Parasite and host determinants of visceral leishmaniasis

by

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A ma famille.

Abstract

Leishmaniasis is a neglected tropical disease caused by *Leishmania* protozoa and transmitted by a sand fly vector. There are three main disease manifestations: self-healing but scarring cutaneous leishmaniasis, mucocutaneous leishmaniasis with destruction of the mucosal tissues in the nose, mouth and throat, and visceral leishmaniasis in which parasites disseminate to the bone marrow, liver and spleen, leading to high fever, hepatosplenomegaly, wasting, and death in the absence of treatment. Visceral leishmaniasis is the second most lethal tropical disease after malaria. A key question of leishmaniasis research is why some *Leishmania* species such as *Leishmania major* remain at the site of the sand fly bite in cutaneous leishmaniasis while other species such as *Leishmania donovani* metastasize to visceral organs. The overall goal of this thesis was to identify factors involved in visceral disease pathogenesis. We hypothesized that parasite factors play a determining role in visceral disease and that host characteristics are also involved.

First, we examined the function of A2, a protein family already implicated in *L. donovani* visceralization. A2 is shown to protect against heat shock and oxidative stress, key host defences against visceral leishmaniasis. Second, we studied an atypical *L. donovani* clinical isolate from Sri Lanka that causes cutaneous rather than visceral leishmaniasis, comparing it to a clinical isolate from a Sri Lankan visceral leishmaniasis patient. Although both strains were equally infective to macrophages *in vitro,* they caused significantly different

disease phenotypes *in vivo* in mice: only the cutaneous isolate caused footpad swelling while only the visceral isolate led to significant liver and spleen parasitemia. A2 expression was lower in the cutaneous isolate and ectopically expressing an additional A2 gene in the cutaneous isolate partially restored virulence in the visceral organs. Therefore, parasite factors are a key determinant of visceral disease.

However, host characteristics and history can also influence the development of visceral leishmaniasis. In Sri Lanka, cutaneous leishmaniasis caused by *L. donovani* is frequent while visceral disease is rare. We show here that immunization with a cutaneous clinical isolate is associated with a mixed Th1/Th2 response and protects BALB/c mice from visceral leishmaniasis, providing a possible rationale for the low incidence of visceral leishmaniasis in Sri Lanka.

Overall, these results represent significant progress into the determinants of visceral leishmaniasis and in particular on the role of the virulence factor A2. A novel candidate for a live-attenuated vaccine against visceral leishmaniasis is also presented here. Given the lack of a human vaccine for leishmaniasis and the limitations of the current treatment options, this work could have a significant impact on disease management in Sri Lanka and on vaccine development.

Résumé

Les leishmanioses sont un groupe de maladies tropicales causées par le protozoaire *Leishmania* et transmises par la piqûre de phlébotomes. Les leishmanioses peuvent être divisées en trois formes cliniques: leishmaniose cutanée qui guérit sans traitement mais laisse des cicatrices, leishmaniose mucocutanée avec destruction des muqueuses du nez, de la bouche et de la gorge, et leishmaniose viscérale où les parasites quittent le site de la piqûre et se propagent jusqu'à la moelle osseuse, le foie et la rate. Les symptômes de la leishmaniose viscérale sont une forte fièvre et une hepatosplénomégalie, et ceci peut être fatal en l'absence de traitement. La leishmaniose viscérale a le deuxième plus haut taux de mortalité parmi les maladies tropicales, après la malaria. Un des enjeux majeurs de la recherche sur les leishmanioses est de comprendre pourquoi certaines espèces de *Leishmania* comme *Leishmania major* restent au site de la piqûre des phlébotomes dans le cas des leishmanioses cutanées, tandis que *Leishmania donovani* se propage jusqu'aux organes viscéraux. Le but de cette thèse est d'identifier les facteurs impliqués dans la pathogénèse de la leishmaniose viscérale. L'hypothèse de recherche est que les caractéristiques du parasite jouent un rôle principal dans la leishmaniose viscérale et que les caractéristiques de l'hôte sont également importantes.

