by their active site motifs, containing either one or two cysteinyl residues. These thiol groups are essential for (1) the reduction of protein disulfides (thioredoxins), (2) protein de-glutathionylation (glutaredoxins), or (3) the reduction of H_2O_2 (peroxiredoxins). Peroxiredoxins use a redox-active cysteine residue (peroxidatic Cys) to reduce a broad spectrum of substrates, including H_2O_2, organic hydroperoxides, and peroxynitrite ($ONOO^-$) (Hanschmann et al. 2013). Upon reduction of the peroxide, the peroxidatic Cys-SH is oxidized to sulfenic acid (Cys-SOH). Peroxiredoxins return to their reduced state upon reduction of the disulfide by an appropriate electron donor. *Leishmania* lack catalase and selenium-glutathione peroxidases. Instead they have a unique oxidoreductase of the thioredoxin superfamily, known as tryparedoxin (TXN). The peroxidoxins of *Leishmania* and other trypanosomatids are commonly referred to as tryparedoxin peroxidases (TXNPxs) (Flohé 2012). Two TXNPxs have been described in *Leishmania* that are localized in the parasite mitochondrion and cytosol (Castro et al. 2004; Jirata et al. 2006; Barr and Gedamu 2003). Overexpression studies and other approaches have revealed some substrate differences between these two variants; cTXNPx preferentially inactivates H_2O_2 and the organic hydroperoxide *t*-bOOH (Castro et al. 2010). In contrast, overexpression of *Li*mTXNPx in promastigotes did not ensure any significant resistance to exogenously added H_2O_2, but sheltered parasites when exposed to *t*-bOOH. The functions for the TXNPx molecules have thus far been linked to their localization within the parasite. Current views are that the mitochondrial variant detoxifies oxidative radicals that are produced primarily during electron transport (Castro et al. 2008). The cytosolic variant functions in response to parasite stress from changes in the parasite's environment such as temperature increase in the mammalian host in contrast to the sand fly gut.

In light of the fact that the mitochondrial and cytosolic TXNPxs are within the parasite, it would appear that the surface of the parasite is still vulnerable to the effects of oxygen radicals unless there are other molecules that are exported to the parasite surface or secreted from the parasite. The generation of superoxide in phagosomal compartments occurs at the limiting membrane from where oxygen

radicals diffuse. ROS diffuse poorly across membranes; translocation across membranes occurs through channels and transporters (Bienert et al. 2006). NO that is produced by the cytosolic enzyme, iNOS, reacts with oxygen to form peroxynitrite and other species that diffuse more readily across membranes (Denicola et al. 1998). Based on an inability to recover peroxidase activity in the supernatant fluid from infective promastigotes, Romao et al. (2009) concluded that it is unlikely that cTXNPx is secreted either from promastigotes or from amastigotes. In studies to identify parasite molecules that are exported from PVs, Kima and colleagues identified a novel TXNPx variant that is released from intracellular parasites and is exported from PVs in the host cell (Kima et al. 2010). Recently, a secreted peroxidase activity was described (Singh et al. 2013) and was proposed to target host molecules in the infected cell cytosol. Although the peroxidase activity of these secreted peroxidases has not been evaluated sufficiently, there is a strong likelihood that a parasite peroxidase is available to detoxify oxygen and nitrogen radicals within the PV and in the host cell.

In addition to their activity on oxygen radicals, peroxiredoxins have been shown to exert other functions that might be just as important in parasite pathogenesis. It was determined in studies where the mTXNPx gene (mtxnpx(−)) was deleted that loss of that peroxidase molecule resulted in reduced virulence (Castro et al. 2011). However, there was no difference in the capacity of the mtxnpx(−) parasites to resist oxidants that were generated throughout the parasite's life cycle or to which parasites were exposed. Interestingly, complementation with a peroxidase defective but otherwise identical mutant restored wild-type infectivity to the genetically ablated parasites. They deduced that the protein conferred thermotolerance to the parasite by stabilizing other critical parasite molecules; basically it acted as a chaperone. Besides their peroxidase function, peroxiredoxins have also been shown to modulate host immune responses partly through their interactions with signaling intermediates in the toll-like receptor 4 (TLR4) signaling pathway (Ishii et al. 2012). Could intermediates in the TLR4 pathway be targets of the secreted TXNPxs?

Other ROS/RNS deactivating molecules: There are other molecules that carry out functions to limit the oxidative burst. These include superoxide dismutases (SODs). SODs are metalloenzymes that play a role in parasite defense mechanisms by detoxifying H_2O_2 and O_2. Three types of SODs, Cu/ZnSOD, MnSOD, and FeSOD, have been identified based on their metal cofactor. Two classes of intracellular FeSODs have been identified in *Leishmania*: FeSODA and FeSODB1&2. Expression of FeSODB1 in contrast to FeSODB2 is low in the early logarithmic stage and increases toward the stationary and amastigote stages (Plewes et al. 2003). Evidence of the important roles of these molecules has come from gene deletion studies. Parasites in which a single allele of the FeSODB1 gene was genetically ablated failed to thrive within macrophages (Plewes et al. 2003). The primary location of FeSODB1 and FeSODB2 is the glycosomes. Based on that location it is unlikely that those molecules would be available to react directly with external O_2^- generated from macrophages. Either glycosomes play a significant role in parasite antioxidant defense or the current understanding of the participation of these

molecules in parasite protection is incomplete. FeSODA is localized to the mitochondrion. Overexpression studies showed that it protects parasites from miltefosine-induced oxidative-induced stress (Getachew and Gedamu 2012). Even though FeSODA is released into the parasite cytosol after prolonged exposure to miltefosine, it is not known whether it is released by intracellular parasites under physiological conditions.

Other molecules have been described that have an indirect effect on ROS detoxification. One of these molecules is the ferrous iron transporter LIT1. Expression of this molecule is controlled by iron availability, but its effect on parasite resistance to ROS is through its regulation of SOD activity. It was also suggested that through its effect on iron levels in the parasite, LIT1 expression serves as trigger for *Leishmania* differentiation from the promastigote to the amastigote form (Mittra et al. 2013). Another molecule that plays a role in ROS detoxification is subtilisin. Subtilisin is an unusual Clan SB, family S8 serine protease (Swenerton et al. 2010). In studies to determine the function of subtilisin, parasites that lacked the subtilisin genes were derived (SUB−/−). The authors found that in SUB−/− parasites the expression of TXNPxs was altered. Moreover, SUB−/− *Leishmania* were found to have increased sensitivity to hydroperoxides compared with wild-type parasites in vitro. It was unexplained why SUB−/− parasites were also not able to transform from the promastigote to the amastigote form.

Parasite molecules that block cytokine signaling: As discussed earlier, immune effectors are released into *Leishmania* lesions. Parasites persist in lesions in part because they modulate the response of infected cells to cytokines and other effectors. Just a handful of *Leishmania* molecules have been implicated in the targeting of signaling intermediates. Some of those *Leishmania* molecules that target intermediates in the signaling pathways of cytokines are profiled below.

Casein kinases target the IFNAR1 receptor. Type I IFNs signal via interaction with the heterodimeric receptor complex composed of two chains (IFNAR1 and IFNAR2). Ligand binding activates receptor-associated members of the JAK family of tyrosine kinases, Jak1 and Tyk2. These kinases phosphorylate and activate the STAT proteins, which increase transcription of the IFN-induced genes whose products exert antiviral, immunomodulatory, and antiproliferative effects. The IFNAR1 subunit of this receptor is essential for IFN-α/β signaling. The levels of IFNAR1 are mainly regulated by ubiquitin-dependent endocytosis and ensuing degradation of this chain and the entire type I IFN receptor. Casein kinase 1α (CK1α) is a major bona fide kinase of IFNAR1 that mediates basal phosphorylation, ubiquitination, and turnover of IFNAR1. Several studies implicated CK1α in ligand-independent degron phosphorylation and degradation of IFNAR1 stimulated by ER stress inducers, including VSV. A *Leishmania* orthologue of casein kinase 1 (L-CK1) was recently identified that is capable of increasing IFNAR1 degron phosphorylation in cells (Liu et al. 2009). Infection of either dendritic cells or macrophages with *L. major* modestly decreased IFNAR1 levels and attenuated cellular responses to IFN-α in vitro. Although it was not shown where L-CK1 encounters IFNAR1, it is likely to be outside the *Leishmania* PV. This implies that

this *Leishmania* molecule is exported from PVs by a mechanism that is yet to be described. Type 1 interferon exerts a regulatory effect on IFNγ. In leishmaniasis, type 1 interferon plays a complex role as shown by Soong and colleagues (Soong 2012). Expression of L-CK1 might exert a greater effect on the course of infection than is presently appreciated.

Leishmania molecules that target NF-κB: NF-κB is a family of transcription factors that regulates the expression of a large variety of genes. Most of those genes are involved in processes such as inflammatory and immune responses of the cell, cell growth, and development. NF-κB is activated by extracellular stimuli. There are five protein members of the NF-κB family: RelA (p65), c-Rel, RelB, p105 (NF-κB1), and p100 (NF-κB2). The latter two are produced as precursor proteins that are cleaved into a p50 and p52 subunits. RelA, RelB, and c-Rel contain a transactivation domain, which means that they can activate gene expression. p50 and p52 can bind DNA but lack transactivation domains. NF-κB proteins are bound to inhibitory IκB proteins that retain associated NF-κB molecules in the cytoplasm. There are three main members of the IκB family, IκBα, IκBβ, and IκBε. IκB are in turn regulated by members of the IκB-kinase (IKK) complex, which consists of two kinase subunits, IKK1 (IKKα) and IKK2 (IKKβ), and a regulatory protein, NEMO (IKKγ). Activation of NF-κB can be induced by proinflammatory cytokines and pathogenic components, through the so-called classical activation pathway. Here the IKK2 subunit of the IKK complex induces phosphorylation of IκB proteins, leading to their polyubiquitination and subsequent degradation by proteasomes. The release of NF-κB from inhibition results in their translocation to the nucleus, where they form dimers that bind to specific gene sequences. An alternate pathway of NF-κB activation occurs when IKK1 homodimers phosphorylate NF-κB2 (p100 precursor) and induces its processing to p52. *Leishmania* infection has been shown to inhibit NF-κB-mediated gene activation. At least two parasite molecules have been implicated. The cysteine proteinase, CPb, is a member of the Clan CA family C1 papain-like group of cysteine peptidases. It exists as multiple isoenzymes, which are encoded by a tandem array of similar *CPB* genes. The CPBs of *L. mexicana*, for example, are stage-regulated and the isoforms present differences in their substrate specificity and catalytic properties. Several of the NF-κB family members were shown to be targets of CPb. Specifically, p65 Rel A and c-Rel that are activated by exposure of cells to lipopolysaccharide (LPS), and IκBα and IκBβ were degraded extensively by LmCPb expressed by *Leishmania* amastigotes (Cameron et al. 2004). This effect on NF-κB resulted in the inability of infected cells to secrete IL-12 in response to LPS activation. These studies were confirmed with gene deletion studies of the LmCPb gene. Interestingly, although infection of macrophages with *Leishmania* promastigotes also inhibited IL-12 production in response to LPS, the pattern of degradation of NF-κB was different. Here, p65 Rel A was degraded to a 35 kDa fragment (Gregory et al. 2008; Abu-Dayyeh et al. 2010). The other *Leishmania* molecule that has been implicated in the degradation of NF-κB was shown to be *Leishmania* metalloprotease gp63. The participation of gp63 in NF-κB degradation as well as other processes was recently reviewed (Isnard et al. 2012).

Given that NF-κB is localized in the cytosol of host cells, a mechanism must exist for LCPb and gp63 to gain access to that compartment. Mottram and colleagues proposed that in infected cells, parasites secrete CPb into the PV lumen (Mottram et al. 2004). Then vesicles containing CPb and other proteins pinch off from the PV. The vesicles rupture in the cytosol, thus releasing parasite molecules that can target host molecules in the cytosol. No better model for parasite protein export from PVs has been proposed. Interestingly, a different mechanism has been proposed for how gp63 gains access to the host cell cytosol. GP63 is expressed most abundantly by promastigote forms. Olivier and colleagues showed that gp63 can be delivered to uninfected cells (Olivier et al. 2012). They suggested that through its interactions with macrophage surface molecules, gp63 can be internalized by a lipid rafts-mediated endocytic process. Once these parasite proteins access the cytosol they may target other host molecules. For example, LPb has been implicated in the degradation of the signal transducer and activator of transcription 1alpha (STAT-1α), and activating protein 1 (AP-1) (Abu-Dayyeh et al. 2010). Multiple other targets for gp63 have been described (Olivier et al. 2012). Going forward, it will be of interest to determine the details of these and other schemes by which parasites are exported from PVs. Furthermore, information of other molecules that are exported should shed light on processes that parasites target for survival.

Molecules that target Activated Protein-1 (*AP-1*). Several studies had shown that AP-1 transcription factor is inactivated by *Leishmania* infection. Activated Protein-1 (AP-1) is an important transcription factor that mediates gene regulation in response to physiological and pathological stimuli, including cytokines and growth factors. AP-1 is formed by homodimers of Jun family members (c-Jun, Jun B, and Jun D), or heterodimers of Jun and Fos family members (c-Fos, Fos B, Fra 1, and Fra 2). In studies to determine how *Leishmania* target the function of AP-1, Olivier and colleagues focused on the *Leishmania* surface-expressed metalloprotease GP63 (Contreras et al. 2010). They performed electro-mobility shift assays (EMSA) to assess DNA binding of AP-1 in the presence of lysates from cells infected with parasites that lacked GP63 or normal GP3 expression. AP-1 binding to DNA was impaired in the presence of GP63 expression. Moreover, incubation of lysates with GP63 resulted in degradation of c-Jun, Jun B, c-FOS, and AP-1 subunits. Intriguingly, parasite internalization was not required for GP63 to exert its effect on AP-1. Could bystander uninfected cells be susceptible to their AP-1s being targeted? Based on this observation, the authors deduced that GP63 that are GPI-anchored molecules and that can also be secreted by promastigotes can gain access into cells and traffick to the nucleus where they encounter AP-1. The details of the trafficking of GP63 to the nucleus are unclear. However, they showed a requirement for the presence of GPI-anchored molecules, which would suggest that endocytic vesicles that participate in their uptake might have special trafficking cues. The expectation should be that when GP63 are expressed by amastigote forms, albeit at much lower levels, those molecules will have to engage a different trafficking path to the nucleus.

Concluding remarks. *Leishmania* survival in its mammalian host is dependent on the outcome of numerous host–parasite interactions. These interactions might determine which cytokines or chemokines are released into *Leishmania* lesions. The presence of cytokines in the vicinity of *Leishmania*-infected cells could be of limited value if the parasites elaborate molecules that limit responsiveness to those cytokines. Only a small number of parasite mediators have been described that target host responses. As to be expected there are many outstanding questions about where and how those parasite mediators interact with their host targets to promote *Leishmania* pathogenesis. There are exciting new studies underway on export schemes of *Leishmania* molecules. Hopefully, those studies will shed light on the export of molecules profiled in this review and also the export and function of other molecules that are yet to be identified.

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References

Abu-Dayyeh I, Hassani K, Westra ER, Mottram JC, Olivier M (2010) Comparative study of the ability of Leishmania mexicana promastigotes and amastigotes to alter macrophage signaling and functions. Infect Immun 78:2438–2445

Alexander J, Brombacher F (2012) T helper1/t helper2 cells and resistance/susceptibility to leishmania infection: is this paradigm still relevant? Front Immunol 3:80. doi:10.3389/fimmu.2012.00080

Barr SD, Gedamu L (2003) Role of peroxidoxins in Leishmania chagasi survival. Evidence of an enzymatic defense against nitrosative stress. J Biol Chem 278(12):10816–10823. doi:10.1074/jbc.M212990200

Ben-Othman R, Dellagi K, Guizani-Tabbane L (2009) Leishmania major parasites induced macrophage tolerance: implication of MAPK and NF-kappaB pathways. Mol Immunol 46(16):3438–3444. doi:10.1016/j.molimm.2009.05.337

Bienert GP, Schjoerring JK, Jahn TP (2006) Membrane transport of hydrogen peroxide. Biochim Biophys Acta 1758(8):994–1003. doi:10.1016/j.bbamem.2006.02.015

Cameron P, McGachy A, Anderson M, Paul A, Coombs GH, Mottram JC, Alexander J, Plevin R (2004) Inhibition of lipopolysaccharide-induced macrophage IL-12 production by Leishmania mexicana amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. J Immunol 173(5):3297–3304

Castro H, Sousa C, Novais M, Santos M, Budde H, Cordeiro-da-Silva A, Flohé L, Tomás AM (2004) Two linked genes of Leishmania infantum encode tryparedoxins localised to cytosol and mitochondrion. Mol Biochem Parasitol 136(2):137–147

Castro H, Romao S, Gadelha FR, Tomás AM (2008) Leishmania infantum: provision of reducing equivalents to the mitochondrial tryparedoxin/tryparedoxin peroxidase system. Exp Parasitol 120(4):421–423. doi:10.1016/j.exppara.2008.09.002

Castro H, Romao S, Carvalho S, Teixeira F, Sousa C, Tomás AM (2010) Mitochondrial redox metabolism in trypanosomatids is independent of tryparedoxin activity. PLoS One 5(9):e12607. doi:10.1371/journal.pone.0012607

Castro H, Teixeira F, Romao S, Santos M, Cruz T, Flórido M, Appelberg R, Oliveira P, Ferreira-da-Silva F, Tomás AM (2011) Leishmania mitochondrial peroxiredoxin plays a crucial peroxidase-unrelated role during infection: insight into its novel chaperone activity. PLoS Pathog 7(10):e1002325. doi:10.1371/journal.ppat.1002325

Channon JY, Roberts MB, Blackwell JM (1984) A study of the differential respiratory burst activity elicited by promastigotes and amastigotes of Leishmania donovani in murine resident peritoneal macrophages. Immunology 53(2):345–355

Contreras I, Gómez MA, Nguyen O, Shio MT, McMaster RW, Olivier M (2010) Leishmania-induced inactivation of the macrophage transcription factor AP-1 is mediated by the parasite metalloprotease GP63. PLoS Pathog 6(10):e1001148. doi:10.1371/journal.ppat.1001148

Denicola A, Souza JM, Radi R (1998) Diffusion of peroxynitrite across erythrocyte membranes. Proc Natl Acad Sci U S A 95(7):3566–3571

Flohé L (2012) The trypanothione system and its implications in the therapy of trypanosomatid diseases. Int J Med Microbiol 302(4–5):216–220. doi:10.1016/j.ijmm.2012.07.008

Getachew F, Gedamu L (2012) Leishmania donovani mitochondrial iron superoxide dismutase A is released into the cytosol during miltefosine induced programmed cell death. Mol Biochem Parasitol 183(1):42–51. doi:10.1016/j.molbiopara.2012.01.005

Gregory DJ, Olivier M (2005) Subversion of host cell signalling by the protozoan parasite Leishmania. Parasitology 130(suppl):S27–S35. doi:10.1017/S0031182005008139

Gregory DJ, Godbout M, Contreras I, Forget G, Olivier M (2008) A novel form of NF-kappaB is induced by Leishmania infection: involvement in macrophage gene expression. Eur J Immunol 38(4):1071–1081. doi:10.1002/eji.200737586

Gupta G, Oghumu S, Satoskar AR (2013) Mechanisms of immune evasion in leishmaniasis. Adv Appl Microbiol 82:155–184. doi:10.1016/B978-0-12-407679-2.00005-3

Hanschmann EM, Godoy JR, Berndt C, Hudemann C, Lillig CH (2013) Thioredoxins, glutaredoxins, and peroxiredoxins-molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. Antioxid Redox Signal 19(13):1539–1605. doi:10.1089/ars.2012.4599

Ishii T, Warabi E, Yanagawa T (2012) Novel roles of peroxiredoxins in inflammation, cancer and innate immunity. J Clin Biochem Nutr 50(2):91–105. doi:10.3164/jcbn.11-109

Isnard A, Shio MT, Olivier M (2012) Impact of Leishmania metalloprotease GP63 on macrophage signaling. Front Cell Infect Microbiol 2:72. doi:10.3389/fcimb.2012.00072

Jirata D, Kuru T, Genetu A, Barr S, Hailu A, Aseffa A, Gedamu L (2006) Identification, sequencing and expression of peroxidoxin genes from Leishmania aethiopica. Acta Trop 99(1):88–96. doi:10.1016/j.actatropica.2006.08.001

Kima PE (2007) The amastigote forms of Leishmania are experts at exploiting host cell processes to establish infection and persist. Int J Parasitol 37(10):1087–1096. doi:10.1016/j.ijpara.2007.04.007

Kima PE, Soong L (2013) Interferon gamma in leishmaniasis. Front Immunol 4:156. doi:10.3389/fimmu.2013.00156

Kima PE, Bonilla JA, Cho E, Ndjamen B, Canton J, Leal N, Handfield M (2010) Identification of Leishmania proteins preferentially released in infected cells using change mediated antigen technology (CMAT). PLoS Negl Trop Dis 4(10). pii: e842. doi:10.1371/journal.pntd.0000842

Liu J, Carvalho LP, Bhattacharya S, Carbone CJ, Kumar KG, Leu NA, Yau PM, Donald RG, Weiss MJ, Baker DP, McLaughlin KJ, Scott P, Fuchs SY (2009) Mammalian casein kinase 1alpha and its leishmanial ortholog regulate stability of IFNAR1 and type I interferon signaling. Mol Cell Biol 29(24):6401–6412. doi:10.1128/MCB.00478-09

Lodge R, Descoteaux A (2006) Phagocytosis of Leishmania donovani amastigotes is Rac1 dependent and occurs in the absence of NADPH oxidase activation. Eur J Immunol 36(10):2735–2744. doi:10.1002/eji.200636089

Lodge R, Diallo TO, Descoteaux A (2006) Leishmania donovani lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane. Cell Microbiol 8(12):1922–1931. doi:10.1111/j.1462-5822.2006.00758.x

Mittra B, Cortez M, Haydock A, Ramasamy G, Myler PJ, Andrews NW (2013) Iron uptake controls the generation of Leishmania infective forms through regulation of ROS levels. J Exp Med 210(2):401–416. doi:10.1084/jem.20121368

Moradin N, Descoteaux A (2012) Leishmania promastigotes: building a safe niche within macrophages. Front Cell Infect Microbiol 2:121. doi:10.3389/fcimb.2012.00121

Mottram JC, Coombs GH, Alexander J (2004) Cysteine peptidases as virulence factors of Leishmania. Curr Opin Microbiol 7(4):375–381. doi:10.1016/j.mib.2004.06.010

Nauseef WM (2008) Nox enzymes in immune cells. Semin Immunopathol 30(3):195–208. doi:10.1007/s00281-008-0117-4

Nylén S, Sacks D (2007) Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol 28(9):378–384. doi:10.1016/j.it.2007.07.004

Olivier M, Atayde VD, Isnard A, Hassani K, Shio MT (2012) Leishmania virulence factors: focus on the metalloprotease GP63. Microbes Infect 14(15):1377–1389. doi:10.1016/j.micinf.2012.05.014

Pham NK, Mouriz J, Kima PE (2005) Leishmania pifanoi amastigotes avoid macrophage production of superoxide by inducing heme degradation. Infect Immun 73(12):8322–8333. doi:10.1128/IAI.73.12.8322-8333.2005

Plewes KA, Barr SD, Gedamu L (2003) Iron superoxide dismutases targeted to the glycosomes of Leishmania chagasi are important for survival. Infect Immun 71(10):5910–5920

Romao S, Castro H, Sousa C, Carvalho S, Tomás AM (2009) The cytosolic tryparedoxin of Leishmania infantum is essential for parasite survival. Int J Parasitol 39(6):703–711. doi:10.1016/j.ijpara.2008.11.009

Singh N, Bajpai S, Kumar V, Gour JK, Singh RK (2013) Identification and functional characterization of Leishmania donovani secretory peroxidase: delineating its role in NRAMP1 regulation. PLoS One 8(1):e53442. doi:10.1371/journal.pone.0053442

Soong L (2012) Subversion and utilization of host innate defense by Leishmania amazonensis. Front Immunol 3:58. doi:10.3389/fimmu.2012.00058

Swenerton RK, Knudsen GM, Sajid M, Kelly BL, McKerrow JH (2010) Leishmania subtilisin is a maturase for the trypanothione reductase system and contributes to disease pathology. J Biol Chem 285(41):31120–31129. doi:10.1074/jbc.M110.114462

Paratransgenic Control of Leishmaniasis: New Developments

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Abstract Leishmaniasis is a devastating neglected tropical disease caused by approximately 20 different species of Leishmania parasites. These obligate intercellular protozoa are spread naturally to humans by female phlebotomine sand flies of the genus Phlebotomus (Old World) or Lutzomyia (New World). Depending on the infecting parasite and the innate host-immune response, the clinical manifestation of leishmaniasis ranges from cutaneous ulcerations to system infections. It is therefore not surprising that treatment of leishmaniasis varies greatly with the type of disease (cutaneous, mucosal, or visceral). Currently, no vaccines are available for any form of leishmaniasis, and control of disease has focused largely on vector eradication through pesticide use. The Visceral Leishmaniasis (VL) Elimination Initiative was launched in the 2005 between the governments of India, Nepal and Bangladesh and the WHO. The aim of this initiative is to reduce annual VL incidence in this region to below 1/10,000 inhabitance by 2015. Despite some early success, recent reports are now citing difficulties in sustaining prolonged spraying campaigns and emerging public concerns regarding insecticide use and environmental toxicity. Further, new populations of sand flies in these endemic regions in India have now gained resistance to DDT. For these reasons, alternative and integrative vector control strategies are needed to reach the VL elimination target date in 2015.

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The paratransgenic strategy was initially developed to control the vectorial transmission of *Trypanosoma cruzi*, the protozoan parasite responsible for Chagas disease, by blood-sucking triatomine bugs. In this strategy, bacterial flora native to disease-transmitting vectors are isolated and genetically transformed in vitro to export molecules that interfere with pathogen transmission. The genetically altered symbionts are then re-introduced into the host vector where expression of engineered molecules affects the host's ability to transmit the pathogen. In this review, we will discuss the strategy that we have adopted to adapt the paratransgenic approach to control vectorial transmission of leishmania in sand flies, and describe how two effector molecules, antimicrobial peptides (AMP's) and single chain antibodies (scFv's), can be utilized to specifically target the parasites in sand fly. Further, we address the evolving concepts related to field dispersal of engineered bacteria as part of the paratransgenic control strategy coupled with the attendant risk assessment evaluation that is critical for field release. We conclude with the development and testing of a "barrier" method of containment and dispersal for paratransgenic technologies.

Keywords Leishmaniasis • Sand flies • Paratransgenesis • Antimicrobial peptides • Single-chain antibodies • Horizontal gene transfer • Microencapsulation

Introduction

Leishmaniasis is a devastating neglected tropical disease caused by obligate intracellular protozoa that belong to the genus *Leishmania*. These parasites are spread naturally to humans by female phlebotomine sand flies of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World). This neglected tropical disease complex is present in more than 90 countries, affecting over two million people per year, with 350 million more at risk for infection (WHO 2012).

More than 20 different *Leishmania* species have been identified to be pathogenic. Depending on the infecting parasite and the innate host-immune response, the clinical manifestations of leishmaniasis range from cutaneous ulcerations to systemic infections. Cutaneous leishmaniasis (CL) results from infection of the dermal macrophages. In the Old World, this disease is mostly cause by *L. major*, and transmitted by *P. papatasi*. CL is prominent in the Middle East, the Indian subcontinent, Asia, Mediterranean, and East Africa. Visceral leishmaniasis (VL) results from infection of the reticuloendothelial system. This disease is often lethal if left untreated. This manifestation of leishmaniasis, also known as "kala-azar," is predominantly transmitted by female *P. argentipes* infected with *L. donovani* in India, Bangladesh, Nepal, and Sudan. Mortality caused by this disease is second only to malaria.

Treatment of Leishmaniasis

Treatment of leishmaniasis varies greatly with the type of disease (cutaneous, mucosal, or visceral), *Leishmania* species, and geographic location. Old World cutaneous disease often self-heals and as a result requires no treatment. New World cutaneous disease is less likely to self-resolve and because of the risk of development of mucosal leishmaniasis with *L. braziliensis*, systemic treatment is more commonly undertaken. Treatment courses are often lengthy and cure is difficult to achieve with many cutaneous and mucocutaneous forms. Treatment modalities for cutaneous leishmaniasis range from local therapy including topical treatments and intralesional injections to systemic therapy with pentavalent antimonials, amphotericin B, or miltefosine being used most commonly.

The treatment of VL has changed substantially over the last decade. Pentavalent antimonial drugs were the standard treatments of choice until the emergence of drug non-responsiveness in highly endemic regions such as Bihar, India (Guerin et al. 2002). Newer modalities, while effective, pose practical limitations as the majority of patients suffering from VL are unable to pay for costly medications and hospitalization. Such therapies include multiple formulations of amphotericin B, paramomycin, and miltefosine. Dose finding studies have suggested significant efficacy with even a single dose of liposomal amphotericin B (Sundar et al. 2010); however, it is unlikely that this single-dose regimen will be adopted into practice because of the risk of drug resistance. Paramomycin, an injectable aminoglycoside medication, has also been studied and found effective in clinical trials (Sundar et al. 2009). Miltefosine, an oral agent with excellent skin penetration, has proven successful in treatment of VL and has the advantage of not requiring hospitalization for administration (Sundar et al. 2006). Most recently, there has been evaluation of combinations of drugs for the treatment of VL. Combination therapy has the distinct advantage of decreasing the risk of drug resistance, decreasing the side effects, and improving cost-effectiveness. Regimens found to be effective include combination therapy with amphotericin B and miltefosine, amphotericin B and paramomycin, and miltefosine in combination with paramomycin (Sundar et al. 2011).

In the absence of a vaccine, control of leishmaniasis has focused largely on vector eradication through pesticides. DDT spraying in India associated with the National Malaria Control Program in the 1960s was very successful in reducing sand fly populations and cases of VL were nearly eliminated (Thakur and Kumar 1992). More recently, the VL Elimination Initiative was launched in 2005 between the governments of India, Nepal, and Bangladesh (World Health Organization 2004) with support by the WHO (World Health Organization 2007), to reduce the annual VL incidence in this region to below 1/10,000 inhabitants by 2015. Indoor residual spraying (IRS) along with long-lasting insecticide-treated nets (LNs) was recommended as the main vector control strategy. While the Regional Technical Advisory Group to the VL Elimination Initiative had suggested standardization of IRS strategies (i.e., spraying calendar, insecticide used), and monitoring the effectiveness of these spraying programs and related problems of human health and

environmental toxicity, protocols varied greatly between the three countries. Different spraying strategies, as well as pesticides—DDT in India, deltamethrin in Bangladesh, and alpha-cypermethrine in Nepal—were adapted by each country. The choice of DDT in India is perplexing, given numerous reports citing resistance amongst *P. argentipes* to DDT in VL endemic regions in Bihar (Kishore et al. 2004; Dhiman et al. 2003; Singh et al. 2001; Mukhopadhyay et al. 1987, 1996). Despite these challenges, the VL Elimination program has seen some success. The use of LNs in three endemic VL villages in India and Nepal showed that the cluster-wide distribution of LNs significantly reduced the *P. argentipes* density/house by 24.9 % (Picado et al. 2010). Although the impact of IRS on VL in these countries remains to be evaluated, it is evident that proper application of IRS significantly reduces density of *P. argentipes* in many areas (Kumar et al. 2009). It is interesting to note while governmental IRS campaigns were nonexistent in VL endemic regions of Bangladesh until 2010, routine IRS activities in India and Nepal were deemed to be suboptimal (Picado et al. 2012). Cuts in public spending, lack of trained manpower, managerial problems, and corruption—including mismanagement of the pesticide stocks and diversion of these chemicals to the black market for agricultural purposes—appear to be the root of this issue (Kishore et al. 2006). There are also reports of “patchy” geographic coverage in spraying, poor user acceptance, and low community participation during the spraying campaigns (Kumar et al. 2009). Finally, several new reports suggest that newer populations of sand flies have gained resistance to DDT (Dinesh et al. 2010; Singh et al. 2012). The difficulties in sustaining these spraying campaigns, the attendant risks of toxicity and evolution of vector resistance, plus the lack of effective vaccines necessitate the development of alternative vector control strategies to reach the VL elimination target date of 2015.

Paratransgenesis to Reduce Vector Competence

For over a decade, our laboratory has developed novel paratransgenic strategies aimed at controlling transmission of several infectious diseases (Beard et al. 1998; Durvasula et al. 1997; Hillesland et al. 2008). In this strategy, bacterial flora native to disease-transmitting vectors are isolated and genetically transformed in vitro to export molecules that interfere with pathogen transmission. The genetically altered symbionts are then reintroduced into the host vector where expression of engineered molecules affects the host’s ability to transmit the pathogen, i.e., its vector competence. There are several requirements for such an approach (Beard et al. 2002): (a) a population of symbiotic bacteria must exist within a given disease-transmitting vector, (b) symbiotic bacteria should be specific to a given vector, (c) bacterial symbionts should be amenable to culture and genetic manipulation, (d) genetically altered symbionts should remain stable, (e) fitness of the genetically altered symbionts to re-infect host vectors should not be compromised, nor should their normal symbiotic functions be altered, (f) transgene products released from the genetically altered symbionts should interact with the target pathogen(s), and (g) a

method must exist for dispersal of the genetically altered symbionts amongst naturally occurring populations of vectors with minimal nontarget spread of foreign genes to environmental bacteria and other arthropods.

The paratransgenic approach was initially optimized to control vectorial transmission of *Trypanosoma cruzi*, the protozoan parasite responsible for Chagas disease. *T. cruzi* is transmitted to humans by blood-sucking triatomine bugs. The symbiotic associations between triatomine vectors, specifically *T. infestans* and *Rhodococcus prolixus*, and nocardiform actinomycetes are well characterized (Baines 1956; Dasch et al. 1984). These bacteria are thought to aid in the processing of B complex vitamins in the restricted blood diets of these insects and are essential to the survival of the triatomine. Newly emerging nymphs of *R. prolixus* are transiently aposymbiotic (lacking in gut-associated symbionts). The actinomycete, *R. rhodnii*, is acquired by early nymphal stages through coprophagy, i.e., the ingestion of feces of other colony insects. Aposymbiotic nymphs of *R. prolixus*, which emerge from surface-sterilized eggs and are reared in sterile chambers without access to *R. rhodnii*, will fail to develop beyond the second instar stage despite regular blood meals. Introduction of *R. rhodnii* to the aposymbiotic nymphs either through a blood meal or via the synthetic fecal preparation, CRUZIGARD, permits normal sexual development without compromise of insect fitness (Durvasula et al. 1999b). In the insect, *R. rhodnii* remains extracellular and reaches its highest concentrations in the hindgut lumen in close proximity to infective forms of *T. cruzi*. Initially, *R. rhodnii* was transformed with pRr1.1, a shuttle plasmid containing a gene encoding resistance to the antibiotic thiostrepton, to support the hypothesis that a transgene-carrying symbiont could be introduced into *R. prolixus*. The modified symbiont was maintained through the insect's development without adverse effects on insect survival and fitness (Beard et al. 1992). Subsequently, we demonstrated that the antimicrobial peptide (AMP), cecropin A, could be expressed in the gut lumen of *R. prolixus* at levels capable of eradication of *T. cruzi* (Durvasula et al. 1997). These studies set the stage for additional work involving triatomine bugs, sharpshooters, and sand flies.

Paratransgenesis in Sand Flies

The paratransgenic approach will work in the sand fly as it has a short life cycle with only a brief window of parasite development. Amastigote-infected macrophages are acquired by female sand flies when the first blood meal is ingested. The parasites differentiate and propagate as promastigotes within the midgut of the sand fly. Within 4–5 days post-blood meal, the parasites migrate to the salivary glands, and are transferred to the mammalian host when the sand fly takes its second blood meal. For the paratransgenic strategy to be successful, the recombinant bacteria should preferably persist within the sand fly gut until the first blood meal. However, it is possible that an anti-leishmania recombinant molecule may remain active in the sand fly gut in the absence of the engineered bacterium itself. Functional

recombinant cecropin A, an antitrypanosomal AMP, persisted for over 6 months in the gut lumen with biological activity in paratransgenic triatomine bugs (Durvasula et al. 1997). Such an outcome would actually be viewed as highly desirable as the intended biological effect would occur with reduced risk of unwanted spread of transgenic bacteria via activities of the adult flies (Hurwitz et al. 2011).

For successful deployment of the paratransgenic approach in this vector system, an understanding of sand fly biology is essential. Though no symbionts have ever been identified in sand flies, numerous commensal microbes have been isolated. *E. coli* and a variety of Gram-positive organisms such as *Bacillus subtilis* and *Micrococcus* spp. were isolated by Rajendran and Modi (1982) in wild-caught and laboratory-bred sand flies. In 1996, Dillon et al. (1996) examined 78 field-caught *P. papatasi* from two different regions of Sinai, Egypt. While over half of these sand flies carried gut microbiota, many were identified to be *Enterobacteriaceae* which are capable of causing human disease. Volf et al. isolated bacteria from the gut of different developmental stages of *P. duboseqi* (Volf et al. 2002). Most were identified to belong to a single strain of *Ochrobactrum*. In females, the highest bacterial counts were observed 2 days after blood feeding, returning to pre-feeding levels 7 days post-blood feeding. In 2008, we detailed an extensive analysis of the gut flora of *P. argentipes* trapped from four VL endemic sites in Bihar, India (Hillesland et al. 2008). In this survey, we identified 28 distinct gut microorganisms through 16S rDNA sequencing. *Staphylococcus* spp., *Escherichia coli*, and *Enterobacter*, all human pathogens, were identified. However, their potential of pathogenicity is precluding their use in the paratransgenic system. We also identified an abundance of nonpathogenic soil bacteria that are used in industry including *Bacillus megaterium*, *Brevibacterium linens*, *Bacillus subtilis*, and *Bacillus pumilis*. The prevalence of these *Bacillus* spp. in *P. papatasi* from sites in India, Turkey, and Tunisia was recently validated by Mukhopadhyay et al. (2012). The occurrence of *Bacillus* spp., specifically *B. subtilis* and *B. megaterium*, in field-caught sand flies may be a reflection of environmental bacteriology. Marketed bio-fertilizers often contain *B. megaterium* and *B. subtilis*. These organisms are highly amenable to genetic manipulation, and have been utilized as microbial factories for production of many recombinant proteins. Further, some strains of *B. subtilis* have inherent probiotic properties. This bacterium is "generally regarded as safe," and was chosen as our vehicle of choice for delivery of anti-leishmania molecules to sand flies.

In natural conditions, sand flies will lay their eggs in very loose soil that is rich in organic matter. Breeding sites for both Old and New World sand flies are somewhat characterized. Using soil emergent traps, Casanova collected three species of *Lutzomyia* from the forest floor and peridomestic area in two municipalities in the Valley of Mogi Guacu River in Brazil where CL is endemic (Casanova 2001). Vieira et al. reported on identification of peridomiciliary breeding sites for *L. intermedia* and *L. migonei* in another CL endemic region in Southeast Brazil (Vieira et al. 2012). Ghosh and Bhattacharya described *P. argentipes* breeding sites in West Bengal (Ghosh and Bhattacharya 1991) and further defined the population biology of this vector (Ghosh et al. 1999). In VL endemic regions of Bihar, India, Kesari et al. successfully isolated sand fly larvae from soil samples of mixed dwellings and isolated

cowsheds (Kesari et al. 2000). Breeding sites for *P. argentipes* and *P. papatasi* were further delineated in another study in Pondicherry, India (Ilango et al. 1994). Immature forms of *P. papatasi* are often associated with burrows of their respective rodent reservoirs—the fat sand rat (*Psammomys obesus*) in Israel (Wasserberg et al. 2003) and gerbils (*Meriones* spp.) in Tunisia (Tabbabi et al. 2011). Chelbi et al. noted that active rabbit holes in peridomestic areas serve as fertile breeding sites for *P. papatasi* in Tunisia (Chelbi et al. 2008), while Moncaz et al. described breeding habitats for *P. sergenti*, the vector for *L. tropica* inside caves that are also home to rock hyraxes (Moncaz et al. 2012).

It is interesting to note that many identified breeding sites are located in close proximity to domesticated livestock or other animal dwellings. Chelbi et al. had, in fact, demonstrated that breeding of rabbits in peridomestic areas resulted in a low indoor abundance of *P. papatasi*. These investigators suggest that active rabbit holes may function well as a zooprophylaxis-based option for long-term control of CL in rural endemic areas (Chelbi et al. 2008). Environmental cues, specifically hexanal and 2-methyl-2-butanol emanating from the rabbit feces, had been shown to be an oviposition attractant for sand flies (Elnaiem and Ward 1992; Dougherty et al. 1995). The complex microbial community in the rabbit feces may also contribute to some of these aromatics. Peterkova-Koci et al. had shown the preference of sand flies to oviposition on rabbit feces containing a live microbial community (Peterkova-Koci et al. 2012). Further, *L. longipalpis* deposits dodecanoic acid on freshly oviposited eggs (Dougherty and Hamilton 1997; Dougherty et al. 1995). This pheromone, coupled with volatile environmental oviposition cues may help gravid females to locate suitable oviposition sites (Mccall and Cameron 1995; Dougherty et al. 1993).

The acquisition of bacteria in sand flies has not been studied. However, the large number of soil and environmental bacteria identified from field-caught sand flies would suggest that these insects are typically colonized by microbes encountered at breeding sites or during sugar meals (Hillesland et al. 2008). To translate these findings about sand fly vector ecology and microbiology to strategies for paratransgenic manipulation, we have devised an approach that may be applicable to the field environment. We propose to deploy large concentrations of transformed *B. subtilis* at sand fly breeding sites to selectively colonize the emerging flies. However, unlike the triatomine bugs, Dipteran sand flies are holometabolous insects; that is, they undergo complete metamorphosis from a soft-bodied, wingless larval stage to a hardened, winged adult. Upon eclosure, the larvae pass through four instars before pupation and adult emergence. Larval development occurs entirely within the soil and continuous ingestion of soil material results in microbial transit within the larval gut. The mechanisms that determine whether a particular bacterial species is sequestered rather than digested during Dipteran development are not well understood. Although the transstadial passage of bacteria from larvae to pupae and adult flies has been reported for *P. duboseqi* (Volf et al. 2002) and *P. argentipes* (Hurwitz et al. 2011), there are ongoing debates as to whether or not all the bacteria in the gut lumen of the larvae are eliminated during metamorphosis. It is likely that specific, yet uncharacterized, insect–microbial interactions impact the transstadial passage of

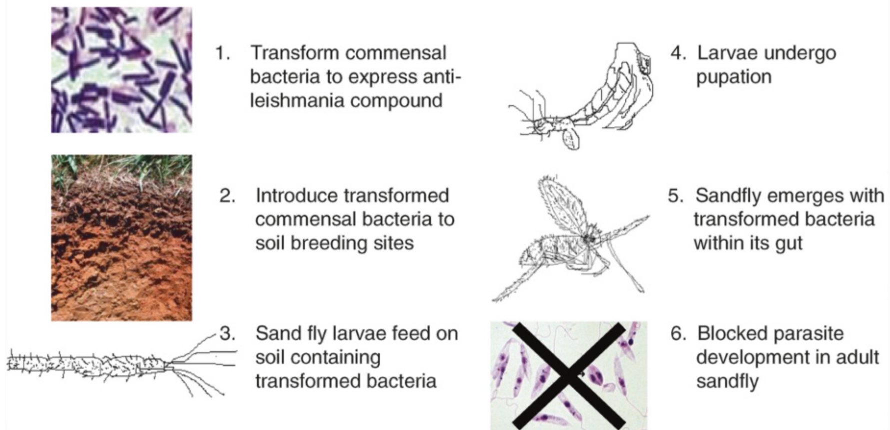

Fig. 1 Paratransgenic strategy for control of leishmaniasis. In this scheme, we propose to transform sand fly commensal bacteria to express anti-leishmania molecules (1), these genetically altered microbes will be introduced to sand fly breeding sites (2) where they will be consumed by sand fly larvae (3). We have demonstrated that the genetically altered bacteria will be retained by the sand fly as it undergoes metamorphosis (4). Expression of the anti-leishmania molecule in the gut of the emergent paratransgenic sand fly (5) would kill invading leishmania species, therefore rendering the sand fly refractory to infection (6), and thus transmission of the parasite

some microbes. Alternatively, it is possible that inundation of breeding sites with a large concentration of a known microbe could drive the organism through metamorphosis to the emerging adult stage. Regardless of mechanism, transstadial passage of bacteria through sand fly development is necessary for the paratransgenic strategy to succeed. Successful delivery of bacteria that express anti-leishmania molecules to soil-dwelling larval stages with retention and transgene expression at the adult stage would render emerging sand flies refractory to *Leishmania* infections, thereby disrupting the cycle of parasite transmission (Fig. 1).

Recently, we demonstrated the transstadial passage of indigenous bacterial flora from larvae to emergent sand fly, to set the stage for paratransgenic manipulation of sand flies with anti-leishmanial molecules (Hurwitz et al. 2011). We added fourth instar sand fly larvae into sterilized sand fly larval chow (rabbit feces) mixed with sterile PBS. We then examined each developmental stage (larvae, pupae, and emergent sand flies) for microbial content. Two microbes, *Lysinibacillus fusiformis* and *B. cereus*, were isolated at each developmental stage, proving that transstadial transit is possible. We then inoculated sterilized larval chow with 10^7 colony forming units (CFU) of *B. subtilis* transformed with green fluorescent protein (GFP). Fourth instar larvae were transferred to the media and allowed to undergo metamorphosis. Seventy-five percent of the emergent paratransgenic sand flies carried, on average, 3.9×10^4 CFU of the GFP-expressing *B. subtilis*. Our results would suggest that under laboratory conditions the indigenous flora could be displaced with a complementary microbe, such as GFP-expressing *B. subtilis*, to generate paratransgenic sand flies (Fig. 1) (Hurwitz et al. 2011). These results suggest that inundation of

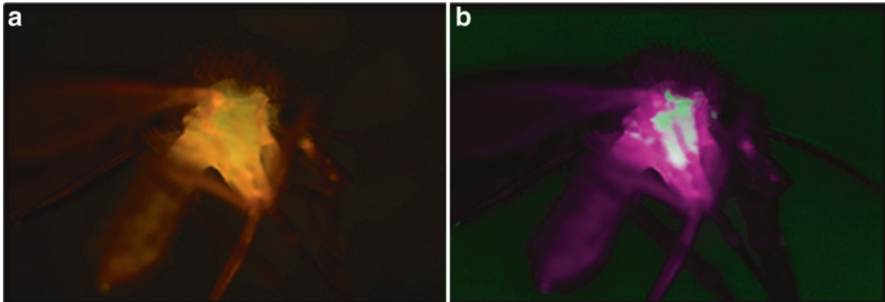

Fig. 2 Whole mount of paratransgenic sand fly. (**a**) The autofluorescence associated with the outer carapace and specific GFP fluorescence within the sand fly. (**b**) GFP-specific fluorescence signal uncoupled from the background. These ×4-images were captured using a Nuance multispectral imaging system. GFP-specific fluorescence is contained to the midgut chamber of the adult sand fly with no evidence of transfer to other regions of the insect. Figure is adapted from Hurwitz et al., The paratransgenic sand fly: a platform for control of Leishmania transmission. *Parasit Vectors*, 2011, 4:e82

sand fly breeding sites with transformed, nonpathogenic commensal bacteria is an option for production of paratransgenic sand flies. Whether or not the addition of transformed *B. subtilis* to known breeding sites in the field would alter environmental oviposition cues for gravid sand flies remains to be assessed.

Effector Molecules for Paratransgenic Control of Leishmania Infections in Sand Flies

Antimicrobial Peptides

AMPs are highly conserved molecules that play a vital role in innate immune defense by providing broad-spectrum antimicrobial activities (Fig. 2). They have been identified in many multicellular organisms, and function as "first-line" defense against invading microbes, including protozoans. AMPs are usually described as small amphipathic and highly basic molecules that can discriminate between host and bacterial membranes by charge and composition. Although their main method of attack is often described as "disrupting membranes of nonhost cells," AMPs have been shown to interfere with host metabolism as well as target cytoplasmic components.

We have examined a number of effector molecules for the paratransgenic control of Chagas disease. In our initial work, we re-populated aposymbiotic *R. prolixus* nymphs with *R. rhodnii* transformed to express the AMP cecropin A (Durvasula et al. 1997). At the end of the trial, 65 % of the paratransgenic *R. prolixus* examined showed complete elimination of *T. cruzi*. The remaining 35 % had a 2- to 3-log reduction in parasite count, demonstrating that the in vivo expression of an AMP from a genetically modified symbiont can significantly reduce carriage of the

parasite by the host vector. We reported on the in vitro activities of several other AMPs against *T. cruzi* (Fieck et al. 2010). Commercially available AMPs, apidaecin, magainin, and melittin, were found to have preferential toxicity against *T. cruzi*, but had minimal effect on the *R. prolixus* symbiont, *R. rhodnii*, and the *E. coli* XL1-blue cloning host. We also showed that pair-wise combinations of these AMPs resulted in much improved *T. cruzi* killing efficiency that were either additive or synergistic in nature (Fieck et al. 2010).

The in vitro activity of AMPs against leishmania parasites had been demonstrated by a number of investigators. The temporins are a large family of small α-helical AMPs produced on the skin of Eurasian and New World ranid frogs (Simmaco et al. 1996). These molecules are usually characterized by a highly variable sequence, dominated by hydrophobic amino acid residues. These α-helical structures function by perturbing the lipid bilayer leading to transient pore formation, which results in membrane disruption at some threshold of peptide accumulation. Mangoni et al. showed the LC_{50} of temporins A and B against promastigote stages of *L. donovani* were 8.4 μM and 8.6 μM, respectively (Mangoni et al. 2005). Temp-SHd, a 17-residue-long peptide, is perhaps the longest temporin known to date. This AMP displays leishmanicidal activity against both promastigotes and amastigotes of *L. infantum*, with an IC_{50} of 16.5 μM and 23.5 μM, respectively. This peptide also displayed similar activities against other *Leishmania* spp. responsible for cutaneous and mucocutaneous forms of leishmaniasis. In addition to its anti-leishmania effect, the spectrum of activity of Temp-SHd extends to other members of the Trypanosomatidae family, i.e., *T. brucei* and *T. cruzi* (Abbassi et al. 2013).

The cathelicidins are a family of AMPs that are produced and stored in the neutrophils of many mammalian species. All members of the cathelicidin family contain a C-terminal cationic domain and an N-terminal cathelin portion that must be cleaved to release the active C-terminal peptide (Zanetti 2004). The bovine myeloid AMPs, BMAP-18 (Haines et al. 2009) and BMAP-28 (Lynn et al. 2011), are members of the cathelicidin family, and have both been demonstrated to be lethal to *L. donovani* and *L. major*, respectively. At low concentrations BMAP-18 disrupts membrane potentials and induces apoptotic cell death with no obvious alterations to the parasite plasma membrane. Higher concentrations of this AMP result in cell lysis as rapidly as 15 min following treatment (Haines et al. 2009). Lynn et al. showed that BMAP-28 triggers a late apoptotic event that results in membrane disruption and osmotic lysis in *L. major* promastigotes (Lynn et al. 2011).

Cecropin A is isolated from the hemolymph of *Hyalophora cecropia*, the Giant Silk moth. The effect of cecropin A on *Leishmania* promastigotes is minimal unless high concentrations are used (Akuffo et al. 1998). The cecropin A-melittin hybrid protein is made up of the cationic N-terminal sequence of cecropin A, followed by the hydrophobic N-terminal sequence of the bee venom toxin, melittin (Andreu et al. 1992). This hybrid protein shows much higher antibiotic activity than cecropin, and acts on organisms otherwise resistant to cecropin A. The cecropin A-melittin (CEMEL) hybrid proteins cause rapid collapse of electrical potential and morphological damage at the surface membrane of *L. donovani* (Diaz-Achirica et al. 1998). Further truncated versions of this hybrid protein were found to be more active against parental peptide (Luque-Ortega et al. 2003).

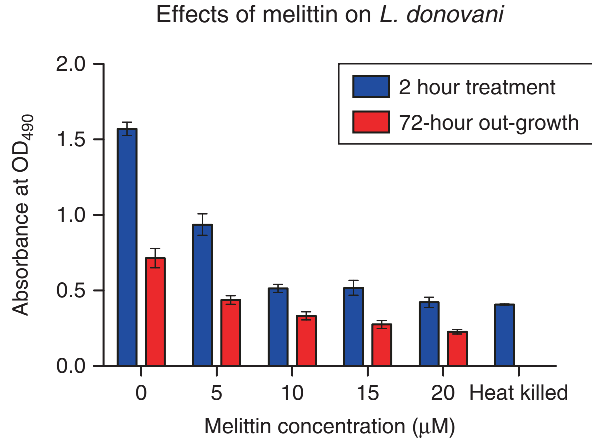

Fig. 3 The effects of melittin on *L. donovani* were compared to that of heat killing of parasites. Each bar represents the mean of three replicates; error bar represents the standard error of the mean

Melittin is the main constituent of honeybee venom. In previous studies, we had demonstrated that this molecule causes rapid lysis of *T. cruzi* (Fieck et al. 2010). We have recently tested the effects of melittin on cell viability and subsequent proliferation of several strains of *Leishmania* promastigotes. In these experiments, parasites were treated with increasing concentrations of melittin for 2 h. Following treatment, aliquots of the treated cells were transferred to fresh media to allow parasite proliferation. The viability of the remaining cells was measured using MTS assays (Promega, Madison, WI, USA). Heat-inactivated parasites, prepared by incubation at 95 °C for 30 min, serve as negative controls for these assays. Assessment of parasite apoptosis was also determined using flow cytometry in separate experiments (data not shown). All experiments were performed in triplicates and repeated at least three times. Figure 3 shows the ability of melittin to inhibit *L. donovani* in a concentration-dependent manner. These preliminary data would further suggest that parasites damaged by melittin do not proliferate well. Morphological changes of these parasites caused by melittin are currently being investigated. The IC_{50} of melittin was computed to be approximately 5μM, suggesting that this would function well as an effector molecule for paratransgenic expression.

The strong inhibitory activity of AMPs against both Gram-negative and Gram-positive bacteria is widely published. For the paratransgenic strategy to be successful, it is imperative that the fitness of a genetically modified commensal bacterium is not compromised. With that in mind, we evaluated the effects of melittin on *B. subtilis*, the “delivery vehicle” for this paratransgenic approach. The MBC of melittin for *B. subtilis* 1012 strain was found to be approximately 5 μm, suggesting that substantial expression of this AMP would be detrimental to the bacterial host (Table 1). In the honeybee, melittin is expressed as an inactive from. Promelittin is slowly hydrolyzed to its active form by dipeptidylpeptidase IV in the venom glands of these insects (Kreil et al. 1980). We have initiated work to express a modified version of promelittin in *B. subtilis*. We have designed this molecule to be “activated” only by enzymes that are present in the blood meal. Lebeau et al. had

Table 1 Antimicrobial activity of melittin against *B. subtilis*

Antimicrobial peptide	MIC	MBC
Melittin	<1 μM	5 μM

We defined the minimal inhibitory concentration (MIC) as the lowest concentration that resulted in the first significant decrease in OD reading, while the minimal bactericidal concentration (MBC) is the lowest AMP concentration that resulted in no growth on the culture plate the following day

employed a similar strategy to activate promelittin with fibroblast-activation protein to targeting of cancer stroma cells (Lebeau et al. 2009). We envision the following scenario for our system—the constitutive expression of promelittin by genetically modified *B. subtilis* in the paratransgenic sand fly would result in a buildup of this pro-effector molecule prior to the blood meal. Highly specific proteolytic enzymes present in the first blood meal will remove the pro-domain of these molecules. Invading *Leishmania* parasites will then be lysed by the activated melittin, thereby breaking the cycle of parasite transmission. If our strategy of expressing melittin in its inactive form is successful, a similar strategy can be applied for use with other AMPs, such as BAMPs for paratransgenic control of leishmaniasis.

Single-Chain Antibodies (scFvs)

Recombinant single-chain antibodies (scFvs) are fusion proteins containing the variable regions of the heavy and light chains of immunoglobulins. These regions are connected to one another with a short linker of about 10 amino acid residues. Despite the absence of the constant regions, these proteins retain specificity to target antigens comparable to that of parent immunoglobulins. Because of their small size, scFvs are readily produced in bacterial cultures. We had genetically modified *R. rhodnii* to produce a functional murine three-domain antibody fragment (rDB3) that binds progesterone (Durvasula et al. 1999a). The scFv-producing cells were subsequently fed to aposymbiotic *R. prolixus*. We showed that the rDB3 antibody fragment was synthesized and secreted into the insect gut lumen by the engineered symbiont in a stable fashion over the 6-month period of the study. We later repeated these studies via the successful transformation of a *Corynebacterium* symbiont of *T. infestans*, with the same shuttle plasmid (Durvasula et al. 2008).

For successful development within the sand fly midgut, the promastigotes must attach to the midgut epithelia to prevent expulsion with the digested blood meal. Lipophosphoglycan (LPG) is the major glycoconjugate on the surface of *Leishmania* promastigotes. This tripartite molecule consists of a repeating phosphoglycan (PG) domain that is linked via a hexasaccharide glycan core to a 1-*O*-alkyl-2-lyso-phosphatidylinositol lipid anchor (Sacks et al. 2000). Branching sugar residues, sialic acids—often presenting as *O*-acetylated sialoglycoconjugates, and carbohydrates in turn decorate the PG backbone. These decorations are highly polymorphic between *Leishmania* spp. (Pimenta et al. 1994; Kamhawi et al. 2000; Mahoney

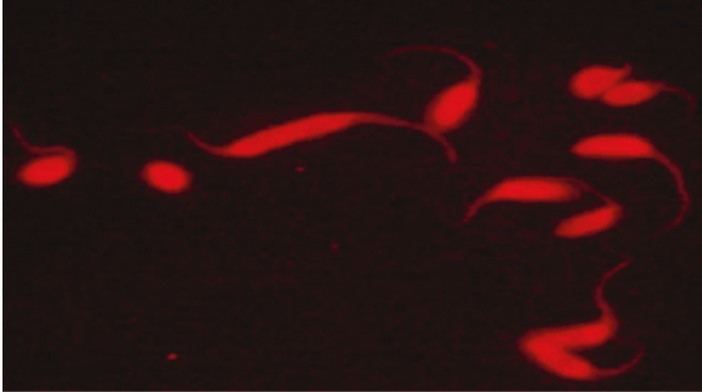

Fig. 4 Confocal image of anti-sialy-Tn REDantibody targeting glycan structures on the surface of *T. cruzi* epimastigotes. Figure adapted from Markiv et al. Module based antibody engineering: a novel synthetic REDantibody. *J Immunol Methods*, 2011, 364:40–49

et al. 1999; Soares et al. 2004) and had been demonstrated to be critical for vectorial competence (Pimenta et al. 1994). For example, *P. papatasi* only supports development of *L. major* but not of any other *Leishmania* species tested. For *L. major* to establish its infectivity, terminal galactosyl residues on *L. major* LPG must interact with the *P. papatasi* midgut receptor PpGalec receptor (Kamhawi et al. 2004). ScFvs that bind with high specificity to these glycoconjugates can be generated to prevent these parasite–vector interactions.

The sialyl-Tn and sialylated Lewis $(Le)_a$ blood group antigen are part of a panel of markers used in cancer diagnostics, and are recognized by B72.3 and CA19.9 antibodies, respectively. Interestingly these same antigens can be detected on the surface of *T. cruzi*. The presence of sialoglycoconjugates on the glycocalyx of *Leishmania* spp., coupled with the inherent outer cell wall similarities shared by *T. cruzi* and *Leishmania* spp., would lead us to predict that the B72.3 and CA19.9 REDantibodies will bind to *Leishmania* parasites. ScFvs for both these antibodies were therefore created. Synthetic DNA sequences of B72.3 and CA19.9 antibody variable domains in V_H–V_L orientation were codon optimized for expression in *E. coli*. We then inserted monomeric red fluorescent protein (mRFP) from *Discosoma* as a rigid linker between the V_H and V_L domains, generating a novel and highly stable REDantibody (Markiv et al. 2011). The resulting recombinant fluorescent molecules were demonstrated to be highly stable, and bind with specificity to *T. cruzi* epimastigotes (Fig. 4). In preliminary work, we have further shown that both these REDantibodies bind with specificity to *Leishmania* promastigotes (Fig. 5). In vitro studies are underway to determine if these constructs can be utilized to block parasite–vector interactions. If successful, these molecules will be added to our armamentarium of molecules for paratransgenic control of leishmania transmission.

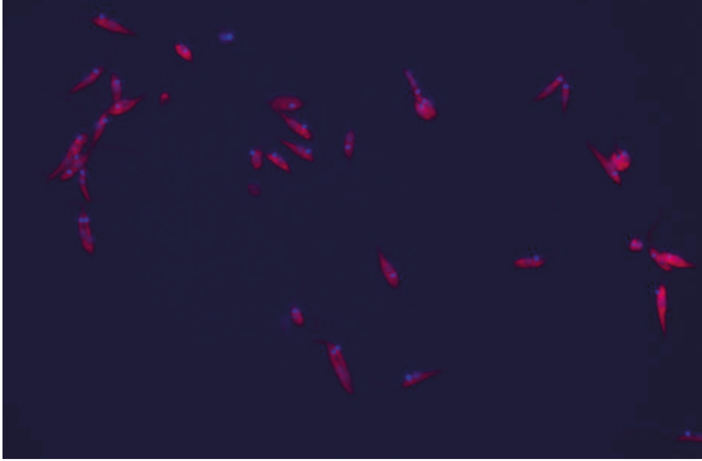

Fig. 5 Fluorescent micrograph of *L. donovani* co-stained with anti-sialy-Tn REDantibody and DAPI

From Bench Top to Field Trials

Deployment of genetically altered lines of bacteria to target field populations of sand flies may have profound environmental consequences. Horizontal gene transfer (HGT), an important process in bacterial evolution, has been implicated in phenomena such as antimicrobial resistance and virulence regulation. Incorporation of a transgene in a nontarget bacterium has the potential to alter fitness of the organism, with potentially disruptive ecological consequences. To address this, we have generated mathematical models predicting HGT between genetically modified *R. rhodnii* and *G. rubropertinctus*, a closely related environmental actinomycete (Matthews et al. 2011). The model predicts an HGT frequency of less than 1.14×10^{-16} per 100,000 generations at the 99 % certainty level. This predicted transfer frequency is less than the estimated average mutation frequency in bacteria, 10^{-1} per gene per 1,000 generations, suggesting that even if HGT were to occur between *R. rhodnii* and *G. rubropertinctus*, the transgene would likely not persist in the recipient organism, and that the likelihood of these unwanted events is low. Similar studies will have to be undertaken with *B. subtilis*.

Beyond reliance on low levels of HGT occurring in naturally occurring parameters, we have also begun the development and testing of a "barrier" method of containment and dispersal for paratransgenic technologies. To this end, we are advancing on a microencapsulation strategy, utilizing biologically derived polymers such as sodium alginates, for delivery of transformed bacteria into the insect gut (Fig. 6) (Forshaw et al. in prep). In this strategy engineered bacteria are encased within a three-dimensional biopolymer matrix where they are contained until ingested by the target organism (Bextine and Thorvilson 2002). Depending on biopolymer selection, the matrix can be "tuned" to pH gradients or enzymes within

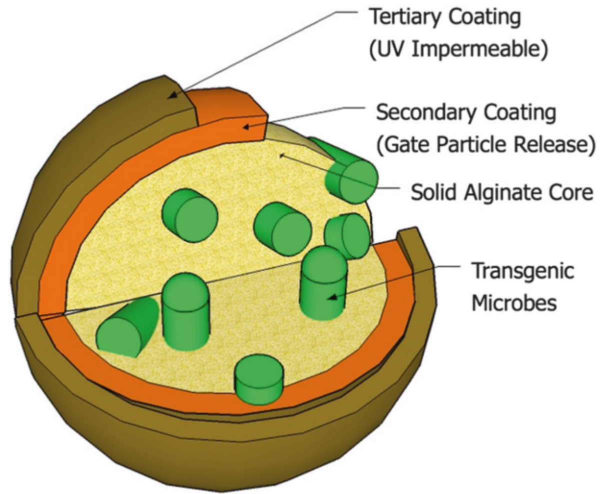

Fig. 6 Cartoon illustration of a multilayered alginate-core microcapsule encapsulating transgenic bacteria. Microcapsules can be "tuned" to various release parameters (directed release) based on underlying polymeric and crosslinking selection. Encapsulants can be bacteria, fungal spores, enzymes, antibodies, etc. Sizes usually range from 10 to 60 μm

the sand fly gut, which will cause the polymer to swell only upon ingestion, releasing the encapsulated bacteria (Lin et al. 2005). Unconsumed matrices will undergo a prolonged biodegradation period that well exceeds the life-span of the encapsulated bacteria. In preliminary studies, we have demonstrated that encapsulated *Pantoea agglomerans* can be contained indefinitely under varying conditions depending on polymer and cross-linker selection (Forshaw et al. in prep). We have further demonstrated that *P. agglomerans* can be delivered into the foregut and midgut of the glassy winged sharpshooter *Homalodisca vitripennis* (GWSS) that were allowed to feed on grapevines painted with bacteria-containing microcapsules (Forshaw et al. in prep). We plan to adapt this strategy to deliver genetically modified *B. subtilis* to sand fly larvae.

Conclusions

Despite great advances in public health worldwide, insect-transmitted infectious diseases remain a leading cause of morbidity and mortality. Currently, the best methods for control of many such diseases involve the use of chemical pesticides. Campaigns, such as the VL Elimination Initiative, have in the short term yielded encouraging results. However, environmental toxicity and adverse effects on human health limit the use of many chemical pesticides. Further, the emergence of insect resistance to a wide variety of insecticides has greatly undermined their efficacy. Finally, the cost of repeated applications of pesticides is often prohibitive.

Here, we describe an alternate methodology, paratransgenesis, for transmission control of leishmaniasis. In this strategy, anti-leishmania molecules are expressed

via transformed commensal bacteria in the insect vectors. Effector molecules, including AMPs and single-chain antibodies, that may be effective against leishmania are currently being studied. Unlike current arthropod eradication strategies with insecticides, the paratransgenic approach does not aim to eliminate sand flies. Rather, the overarching goal is to modulate the insect's ability to transmit a parasite. Such an approach could provide valuable tools for control of leishmaniasis in highly endemic regions of the world.

References

Abbassi F, Raja Z, Oury B et al (2013) Antibacterial and leishmanicidal activities of temporin-SHd, a 17-residue long membrane-damaging peptide. Biochimie 95:388–399

Akuffo H, Hultmark D, Engstom A et al (1998) Drosophila antibacterial protein, cecropin A, differentially affects non-bacterial organisms such as *Leishmania* in a manner different from other amphipathic peptides. Int J Mol Med 1:77–82

Andreu D, Ubach J, Boman A et al (1992) Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity. FEBS Lett 296:190–194

Baines S (1956) The role of the symbiotic bacteria in the nutrition of *Rhodnius prolixus* (Hemiptera). J Exp Biol 33:533–541

Beard CB, Mason PW, Aksoy S et al (1992) Transformation of an insect symbiont and expression of a foreign gene in the Chagas' disease vector *Rhodnius prolixus*. Am J Trop Med Hyg 46:195–200

Beard CB, Durvasula RV, Richards FF (1998) Bacterial symbiosis in arthropods and the control of disease transmission. Emerg Infect Dis 4:581–591

Beard CB, Cordon-Rosales C, Durvasula RV (2002) Bacterial symbionts of the triatominae and their potential use in control of Chagas disease transmission. Annu Rev Entomol 47:123–141

Bextine B, Thorvilson H (2002) Field applications of bait-formulated *Beauveria bassiana* alginate pellets for biological control of the red imported fire ant. Environ Entomol 31:746–752

Casanova C (2001) A soil emergence trap for collections of phlebotomine sand flies. Mem Inst Oswaldo Cruz 96:273–275

Chelbi I, Kaabi B, Derbali M et al (2008) Zooprophylaxis: impact of breeding rabbits around houses on reducing the indoor abundance of *Phlebotomus papatasi*. Vector Borne Zoonotic Dis 8:741–747

Dasch GA, Weiss E, Chang S (1984) Endosymbionts in insects. Williams & Wilkins, Baltimore, MD

Dhiman RC, Raghavendra K, Kumar V et al (2003) Susceptibility status of *Phlebotomus argentipes* to insecticides in districts Vaishaii and Patna (Bihar). J Commun Dis 35:49–51

Diaz-Achirica P, Ubach J, Guinea A et al (1998) The plasma membrane of *Leishmania donovani* promastigotes is the main target for CA(1-8)M(1-18), a synthetic cecropin A-melittin hybrid peptide. Biochem J 330:453–460

Dillion RJ, El Kordy E, Lane RP (1996) The prevalence of a microbiota in the digestive tract of *Phlebotomus papatasi*. Ann Trop Med Parasitol 90:669–673

Dinesh DS, Das ML, Picado A et al (2010) Insecticide susceptibility of *Phlebotomus argentipes* in visceral leishmaniasis endemic districts in India and Nepal. PLoS Negl Trop Dis 4:e859

Dougherty MJ, Hamilton JGC (1997) Dodecanoic acid is the oviposition pheromone of *Lutzomyia longipalpis*. J Chem Ecol 23:2657–2671

Dougherty MJ, Hamilton JG, Ward RD (1993) Semiochemical mediation of oviposition by the phlebotomine sandfly *Lutzomyia longipalpis*. Med Vet Entomol 7:219–224

Dougherty M, Guerin PM, Ward RD (1995) Identification of oviposition attractants for the sand fly *Lutzomyia longipalpis* (Diptera, Psychodidae) in volatiles of feces from vertebrates. Physiol Entomol 20:23–32

Durvasula RV, Gumbs A, Panackal A et al (1997) Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc Natl Acad Sci U S A 94:3274–3278

Durvasula RV, Gumbs A, Panackal A et al (1999a) Expression of a functional antibody fragment in the gut of *Rhodnius prolixus* via transgenic bacterial symbiont *Rhodococcus rhodnii*. Med Vet Entomol 13:115–119

Durvasula RV, Kroger A, Goodwin M et al (1999b) Strategy for introduction of foreign genes into field populations of Chagas disease vectors. Ann Entomol Soc Am 92:937–943

Durvasula RV, Sundaram RK, Kirsch P et al (2008) Genetic transformation of a *Corynebacterial* symbiont from the Chagas disease vector *Triatoma infestans*. Exp Parasitol 119:94–98

Elnaiem DE, Ward RD (1992) Oviposition attractants and stimulants for the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae). J Med Entomol 29:5–12

Fieck A, Hurwitz I, Kang AS et al (2010) *Trypanosoma cruzi*: synergistic cytotoxicity of multiple amphipathic anti-microbial peptides to *T. cruzi* and potential bacterial hosts. Exp Parasitol 125:342–347

Ghosh KN, Bhattacharya A (1991) Breeding places of *Phlebotomus argentipes* Annandale and Brunetti (Diptera: Psychodidae) in West Bengal, India. Parassitologia 33:267–272

Ghosh KN, Mukhopadhyay JM, Guzman H et al (1999) Interspecific hybridization and genetic variability of *Phlebotomus sandflies*. Med Vet Entomol 13:78–88

Guerin PJ, Olliaro P, Sundar S et al (2002) Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. Lancet Infect Dis 2:494–501

Haines LR, Thomas JM, Jackson AM et al (2009) Killing of trypanosomatid parasites by a modified bovine host defense peptide, BMAP-18. PLoS Negl Trop Dis 3:e373

Hillesland H, Read A, Subhadra B et al (2008) Identification of aerobic gut bacteria from the kala azar vector, *Phlebotomus argentipes*: a platform for potential paratransgenic manipulation of sand flies. Am J Trop Med Hyg 79:881–886

Hurwitz I, Hillesland H, Fieck A et al (2011) The paratransgenic sand fly: a platform for control of *Leishmania* transmission. Parasit Vectors 4:82

Ilango K, Dhanda V, Srinivasan R et al (1994) *Phlebotomine* sandflies (Diptera: Psychodidae) of Tamil Nadu and Pondicherry, southern India, in relation to visceral leishmaniasis. Ann Trop Med Parasitol 88:413–431

Kamhawi S, Modi GB, Pimenta PF et al (2000) The vectorial competence of *Phlebotomus sergenti* is specific for *Leishmania tropica* and is controlled by species-specific, lipophosphoglycan-mediated midgut attachment. Parasitology 121:25–33

Kamhawi S, Ramalho-Ortigao M, Pham VM et al (2004) A role for insect galectins in parasite survival. Cell 119:329–341

Kesari S, Kishore K, Palit A et al (2000) An entomological field evaluation of larval biology of sandfly in Kala-azar endemic focus of Bihar—exploration of larval control tool. J Commun Dis 32:284–288

Kishore K, Kumar V, Kesari S et al (2004) Susceptibility of *Phlebotomus argentipes* against DDT in endemic Districts of North Bihar, India. J Commun Dis 36:41–44

Kishore K, Kumar V, Kesari S et al (2006) Vector control in leishmaniasis. Indian J Med Res 123:467–472

Kreil G, Haiml L, Suchanek G (1980) Stepwise cleavage of the pro part of promelittin by dipeptidylpeptidase IV. Evidence for a new type of precursor–product conversion. Eur J Biochem 111:49–58

Kumar V, Kesari S, Dinesh DS et al (2009) A report on the indoor residual spraying (IRS) in the control of *Phlebotomus argentipes*, the vector of visceral leishmaniasis in Bihar (India): an initiative towards total elimination targeting 2015 (series-1). The Free Library [Online]. Available: http://www.thefreelibrary.com/

Lebeau AM, Brennen WN, Aggarwal S et al (2009) Targeting the cancer stroma with a fibroblast activation protein-activated promelittin protoxin. Mol Cancer Ther 8:1378–1386

Lin WC, Yu DG, Yang MC (2005) pH-sensitive polyelectrolyte complex gel microspheres composed of chitosan/sodium tripolyphosphate/dextran sulfate: swelling kinetics and drug delivery properties. Colloids Surf B Biointerfaces 44:143–151

Luque-Ortega JR, Saugar JM, Chiva C et al (2003) Identification of new leishmanicidal peptide lead structures by automated real-time monitoring of changes in intracellular ATP. Biochem J 375:221–230

Lynn MA, Kindrachuk J, Marr AK et al (2011) Effect of BMAP-28 antimicrobial peptides on *Leishmania major* promastigote and amastigote growth: role of leishmanolysin in parasite survival. PLoS Negl Trop Dis 5:e1141

Mahoney AB, Sacks DL, Saraiva E et al (1999) Intra-species and stage-specific polymorphisms in lipophosphoglycan structure control *Leishmania donovani*-sand fly interactions. Biochemistry 38:9813–9823

Mangoni ML, Saugar JM, Dellisanti M et al (2005) Temporins, small antimicrobial peptides with leishmanicidal activity. J Biol Chem 280:984–990

Markiv A, Anani B, Durvasula RV et al (2011) Module based antibody engineering: a novel synthetic REDantibody. J Immunol Methods 364:40–49

Matthews S, Sreehari Rao V, Durvasula RV (2011) Modeling horizontal gene transfer (HGT) in the gut of the Chagas disease vector *Rhodnius prolixus*. Parasit Vectors 4:77

Mccall PJ, Cameron MM (1995) Oviposition pheromones in insect vectors. Parasitol Today 11:352–355

Moncaz A, Faiman R, Kirstein O et al (2012) Breeding sites of *Phlebotomus sergenti*, the sand fly vector of cutaneous leishmaniasis in the Judean Desert. PLoS Negl Trop Dis 6:e1725

Mukhopadhyay AK, Chakravarty AK, Kureel VR et al (1987) Resurgence of *Phlebotomus argentipes & Ph. papatasi* in parts of Bihar (India) after DDT spraying. Indian J Med Res 85:158–160

Mukhopadhyay AK, Hati AK, Chakraborty S et al (1996) Effect of DDT on *Phlebotomus* sandflies in Kala-Azar endemic foci in West Bengal. J Commun Dis 28:171–175

Mukhopadhyay J, Braig HR, Rowton ED et al (2012) Naturally occurring culturable aerobic gut flora of adult *Phlebotomus papatasi*, vector of *Leishmania major* in the Old World. PLoS One 7:e35748

Peterkova-Koci K, Robles-Murguia M, Ramalho-Ortigao M et al (2012) Significance of bacteria in oviposition and larval development of the sand fly *Lutzomyia longipalpis*. Parasit Vectors 5:145

Picado A, Das ML, Kumar V et al (2010) Effect of village-wide use of long-lasting insecticidal nets on visceral Leishmaniasis vectors in India and Nepal: a cluster randomized trial. PLoS Negl Trop Dis 4:e587

Picado A, Dash AP, Bhattacharya S et al (2012) Vector control interventions for visceral leishmaniasis elimination initiative in South Asia, 2005-2010. Indian J Med Res 136:22–31

Pimenta PF, Saraiva EM, Rowton E et al (1994) Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. Proc Natl Acad Sci U S A 91:9155–9159

Rajendran P, Modi GB (1982) Bacterial flora of sandfly gut (Diptera: Psychodidae). Indian J Public Health 26:49–52

Sacks DL, Modi G, Rowton E et al (2000) The role of phosphoglycans in *Leishmania*-sand fly interactions. Proc Natl Acad Sci U S A 97:406–411

Simmaco M, Mignogna G, Canofeni S et al (1996) Temporins, antimicrobial peptides from the European red frog *Rana temporaria*. Eur J Biochem 242:788–792

Singh R, Das RK, Sharma SK (2001) Resistance of sandflies to DDT in Kala-azar endemic districts of Bihar, India. Bull World Health Organ 79:793

Singh RK, Mittal PK, Dhiman RC (2012) Insecticide susceptibility status of *Phlebotomus argentipes*, a vector of visceral leishmaniasis in different foci in three states of India. J Vector Borne Dis 49:254–257

Soares RP, Barron T, Mccoy-Simandle K et al (2004) *Leishmania tropica*: intraspecific polymorphisms in lipophosphoglycan correlate with transmission by different *Phlebotomus* species. Exp Parasitol 107:105–114

Sundar S, Jha TK, Thakur CP et al (2006) Oral miltefosine for the treatment of Indian visceral leishmaniasis. Trans R Soc Trop Med Hyg 100(suppl 1):S26–S33

Sundar S, Agrawal N, Arora R et al (2009) Short-course paromomycin treatment of visceral leishmaniasis in India: 14-day vs 21-day treatment. Clin Infect Dis 49:914–918
Sundar S, Chakravarty J, Agarwal D et al (2010) Single-dose liposomal amphotericin B for visceral leishmaniasis in India. N Engl J Med 362:504–512
Sundar S, Sinha PK, Rai M et al (2011) Comparison of short-course multidrug treatment with standard therapy for visceral leishmaniasis in India: an open-label, non-inferiority, randomised controlled trial. Lancet 377:477–486
Tabbabi A, Ghrab J, Aoun K et al (2011) Habitats of the sandfly vectors of *Leishmania tropica* and *L. major* in a mixed focus of cutaneous leishmaniasis in southeast Tunisia. Acta Trop 119:131–137
Thakur CP, Kumar K (1992) Post kala-azar dermal leishmaniasis: a neglected aspect of kala-azar control programmes. Ann Trop Med Parasitol 86:355–359
Vieira VP, Ferreira AL, Biral Dos Santos C et al (2012) Peridomiciliary breeding sites of phlebotomine sand flies (Diptera: Psychodidae) in an endemic area of American cutaneous leishmaniasis in southeastern Brazil. Am J Trop Med Hyg 87:1089–1093
Volf P, Kiewegova A, Nemec A (2002) Bacterial colonisation in the gut of *Phlebotomus duboseqi* (Diptera: Psychodidae): transtadial passage and the role of female diet. Folia Parasitol (Praha) 49:73–77
Wasserberg G, Abramsky Z, Kotler BP et al (2003) Anthropogenic disturbances enhance occurrence of cutaneous leishmaniasis in Israel Deserts: patterns and mechanisms. Ecol Appl 13:868–881
World Health Organization (2004) Regional strategic framework for elimination of kala-azar from the South-East Asia Region (2005–2015). Regional Office for South-East Asia, New Delhi
World Health Organization (2007) Regional Technical Advisory Group on kala-azar elimination. Report of the second meeting, Kathmandu, Nepal, 20 October–2 November 2006 [online]
World Health Organization (2012) Leishmaniasis: worldwide epidemiological and drug access update. World Health Organization, Geneva. http://www.who.int/leishmaniasis/resources/Leishmaniasis_worldwide_epidemiological_and_drug_access_update.pdf
Zanetti M (2004) Cathelicidins, multifunctional peptides of the innate immunity. J Leukoc Biol 75:39–48

Current Status and Future Challenges for the Development of Genetically Altered Live Attenuated *Leishmania* Vaccines

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Abstract Leishmaniasis is a protozoan parasitic disease endemic to the tropical and subtropical regions of the world, with three major clinical forms, self-healing cutaneous leishmaniasis, mucocutaneous leishmaniasis, and fatal visceral leishmaniasis (VL). Drug treatment is expensive, and often results in the development of resistance due to lack of compliance and prolonged use. No vaccine is available against leishmaniasis. Immunization with first- and second-generation *Leishmania* vaccines has shown some efficacy in animal models but little or none in humans. However, individuals who recover from a natural infection are protected from reinfection and develop lifelong protection, suggesting infection may be a prerequisite for creating immunological memory. Genetically altered live attenuated parasites with controlled infectivity could achieve such immunological memory and yield protection without overt disease. In this chapter, we discuss development and characteristics of genetically altered live attenuated *Leishmania donovani* parasites and their possible use as vaccine candidates against VL. In addition, we discuss the challenges with regard to safety and immunogenicity of the live attenuated parasites.

Keywords Live attenuated parasites • Sand fly • Memory T cells • Vaccine

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Introduction

Leishmaniasis is a broad spectrum of diseases caused by protozoan parasites of the genus *Leishmania*. It infects about 12 million individuals globally in tropical and subtropical regions, with ~2 million new clinical cases [0.5 million visceral leishmaniasis (VL) and 1.5 million cutaneous leishmaniasis (CL)] reported annually with an estimated death toll of ~60,000 persons/year (Desjeux 2004). *Leishmania* infection occurs in five continents and is endemic in 98 countries. The three major clinical forms of leishmaniasis, VL, CL, and mucocutaneous leishmaniasis (MCL), are the result of infection by different species of the parasite, tissue tropism, and differential immune response of the host (Selvapandiyan et al. 2012; Murray et al. 2005). The clinical manifestation of VL, which is fatal if untreated, is characterized by prolonged fever and hepato-splenomegaly. The causative agent for VL is *L. donovani* in the Old World or *L. infantum* or *L. chagasi* in the New World. CL manifests as mostly painless dermal lesions that are caused by *L. major*, *L. aethopica*, or *L. tropica* in the Old World and by *L. mexicana* or *L. braziliensis* complex in the New World. MCL affects the mucosal region of the host, and is caused by *L. braziliensis*, *L. amazonensis*, *L. panamensis*, or *L. guyanensis* (Harhay et al. 2011; Herwaldt 1999).

Leishmania is transmitted to the host by an infected sand fly bite. Female sand flies (*Phlebotomus* species in the Old World, *Lutzomyia* species in the New World) acquire *Leishmania* parasites when they feed on infected mammalian hosts. While they take the blood meal the metacyclic form of *Leishmania* is regurgitated into the blood circulation (Sacks and Kamhawi 2001; Bates 2006, 2007; Rogers et al. 2004). In the feeding process sand fly saliva plays an important role (Anderson et al. 2006; Oliveira et al. 2009). Parasites taken up by the neutrophils, that are in turn ingested by the host macrophages, differentiate into the nonmotile amastigote form and reside and multiply in the hostile environment of phagolysosomes of the host macrophages (Peters et al. 2008; van Zandbergen et al. 2004).

The only treatment for leishmaniasis is drug therapy that includes pentavalent antimonials, miltefosine, paromomycin, and amphotericin B as the standard drugs. However, either prolonged use or inefficient drug therapy has resulted in widespread drug resistance. Currently most of the clinical cases are resistant to the first-line drug, antimony (Sundar and Chatterjee 2006; Srivastava et al. 2011; Croft et al. 2006). There is concern about the possibility of development of resistance to the new oral drug miltefosine in the near future as there are already reports of relapse among miltefosine-treated cases (Bhandari et al. 2012). There are no vaccines available at present against any form of leishmaniasis. Attempts to develop vaccines for such parasitic agents such as heat-killed, subunit, or DNA vaccines have not resulted in a successful vaccine candidate that could be applicable to humans. It has been estimated that a *Leishmania* vaccine even with 50 % efficacy and 5-year protection is cost effective compared with drug therapy (Bacon et al. 2013).

Immunity Against Leishmaniasis

Understanding the protective immune response against *Leishmania* is important for the design of effective vaccines and to obtain enhanced vaccine-induced immunity. Immunity to leishmaniasis is mediated by both arms of mammalian cellular immune system; innate (by neutrophils, macrophages, and dendritic cells) and adaptive (T cells) responses (Kedzierski 2010). The role of B cells in regulating immunity to leishmaniasis has been studied mostly in the models of cutaneous leishmaniasis (Scott et al. 1986; Sacks et al. 1984; Wanasen et al. 2008). Experiments with B cell-deficient mice suggested suppression of early protective T cell responses by marginal zone B cells upon *L. donovani* infection (Bankoti et al. 2012). Further, in VL a recent report described the role of B cells in hepatic granulomas and their importance in controlling the experimental VL in mice (Moore et al. 2012).

The sand fly bite causes minimal tissue damage that promotes recruitment of neutrophils to the site of injury as a primary immune defense mechanism of the host (Peters et al. 2008; van Zandbergen et al. 2004). However, regardless of *Leishmania* spp. these neutrophils are the primary target of this parasite. Eventually *Leishmania* hijacks the whole immune system for its own survival (Wanasen et al. 2008). The immune response and pathology of visceral leishmaniasis are complex involving a number of genetic and cellular factors that together determine susceptibility or resistance to parasites (Cummings et al. 2010). A clear dichotomy between Th1-mediated protection (mediated by major cytokines IFNγ, IL2, TNFα) and Th2-mediated disease progression (mediated by major cytokines IL10, IL4) has been demonstrated in mice against CL (Alexander and Bryson 2005; Selvapandiyan et al. 2006; Scott 2003). However, this Th1/Th2 dichotomy is not as clear in visceral infection of mice and even less so in human VL (Nylen and Sacks 2007).

In VL (both in murine and human), resolution of infection depends on the production of Th1 cytokines (Selvapandiyan et al. 2006, 2009; Dey et al. 2007a, b; Banerjee et al. 2008; Ghalib et al. 1995; Kenney et al. 1998; Duthie et al. 2012). Production of IL12 by antigen presenting cells and IFNγ by T cells is crucial for controlling the parasite growth and development of host immunity (Dey et al. 2007a; Murray 1997). Susceptibility to visceral leishmaniasis is correlated with the presence of a Th2 response (Nylen and Sacks 2007). *L. donovani* infection stimulates expression of Th2-associated cytokines (IL10, IL4, IL5, and IL13). It appears that IL10 plays dual and opposing roles in VL. On one side, IL10 suppresses host immunity and helps parasite survival; on the flip side, IL10 also protects the host from tissue damage by exaggerated inflammation (Duthie et al. 2012). Recent findings suggest that some IFNγ producing cells are a crucial source of IL10, which act as a negative feedback mechanism to control tissue damage (Murray 1997; Murphy et al. 2001). A study with splenic T cells derived from VL patients showed elevated expression of both IFNγ and IL10 (Murray et al. 2003) suggesting that at least some cells are producing both cytokines as part of a strong inflammatory response and

potentially limiting the tissue damage by a feedback control mechanism (Nylen and Sacks 2007). Further, elevated levels of IL10 in serum and enhanced levels of IL10 mRNA expression in lesion tissue are a direct indication of severe visceral leishmaniasis in mice and humans (Kenney et al. 1998; Murphy et al. 2001; Murray et al. 2003; Nylen et al. 2007). IL10 plays a crucial role in the establishment and maintenance of Th2-dependent immune responses by suppressing Th1-dependent cell-mediated immunity (Fiorentino et al. 1989; Mosmann and Moore 1991). In the murine VL model, IL10 directly inhibits the antimicrobial machinery of macrophages by modulating normal signal transduction mechanisms (Bhattacharyya et al. 2001). IL10 inhibits killing of amastigotes by down-regulating the production of TNFα and nitric oxide by macrophages and dendritic cells and thereby strongly impairs their antimicrobial activity (Bhattacharyya et al. 2001; Vouldoukis et al. 1997). IL10 also inhibits the production of IFNγ, one of the major pro-inflammatory cytokines (Moore et al. 1990). However, in visceral leishmaniasis, it has been suggested that the impaired function of cellular immunity that correlates with progression of active disease may be due to the inhibitory effects of IL10 independent of the IFNγ level (Holaday et al. 1993). IL4 has generally been considered a Th2 cytokine that helps in the proliferation of the Th2 cell population and consequently a significant down-regulator of Th1 cell response (Cua and Stohlman 1997; Lehn et al. 1989). The role of IL4 is not very clear in CL but in VL is necessary for an effective chemotherapy (Alexander and Bryson 2005; Alexander et al. 2000). Pro-inflammatory cytokine TNFα also shares a significant role in resistance because TNFα$^{-/-}$ mice readily succumb to infection with *Leishmania* sp. (Murray et al. 2000; Tumang et al. 1994). In addition, TNFα was also shown to stimulate the action of IFNγ in the induction of nitric oxide (NO) production in macrophages to kill the parasite (Liew et al. 1990).

Besides Th1 and Th2, a third subset of T helper cells of CD4+ T cell lineage called Th17 cells has been described recently that produces IL17 family of cytokines (Harrington et al. 2006). They are pro-inflammatory cytokines that stimulate the production of IL6, TNFα, and chemokines. Recent studies have shown a role for Th17 responses in chronic inflammation associated with parasitic infection and importantly vaccine-induced memory responses in certain bacterial vaccines (Lin et al. 2010). A very recent study suggested that *L. donovani* strongly induced IL17 and IL22 in human peripheral blood mononuclear cells (PBMCs), and enhanced secretion of these cytokines correlated with protection (Pitta et al. 2009).

The role of CD8+ cytotoxic T cells in the control of experimental CL and VL has been described (Ruiz and Becker 2007). In murine VL, control of parasite multiplication and leishmanicidal activity are both associated with development of a granuloma in the liver, which requires both CD4 and CD8 T cells that produce IL12, IFNγ, and IL2 (Murray 1997; Polley et al. 2006; Squires et al. 1989; Stager et al. 2000; Stern et al. 1988; Engwerda et al. 1998).

Thus, in addition to the protective T-cell response, the role of other T-cell subsets, including regulatory T and Th17 cells in either susceptibility or resistance to experimental and human visceral leishmaniasis, is not clearly understood and requires detailed investigations to help in vaccine designs. Finally, complexity of

immune response in humans is not faithfully reproduced in small animal models, thus limiting our ability to predict the efficacy of a laboratory-tested vaccine candidate in the field.

Leishmania Vaccines

1. *Past approaches to vaccination*: Leishmanization, deliberate infection with inoculum from CL patients which conferred a lifelong immunity against re-challenge with the parasite, was long practiced in CL endemic regions. However this turned to be unsafe because the wild type parasite caused full-blown disease in immune-compromised subjects thus leading to discontinuation of this practice. Advances in biotechnology have now opened up new methods for the development of vaccine candidates. Following is the outcome of various strategies that were used to develop *Leishmania* vaccine candidates.
2. *First-generation vaccine*; *vaccination with whole killed or crude antigens of the Leishmania parasites*: Early vaccination involved the use of autoclaved *L. major* (ALM) along with BCG in a clinical trial in Iran (Bahar et al. 1996; Dowlati et al. 1996). However the vaccine was not adequately immunogenic. Addition of alum (aluminum hydroxide) increased the immunogenicity of ALM + BCG, but failed to protect vervet monkeys against *L. major* infection (Gicheru et al. 2001). However, Alum-ALM + BCG protected langur monkeys against VL (Misra et al. 2001). Clinical trials have been conducted with inactivated whole *Leishmania* parasite as a vaccine in Ecuador and Columbia against CL (Armijos et al. 1998, 2004; Convit et al. 2003). The results of this vaccination showed that the vaccine was safe; however, the level of protection was not significant in the CL endemic patients.
3. *Second-generation vaccine*: *vaccination with DNA*, *purified fractions*, *or recombinant proteins with various adjuvants*: A variety of DNA vaccines coding for antigenic proteins and purified recombinant proteins have been investigated as *Leishmania* vaccine antigen candidates in laboratory animal models (Stager et al. 2000; Rafati et al. 2005, 2006; Soong et al. 1995; Basu et al. 2005; Tewary et al. 2005; Sjolander et al. 1998). Among them LACK (*Leishmania* receptor for activated C kinase) has been demonstrated very efficacious against CL, but failed to protect against experimental VL (Mougneau et al. 1995; Melby et al. 2001). Later vaccine candidates involved a combination of protein antigens such as Leish-111f comprising the *L. major* homologue of eukaryotic thiol-specific antioxidant (TSA), the *L. major* stress-inducible protein 1, and the *L. braziliensis* elongation and initiation factor, together with monophosphoryl lipid A formulated in oil-in-water emulsion (MPL–SE). This vaccine candidate showed protection in mice, hamsters, and monkeys (Skeiky et al. 2002; Coler et al. 2007; Campos-Neto et al. 2001), and was found to be safe and immunogenic in healthy individuals with and without the history of previous infection with *L. donovani* (Chakravarty et al. 2011). Recently another vaccine candidate (KSAC) was

tested in animal model and shown to infer protection against *L. major* transmitted through sand fly bite (Gomes et al. 2012). KSAC comprises kinetoplast membrane protein-11 (KMP-11), sterol 24-c-methyl transferase (SMT), A2, and cysteine proteinase-B (CPB), and each component of this fusion protein has been demonstrated to individually confer protection against experimental VL in mice (Rafati et al. 2005; Basu et al. 2005; Ghosh et al. 2001; Goto et al. 2009). However, so far no candidate vaccine had clinical efficacy in *Leishmania* endemic regions.

Live Attenuated Vaccine

1. *Generation of attenuated parasites*: In this chapter we will focus on the advantages and challenges of the live attenuated *Leishmania* parasite vaccines constructed by genetic manipulations. Targeted gene deletions have been carried out to develop *Leishmania* vaccine candidates that have attenuated virulence (Table 1). Among the vaccine candidates developed for CL, *L. major* dihydrofolate reductase-thymidylate synthase (*dhfr-ts*-) knockout parasites protected mice (Titus et al. 1995) but not rhesus monkeys (Amaral et al. 2002). *L. major* deficient in surface and secreted phosphoglycans (*lpg2*$^{-/-}$), although unable to survive in sand flies and macrophages, retained the ability to persist indefinitely in mice and conferred protection against virulent challenge, even in the absence of a strong Th1 response (Spath et al. 2003; Uzonna et al. 2004). However, *lpg2*$^{-/-}$ parasites over time unexpectedly regained virulence (Spath et al. 2004). *L. major* deleted for phosphomannomutase (*PMM*) protected mice, despite no increase in either effector or memory response (Kedzierski et al. 2008). Additionally, *L. mexicana* deficient in cysteine proteinase genes (Δ*cpa* and Δ*cpb*) conferred protection in mice and hamsters against homologous challenge (Alexander et al. 1998; Saravia et al. 2006). Among the vaccination studies in VL (Table 1), mice immunized with an *L. donovani* strain deleted for biopterin transporter (*BT1*) were also similarly protected from virulent challenge (Papadopoulou et al. 2002). Recent attempts using partial knockout parasites for the A2-A2rel gene cluster in *L. donovani* (Zhang and Matlashewski 2001) and SIR2 gene in *L. infantum* (Silvestre et al. 2007) as immunogens induced protection against virulent challenge in BALB/c mice. However, such mutants cannot be used as vaccine candidates, because they still carry wild type allele/s and could cause disease. Efforts also were made to enhance the vaccine safety by including drug-sensitive *L. major* mutants with suicide genes for controlled infection (Davoudi et al. 2005; Muyombwe et al. 1997). These mutant strains, despite limitations demonstrated, shared the advantages and disadvantages of live attenuated vaccines generated by targeted gene disruptions. We think that it is critical to develop attenuated lines through complete gene knockouts that generate organisms with controlled infectivity, do not persist for prolonged period in the host, and provide sufficient sustained antigenic load to induce immunological memory. Such vaccine

Table 1 Genetically altered live attenuated vaccine candidates against leishmaniasis

Parasite	Characterization of attenuation	Animal model	Results of immunization	References
L. donovani	Biopterin transporter gene-deleted parasite ($BT1^{-/-}$)	BALB/c mice	Protective immunity, antigen-specific IFNγ secretion	Papadopoulou et al. (2002)
L. tarentolae	Nonpathogenic strain expressing *L. donovani* A2 antigen	BALB/c mice	Protective immunity against *L. infantum* challenge, high IFNγ, low IL-5	Mizbani et al. (2009)
L. donovani	Replication-deficient centrin gene deleted ($Cen^{-/-}$)	BALB/c mice, Syrian hamster	Protective immunity against *L. donovani* and *L. braziliensis* challenge. Increased IFNγ, IL-2, and TNF producing cells and IFNγ/IL-10 ratio	Selvapandiyan et al. (2009)
L. infantum	Silent information regulatory 2 single allele deletion ($SIR2^{+/-}$)	BALB/c mice	Protective immunity, increased antigen-specific IFNγ/IL-10 ratio	Silvestre et al. (2007)
L. donovani	Cytochrome c oxidase complex component p27 gene-deleted cell line ($Ldp27^{-/-}$)	BALB/c mice	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. (2010, 2013)
L. major	Phosphoglycan (PG)-deficient $lpg2^{-/-}$	BALB/c mice	Persists without pathology, and protects against virulent challenge without inducing a strong Th1 response	Spath et al. (2003), Liu et al. (2013)
L. major	Dihydrofolate reductase-thymidylate synthetase $Dhfr\text{-}ts^{-/-}$	Mice and primates	Protective in mice but not in primates	Titus et al. (1995), Amaral et al. (2002)
L. mexicana	Cysteine proteinase-deficient mutant (delta cpa)	Mice	Induces Th1 response and protective	Alexander et al. (1998)

candidates have the potential to be safe and efficacious compared to Leishmanization vaccine approach (Selvapandiyan et al. 2012). Towards this concept, we have developed several *L. donovani* mutant parasites (Selvapandiyan et al. 2009), which lack virulence-specific genes, e.g., *L. donovani* centrin gene (*LdCen*$^{-/-}$), *L. donovani* p27 gene (*Ldp27*$^{-/-}$) (Dey et al. 2013), Ufm1 (*LdUfm1*$^{-/-}$) (Gannavaram et al. 2012), and Ufsp (*LdUfps*$^{-/-}$) (Gannavaram et al. unpublished). Latter two genes are associated with ubiquitin-like protein modification system in *Leishmania* (Gannavaram et al. 2012). Centrin is a growth regulating gene in the protozoan parasites *Leishmania* (Selvapandiyan et al. 2001), *Trypanosoma* (Selvapandiyan et al. 2007), and *Plasmodium* (Mahajan et al. 2008) and higher eukaryotes (Salisbury 1995). *LdCen*$^{-/-}$ is specifically attenuated at the amastigote stage and not at the promastigote (Selvapandiyan et al. 2004). The *LdCen*$^{-/-}$ 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 (Selvapandiyan et al. 2009). *LdCen*$^{-/-}$ was found to be safe and protective in mice and hamster models against virulent *L. donovani* challenge (Selvapandiyan et al. 2009). *Leishmania donovani* 27 kDa mitochondrial inner membrane protein preferentially expressed in the amastigote stage is an essential component of cytochrome c oxidase complex involved in oxidative phosphorylation (Dey et al. 2010). The *Ldp27*$^{-/-}$ cell line is attenuated for virulence in mice (Dey et al. 2010) and induces a strong Th1 type response against wild type infection (Dey et al. 2013). In addition, immunization with *Ldp27*$^{-/-}$ confers a long lasting protection against *L. donovani* infection (Dey et al. 2013).

2. *Memory T cells in live attenuated parasite vaccines*: Generation and preservation of the immunological memory seems to be an important aspect of antiparasitic vaccine development. In CL, the role of memory cells has been well studied and suggests generation of both effector memory (EM) and central memory (CM) T cells during infection (Okwor et al. 2009; Zaph et al. 2004). After antigenic stimulation, a few pathogen-specific T_{EM} cells become T_{CM} cells, which live for longer periods of time and stay in secondary lymphoid organs. Upon antigenic re-stimulation, CM cells become EM T cells and mediate protection (Kaech et al. 2002). Mice immunized with live attenuated *L. major DHFR*$^{-/-}$ parasites showed significant protection upon virulent parasite challenge, even after 25 weeks of immunization (in the absence of gene-deleted parasites), which was due to the presence of T_{CM} cells (Zaph et al. 2004). Similarly, mice immunized with *LdCen*$^{-/-}$ parasites, that do not survive beyond 5 weeks post-injection in mice, showed protection after 24 weeks post-immunization (Selvapandiyan et al. 2009) suggesting *LdCen*$^{-/-}$ parasites also could generate a memory response that can be recalled upon challenge as was shown for immunization with *L. major DHFR*$^{-/-}$ parasites (Zaph et al. 2004). Similarly, studies with *Ldp27*$^{-/-}$ showed that there was a strong recall of cellular immune response both during the persistence of attenuated parasites and also after the parasites were cleared from the animals, i.e., in the absence of persisting antigen. Adoptive transfer of T cells

harvested from *Ldp27*$^{-/-}$ post-immunized (20 weeks) mice to naïve mice showed protection against wild type *Leishmania* parasite infection, suggesting generation of memory T cells (Dey et al. 2013). Further studies are needed to analyze the mechanism and the type of memory T cells generated by the live attenuated parasites.

3. *Multifunctional T cells in live attenuated parasites*: Induction of multifunctional T cells is an important determinant of vaccine efficacy (Darrah et al. 2007). Most vaccine studies measure the frequency of IFNγ producing cells as the primary correlates of protection. However single immune parameters may not always be sufficient to predict protection. For example, it has been shown that high levels of pro-inflammatory cytokines such as IFNγ (Oliveira et al. 2011) and TNFα (Lessa et al. 2001) correlate with the development of human CL and MCL. Recently several studies have now reported that multifunctional T cells are good predictors of vaccine efficacy against some pathogens including *Leishmania* (Coler et al. 2007; Lindenstrom et al. 2009; Peters et al. 2012). In our studies with live attenuated parasite vaccine we also observed significant enhancement of multifunctional T cells against wild type *Leishmania* challenge that correlated with protection (Selvapandiyan et al. 2009; Dey et al. 2013). Taken together T cells that are multifunctional and simultaneously produce IFNγ, TNFα, and IL2 provide optimal effector function that leads to protection. Therefore, it is important to analyze multifunctionality of T cells to evaluate the efficacy of *Leishmania* vaccines.
4. *Parasite persistence and memory response*: Live attenuated parasites elicit memory response both in the presence and absence of parasite persistence and the antigen-specific cell-mediated immunity correlates with robust NO generation and humoral response (Selvapandiyan et al. 2009; Dey et al. 2013; Okwor et al. 2009). However, the observation that short-term immunized mice show a higher level of protection than long-term immunized mice (Selvapandiyan et al. 2009; Dey et al. 2013) suggests that with persistence there is a pool of effector cells that rapidly respond to infection whereas in the absence of parasite persistence when there is little antigen present there is a small pool of central memory cells which respond slowly to infection by differentiation into effector memory cells that eventually help in controlling the parasite burden. This suggested that persistence of parasite antigen and antigen-specific effector T cells are probably more effective than central memory cells alone. Similarly, effector memory cells at the mucosal sites can proliferate and can play roles functionally similar to central memory cells in mediating protection (Masopust et al. 2006). Further adoptive transfer experiments confirmed the generation of antigen-specific CD4 and CD8 T cells in immunized mice which confer protection during reinfection with wild type parasites (Zaph et al. 2004). So far, the strategies for targeted delivery of vaccines in combination with immunomodulatory molecules to enhance the magnitude of immune response and memory have been mostly empirical. Further studies are needed to analyze the type of T cells (potentially memory T cells) and their specific role in live attenuated parasite immunization.

Taken together the examples of genetically altered parasites described above provide opportunities for live attenuated vaccine candidates to be evaluated in preclinical and clinical conditions. However, various challenges remain before such vaccines become a reality.

5. *Live attenuated Leishmania donovani parasites as pan Leishmania vaccines*: Our previous studies with *LdCen*$^{-/-}$ and *Ldp27*$^{-/-}$ demonstrated that immunization with live attenuated parasites provides a significant protection against infection with homologous as well as heterologous species of *Leishmania* parasites (Selvapandiyan et al. 2009; Dey et al. 2013). For example, we found that immunization with these mutant parasites cross protected mice against challenge with *L. braziliensis* that causes MCL (Selvapandiyan et al. 2009) and against *L. mexicana* that causes cutaneous leishmaniasis (CL) (Dey et al. unpublished data). Immunization with *Ldp27*$^{-/-}$ conferred protection against *L. major*, *L. braziliensis*, or *L. mexicana* challenge, indicating that these mutant parasites are safe and effective vaccine candidates against VL, MCL, and CL (Dey et al. unpublished data). Recently we observed that *LdCen*$^{-/-}$ parasite immunization induces both humoral and cellular immune response in dogs (Fiuza et al. 2013). Importantly, immunity induced by *LdCen*$^{-/-}$ parasite is comparable to a licensed canine vaccine (Leishmune, a recombinant vaccine) available in Brazil and is protective against *L. infantum chagasi* challenge (Fiuza et al. unpublished data). Taken together, these studies strongly suggest that the *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ mutant parasite vaccines are safe and effective and can confer protection against *Leishmania* species in addition to *L. donovani* that are circulating in endemic areas.

Challenges for Genetically Modified Live Attenuated *Leishmania* Vaccines

Safety

1. *Stability of the attenuated parasites*: A major concern with live attenuated parasite vaccines is the risk of reversion to a virulent phenotype either during manufacturing or following immunization in humans. Hence biochemical or molecular markers are needed to assess the genetic and phenotypic traits of the attenuated parasites and to monitor long-term safety. To date there are no known defined markers of attenuation for genetically altered parasites. To that end, our laboratory is working to identify such biochemical or molecular markers. For example, to assess genes whose expression patterns are markers of attenuation, the *LdCen*$^{-/-}$ line was compared to wild type parasites by gene expression microarray (Srividya et al. 2007; Duncan et al. 2004). Two genes, one coding for the mitochondrial inner membrane protein (27 kDa protein) and another coding for putative argininosuccinate synthase (ASS), that normally express a higher RNA

level in the amastigote stage than in the promastigote stage of wild type cells, were found down-regulated in their RNA levels in *LdCen*$^{-/-}$ amastigote cells (Duncan et al. 2009). Northern blot analysis of p27 and ASS genes from *LdCen*$^{-/-}$ parasites recovered from mice after five weeks of infection had the same expression level as prior to infection in animals. Therefore these two genes could be used as markers of attenuation to monitor the safety of the *LdCen*$^{-/-}$ cell line as it is developed as a potential vaccine. Also further genetic evaluation of such live attenuated parasites is needed to monitor alterations in the parasite genome that could arise following long-term cultivation during manufacturing or vaccination in human volunteers in clinical studies. Such an evaluation is essential for evaluation of safety of the live attenuated parasites.

2. *Development of biomarkers of protection using bioinformatics tools*: In general, a major shortcoming in the vaccine development is the inadequate predictive power of the immune responses mounted in the host against the vaccines as previously discussed. For the live attenuated parasite vaccines, the primary barrier against widespread use remains the absence of markers of safety and efficacy. Recent studies involving human yellow fever vaccine and human influenza vaccine have elegantly demonstrated that applying systems biology approaches allows to uncover not only an unbiased set of markers associated with protection but also a mechanistic understanding of the immune protection (Nakaya et al. 2011; Querec et al. 2009). Obtaining such information via "systems biology" approaches in *Leishmania* vaccination would entail testing the gene expression profiles in isolated human PBMCs and ascertaining genes uniquely expressed upon infection with the genetically attenuated parasites. Such testing in PBMCs isolated from healed VL (HVL) cases and comparing them to PBMCs from VL cases might enhance the validity of gene markers, since it is expected that HVL cases might be resistant to reinfection and might induce an immune response consistent with a protective phenotype. Such systematic evaluation would allow understanding of how vaccine-induced immune responses are coordinately regulated in normal, infected, and individuals recovered from infection. This will provide information regarding correlates of protection as well as biomarkers of safety. Vaccine trials in leishmaniasis thus far have relied on the evaluation of a limited number of qualitative and quantitative features of effector adaptive immune responses. The failure to identify such predictors has been primarily a consequence of the lack of assays that can measure integrated immune responses in the vaccine trials (Trautmann and Sekaly 2011). Studies of the immune response in humans analyzed by systems biology approaches generate new hypotheses, new biomarkers of pathogenesis, and new signatures of protection that can be further validated in suitable animal models. The stages involving development of candidate vaccines should include mechanistic studies involving immune correlates of protection, route of administration, adjuvants, mode of challenge in mice and hamsters, and studies in outbred models such as dogs and monkeys (Fig. 1). Some of these studies are already completed in our laboratory (indicated with a black arrow in Fig. 1). New approaches to systematic analysis

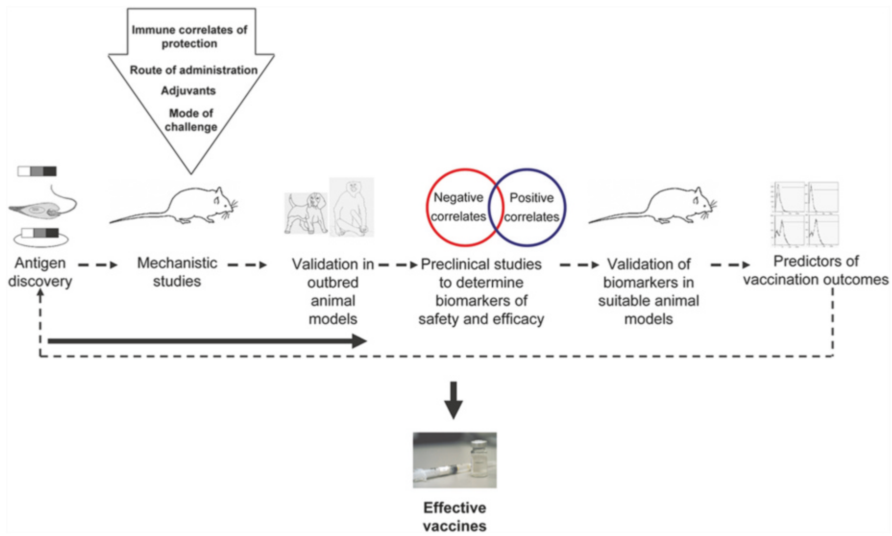

Fig. 1 The outline of activities involved in vaccine development is depicted in the schematic diagram

and discovery of markers of immune protection of vaccines are facilitated by recent advancements in computational tools. Such studies will allow for unbiased discovery of markers of safety and efficacy in human trials, which has been so far limited to cytokine profiles with the current vaccines.

3. *Manufacturing of genetically modified live attenuated vaccines*: Live attenuated parasite vaccines intended for immunization in clinical trials will need to be manufactured under strictly controlled conditions, i.e., good manufacturing practices (cGMP) avoiding ingredients that could be hazardous for human use. Since *Leishmania* parasites are auxotrophs for several key metabolites, bovine serum is normally added in culture, to support parasite growth. Addition of bovine serum may not be suitable for human use. Even though in most cases use of serum from BSE-negative animals is routine, there can be situations where it is not practical. Therefore, culture conditions for the genetically defined, attenuated parasites in serum-free medium (SFM) are needed. For example, in our studies *LdCen*$^{-/-}$, *Ldp27*$^{-/-}$, and *Ld1S2D* (the parent wild type strain of these genetically altered parasites) were adapted to growth in chemically defined medium (SFM). SFM supported the growth of all tested *L. donovani* parasites at rates comparable with those obtained with serum-supplemented M199 medium (Fig. 2). Moreover, growth in such medium did not affect phenotypic characteristics of the parasites. We did not observe any difference in growth of mutant parasites compared to its wild type counterpart (Fig. 2). Parasites remained viable up to 6 days in culture (Fig. 2). Taken together, these results demonstrate the feasibility of cultivating *L. donovani* promastigotes in SFM and provide an alternative to use of serum for growing such parasites in abundant quantities. Consideration should be given to

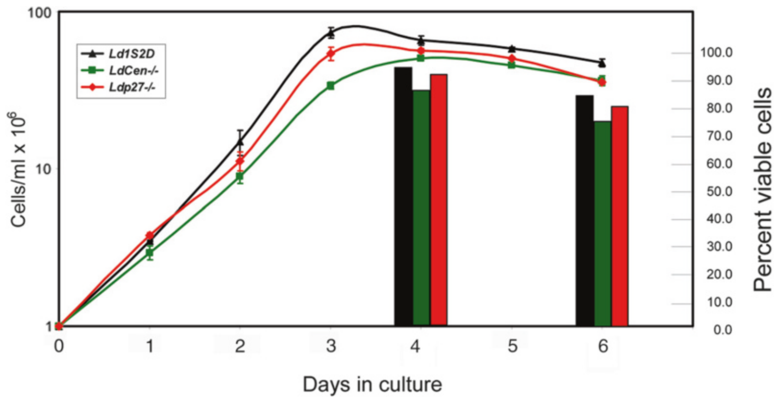

Fig. 2 Plots of the in vitro growth kinetics of *L. donovani* promastigotes grown in serum-free medium (SFM). Mean population densities were calculated from triplicate cultures represented by different colors on line graphs as indicated in the legend. The values represent mean ± SD. Adapted *Ldp27*$^{-/-}$, *LdCen*$^{-/-}$, and *Ld1S2D* (wild type) parasites were inoculated to a final concentration of 10^6 cells/mL in 5 mL fresh SFM in 25 cm^2 flasks. Twenty-microliter aliquots were taken daily and parasite density was determined using a Coulter Counter. Parasite viability was determined at days 4, 6, and 8, microscopically by counting the number of dead (D) and live (L) parasites and then calculating % viable using this formula: ([**L**/(**D** + **L**)] × 100) and is shown as bars with the same color coding as the growth curves

explore other conditions of parasite growth which are independent of materials derived from animal sources such as plant-derived materials.

4. *Environmental safety*: Another safety concern for genetically altered parasites to be used as vaccine candidates is whether such parasites persist in humans, are picked up by sand flies, are able to survive in the sand fly gut, and develop into infectious metacyclic cells. The alterations made to date have been optimized for disrupting survival of the parasites as amastigotes; yet allowing propagation as cultured promastigotes. Whether or not this will disrupt survival in sand flies cannot be predicted, but demonstration that the attenuated parasites do not survive or differentiate in the insect will be an important measure of safety against the potential for genetic recombination with the wild type parasites (Akopyants et al. 2009). Preliminary studies from our laboratory suggest that genetically modified parasites may not be able to survive in the sand fly gut (Dey et al. unpublished data).

Efficacy

1. *Route of administration*: Studies to date with genetically targeted live attenuated parasites have primarily delivered the vaccine parasites intravenously (Selvapandiyan et al. 2009; Silvestre et al. 2007; Zhang and Matlashewski 1997). If the live attenuated vaccines are to be developed for human use, more acceptable

routes of injection such as intramuscular, subcutaneous, or intradermal must be optimized. The intradermal infection route has been studied extensively for cutaneous *Leishmania* (Belkaid et al. 1998); visceralization of *L. donovani* has been demonstrated after intradermal injection (Kaur et al. 2008) and is being further investigated by us and others as a route of administration. Subcutaneous injection of *L. infantum chagasi*-attenuated parasites has been tested, but failed to protect (Streit et al. 2001), though the nature of the vaccine strain, not the route of injection, may be the proximate cause of protection failure (Streit et al. 2001). Efforts should be made to test the vaccine candidates using the feasible using modes of immunization that are feasible for humans.

2. *Role of sand fly salivary proteins in Leishmania vaccine immunogenicity*: *Leishmania* parasites are transmitted by the bites of the infected sand flies, and studies have established that saliva of sand flies has important role in the establishment of disease (Rogers et al. 2004; Monteiro et al. 2007). Saliva contains a variety of potent and pharmacologically active components that favorably change the bite site for smooth feeding (Charlab et al. 1999). Exposure to sand fly bites or salivary proteins results in strong cellular and humoral immunity to sand fly saliva (Oliveira et al. 2006, 2008; Collin et al. 2009; Kamhawi et al. 2000). Salivary protein components are potential targets for vaccine development to control *Leishmania* infection both in CL and VL. A salivary protein from *Lutzomyia longipalpis* (LJM19) confers significant protection against the fatal outcome of *L. infantum chagasi* infection in animal model (Gomes et al. 2008). Therefore, it is important to explore the role of sand fly salivary gland proteins in the immunogenicity of *Leishmania* vaccine candidates including genetically altered live attenuated parasites.
3. *Protection against natural mode of challenge*: The traditional challenge models for visceral leishmaniasis are intravenous injection in the BALB/c mouse and intracardiac injection of the hamster utilizing millions of stationary phase-cultured virulent promastigotes or spleen-derived amastigotes. The metacyclogenesis in vitro culture is only partial; so stationary phase promastigotes are an imperfect model of the natural infection, but ficoll gradient separation of metacyclics is a definite improvement (Selvapandiyan et al. 2009). The advantage of intravenous and intracardiac challenge models is that they are well characterized, the disease outcome in a naïve animal is documented, and comparison of a current vaccine under evaluation can be made to earlier vaccine candidates. However these challenge models cannot be used in clinical trials. On the other hand low dose needle intradermal injection challenge model is closer to the natural infection; however, the uncertainties of visceralization and the long delay to disease symptoms may pose serious limitations, especially in light of recent demonstration that vaccines that protect against needle challenge do not protect against infected sand fly bites (Peters et al. 2009). The efficacy of a *Leishmania* vaccine of any type is finally judged by its ability to protect recipients from natural infection which is through the sand fly bite. Sand fly bites deliver approximately 1,000 metacyclic promastigotes in the context of sand fly saliva, which is a potent modulator of the infection process (Oliveira et al. 2008; Gomes et al. 2008). Therefore it is important to test the efficacy of *Leishmania* vaccines

including genetically altered live attenuated candidates against sand fly infection in preclinical models before testing them in clinical trials. Towards that end we and others have initiated studies to develop visceralization animal models (Aslan et al. 2013) and the genetically altered live attenuated parasites are being tested against infected sand fly challenge.

Efficacy of Live Attenuated Vaccine in Different Age Groups

High morbidity and mortality rates associated with disease in the elderly are common across a spectrum of pathogens. During aging, the immune system starts slowly declining in its efficiency to fight off infectious agents which in turn results in severe symptoms and prolonged duration of the infection (Singh et al. 2007). Previously, studies have shown that accumulation of functional T regulatory (Treg) cells in aged host promotes reactivation of infectious diseases like *Leishmania* infection (Lages et al. 2008). However there are other reports which suggest that aged BALB/c mice develop resistance to *Leishmania* infection by developing Th1 T cell response, i.e., healing response (Ehrchen et al. 2004). Therefore studies are needed to develop correlates of immune protection and to evaluate the efficacy of live attenuated vaccine candidates in aged populations.

Malnutrition

The protein energy malnutrition (PEM) and visceral leishmaniasis are growing problems of public health in VL endemic regions. PEM is one of the major decisive factors of fatal outcome of the VL (Malafaia 2009). The nutritional status of individuals infected with *Leishmania* has a significant role in the clinical outcome of VL (Pearson et al. 1992; Gomes et al. 2007). However, the impact of malnutrition specifically on immune response against *Leishmania* infection is not currently well understood. Malnutrition impairs elements of adaptive and innate immunity which would be important for protection against *Leishmania* infection (Anstead et al. 2001; Maciel et al. 2008). Vaccine efficacy depends on the ability of individuals to mount an appropriate immune response. Understanding of the immune mechanism of live attenuated parasites in malnourished subjects should provide clues about the efficacy of such vaccine candidates.

Conclusion

Leishmaniasis is one of the major public health problems worldwide and there are no effective preventions available including vaccines. With the advent of advancements in several “omics” technologies, there is rapid progress in our understanding

of the molecular nature of potential *Leishmania* vaccine candidates. However most of the candidates which include recombinant protein, DNA vaccines, or whole lysates of *Leishmania* failed to protect humans in endemic areas. On the other hand genetically modified live attenuated vaccines offer several advantages such as they mimic natural infection without causing any disease and provide whole repertoire of parasite antigens which can persist to induce memory response. In addition, since live attenuated parasites have the capability to present antigen profile of the whole parasite, they also have the potential to be pan *Leishmania* vaccines as was demonstrated in our animal studies. Finally, for the genetically altered live attenuated parasites to be vaccine candidates that can be used in humans, it is essential, using bioinformatics tools, to develop markers that can assess their safety and immune correlates of protection.

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References

Akopyants NS et al (2009) Demonstration of genetic exchange during cyclical development of *Leishmania* in the sand fly vector. Science 324(5924):265–268

Alexander J, Bryson K (2005) T helper (h)1/Th2 and *Leishmania*: paradox rather than paradigm. Immunol Lett 99(1):17–23

Alexander J, Coombs GH, Mottram JC (1998) *Leishmania mexicana* cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. J Immunol 161(12):6794–6801

Alexander J et al (2000) Endogenous IL-4 is necessary for effective drug therapy against visceral leishmaniasis. Eur J Immunol 30(10):2935–2943

Amaral VF et al (2002) 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 97(7):1041–1048

Anderson JM et al (2006) Comparative salivary gland transcriptomics of sandfly vectors of visceral leishmaniasis. BMC Genomics 7:52

Anstead GM et al (2001) Malnutrition alters the innate immune response and increases early visceralization following *Leishmania donovani* infection. Infect Immun 69(8):4709–4718

Armijos RX et al (1998) Field trial of a vaccine against New World cutaneous leishmaniasis in an at-risk child population: safety, immunogenicity, and efficacy during the first 12 months of follow-up. J Infect Dis 177(5):1352–1357

Armijos RX et al (2004) Safety, immunogenicity, and efficacy of an autoclaved *Leishmania amazonensis* vaccine plus BCG adjuvant against New World cutaneous leishmaniasis. Vaccine 22(9–10):1320–1326

Aslan H et al (2013) A new model of progressive visceral Leishmaniasis in Hamsters by natural transmission via bites of vector sand flies. J Infect Dis 207(8):1328–1338

Bacon KM et al (2013) The potential economic value of a cutaneous leishmaniasis vaccine in seven endemic countries in the Americas. Vaccine 31(3):480–486

Bahar K et al (1996) Comparative safety and immunogenicity trial of two killed *Leishmania major* vaccines with or without BCG in human volunteers. Clin Dermatol 14(5):489–495

Banerjee A, De M, Ali N (2008) Complete cure of experimental visceral leishmaniasis with amphotericin B in stearylamine-bearing cationic liposomes involves down-regulation of IL-10 and favorable T cell responses. J Immunol 181(2):1386–1398

Bankoti R et al (2012) Marginal zone B cells regulate antigen-specific T cell responses during infection. J Immunol 188(8):3961–3971

Basu R et al (2005) 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 174(11):7160–7171

Bates PA (2006) Housekeeping by *Leishmania*. Trends Parasitol 22(10):447–448

Bates PA (2007) Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol 37(10):1097–1106

Belkaid Y et al (1998) 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 188(10):1941–1953

Bhandari V et al (2012) Drug susceptibility in *Leishmania* isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. PLoS Negl Trop Dis 6(5):e1657

Bhattacharyya S et al (2001) Immunomodulatory role of interleukin-10 in visceral leishmaniasis: defective activation of protein kinase C-mediated signal transduction events. Infect Immun 69(3):1499–1507

Campos-Neto A et al (2001) Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. Infect Immun 69(6):4103–4108

Chakravarty J et al (2011) 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 29(19):3531–3537

Charlab R et al (1999) Toward an understanding of the biochemical and pharmacological complexity of the saliva of a hematophagous sand fly Lutzomyia longipalpis. Proc Natl Acad Sci USA 96(26):15155–15160

Coler RN et al (2007) Leish-111f, a recombinant polyprotein vaccine that protects against visceral Leishmaniasis by elicitation of CD4+ T cells. Infect Immun 75(9):4648–4654

Collin N et al (2009) Sand fly salivary proteins induce strong cellular immunity in a natural reservoir of visceral leishmaniasis with adverse consequences for *Leishmania*. PLoS Pathog 5(5):e1000441

Convit J et al (2003) Immunotherapy of American cutaneous leishmaniasis in Venezuela during the period 1990–99. Trans R Soc Trop Med Hyg 97(4):469–472

Croft SL, Sundar S, Fairlamb AH (2006) Drug resistance in leishmaniasis. Clin Microbiol Rev 19(1):111–126

Cua DJ, Stohlman SA (1997) In vivo effects of T helper cell type 2 cytokines on macrophage antigen-presenting cell induction of T helper subsets. J Immunol 159(12):5834–5840

Cummings HE, Tuladhar R, Satoskar AR (2010) Cytokines and their STATs in cutaneous and visceral leishmaniasis. J Biomed Biotechnol 2010:294389

Darrah PA et al (2007) Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. Nat Med 13(7):843–850

Davoudi N et al (2005) Development of a recombinant *Leishmania major* strain sensitive to ganciclovir and 5-fluorocytosine for use as a live vaccine challenge in clinical trials. Vaccine 23(9):1170–1177

Desjeux P (2004) Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis 27(5):305–318

Dey R et al (2007a) Induction of host protective Th1 immune response by chemokines in *Leishmania donovani*-infected BALB/c mice. Scand J Immunol 66(6):671–683
Dey R et al (2007b) Functional paradox in host-pathogen interaction dictates the fate of parasites. Future Microbiol 2(4):425–437
Dey R et al (2010) Characterization of a *Leishmania* stage-specific mitochondrial membrane protein that enhances the activity of cytochrome c oxidase and its role in virulence. Mol Microbiol 77(2):399–414
Dey R et al (2013) Live attenuated *Leishmania donovani* p27 gene knockout parasites are nonpathogenic and elicit long-term protective immunity in BALB/c mice. J Immunol 190(5):2138–2149
Dowlati Y et al (1996) Stepwise safety trial of a killed *Leishmania* vaccine in Iran. Clin Dermatol 14(5):497–502
Duncan RC et al (2004) The application of gene expression microarray technology to kinetoplastid research. Curr Mol Med 4(6):611–621
Duncan R et al (2009) Biomarkers of attenuation in the *Leishmania donovani* centrin gene deleted cell line-requirements for safety in a live vaccine candidate. Open Parasitology 3:32–41
Duthie MS et al (2012) The development and clinical evaluation of second-generation leishmaniasis vaccines. Vaccine 30(2):134–141
Ehrchen J et al (2004) Senescent BALB/c mice are able to develop resistance to *Leishmania major* infection. Infect Immun 72(9):5106–5114
Engwerda CR et al (1998) Neutralization of IL-12 demonstrates the existence of discrete organ-specific phases in the control of *Leishmania donovani*. Eur J Immunol 28(2):669–680
Fiorentino DF, Bond MW, Mosmann TR (1989) Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 170(6):2081–2095
Fiuza JA et al (2013) Induction of immunogenicity by live attenuated *Leishmania donovani* centrin deleted parasites in dogs. Vaccine 31(14):1785–1792
Gannavaram S et al (2012) 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 86(1):187–198
Ghalib HW et al (1995) IL-12 enhances Th1-type responses in human *Leishmania donovani* infections. J Immunol 154(9):4623–4629
Ghosh A, Zhang WW, Matlashewski G (2001) Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against *Leishmania donovani* infections. Vaccine 20(1–2):59–66
Gicheru MM et al (2001) Vervet monkeys vaccinated with killed *Leishmania major* parasites and interleukin-12 develop a type 1 immune response but are not protected against challenge infection. Infect Immun 69(1):245–251
Gomes CM et al (2007) Correlation between the components of the insulin-like growth factor I system, nutritional status and visceral leishmaniasis. Trans R Soc Trop Med Hyg 101(7):660–667
Gomes R et al (2008) 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 USA 105(22):7845–7850
Gomes R et al (2012) KSAC, a defined *Leishmania antigen*, plus adjuvant protects against the virulence of *L. major* transmitted by its natural vector Phlebotomus duboscqi. PLoS Negl Trop Dis 6(4):e1610
Goto Y et al (2009) *Leishmania infantum* sterol 24-c-methyltransferase formulated with MPL-SE induces cross-protection against *L. major* infection. Vaccine 27(21):2884–2890
Harhay MO et al (2011) Who is a typical patient with visceral leishmaniasis? Characterizing the demographic and nutritional profile of patients in Brazil, East Africa, and South Asia. Am J Trop Med Hyg 84(4):543–550
Harrington LE, Mangan PR, Weaver CT (2006) Expanding the effector CD4 T-cell repertoire: the Th17 lineage. Curr Opin Immunol 18(3):349–356

Herwaldt BL (1999) Leishmaniasis. Lancet 354(9185):1191–1199

Holaday BJ et al (1993) Potential role for interleukin-10 in the immunosuppression associated with kala azar. J Clin Invest 92(6):2626–2632

Kaech SM, Wherry EJ, Ahmed R (2002) Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol 2(4):251–262

Kamhawi S et al (2000) Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies. Science 290(5495):1351–1354

Kaur S et al (2008) Effect of dose and route of inoculation on the generation of CD4+ Th1/Th2 type of immune response in murine visceral leishmaniasis. Parasitol Res 103(6):1413–1419

Kedzierski L (2010) Leishmaniasis vaccine: where are we today? J Glob Infect Dis 2(2):177–185

Kedzierski L et al (2008) 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 38(11):3090–3100

Kenney RT et al (1998) Splenic cytokine responses in Indian kala-azar before and after treatment. J Infect Dis 177(3):815–818

Lages CS et al (2008) Functional regulatory T cells accumulate in aged hosts and promote chronic infectious disease reactivation. J Immunol 181(3):1835–1848

Lehn M et al (1989) IL-4 inhibits H_2O_2 production and antileishmanial capacity of human cultured monocytes mediated by IFN-gamma. J Immunol 143(9):3020–3024

Lessa HA et al (2001) Successful treatment of refractory mucosal leishmaniasis with pentoxifylline plus antimony. Am J Trop Med Hyg 65(2):87–89

Liew FY, Li Y, Millott S (1990) Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of *Leishmania major* through the induction of nitric oxide. J Immunol 145(12):4306–4310

Lin Y, Slight SR, Khader SA (2010) Th17 cytokines and vaccine-induced immunity. Semin Immunopathol 32(1):79–90

Lindenstrom T et al (2009) Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. J Immunol 182(12):8047–8055

Liu D et al (2013) Deficiency of *Leishmania* phosphoglycans influences the magnitude but does not affect the quality of secondary (memory) anti-*Leishmania* immunity. PLoS One 8(6):e66058

Maciel BL et al (2008) Association of nutritional status with the response to infection with *Leishmania chagasi*. Am J Trop Med Hyg 79(4):591–598

Mahajan B et al (2008) Centrins, cell cycle regulation proteins in human malaria parasite *Plasmodium falciparum*. J Biol Chem 283(46):31871–31883

Malafaia G (2009) Protein-energy malnutrition as a risk factor for visceral leishmaniasis: a review. Parasite Immunol 31(10):587–596

Masopust D et al (2006) Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. J Immunol 177(2):831–839

Melby PC et al (2001) *Leishmania donovani* p36(LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. Infect Immun 69(8):4719–4725

Misra A et al (2001) Successful vaccination against *Leishmania donovani* infection in Indian langur using alum-precipitated autoclaved *Leishmania major* with BCG. Vaccine 19(25–26):3485–3492

Mizbani A et al (2009) Recombinant *Leishmania tarentolae* expressing the A2 virulence gene as a novel candidate vaccine against visceral leishmaniasis. Vaccine 28(1):53–62

Monteiro MC et al (2007) Effect of Lutzomyia longipalpis salivary gland extracts on leukocyte migration induced by *Leishmania major*. Am J Trop Med Hyg 76(1):88–94

Moore KW et al (1990) Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. Science 248(4960):1230–1234

Moore JW et al (2012) B cell: T cell interactions occur within hepatic granulomas during experimental visceral leishmaniasis. PLoS One 7(3):e34143

Mosmann TR, Moore KW (1991) The role of IL-10 in crossregulation of TH1 and TH2 responses. Immunol Today 12(3):A49–A53

Mougneau E et al (1995) Expression cloning of a protective *Leishmania antigen*. Science 268(5210):563–566
Murphy ML et al (2001) IL-10 mediates susceptibility to *Leishmania donovani* infection. Eur J Immunol 31(10):2848–2856
Murray HW (1997) Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis. J Infect Dis 175(6):1477–1479
Murray HW et al (2000) Visceral leishmaniasis in mice devoid of tumor necrosis factor and response to treatment. Infect Immun 68(11):6289–6293
Murray HW et al (2003) Determinants of response to interleukin-10 receptor blockade immunotherapy in experimental visceral leishmaniasis. J Infect Dis 188(3):458–464
Murray HW et al (2005) Advances in leishmaniasis. Lancet 366(9496):1561–1577
Muyombwe A et al (1997) Selective killing of *Leishmania* amastigotes expressing a thymidine kinase suicide gene. Exp Parasitol 85(1):35–42
Nakaya HI et al (2011) Systems biology of vaccination for seasonal influenza in humans. Nat Immunol 12(8):786–795
Nylen S, Sacks D (2007) Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol 28(9):378–384
Nylen S et al (2007) Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. J Exp Med 204(4):805–817
Okwor I et al (2009) Inoculation of killed *Leishmania major* into immune mice rapidly disrupts immunity to a secondary challenge via IL-10-mediated process. Proc Natl Acad Sci USA 106(33):13951–13956
Oliveira F et al (2006) From transcriptome to immunome: identification of DTH inducing proteins from a Phlebotomus ariasi salivary gland cDNA library. Vaccine 24(3):374–390
Oliveira F et al (2008) Immunity to distinct sand fly salivary proteins primes the anti-*Leishmania* immune response towards protection or exacerbation of disease. PLoS Negl Trop Dis 2(4):e226
Oliveira F et al (2009) Sand flies, *Leishmania*, and transcriptome-borne solutions. Parasitol Int 58(1):1–5
Oliveira F et al (2011) Lesion size correlates with *Leishmania* antigen-stimulated TNF-levels in human cutaneous leishmaniasis. Am J Trop Med Hyg 85(1):70–73
Papadopoulou B et al (2002) Reduced infectivity of a *Leishmania donovani* biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. Infect Immun 70(1):62–68
Pearson RD et al (1992) Visceral leishmaniasis: a model for infection-induced cachexia. Am J Trop Med Hyg 47(1 Pt 2):8–15
Peters NC et al (2008) In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science 321(5891):970–974
Peters NC et al (2009) Vector transmission of *Leishmania* abrogates vaccine-induced protective immunity. PLoS Pathog 5(6):e1000484
Peters NC et al (2012) Evaluation of recombinant *Leishmania* polyprotein plus glucopyranosyl lipid A stable emulsion vaccines against sand fly-transmitted *Leishmania major* in C57BL/6 mice. J Immunol 189(10):4832–4841
Pitta MG et al (2009) IL-17 and IL-22 are associated with protection against human kala azar caused by *Leishmania donovani*. J Clin Invest 119(8):2379–2387
Polley R et al (2006) Adoptive immunotherapy against experimental visceral leishmaniasis with CD8+ T cells requires the presence of cognate antigen. Infect Immun 74(1):773–776
Querec TD et al (2009) Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. Nat Immunol 10(1):116–125
Rafati S et al (2005) 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 23(28):3716–3725
Rafati S, Zahedifard F, Nazgouee F (2006) Prime-boost vaccination using cysteine proteinases type I and II of *Leishmania infantum* confers protective immunity in murine visceral leishmaniasis. Vaccine 24(12):2169–2175

Rogers ME et al (2004) Transmission of cutaneous leishmaniasis by sand flies is enhanced by regurgitation of fPPG. Nature 430(6998):463–467

Ruiz JH, Becker I (2007) CD8 cytotoxic T cells in cutaneous leishmaniasis. Parasite Immunol 29(12):671–678

Sacks D, Kamhawi S (2001) Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. Annu Rev Microbiol 55:453–483

Sacks DL et al (1984) Cutaneous leishmaniasis in anti-IgM-treated mice: enhanced resistance due to functional depletion of a B cell-dependent T cell involved in the suppressor pathway. J Immunol 132(4):2072–2077

Salisbury JL (1995) Centrin, centrosomes, and mitotic spindle poles. Curr Opin Cell Biol 7(1):39–45

Saravia NG et al (2006) Pathogenicity and protective immunogenicity of cysteine proteinase-deficient mutants of *Leishmania mexicana* in non-murine models. Vaccine 24(19):4247–4259

Scott P (2003) Development and regulation of cell-mediated immunity in experimental leishmaniasis. Immunol Res 27(2–3):489–498

Scott P, Natovitz P, Sher A (1986) B lymphocytes are required for the generation of T cells that mediate healing of cutaneous leishmaniasis. J Immunol 137(3):1017–1021

Selvapandiyan A et al (2001) Expression of a mutant form of *Leishmania donovani* centrin reduces the growth of the parasite. J Biol Chem 276(46):43253–43261

Selvapandiyan A et al (2004) Centrin gene disruption impairs stage-specific basal body duplication and cell cycle progression in *Leishmania*. J Biol Chem 279(24):25703–25710

Selvapandiyan A et al (2006) Genetically modified live attenuated parasites as vaccines for leishmaniasis. Indian J Med Res 123(3):455–466

Selvapandiyan A et al (2007) Centrin1 is required for organelle segregation and cytokinesis in *Trypanosoma brucei*. Mol Biol Cell 18:3290–3301

Selvapandiyan A et al (2009) Intracellular replication-deficient *Leishmania donovani* induces long lasting protective immunity against visceral leishmaniasis. J Immunol 183(3):1813–1820

Selvapandiyan A et al (2012) Immunity to visceral leishmaniasis using genetically defined live-attenuated parasites. J Trop Med 2012:631460

Silvestre R et al (2007) SIR2-deficient *Leishmania infantum* induces a defined IFN-gamma/IL-10 pattern that correlates with protection. J Immunol 179(5):3161–3170

Singh N et al (2007) Age-influenced population kinetics and immunological responses of *Leishmania donovani* in hamsters. Parasitol Res 101(4):919–924

Sjolander A et al (1998) Vaccination with recombinant parasite surface antigen 2 from *Leishmania major* induces a Th1 type of immune response but does not protect against infection. Vaccine 16(20):2077–2084

Skeiky YA et al (2002) Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. Vaccine 20(27–28):3292–3303

Soong L et al (1995) *Leishmania* pifanoi amastigote antigens protect mice against cutaneous leishmaniasis. Infect Immun 63(9):3559–3566

Spath GF et al (2003) Persistence without pathology in phosphoglycan-deficient *Leishmania major*. Science 301(5637):1241–1243

Spath GF et al (2004) 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 72(6):3622–3627

Squires KE et al (1989) Experimental visceral leishmaniasis: role of endogenous IFN-gamma in host defense and tissue granulomatous response. J Immunol 143(12):4244–4249

Srivastava P et al (2011) Unusual case of resistance to amphotericin B in visceral leishmaniasis in a region in India where leishmaniasis is not endemic. J Clin Microbiol 49(8):3088–3091

Srividya G et al (2007) Transcriptome analysis during the process of in vitro differentiation of *Leishmania donovani* using genomic microarrays. Parasitology 134(Pt 11):1527–1539

Stager S, Smith DF, Kaye PM (2000) Immunization with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. J Immunol 165(12):7064–7071

Stern JJ et al (1988) Role of L3T4+ and LyT-2+ cells in experimental visceral leishmaniasis. J Immunol 140(11):3971–3977

Streit JA et al (2001) Protective immunity against the protozoan *Leishmania chagasi* is induced by subclinical cutaneous infection with virulent but not avirulent organisms. J Immunol 166(3):1921–1929

Sundar S, Chatterjee M (2006) Visceral leishmaniasis—current therapeutic modalities. Indian J Med Res 123(3):345–352

Tewary P et al (2005) A heterologous prime-boost vaccination regimen using ORFF DNA and recombinant ORFF protein confers protective immunity against experimental visceral leishmaniasis. J Infect Dis 191(12):2130–2137

Titus RG et al (1995) Development of a safe live *Leishmania* vaccine line by gene replacement. Proc Natl Acad Sci USA 92(22):10267–10271

Trautmann L, Sekaly RP (2011) Solving vaccine mysteries: a systems biology perspective. Nat Immunol 12(8):729–731

Tumang MC et al (1994) Role and effect of TNF-alpha in experimental visceral leishmaniasis. J Immunol 153(2):768–775

Uzonna JE et al (2004) Vaccination with phosphoglycan-deficient *Leishmania major* protects highly susceptible mice from virulent challenge without inducing a strong Th1 response. J Immunol 172(6):3793–3797

van Zandbergen G et al (2004) Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. J Immunol 173(11):6521–6525

Vouldoukis I et al (1997) Interleukin-10 and interleukin-4 inhibit intracellular killing of *Leishmania infantum* and *Leishmania major* by human macrophages by decreasing nitric oxide generation. Eur J Immunol 27(4):860–865

Wanasen N, Xin L, Soong L (2008) Pathogenic role of B cells and antibodies in murine *Leishmania amazonensis* infection. Int J Parasitol 38(3–4):417–429

Zaph C et al (2004) Central memory T cells mediate long-term immunity to *Leishmania major* in the absence of persistent parasites. Nat Med 10(10):1104–1110

Zhang WW, Matlashewski G (1997) Loss of virulence in *Leishmania donovani* deficient in an amastigote-specific protein, A2. Proc Natl Acad Sci USA 94(16):8807–8811

Zhang WW, Matlashewski G (2001) Characterization of the A2-A2rel gene cluster in *Leishmania donovani*: involvement of A2 in visceralization during infection. Mol Microbiol 39(4):935–948

Reservoir Control Strategies for Leishmaniasis: Past, Present, and Future

Christine A. Petersen and Kevin J. Esch

Abstract Visceral leishmaniasis (VL) caused by *Leishmania* spp. is zoonotic. In many regions dogs serve as the predominant domestic reservoir of disease. This reservoir status has led to multiple efforts to control disease via reservoir control. Historically, public health-oriented strategies have focused on dog culling. Canine culling was established in Palestine in the 1940s and both China and former central republics of the Soviet Union in the 1950s. In these instances, with ample public health infrastructure funded by a centralized state, along with ability to use widespread spraying of DDT, VL transmission was halted but disease was not eradicated in any instance. Canine culling continues to be the predominant policy for VL control in Brazil, despite limited effectiveness. In well-developed countries of Europe with long average life expectancy and overall good health care, education, and nutrition, VL is predominantly found in immunocompromised patients. This is in spite of concurrently high levels of canine VL (cVL) in the same geographic locale. Similar trends are beginning to be seen in areas of rapid economic advancement within Brazil, demonstrating the importance of general health and environmental conditions in prevention of VL. As palatability for widespread canine culling wanes within areas endemic for cVL, combined alternate approaches including topical insecticides, reproductive control, and vaccination should be strongly considered.

Keywords Canine • Culling • Reproductive control • Topical insecticides • *Leishmania* • Zoonotic

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Introduction

It has been projected that over 2.5 million dogs are infected with *Leishmania infatum* in Europe (Moreno and Alvar 2002). Many of these dogs become symptomatic and are treated for their disease. Despite this, and with evidence for additional reservoirs (Jimenez et al. 2013), cases of human visceral leishmaniasis (hVL) are uncommon and found predominantly in immunocompromised patients (Alvar et al. 1997; Molina et al. 2003). *L. infantum* is also the causative agent of visceral leishmaniasis (VL) in Brazil, where it has developed an urban endemic cycle due to both the dense population of domestic dogs and acclimation of the vector species *Lutzomyia longipalpis* to the peri-urban environment (Werneck 2008; Romero and Boelaert 2010). Cases of hVL in Brazil have remained relatively constant since the year 2000 with an incidence of approximately 2.5–3 cases per 100,000 people (Barreto et al. 2011). Although there have been recent surges in incidence in southern and western states, 60 % of hVL cases are reported in northeastern states of Brazil (Werneck 2008). Within these states are hyperendemic foci with much higher incidence (Romero and Boelaert 2010), predominantly in areas of lower education and overall health, demonstrated to be risk factors of infection (Esch et al. 2012). Domestic dogs are considered the predominant reservoir of *L. infantum* in hyperendemic foci with canine seroprevalence between 8 and 40 % (Queiroz et al. 2009; Tasca et al. 2009; Nunes et al. 2010; Romero and Boelaert 2010). Household presence of *L. infantum*-infected dogs in Brazil is a major risk factor for human infection. Differences in the rate of canine to human *Leishmania* transmission between continents may be closing due to socioeconomic gains being made in Brazil, with potential VL control policy changes to follow (Dantas-Torres et al. 2012).

Historical Approaches to Reservoir Control

Elimination of dogs to control hVL has been conducted in China, republics of the former Soviet Union, and Palestine (Costa 2011; Hamarsheh et al. 2012). In China VL was endemic within lowland areas and access to treatment was minimal (Costa 2011). Due to limited access to hVL treatment, during the 1950s canine culling methods were strongly supported by the state for control of zoonoses, with vigorous efforts to eliminate all dogs that had any evidence of illness, treat human patients, and to spray dichlorodiphenyltrichloroethane (DDT) to eliminate any potential infected vector species. Not surprisingly these efforts did interrupt transmission in areas of anthropozoonotic transmission and by the 1970s hVL was relatively rare. In areas of zoonotic transmission disease transmission was also halted, but ascribing specific success to the culling effort versus human treatment and environmental spraying is difficult. An outbreak of VL occurred in 2008, suggesting that these robust efforts as a whole prevented disease transmission temporarily, but did not provide disease eradication.

Systematic canine culling of all dogs that appeared diseased has occurred in Palestine for close to 70 years. In a recent study within the Palestinian territories, intensive culling of any diseased dog made discovery of dogs symptomatic with VL impossible (Hamarsheh et al. 2012). Cases of hVL have been low, but still occur. Asymptomatic-infected dogs certainly could be serving as a reservoir in this area (Hamarsheh et al. 2012), as well as other potential reservoirs (Amro et al. 2009; Talmi-Frank et al. 2010). VL within the former Soviet Union was predominantly an urban disease, found within capital cities including Tbilisi and Samarkand. Control programs including mass DDT spraying within central republics of the former Soviet Union were also implemented with strong support from the centralized government. These programs reduced VL incidence dramatically with exception of the Kyzlorda oblast where there was a large, *L. infantum*-infected population of coyotes. The presence of this significant sylvatic reservoir population, willing to range into peri-domestic areas, facilitated transmission (Costa 2011; Kovalenko et al. 2011). Once the centralized public health infrastructure was eliminated, hVL cases within these areas have flourished (Kovalenko et al. 2011).

Current Approaches to Reservoir VL Control

Treatment, and to a lesser extent vaccination of dogs for VL, is common practice throughout Europe. Treatment results in more dogs surviving infection, but with a high rate of eventual recrudescence, and potential for transmission (Ortranto and Dantes-Torres 2010, 2013). Presence of municipal animal shelters and kennels where unowned dogs are maintained for their life with exposure to vector species and often inadequate protection against VL presents a barrier to control of reservoir VL in these areas (Otranto et al. 2013). Despite a large population of infected dogs in Europe, there is a low incidence of hVL, predominantly in HIV-positive or otherwise immunocompromised individuals (Alvar et al. 2012). Current public health policy in Brazil is aimed at early recognition and treatment of human cases, and surveillance and removal of seropositive dogs (Fig. 1) (Oliveira et al. 2008). Euthanasia of positive dogs is considered to have limited efficacy for the following reasons: (1) significant delays between testing and removal of seropositive dogs, (2) a constant influx of naïve animals into the dog population with little reproductive management, (3) vertical transmission between dam and pups as a means of maintaining canine VL (cVL) within the canine population (Boggiatto et al. 2011), and (4) limitations to current serologic testing in sensitivity and specificity to detect all dogs that could be acting as the disease reservoir. These factors increase the number of infectious dogs, creating a constant influx of susceptible animals and a means of maintaining infection even in the absence of a robust vector. As a result, improved methods of VL management, both in terms of efficacy and cost, are needed to address the incidence of VL in humans and dogs in Brazil.

Risk factors for ZVL in large part relate directly to the differences in incidence of human disease in Brazil and Europe. Numerous studies have demonstrated risk

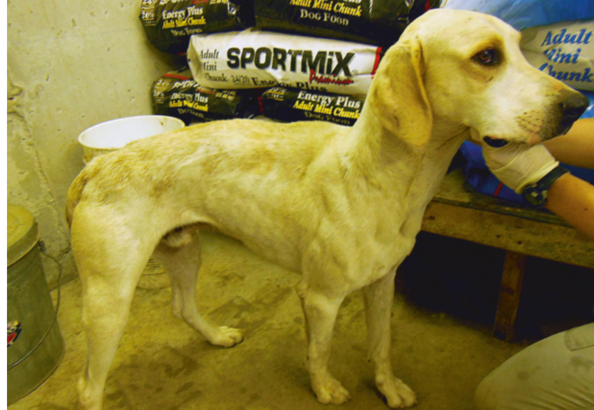

Fig. 1 Dog with seropositive VL culled based on disease status. Historically in China, Russia, and Pakistan widespread culling of dogs along with other methods has been used to control VL transmission. These practices were more recently instated in Brazil with limited efficacy

for ZVL based on the presence of dogs within the household, the housing type (comparing mud-walled housing to concrete or brick housing), education level, income, and disease knowledge (Coura-Vital et al. 2011; de Almeida et al. 2011; Harhay et al. 2011a, b; Lima et al. 2012). Many of the same factors associated with human ZVL also apply to owner characteristics associated with risk for cVL (Coura-Vital et al. 2011; Esch et al. 2012; Lima et al. 2012). Vertical transmission has been characterized in the dog (Pagliano et al. 2005; Boggiatto et al. 2011). In some instances, this means of transmission is sufficient to maintain a population of infected dogs in the absence of vector species (Boggiatto et al. 2011), indicating that reproductive control of dogs is also very important to cVL control. The risks for dogs also include coinfection with other parasites, rickettsial diseases, heartworm disease, or immune suppression (Fig. 2) (Aresu et al. 2007; Cortese et al. 2011). This suggests a complex evolutionary interplay of parasite and host factors, which are likely associated with disease susceptibility.

Review and Consideration of Canine Treatment with Topical Insecticides to Prevent VL

There is now a considerable literature investigating myriad delivery methods of topical insecticide treatment to dogs to prevent cVL and hVL (Mutinga et al. 1993; Gavgani et al. 2002; Alexander et al. 2009; Courtenay et al. 2009; Otranto et al. 2010, 2013). As compared to environmental spraying, these topical treatments have a much more targeted focus, allowing minimal ecological damage and decreased cost to implement (Otranto et al. 2013). Use of synthetic pyrethroids, which act via specific toxic and neurological effect on sand flies, prevents sand fly feeding

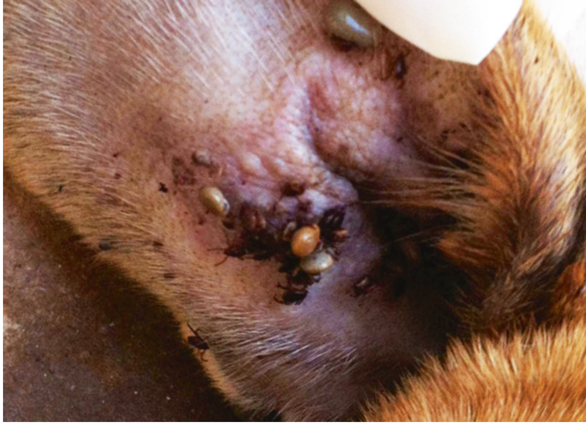

Fig. 2 Multiple comorbidities predispose to infection with *Leishmania infantum* in dogs. Pinna of an ear of a euthanized dog in Brazil infested with various *Rhipicephalus sanguineus* life stages. Tick-borne disease, including Ehrlichiosis, Babesiosis, Rocky Mountain spotted fever and others, is common in outdoor dogs and often predicates clinical VL. Other comorbidities include malnutrition, helminth parasitism, pregnancy, and viral infection

(Mencke et al. 2003; Otranto et al. 2007). Based on promising studies in multiple experimental settings, larger field trials have ensued (Gavgani et al. 2002; Otranto et al. 2013; Ribeiro et al. 2013). These trials for the most part have only emphasized that this means of VL prevention can be effective, although more large scale studies are needed to validate the human public health impact.

Outlook for VL Prevention Via Canine Vaccination

Much focus and effort has been poured into developing a vaccine to prevent transmission of VL from the dog reservoir to humans (Gradoni 2001; Vanloubbeeck and Jones 2004; Tabbara 2006; Paape and Aebischer 2011; Duthie et al. 2012; Raman et al. 2012). Multiple efforts at inactivated parasite vaccines failed, refocusing efforts to recombinant protein antigen constructs or secreted–excreted products of the parasite (da Silva et al. 2000; Borja-Cabrera et al. 2002; Trigo et al. 2010). There are now two vaccines available commercially in Brazil (Palatnik-de-Sousa et al. 2009; Martins et al. 2013), and a handful of vaccines are now licensed for use in dogs in Europe based on these technologies (Lemesre et al. 2005). Several of these require robust adjuvants, such as saponin, which are known to cause high rates of localized vaccine site reactions. Although these vaccines may provide important reduction of canine parasite load, and therefore lead to reduction of canine

reservoir-based transmission, the widespread impact of such products is not yet appreciated, due to concerns with potential adverse reactions and cost.

Looking to Best Practices, Future Reservoir VL Prevention

Prevention of leishmaniasis requires blocking a step in the parasite's life cycle. Interrupting sand fly transmission is of primary importance for individual and community protection from VL. Approaches to address reservoir populations have also been implemented in an attempt to reduce ZVL. Use of repellants including synthetic pyrethroids either topically on dogs or in collars may be the most effective tool for prevention of *L. infantum* infection in dogs and secondarily humans (Otranto et al. 2013). These approaches deter both sand flies from feeding (thought to be 84–96 % effective) and insecticidal activity should the sand fly feed (100 % effective) (Otranto et al. 2013). Although these experimental trials have been encouraging, larger trials in endemic areas are needed to evaluate protection of people provided by use of these methods.

Summary

In many areas dogs are a predominant domestic reservoir for VL. In the past, well-funded, centralized efforts in China and other countries that included dog culling have provided temporary breaks in VL transmission. Brazil has implemented public health policy utilizing voluntary surveillance and culling of positive dogs to reduce the burden of VL (Tesh 1995). While studies have shown that vigilant surveillance and culling can reduce the canine prevalence of VL to a degree, impacts on human infection are more difficult to ascertain (Costa 2011). Limitations in diagnostic sensitivity likely lead to false-negative diagnosis in a large number of asymptomatic dogs, delays between testing and dog removal increase the likelihood of transmission, and the financial and emotional cost of the implemented policy in Brazil is high (Costa 2011; Harhay et al. 2011a, b; Esch et al. 2012). The use of permethrin or deltamethrin collars or topical applications has shown efficacy in reducing sand fly feeding and transmission in endemic areas (Courtenay et al. 2009; Quinnell and Courtenay 2009). However, the cost and necessity for reapplication make these interventions more difficult to utilize in many endemic regions. Despite the cost, in a low-income area of Northeastern Brazil, pet attachment as compared to expense was significantly associated with willingness to voluntarily purchase cVL preventatives (Esch et al. 2012). There are multiple vaccines on the market for cVL in both Brazil and Europe (Lemesre et al. 2005; Dantas-Torres 2006; Martins et al. 2013). There is currently no commercially available vaccine for human leishmaniasis, although there are many in the running to be the first. Given that reservoir control will continue to be a predominant approach to

controlling hVL, a considered approach including topical insecticides, canine vaccination, and canine reproductive control is likely to provide the best control of reservoir VL now and into the future.

References

Alexander B, Barros VC et al (2009) Susceptibility to chemical insecticides of two Brazilian populations of the visceral leishmaniasis vector Lutzomyia longipalpis (Diptera: Psychodidae). Trop Med Int Health 14(10):1272–1277

Alvar J, Canavate C et al (1997) Leishmania and human immunodeficiency virus coinfection: the first 10 years. Clin Microbiol Rev 10(2):298–319

Alvar J, Velez ID et al (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7(5):e35671

Amro A, Schonian G et al (2009) Population genetics of Leishmania infantum in Israel and the Palestinian Authority through microsatellite analysis. Microbes Infect 11(4):484–492

Aresu L, Valenza F et al (2007) Membranoproliferative glomerulonephritis type III in a simultaneous infection of Leishmania infantum and Dirofilaria immitis in a dog. J Vet Diagn Invest 19(5):569–572

Barreto ML, Teixeira MG et al (2011) Successes and failures in the control of infectious diseases in Brazil: social and environmental context, policies, interventions, and research needs. Lancet 377(9780):1877–1889

Boggiatto PM, Gibson-Corley KN et al (2011) Transplacental transmission of Leishmania infantum as a means for continued disease incidence in North America. PLoS Negl Trop Dis 5(4):e1019

Borja-Cabrera GP, Correia Pontes NN et al (2002) Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (Sao Goncalo do Amarante, RN). Vaccine 20(27–28):3277–3284

Cortese L, Terrazzano G et al (2011) Prevalence of anti-platelet antibodies in dogs naturally co-infected by Leishmania infantum and Ehrlichia canis. Vet J 188(1):118–121

Costa CH (2011) How effective is dog culling in controlling zoonotic visceral leishmaniasis? A critical evaluation of the science, politics and ethics behind this public health policy. Rev Soc Bras Med Trop 44(2):232–242

Coura-Vital W, Marques MJ et al (2011) 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 5(8):e1291

Courtenay O, Kovacic V et al (2009) A long-lasting topical deltamethrin treatment to protect dogs against visceral leishmaniasis. Med Vet Entomol 23(3):245–256

da Silva VO, Borja-Cabrera GP et al (2000) A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (Sao Goncalo do Amaranto, RN). Vaccine 19(9–10):1082–1092

Dantas-Torres F (2006) Leishmune vaccine: the newest tool for prevention and control of canine visceral leishmaniosis and its potential as a transmission-blocking vaccine. Vet Parasitol 141(1–2):1–8

Dantas-Torres F, Solano-Gallego L et al (2012) Canine leishmaniosis in the Old and New Worlds: unveiled similarities and differences. Trends Parasitol 28(12):531–538

de Almeida AS, Medronho Rde A et al (2011) Identification of risk areas for visceral leishmaniasis in Teresina, Piaui State, Brazil. Am J Trop Med Hyg 84(5):681–687

Duthie MS, Raman VS et al (2012) The development and clinical evaluation of second-generation leishmaniasis vaccines. Vaccine 30(2):134–141

Esch KJ, Pontes NN et al (2012) Preventing zoonotic canine leishmaniasis in northeastern Brazil: pet attachment and adoption of community leishmania prevention. Am J Trop Med Hyg 87(5):822–831

Gavgani AS, Hodjati MH et al (2002) Effect of insecticide-impregnated dog collars on incidence of zoonotic visceral leishmaniasis in Iranian children: a matched-cluster randomised trial. Lancet 360(9330):374–379

Gradoni L (2001) An update on antileishmanial vaccine candidates and prospects for a canine Leishmania vaccine. Vet Parasitol 100(1–2):87–103

Hamarsheh O, Nasereddin A et al (2012) Serological and molecular survey of Leishmania parasites in apparently healthy dogs in the West Bank, Palestine. Parasit Vectors 5:183

Harhay MO, Olliaro PL et al (2011a) Urban parasitology: visceral leishmaniasis in Brazil. Trends Parasitol 27(9):403–409

Harhay MO, Olliaro PL et al (2011b) Who is a typical patient with visceral leishmaniasis? Characterizing the demographic and nutritional profile of patients in Brazil, East Africa, and South Asia. Am J Trop Med Hyg 84(4):543–550

Jimenez M, Gonzalez E et al (2013) Detection of Leishmania infantum and identification of blood meals in Phlebotomus perniciosus from a focus of human leishmaniasis in Madrid, Spain. Parasitol Res 112(7):2453–2459

Kovalenko DA, Razakov SA et al (2011) Canine leishmaniosis and its relationship to human visceral leishmaniasis in Eastern Uzbekistan. Parasit Vectors 4:58

Lemesre JL, Holzmuller P et al (2005) Protection against experimental visceral leishmaniasis infection in dogs immunized with purified excreted secreted antigens of Leishmania infantum promastigotes. Vaccine 23(22):2825–2840

Lima ID, Queiroz JW et al (2012) Leishmania infantum chagasi in northeastern Brazil: asymptomatic infection at the urban perimeter. Am J Trop Med Hyg 86(1):99–107

Martins VT, Chavez-Fumagalli MA et al (2013) Antigenicity and protective efficacy of a Leishmania amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. PLoS Negl Trop Dis 7(3):e2148

Mencke N, Volf P et al (2003) Repellent efficacy of a combination containing imidacloprid and permethrin against sand flies (Phlebotomus papatasi) in dogs. Parasitol Res 90(suppl 3):S108–S111

Molina R, Gradoni L et al (2003) HIV and the transmission of Leishmania. Ann Trop Med Parasitol 97(suppl 1):29–45

Moreno J, Alvar J (2002) Canine leishmaniasis: epidemiological risk and the experimental model. Trends Parasitol 18(9):399–405

Mutinga MJ, Renapurkar DM et al (1993) A bioassay to evaluate the efficacy of permethrin-impregnated screens used against phlebotomine sandflies (Diptera: Psychodidae) in Baringo District of Kenya. East Afr Med J 70(3):168–170

Nunes CM, Pires MM et al (2010) Relationship between dog culling and incidence of human visceral leishmaniasis in an endemic area. Vet Parasitol 170(1–2):131–133

Oliveira TM, Furuta PI et al (2008) A study of cross-reactivity in serum samples from dogs positive for Leishmania sp., Babesia canis and Ehrlichia canis in enzyme-linked immunosorbent assay and indirect fluorescent antibody test. Rev Bras Parasitol Vet 17(1):7–11

Otranto D, Dantas-Torres F (2010) Canine and feline vector-borne diseases in Italy: current situation and perspectives. Parasit Vectors 3:2

Otranto D, Dantas-Torres F (2013) The prevention of canine leishmaniasis and its impact on public health. Trends Parasitol 29(7):339–345

Otranto D, Paradies P et al (2007) Efficacy of a combination of 10% imidacloprid/50% permethrin for the prevention of leishmaniasis in kennelled dogs in an endemic area. Vet Parasitol 144(3–4):270–278

Otranto D, de Caprariis D et al (2010) Prevention of endemic canine vector-borne diseases using imidacloprid 10% and permethrin 50% in young dogs: a longitudinal field study. Vet Parasitol 172(3–4):323–332

Otranto D, Dantas-Torres F et al (2013) Prevention of canine leishmaniosis in a hyper-endemic area using a combination of 10% imidacloprid/4.5% flumethrin. PLoS One 8(2):e56374
Paape D, Aebischer T (2011) Contribution of proteomics of Leishmania spp. to the understanding of differentiation, drug resistance mechanisms, vaccine and drug development. J Proteomics 74(9):1614–1624
Pagliano P, Carannante N et al (2005) Visceral leishmaniasis in pregnancy: a case series and a systematic review of the literature. J Antimicrob Chemother 55(2):229–233
Palatnik-de-Sousa CB, Silva-Antunes I et al (2009) Decrease of the incidence of human and canine visceral leishmaniasis after dog vaccination with Leishmune in Brazilian endemic areas. Vaccine 27(27):3505–3512
Queiroz PV, Monteiro GR et al (2009) Canine visceral leishmaniasis in urban and rural areas of Northeast Brazil. Res Vet Sci 86(2):267–273
Quinnell RJ, Courtenay O (2009) Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology 136(14):1915–1934
Raman VS, Duthie MS et al (2012) Adjuvants for Leishmania vaccines: from models to clinical application. Front Immunol 3:144
Ribeiro VM, da Silva SM et al (2013) Control of visceral leishmaniasis in Brazil: recommendations from Brasileish. Parasit Vectors 6(1):8
Romero GA, Boelaert M (2010) Control of visceral leishmaniasis in Latin America—a systematic review. PLoS Negl Trop Dis 4(1):e584
Tabbara KS (2006) Progress towards a Leishmania vaccine. Saudi Med J 27(7):942–950
Talmi-Frank D, Kedem-Vaanunu N et al (2010) Leishmania tropica infection in Golden Jackals and Red Foxes, Israel. Emerg Infect Dis 16(12):1973–1975
Tasca KI, Buzetti WA et al (2009) Parasitological, immunohistochemical and histopathological study for Leishmania chagasi detection in splenic tissues of dogs with visceral leishmaniasis. Rev Bras Parasitol Vet 18(1):27–33
Tesh RB (1995) Control of zoonotic visceral leishmaniasis: is it time to change strategies? Am J Trop Med Hyg 52(3):287–292
Trigo J, Abbehusen M et al (2010) Treatment of canine visceral leishmaniasis by the vaccine Leish-111f+MPL-SE. Vaccine 28(19):3333–3340
Vanloubbeeck Y, Jones DE (2004) The immunology of Leishmania infection and the implications for vaccine development. Ann N Y Acad Sci 1026:267–272
Werneck GL (2008) Forum: geographic spread and urbanization of visceral leishmaniasis in Brazil. Introduction. Cad Saude Publica 24(12):2937–2940

Treatment Modalities for Cutaneous and Visceral Leishmaniasis

Bradford S. McGwire

Abstract The annual incidence of cutaneous and visceral leishmaniasis is at least two million people, with 350 million people in 98 countries at risk for acquiring infection. These are diseases mostly of the impoverished and are considered in the top five of neglected infectious diseases worldwide, making prevention, diagnosis, and treatment difficult. Therapy of leishmaniasis ranges from local treatment of cutaneous lesions to systemic, often toxic, therapy for disseminated cutaneous, mucocutaneous, and deadly visceral disease. This chapter discusses the current treatment regimens commonly used for various forms of leishmaniasis with attention to some promising newer experimental approaches.

Keywords *Leishmania* • Cutaneous leishmaniasis • Mucocutaneous leishmaniasis • Visceral leishmaniasis • Therapy

Introduction

The protozoans of the genus *Leishmania* are a diverse group of parasites which are transmitted between mammalian hosts by blood-sucking sandflies. *Leishmania* alternately infect female sandflies, which suck blood for nutritional support for egg-laying, and various mammalian species including humans. A variety of animals serve as natural reservoirs and include both domestic and feral dogs, rodents, foxes, jackals, wolves, raccoons, and hyraxes, sloths, aardvarks, opossums, and rodents such as rats and mice. Humans are thought to constitute the main reservoir in India.

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Human infection by pathogenic leishmanial species causes diverse chronic infections of the skin and viscera and is present in both the Old (in regions of the Far and Middle East, Central Western and Eastern Europe, and Africa) and New (regions of Central and South America) Worlds and is mainly found in rural impoverished areas. Overall there are over 20 species of *Leishmania* worldwide and the subtype of disease relates to the species of infecting *Leishmania* and the interplay of the genetic background and immune status of the host. Worldwide there is an estimated annual incidence of two million cases across 98 countries with an additional 350 million at risk of infection (World Health Organization 2010).

Leishmania *Life Cycle*

Leishmania have two main life cycle stages; the motile flagellated promastigote, which is present in the sandfly vector, and intracellular nonflagellated amastigote, which is present within mammalian host cells (Fig. 1). *Leishmania* are parasites of professional phagocytes (macrophages and dendritic cells) which initiate infection through receptor-mediated binding of infective promastigotes delivered into host tissue during the feeding of infected sandflies (Ueno and Wilson 2012). Parasites housed in parasitophorous vacuoles fuse with lysosomes to form phagolysosomes wherein promastigotes transform into and replicate as amastigotes (Chang and Dwyer 1976). Eventually the parasite burden increases physically disrupting infected host macrophages delivering extracellular amastigotes into surrounding tissue where they are engulfed by uninfected macrophages. Parasites and infected macrophages can metastasize within the skin and visceral organs. Host control of infection is a complex interplay of innate and adaptive immune factors which are incompletely understood (Liese et al. 2008; Chang et al. 2003).

Clinical Syndromes

Leishmanial disease causes three main human syndromes, and some lesser prevalent clinical entities (Fig. 1). The outcome of each is determined by the species of infecting parasite and the genetic susceptibility of the host.

Cutaneous disease: Cutaneous leishmaniasis (CL) is the least severe form of disease and is caused by several species such as *L. major* and *L. tropica* in the Old World and *L. mexicana*, *L. amazonensis*, *L. guyanensis*, *L. panamensis*, and *L. braziliensis* in various regions of Central and South America (Goto and Lauletta Lindoso 2012). Simple cutaneous disease presents as singular ulcerative or nodular lesions at or near the site of insect exposure. These are usually found on uncovered areas of the body such as the face, forearms, and lower legs and evolve over weeks to months. In diffuse cutaneous disease, such as that caused by *L. amazonensis*, nodular lesions of

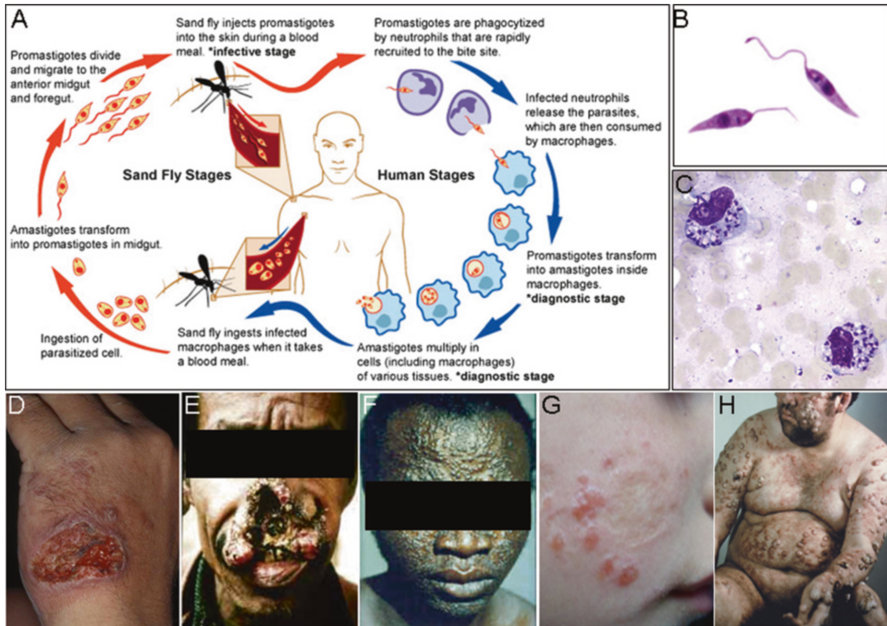

Fig. 1 *Leishmania* life cycle and clinical syndromes. (**A**) Diagrammatic depiction of life cycle of *Leishmania*. (**B**) Giemsa-stained preparation of *L. donovani* promastigotes, (**C**) Giemsa staining of touch preparation of a cutaneous lesion showing the presence of intracellular parasites inside macrophages, (**D**) cutaneous leishmaniasis (CL) lesion on the hand, (**E**) mucocutaneous lesion (MCL) of the mouth and nose, (**F**) facial lesions in a case of post-kala-azar dermal leishmaniasis (PKDL), (**G**) emerging lesions within the old CL scar in leishmaniasis recidivans (LR), (**H**) lesions throughout in diffuse cutaneous leishmaniasis (DCL). Credit for pictures: Panels: (**A**), obtained from NIAID; (**B**), www.lookfordiagnosis.com; (**C**), Centers for Disease Control, www.CDC.gov; (**D**), www.Dermnet.com; (**E**), www.Drugline.org; (**F**), www.WHO.int; (**G**), www.globalskinatlas.com; (**H**), Medical Books Online, www.cixip.com

variable size erupt at various locations, often distant from the site of inciting insect exposure. Simple cutaneous lesions are most often self-healing but in some cases, such as those caused by *L. panamensis* and *L. braziliensis*, can progress to involve mucocutaneous tissue. The resolution of cutaneous lesions can often be hastened by treatment (David and Craft 2009).

Mucocutaneous disease: Mucocutaneous leishmaniasis (MCL) is caused by *L. braziliensis* which can be due to extension of, or parasite metastasis from, local skin disease into the mucocutaneous tissue. MCL can present months to years after resolution of primary lesions. This is often a horribly disfiguring infection resulting from the chronic local destruction of tissue of the nose, mouth, and oro- and nasopharynx and can progress to affect respiratory function and hamper nutrition. The underlying pathogenesis resulting in MCL is not well understood and is probably a result of a complex interplay of host and parasite factors (de Oliveira and Brodskyn 2012). The disease is often refractory to chemotherapy and patients usually die from

secondary superinfections and malnutrition. MCL is found in countries in South America, with the majority of disease found in Brazil, Peru, and Bolivia but is also found in lesser degrees in Colombia, Ecuador, Paraguay, and Venezuela. In Ecuador, most cases are found in the Amazonian lowlands, with lesser incidence in the inter-Andean and Pacific coastal regions (Calvopina et al. 2004).

Visceral disease: Visceral leishmaniasis (VL, also known as kala-azar) results from the infection of phagocytes within the reticuloendothelial system due to metastasis of parasites and parasite-infected macrophages from the initial site of cutaneous infection. In the Old World VL is caused by *L. donovani* (in regions of India, Pakistan, China, and Africa) and by *L. infantum* (in the Mediterranean region). In the New World VL is also caused by *L. infantum* (also known as *L. chagasi* or *L. infantum chagasi*), which is found primarily in Brazil. Visceral disease has been reported in the Middle East caused by viscerotropic strains of *L. tropica* (Center for Disease Control 1992), which has been classically thought of as an agent of CL. The proliferation of parasites in macrophages in the liver, spleen, and bone marrow of patients with VL gives rise to progressive hepatosplenomegaly and bone marrow suppression. Unless treated patients develop pancytopenia and immunosuppression and are prone to superinfections with other microbes. Without therapy patients with VL will eventually succumb to their disease. Individuals co-infected with HIV have a particular susceptibility to developing atypical presentations, and increased severity, of VL and the development of VL in HIV patients is an AIDS-defining illness (ter Horst et al. 2008). This is probably due to the dysregulation effects of both agents on the immune system of the host (Okwor and Uzonna 2013).

Leishmaniasis recidivans (*LR*): This is characterized by a relapse of cutaneous disease within the sites of previous healed CL lesions (Oliveira-Neto et al. 1998). This can occur decades after resolution of the primary lesions and often form within the edge of the previous scar (Marovich et al. 2001). The lesions in LR are reminiscent of those in discoid lupus or lupoid leishmaniasis and require treatment often with dual therapy.

Post-kala-azar dermal leishmaniasis (*PKDL*): A subset of patients successfully treated for VL, and who remain asymptomatic for months to years, develop a fulminant and progressive proliferation of parasites within the skin which give rise to diffuse macular, maculopapular, or nodular lesions. PKDL occurs mainly in India and Sudan in patients infected with *L. donovani* (Zijlstra et al. 2003). In Sudan PKDL can arise in up to 50 % of patients and occurs sooner (in up to 6 months) than in patients in India where it has an incidence of ~5–10 % within 2–3 years after clearance of VL. The pathogenesis of PKDL is not fully understood but appears related to an aggressive interferon γ-driven host immune response generated against lingering dermal parasites. Biopsy of lesions shows macrophages heavily laden with amastigotes which are genotypically identical to the parasites generating the inciting VL. The development of PKDL seen in HIV/AIDS patients undergoing HAART may be a manifestation of immune-reconstitution syndrome (Antinori et al. 2007).

Treatment

There are number of therapies for various forms of leishmaniasis and the preferences for first-line and second-line treatments vary based on the type of disease and are often guided by regional practice in relation to what is currently most effective and available. Pentavalent antimony has been considered the mainstay of therapy in leishmaniasis for decades; however, this agent has multiple toxicities and is increasingly ineffective due to development of parasite resistance. Other alternative agents are utilized in different clinical situations and guided by availability and effectiveness in different localities. The following is general description of the drugs used now in clinical practice (World Health Organization 2010). See Table 1 for summary.

Pentavalent antimony: An agent of choice for most forms of leishmaniasis is systemic use of pentavalent antimony (Sb), which is present in meglumine antimoniate (trade name *Glucantime* or *Glucantim*) or sodium stibogluconate (trade name *Pentostam*). The precise mechanism of action of Sb in leishmaniasis is not well understood but is probably multifactorial acting directly on molecular processes of the parasite as well as influencing macrophage parasiticidal activity (Baiocco et al. 2009). For systemic use, these compounds can be administered intravenously (iv) or intramuscularly (im), but are also used intra-lesionally (il) for CL. For VL and LR, 20 mg Sb/kg body weight per day is given daily for 28–30 days and is used as a first-line agent for up to 60 days in East African PKDL. When used as systemic therapy for CL the treatment length is shorter, between 10 and 20 days, and when used for MCL the length of therapy is extended up to 30 days. Systemic therapy is used in combination with other agents in some cases, including East African VL where it is combined with paromomycin (15 mg/kg/day im) to shorten the overall course of therapy to 17 days, and in CL and DCL caused by *L. aethiopica*. It has been used together with pentoxifylline, a TNF-α inhibitor (400 mg orally/per day), for treatment of CL caused by *L. major* and in MCL where it has been shown to be more effective than antimony alone in leading to complete cure and for shortening the time to cure (Lessa et al. 2001). Allopurinol (20 mg orally/kg/day for 30 days) has also been used as an adjunct with systemic antimony in CL and LR caused by *L. tropica* (Esfandiarpour and Dabiri 2007). Side-effects are common in the use of systemic antimony therapy, the most serious of which is cardiotoxicity (arrhythmias, Q-T prolongation, and sudden death) but multiple others occur (elevation of liver and pancreatic enzymes, pancytopenia, and electrolyte abnormalities) requiring careful monitoring of patients during the course of therapy.

Amphotericin B: The polyene amphotericin B is an effective anti-leishmanial agent which works by binding to membrane ergosterol causing membrane instability. Both the deoxycholate and the liposomally encapsulated (trade name Ambisome) formulations of amphotericin B have been used clinically in leishmaniasis (Barratt and Legrand 2005). Amphotericin B deoxycholate is used iv at a dose of 0.75–1 mg/kg/day for 15–20 doses for VL caused by *L. donovani* or up to 30 days for VL caused by *L. infantum*.

Table 1 Leishmanial diseases, parasites, and treatment regimens by regions

Species	Disease	Geographic location[a]	Treatment[b]
Old World			
L. major	CL	Middle East, Africa	*Local*: t-paro, il-Sb, il-Sb + CT, HT, CT
			Systemic: Flu, s-Sb, s-Sb + ptxf
L. tropica, L. infantum	CL	Middle East, Mediterranean, Africa	*Local*: t-paro, il-Sb, il-Sb + CT, HT, CT
			Systemic: s-Sb,
	LR		s-Sb + Allo
L. aethiopica	CL	Africa	s-Sb + im-paro
L. donovani	VL	Far East	L-AmB, L-AmB + Mil, L-AmB + im-paro, Mil + im-paro, AmB, Mil, im-paro, s-Sb
		Africa	s-Sb + im-paro, s-Sb, L-AmB, AmpB, Mil
	PKDL	Far East	AmB, Mil
	PKDL	African	L-AmB, s-Sb
L. infantum	VL	Mediterranean, Eastern Europe, Far East	L-AmB, AmB, s-Sb
New World			
L. infantum/chagasi	VL	Central and South America	L-AmB, AmB, s-Sb
L. mexicana	CL	North, Central, and South America	*Local*: t-paro, HT, il-Sb
			Systemic: Ket, Mil
L. amazonensis	CL, DCL	South America	*Local*: t-paro, HT, il-Sb
			Systemic: s-Sb
L. panamensis	CL	Central and South America	*Local*: t-paro, HT, il-Sb
			Systemic: Pent, s-Sb, Mil
L. braziliensis	CL, MCL	Central and South America	*Local*: t-paro, HT, il-Sb
			Systemic: AmB, L-AmB, s-Sb
L. guyanensis	CL	South America	*Local*: t-paro, HT, il-Sb
			Systemic: Pent, s-Sb, Mil
L. peruviana, L. venezuelensis	CL	South America	*Local*: t-paro, HT, il-Sb
			Systemic: s-Sb
All species	Relapsed CL, DCL, or MCL		AmB, L-AmB, s-Sb + t-imi, or immunotherapy

CL, cutaneous leishmaniasis; VL, visceral leishmaniasis; LR, leishmaniasis recidivans; PKDL, post kala azar dermal leishmaniasis; MCL, mucocutaneous leishmaniasis

[a]Countries in regional designation where disease has been found: Middle East (Afghanistan, Cyprus, Iran, Iraq, Jordan, Israel, Kuwait, Lebanon, Macedonia, Montenegro, Palestine, Saudi Arabia, Syria, Yemen); Africa (Cameroon, Central African Republic, Egypt, Gambia, Ghana,

Table 1 (continued)

Guinea, Guinea-Bissau, Kenya, Libya, Mali, Mauritania, Morocco, Namibia, Niger, Nigeria, Oman, Senegal, Somalia, Sudan, Tunisia, Uganda), Far East (Bangladesh, China, India, Pakistan, Nepal, Sri Lanka), Eastern Europe (Albania, Algeria, Armenia, Azerbaijan, Bulgaria, Bosnia and Herzegovina, Croatia, Georgia, Kazakhstan, Kyrgyzstan, Romania, Slovenia, Ukraine, Uzbekistan, Turkmenistan), Western Europe/Mediterranean (Greece, France, Italy, Malta, Monaco, Portugal, Spain, Turkey), Central America (Costa Rica, Dominican Republic, Guatemala, Honduras, Mexico, Nicaragua, Panama), North America (United States), South America (Argentina, Belize, Bolivia, Brazil, Columbia, Ecuador, French Guiana, Guyana, Paraguay, Peru, Suriname, Venezuela). Not included are countries where leishmanial disease is found without definitive speciation

[b]Treatment options used for these disease syndromes: il-Sb, intra-lesional stibogluconate (Sb); s-Sb, systemic Sb; Ket, ketoconazole; Flu, fluconazole; Mil, miltefosine; t-paro, topical paromomycin; im-paro, intramuscular paromomycin; L-AmB, liposomal amphotericin B; AmB, amphotericin deoxycholate; CT, cryotherapy; HT, heat therapy; Pent, pentamidine; Ptxf, pentoxyfylline; Allo, allopurinol; t-imi, topical imiquimod

Dosing in PKDL is daily or on alternate days for up to 4 months of therapy. For CL caused by *L. braziliensis* the length of therapy is 25–30 days and for MCL is up to 45 days. Liposomal amphotericin B is typically used at a dose of 2.5–5 mg/kg/day and this formulation reduces the overall length of therapy to one-half that of the non-liposomal form. Single dose liposomal amphotericin B at a dose of 10 mg/kg in VL caused by *L. donovani* or at 5 mg/kg in combination with miltefosine daily for 10 days or paromomycin has been shown to be effective in treatment (see sections below) (Sundar et al. 2011). Common side-effects of amphotericin B are renal insufficiency and electrolyte abnormalities, which are both less with the liposomal form; however, this formulation is more costly which limits its widespread use.

Paromomycin: Paromomycin is an aminoglycoside antibiotic which works by blocking protein synthesis by binding to 16S ribosomal RNA. In leishmaniasis it is used im for systemic use (at 15 mg/kg) alone for up to 21 days in Indian VL or in combination with liposomal amphotericin B for Indian or East African VL (Sundar et al. 2011). Paromomycin can be used topically in New and Old World CL in a 15 % ointment together with 12 % methyl benzothonium chloride daily for 20 days (Arana et al. 2001; Gonzalez et al. 2008). Recently, topical paromomycin with and without gentamicin has been shown to be efficacious in speeding resolution of lesions caused by *L. major* (Ben Salah et al. 2013). Topical therapy is not indicated in cases of MCL. The side-effects of systemic paromomycin are similar to other aminoglycosides such as ototoxicity, vestibular instability, and nephrotoxicity.

Pentamidine: The precise antimicrobial mechanisms of pentamidine (trade names *Pentacrinat, Pentam*) are unknown but the drug interferes with the biosynthesis of macromolecules such as DNA, RNA, phospholipids, and proteins. It has been used (iv doses every other day for 3–5 days at 4 mg/kg of body weight) for the treatment of South American CL caused by *L. guyanensis* and *L. panamensis* and there is some data to show that it is also effective in Old World CL caused by *L. tropica* and *L. major* as well as cutaneous lesions of *L. infantum* (Hellier et al. 2000).

A wide array of adverse reactions have been reported with pentamidine use; most notable are hypoglycemia and/or worsening of diabetes, liver enzyme abnormalities, bone marrow effects such as leukopenia and anemia, nephrotoxicity and cardiotoxicity such as arrhythmias and heart failure, and hypotension. Patients on this drug require careful observation.

Miltefosine: Originally investigated as an antineoplastic agent, the alkylphosphocholine miltefosine (trade names *Impavido* and *Miltex*) is the only oral agent in use for Indian and East African VL both in children and adults where the dose is incrementally weight-based (ages ≤12 years old at 2.5 mg/kg daily; in those >12 years of age and <25 kg of weight the dose is 50 mg/kg; those with weights of 25–50 kg dosing is 100 mg/kg; in those >50 kg is 150 mg/kg for a total of 28 days) (Jha et al. 1999; Sundar et al. 2006). Miltefosine has also been used in combination with paromomycin for Indian VL (see above) (Sundar et al. 2011). Recently it was shown that miltefosine is effective in PKDL; the length of therapy extended up to 12 weeks (Sundar et al. 2013). In New World CL caused by *L. mexicana*, *L. guyanensis*, and *L. panamensis* miltefosine can be effective at a dose of 2.5 mg/kg for 28 days (Soto et al. 2004) but is not of benefit for *L. braziliensis* cutaneous infection. However, Bolivian MCL responds to miltefosine therapy for 4–6 weeks with cure rates of up to 75 % (Soto et al. 2007, 2008, 2009). This drug is very well tolerated and the main adverse effects are nonspecific nausea and vomiting. This agent is teratogenic and contraindicated in pregnancy.

Imiquimod: Imiquimod (trade names *Aldara* or *Zyclara*) is a topical imidazole quinolone that induces macrophage activation through production of pro-inflammatory cytokines such as IL-2, IFN-γ, and TNF-α. It can be used in conjunction with pentavalent antimony in relapsed New World CL (Miranda-Verastegui et al. 2005; Arevalo et al. 2007) and may be effective in antimony-refractory cases (Arevalo et al. 2001). Imiquimod has not proven effective in cases of *L. tropica* (Firooz et al. 2006) but a recent report suggests response in a case of Old World CL caused by *L. chagasi* (Hervas et al. 2012). This agent is generally well tolerated with the main adverse effect being irritation at the site of application.

Azoles: While azoles (ergosterol synthesis inhibitors) have in vitro parasitistatic activity against a variety of leishmanial species they have not been shown to be as effective as other agents for clinical use. Oral ketoconazole (trade name *Nizoral*) (600 mg orally daily for 28–30 days) and fluconazole (trade name *Diflucan*) (a daily dose of 200 mg orally for 6 weeks) have been shown to hasten healing of CL lesions caused by *L. mexicana* (Gonzalez et al. 2009) and *L. major* (Gonzalez et al. 2008; Alrajhi et al. 2002), respectively. Patients on long-term azole therapy require routine blood tests for liver function abnormalities.

Cryotherapy: Liquid nitrogen application directly to CL lesions either once or multiple times, up to five times, every 3–7 days has been used in Old World CL caused by *L. tropica*, *L. aethiopica*, and *L. infantum* or in combination with intra-lesional antimony for *L. major* (Negera et al. 2012; Asilian et al. 2003, 2004).

Heat therapy: Heating lesions to 50 °C for 30 s up to three times can be used to hasten resolution of Old and New World CL lesions and is comparable to intralesional or systemic antimony therapy (Bumb et al. 2013; Reithinger et al. 2005). This treatment has also been found to be effective against CL in HIV-infected patients who do not respond to antimonial therapy (Prasad et al. 2011).

Immunotherapy: Vaccines consisting of heat-killed *Leishmania* plus BCG or defined recombinant antigens together with GM-CSF have been used in small numbers of patients with refractory MCL or DCL (Garcia Bustos et al. 2011; Convit et al. 2003, 2004; Badaro et al. 2001, 2006).

Newer Experimental Approaches

As of this writing, survey of the literature using PubMed and the search terms "leishmaniasis, novel therapy" generates 258 papers, with 141 of these published since 2008. The majority of these reports document anti-leishmanial activity of a diverse range of natural products and synthetic agents in vitro against axenic promastigotes and/or amastigotes and amastigotes in macrophages and in some cases show reductions of parasite burden in murine models. As is the nature of the drug discovery, it is difficult to predict the efficacy of these agents in human trials despite promising preclinical activity and low toxicity. In some cases, these reports document novel preparations for enhanced drug delivery (including nanoparticle delivery) or diminishment of toxicity of compounds that are already used in clinical practice (for example, amphotericin B and antimonials) or optimization of compounds within the same class of compounds which are used clinically. In some cases, agents which are used clinically for other indications have been shown to have anti-leishmanial activity and are under active investigation. It is beyond the scope of this review to discuss the entire breadth of these experimental agents other than to mention some generalized concepts.

Chemical targeting of several specific and unique pathways and enzymes vital to parasite viability is a suitable approach; such targets include proteolytic enzyme systems of the aspartic-, cysteine-, metallo-, serine-, and threonine-protease classes. Aspartic protease inhibitors used in HIV therapy have anti-leishmanial activity (van Griensven et al. 2013). Effect of these agents on parasites may be directly related to activity against the A2 family of leishmanial aspartic proteases. Indirect effects of these inhibitors include induction of mitochondrial dysfunction, apoptosis, and induction of oxidative stress (Krylov and Koonin 2001; Santos et al. 2013). Guidelines for the clinical use of these agents have not yet been established. Several cysteine proteases, of both the cathepsin-L- and B-like classes, are expressed in *Leishmania* and the vinyl sulfone, cathepsin-L-specific inhibitor, K11777, reduces *L. tropica*-related murine infection (Mahmoudzadeh-Niknam and McKerrow 2004) which supports the importance in CP-B in parasite virulence (Williams et al. 2006). Serine- and threonine-proteases are critical to leishmanial endosome and proteosome function, respectively, and inhibitors of these enzymes represent potential

therapeutic agents (Silva-Lopez et al. 2007; Silva-Jardim et al. 2004). Other potential systems include targeting of topoisomerases activity, specifically topoisomerase II which is important in the maintenance of the kinetoplast DNA biogenesis (Das et al. 2006; Motta 2008). There is precedence for use of such inhibitors in treatment of bacterial infections and in cancer chemotherapy. Several other potential drug targets in the *Leishmania* mitochondria include tRNA import from the cytosol, the mitochondrial fatty acid synthesis pathway, disruption of the mitochondrial membrane potential through any number of steps, and the disruption of the electron transport chain to produce reactive oxygen species. Interruption of these processes may lead to mitochondrial dysfunction that culminate in the induction of parasite apoptosis, such as increase in hydrogen peroxide and or superoxide, depletion of membrane potential and ATP production, and increased delocalization of intracellular calcium (Fidalgo and Gille 2011).

Targeting of sterol biosynthesis enzymes such as 14-α-demethylase and 24-methyltransferase may be of benefit in future therapy; although azoles targeting the demethylase have not proved efficacious in monotherapy, they may have a role in combination therapy (Paniz Mondolfi et al. 2011; Marrapu et al. 2011; Kulkarni et al. 2013). Also important is the identification of agents for dual targeting of dihydrofolate reductase-thymidylate synthase and pteridine reductase pathways, which are critical for folate and biopterin scavenging for synthesis of precursors of DNA metabolism (Cunningham and Beverley 2001). The glycosome is a unique structure which compartmentalizes enzymes involved in the first seven steps of glycolysis that are important for regulation of hexokinase and phosphofructokinase activity. Interference with the sorting of enzymes to the glycosome represents a potential chemotherapeutic target (Bringaud et al. 2006; Mannion-Henderson et al. 2000). Additionally, the activities of intracellular iron-superoxide dismutases that are critical to the maintenance of glycosomal integrity from free radical generation also represent putative targets (Plewes et al. 2003). Similarly important is trypanothione reductase, a crucial enzyme for production of trypanothione and tryparedoxin and which is a critical cofactor for tryparedoxin peroxidase (Fairlamb et al. 1985). This system is essential for the neutralization of intracellular nitric oxide, hydrogen peroxide, and iron which is critical for cellular viability; thus, chemically disrupting this system has potential chemotherapeutically (Krauth-Siegel and Inhoff 2003).

Another approach to therapy is augmentation of the host immune system for enhanced elimination of parasites. Agents with immunomodulatory effects may alter host T-cell profiles in favor of pro-inflammatory cascades leading to more robust production of microbicidal effectors for the elimination of intracellular amastigotes within infected macrophages. A variety of new agents are currently under experimental investigation as well as already established agents used for other maladies (Hartley et al. 2013). It is increasingly clear that treatment for diffuse cutaneous, visceral, and mucosal leishmaniasis will likely require multidrug therapy similar to that used in tuberculosis and HIV (Shakya et al. 2011; Olliaro 2010). This approach lessens the development of drug resistance and can diminish toxicities of single agents at higher dose and for longer durations.

Conclusions

The diseases caused by the intracellular protozoan parasite *Leishmania* represent a major global health problem, and are considered a WHO-classified neglected tropical disease. Nearly 10 % of the world's population is at risk of acquiring a form of leishmaniasis. Worldwide it is estimated that there are 12 million active cases of leishmaniasis, with approximately two million new cases occurring each year. Among parasitic infections, this disease is responsible for the highest number of DALYs (disability adjusted life years; a measure of health burden) after malaria. Several treatments including systemic antimonials, liposomal amphotericin B, and miltefosine are currently available for leishmaniasis. However, these chemotherapeutic interventions are toxic and have poor patient compliance because many of them require daily systemic (iv or im) administration for periods ranging from 3 to 5 weeks. Furthermore, the emergence of drug-resistant strains is rapidly increasing worldwide and these treatments fail to induce a sterile cure because they do not eliminate persistent parasites from the host. Therefore, there is a continued need for new therapies against leishmaniasis that are safe, effective in inducing long-term cure, and that are easier to administer. The treatment of leishmaniasis will most likely evolve into an approach using multiple drugs simultaneously to reduce the development of drug resistance. There is an ongoing effort of drug discovery for leishmaniasis but this is mostly at the level of individual investigators. There is a clear need for increased funding in this area and the involvement of the pharmaceutical industry in order to focus efforts on development of chemotherapeutic agents and vaccines for these and other neglected tropical diseases.

References

Alrajhi AA, Ibrahim EA, De Vol EB, Khairat M, Faris RM, Maguire JH (2002) Fluconazole for the treatment of cutaneous leishmaniasis caused by *Leishmania major*. N Engl J Med 346:891–895

Antinori S, Longhi E, Bestetti G, Piolini R, Acquaviva V, Foschi A et al (2007) Post-kala-azar dermal leishmaniasis as an immune reconstitution inflammatory syndrome in a patient with acquired immune deficiency syndrome. Br J Dermatol 157:1032–1036

Arana BA, Mendoza CE, Rizzo NR, Kroeger A (2001) Randomized, controlled, double-blind trial of topical treatment of cutaneous leishmaniasis with paromomycin plus methylbenzethonium chloride ointment in Guatemala. Am J Trop Med Hyg 65:466–470

Arevalo I, Ward B, Miller R, Meng TC, Najar E, Alvarez E et al (2001) Successful treatment of drug-resistant cutaneous leishmaniasis in humans by use of imiquimod, an immunomodulator. Clin Infect Dis 33:1847–1851

Arevalo I, Tulliano G, Quispe A, Spaeth G, Matlashewski G, Llanos-Cuentas A et al (2007) Role of imiquimod and parenteral meglumine antimoniate in the initial treatment of cutaneous leishmaniasis. Clin Infect Dis 44:1549–1554

Asilian A, Sadeghinia A, Faghihi G, Momeni A, Amini Harandi A (2003) The efficacy of treatment with intralesional meglumine antimoniate alone, compared with that of cryotherapy combined with the meglumine antimoniate or intralesional sodium stibogluconate, in the treatment of cutaneous leishmaniasis. Ann Trop Med Parasitol 97:493–498

Asilian A, Sadeghinia A, Faghihi G, Momeni A (2004) Comparative study of the efficacy of combined cryotherapy and intralesional meglumine antimoniate (Glucantime) vs. cryotherapy and

intralesional meglumine antimoniate (Glucantime) alone for the treatment of cutaneous leishmaniasis. Int J Dermatol 43:281–283

Badaro R, Lobo I, Nakatani M, Muinos A, Netto EM, Coler RN et al (2001) Successful use of a defined antigen/GM-CSF adjuvant vaccine to treat mucosal Leishmaniasis refractory to antimony: a case report. Braz J Infect Dis 5:223–232

Badaro R, Lobo I, Munos A, Netto EM, Modabber F, Campos-Neto A et al (2006) Immunotherapy for drug-refractory mucosal leishmaniasis. J Infect Dis 194:1151–1159

Baiocco P, Colotti G, Franceschini S, Ilari A (2009) Molecular basis of antimony treatment in leishmaniasis. J Med Chem 52:2603–2612

Barratt G, Legrand P (2005) Comparison of the efficacy and pharmacology of formulations of amphotericin B used in treatment of leishmaniasis. Curr Opin Infect Dis 18:527–530

Ben Salah A, Ben Messaoud N, Guedri E, Zaatour A, Ben Alaya N, Bettaieb J et al (2013) Topical paromomycin with or without gentamicin for cutaneous leishmaniasis. N Engl J Med 368:524–532

Bringaud F, Riviere L, Coustou V (2006) Energy metabolism of trypanosomatids: adaptation to available carbon sources. Mol Biochem Parasitol 149:1–9

Bumb RA, Prasad N, Khandelwal K, Aara N, Mehta RD, Ghiya BC et al (2013) Long-term efficacy of a single-dose Radio Frequency Heat therapy versus intralesional antimonials for cutaneous leishmaniasis in India. Br J Dermatol 168(5):1114–1119

Calvopina M, Armijos RX, Hashiguchi Y (2004) Epidemiology of leishmaniasis in Ecuador: current status of knowledge—a review. Mem Inst Oswaldo Cruz 99:663–672

Center for Disease Control (1992) Viscerotropic leishmaniasis in persons returning from Operation Desert Storm—1990–1991. MMWR Morb Mortal Wkly Rep 41:131–134

Chang KP, Dwyer DM (1976) Multiplication of a human parasite (*Leishmania donovani*) in phagolysosomes of hamster macrophages in vitro. Science 193:678–680

Chang KP, Reed SG, McGwire BS, Soong L (2003) *Leishmania* model for microbial virulence: the relevance of parasite multiplication and pathoantigenicity. Acta Trop 85:375–390

Convit J, Ulrich M, Zerpa O, Borges R, Aranzazu N, Valera M et al (2003) Immunotherapy of American cutaneous leishmaniasis in Venezuela during the period 1990–99. Trans R Soc Trop Med Hyg 97:469–472

Convit J, Ulrich M, Polegre MA, Avila A, Rodriguez N, Mazzedo MI et al (2004) 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 99:57–62

Cunningham ML, Beverley SM (2001) Pteridine salvage throughout the *Leishmania* infectious cycle: implications for antifolate chemotherapy. Mol Biochem Parasitol 113:199–213

Das BB, Sengupta T, Ganguly A, Majumder HK (2006) Topoisomerases of kinetoplastid parasites: why so fascinating? Mol Microbiol 62:917–927

David CV, Craft N (2009) Cutaneous and mucocutaneous leishmaniasis. Dermatol Ther 22:491–502

de Oliveira CI, Brodskyn CI (2012) The immunobiology of *Leishmania* braziliensis infection. Front Immunol 3:145

Esfandiarpour I, Dabiri SH (2007) Treatment of cutaneous leishmaniasis recidivans with a combination of allopurinol and meglumine antimoniate: a clinical and histologic study. Int J Dermatol 46:848–852

Expert Committee, Control of the leishmaniases. WHO Technical Report Series 949. World Health Organization, Geneva, Switzerland, 2010, pp 1–186

Fairlamb AH, Blackburn P, Ulrich P, Chait BT, Cerami A (1985) Trypanothione: a novel bis(glutathionyl)spermidine cofactor for glutathione reductase in trypanosomatids. Science 227:1485–1487

Fidalgo LM, Gille L (2011) Mitochondria and trypanosomatids: targets and drugs. Pharm Res 28:2758–2770

Firooz A, Khamesipour A, Ghoorchi MH, Nassiri-Kashani M, Eskandari SE, Khatami A et al (2006) Imiquimod in combination with meglumine antimoniate for cutaneous leishmaniasis: a randomized assessor-blind controlled trial. Arch Dermatol 142:1575–1579

Garcia Bustos MF, Barrio AB, Parodi Ramoneda CM, Ramos F, Mora MC, Convit J et al (2011) Immunological correlates of cure in the first American cutaneous leishmaniasis patient treated by immunotherapy in Argentina. A case report. Invest Clin 52:365–375

Gonzalez U, Pinart M, Reveiz L, Alvar J (2008) Interventions for Old World cutaneous leishmaniasis. Cochrane Database Syst Rev (4):CD005067

Gonzalez U, Pinart M, Rengifo-Pardo M, Macaya A, Alvar J, Tweed JA (2009) Interventions for American cutaneous and mucocutaneous leishmaniasis. Cochrane Database Syst Rev:CD004834

Goto H, Lauletta Lindoso JA (2012) Cutaneous and mucocutaneous leishmaniasis. Infect Dis Clin North Am 26:293–307

Hartley MA, Kohl K, Ronet C, Fasel N (2013) The therapeutic potential of immune cross-talk in leishmaniasis. Clin Microbiol Infect 19:119–130

Hellier I, Dereure O, Tournillac I, Pratlong F, Guillot B, Dedet JP et al (2000) Treatment of Old World cutaneous leishmaniasis by pentamidine isethionate. An open study of 11 patients. Dermatology 200:120–123

Hervas JA, Martin-Santiago A, Hervas D, Rojo E, Mena A, Rocamora V et al (2012) Old world *Leishmania infantum* cutaneous leishmaniasis unresponsive to liposomal amphotericin B treated with topical imiquimod. Pediatr Infect Dis J 31:97–100

Jha TK, Sundar S, Thakur CP, Bachmann P, Karbwang J, Fischer C et al (1999) Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. N Engl J Med 341:1795–1800

Krauth-Siegel RL, Inhoff O (2003) Parasite-specific trypanothione reductase as a drug target molecule. Parasitol Res 90(suppl 2):S77–S85

Krylov DM, Koonin EV (2001) A novel family of predicted retroviral-like aspartyl proteases with a possible key role in eukaryotic cell cycle control. Curr Biol 11:R584–R587

Kulkarni MM, Reddy N, Gude T, McGwire BS (2013) Voriconazole suppresses the growth of *Leishmania* species in vitro. Parasitol Res 112:2095–2099

Lessa HA, Machado P, Lima F, Cruz AA, Bacellar O, Guerreiro J et al (2001) Successful treatment of refractory mucosal leishmaniasis with pentoxifylline plus antimony. Am J Trop Med Hyg 65:87–89

Liese J, Schleicher U, Bogdan C (2008) The innate immune response against *Leishmania* parasites. Immunobiology 213:377–387

Mahmoudzadeh-Niknam H, McKerrow JH (2004) *Leishmania tropica*: cysteine proteases are essential for growth and pathogenicity. Exp Parasitol 106:158–163

Mannion-Henderson J, Flaspohler JA, Lemley KR, Rickoll WL, Parsons M (2000) Isolation and characterization of *Leishmania* mutants defective in glycosomal protein import. Mol Biochem Parasitol 106:225–237

Marovich MA, Lira R, Shepard M, Fuchs GH, Kruetzer R, Nutman TB et al (2001) Leishmaniasis recidivans recurrence after 43 years: a clinical and immunologic report after successful treatment. Clin Infect Dis 33:1076–1079

Marrapu VK, Srinivas N, Mittal M, Shakya N, Gupta S, Bhandari K (2011) Design and synthesis of novel tetrahydronaphthyl azoles and related cyclohexyl azoles as antileishmanial agents. Bioorg Med Chem Lett 21:1407–1410

Miranda-Verastegui C, Llanos-Cuentas A, Arevalo I, Ward BJ, Matlashewski G (2005) Randomized, double-blind clinical trial of topical imiquimod 5% with parenteral meglumine antimoniate in the treatment of cutaneous leishmaniasis in Peru. Clin Infect Dis 40:1395–1403

Motta MC (2008) Kinetoplast as a potential chemotherapeutic target of trypanosomatids. Curr Pharm Des 14:847–854

Negera E, Gadisa E, Hussein J, Engers H, Kuru T, Gedamu L et al (2012) Treatment response of cutaneous leishmaniasis due to *Leishmania aethiopica* to cryotherapy and generic sodium stibogluconate from patients in Silti, Ethiopia. Trans R Soc Trop Med Hyg 106:496–503

Okwor I, Uzonna JE (2013) The immunology of *Leishmania*/HIV co-infection. Immunol Res 56:163–171

Oliveira-Neto MP, Mattos M, Souza CS, Fernandes O, Pirmez C (1998) Leishmaniasis recidiva cutis in New World cutaneous leishmaniasis. Int J Dermatol 37:846–849

Olliaro PL (2010) Drug combinations for visceral leishmaniasis. Curr Opin Infect Dis 23:595–602

Paniz Mondolfi AE, Stavropoulos C, Gelanew T, Loucas E, Perez Alvarez AM, Benaim G et al (2011) Successful treatment of Old World cutaneous leishmaniasis caused by Leishmania infantum with posaconazole. Antimicrob Agents Chemother 55:1774–1776

Plewes KA, Barr SD, Gedamu L (2003) Iron superoxide dismutases targeted to the glycosomes of *Leishmania chagasi* are important for survival. Infect Immun 71:5910–5920

Prasad N, Ghiya BC, Bumb RA, Kaushal H, Saboskar AA, Lezama-Davila CM et al (2011) Heat, oriental sore, and HIV. Lancet 377:610

Reithinger R, Mohsen M, Wahid M, Bismullah M, Quinnell RJ, Davies CR et al (2005) Efficacy of thermotherapy to treat cutaneous leishmaniasis caused by *Leishmania tropica* in Kabul, Afghanistan: a randomized, controlled trial. Clin Infect Dis 40:1148–1155

Santos LO, Garcia-Gomes AS, Catanho M, Sodre CL, Santos AL, Branquinha MH et al (2013) Aspartic peptidases of human pathogenic trypanosomatids: perspectives and trends for chemotherapy. Curr Med Chem 20:3116–3133

Shakya N, Sane SA, Gupta S (2011) Antileishmanial efficacy of fluconazole and miltefosine in combination with an immunomodulator—picroliv. Parasitol Res 108:793–800

Silva-Jardim I, Horta MF, Ramalho-Pinto FJ (2004) The *Leishmania chagasi* proteasome: role in promastigotes growth and amastigotes survival within murine macrophages. Acta Trop 91:121–130

Silva-Lopez RE, Morgado-Diaz JA, Chavez MA, Giovanni-De-Simone S (2007) Effects of serine protease inhibitors on viability and morphology of *Leishmania* (*Leishmania*) amazonensis promastigotes. Parasitol Res 101:1627–1635

Soto J, Arana BA, Toledo J, Rizzo N, Vega JC, Diaz A et al (2004) Miltefosine for new world cutaneous leishmaniasis. Clin Infect Dis 38:1266–1272

Soto J, Toledo J, Valda L, Balderrama M, Rea I, Parra R et al (2007) Treatment of Bolivian mucosal leishmaniasis with miltefosine. Clin Infect Dis 44:350–356

Soto J, Rea J, Balderrama M, Toledo J, Soto P, Valda L et al (2008) Efficacy of miltefosine for Bolivian cutaneous leishmaniasis. Am J Trop Med Hyg 78:210–211

Soto J, Rea J, Valderrama M, Toledo J, Valda L, Ardiles J et al (2009) Efficacy of extended (six weeks) treatment with miltefosine for mucosal leishmaniasis in Bolivia. Am J Trop Med Hyg 81:387–389

Sundar S, Jha TK, Thakur CP, Bhattacharya SK, Rai M (2006) Oral miltefosine for the treatment of Indian visceral leishmaniasis. Trans R Soc Trop Med Hyg 100(suppl 1):S26–S33

Sundar S, Sinha PK, Rai M, Verma DK, Nawin K, Alam S et al (2011) Comparison of short-course multidrug treatment with standard therapy for visceral leishmaniasis in India: an open-label, non-inferiority, randomised controlled trial. Lancet 377:477–486

Sundar S, Sinha P, Jha TK, Chakravarty J, Rai M, Kumar N et al (2013) Oral miltefosine for Indian post-kala-azar dermal leishmaniasis: a randomised trial. Trop Med Int Health 18:96–100

ter Horst R, Collin SM, Ritmeijer K, Bogale A, Davidson RN (2008) Concordant HIV infection and visceral leishmaniasis in Ethiopia: the influence of antiretroviral treatment and other factors on outcome. Clin Infect Dis 46:1702–1709

Ueno N, Wilson ME (2012) Receptor-mediated phagocytosis of *Leishmania*: implications for intracellular survival. Trends Parasitol 28:335–344

van Griensven J, Diro E, Lopez-Velez R, Boelaert M, Lynen L, Zijlstra E et al (2013) HIV-1 protease inhibitors for treatment of visceral leishmaniasis in HIV-co-infected individuals. Lancet Infect Dis 13:251–259

Williams RA, Tetley L, Mottram JC, Coombs GH (2006) Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*. Mol Microbiol 61:655–674

Zijlstra EE, Musa AM, Khalil EA, el-Hassan IM, el-Hassan AM (2003) Post-kala-azar dermal leishmaniasis. Lancet Infect Dis 3:87–98

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