Nous examinons la fonction de A2, une famille de protéines qui sont impliquées dans la viscéralisation de *L. donovani*. Nous montrons que A2 protège contre le choc thermique et contre les oxydants, deux défenses clé du système

immunitaire contre la leishmaniose viscérale. Ensuite, nous étudions un isolat clinique atypique de *L. donovani* venu du Sri Lanka qui cause des leishmanioses cutanées au lieu de provoquer des leishmanioses viscérales. Nous comparons cet isolat à un isolat clinique provenant d'un patient sri lankais souffrant de leishmaniose viscérale. Quoique ces deux isolats soient tout aussi infectieux l'un que l'autre *in vitro,* ils causent des symptômes différents *in vivo*: seul l'isolat cutané cause l'enflure du coussinet plantaire après injection sous-cutanée chez les souris et seul l'isolat viscéral peut se multiplier dans le foie et la rate. Les niveaux de A2 sont plus bas dans l'isolat cutané et nous démontrons que cela constitue un facteur déterminant de son atténuation. Ces résultats prouvent donc que les caractéristiques du parasite ont une influence majeure dans la pathogénèse des leishmanioses viscérales.

Cependant, les caractéristiques de l'hôte et son historique médical peuvent également influencer le développement de cette maladie. Les leishmanioses cutanées sont beaucoup plus fréquentes au Sri Lanka que les leishmanioses viscérales. Nous prouvons ici que la vaccination avec l'isolat cutané mène à une réponse du système immunitaire de type Th1/Th2 mixte et protège contre la leishmaniose viscérale dans un modèle *in vivo* d'infection de souris BALB/c. Ces résultats proposent un modèle pour expliquer la rareté de la leishmaniose viscérale au Sri Lanka.

En conclusion, ces résultats éclaircissent plusieurs facteurs impliqués dans le développement de la leishmaniose viscérale et en particulier le rôle du facteur de virulence A2. Nous présentons également un nouveau candidat de vaccin

atténué contre la leishmaniose viscérale. Etant donné l'absence de vaccin humain pour cette maladie et les limites des traitements actuels, cette étude pourrait avoir un impact majeur sur la santé publique au Sri Lanka et sur le développement de vaccins contre la leishmaniose viscérale.

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Contributions to original knowledge

The work presented in this thesis contributes to original knowledge in the field of *Leishmania* pathogenesis and vaccine design as follows:

1. We showed that the *L. donovani* virulence factor A2 is not merely amastigote-specific as was previously thought, but can also be induced in promastigotes by stress.

2. We determined the localization of A2 and showed that it interacts with a key endoplasmic reticulum chaperone, BiP.

3. We generated the first insight into the function of A2, showing that it protects the parasite from heat shock and oxidative stress. These results provide a rationale to explain the previously-described essential role of A2 in parasite visceralization and help to explain the dichotomy between cutaneous and visceral *Leishmania* species.

4. We compared clinical isolates from Sri Lanka and showed that the main determinant of cutaneous *versus* visceral disease phenotype in Sri Lanka is the characteristics of the parasite strains involved, rather than host genetic background.

5. We showed that decreased A2 expression in the cutaneous Sri Lanka isolate is a major determinant of its attenuated virulence.

6. We showed that immunization with the cutaneous isolate from Sri Lanka protects against visceral leishmaniasis. This result provides a rationale for the low levels of visceral leishmaniasis observed in Sri Lanka.

7. Protection by subcutaneous immunization with the cutaneous isolate from Sri Lanka was associated with a mixed Th1/Th2 response prior to challenge infection.

Contributions of authors

As indicated by the Faculty of Graduate and Postdoctoral Studies of McGill University, the candidate has chosen to present a manuscript-based thesis. This thesis comprises four manuscripts and is in accordance with the "Guidelines for Thesis Preparation". LI McCall is recognized as the principal author and to have performed the majority of the work of the manuscripts presented; the specific contributions of authors are as follows:

Chapter 1:

Contains extracts from **McCall LI,** Zhang WW, Matlashewski G (2012) Determinants for the development of visceral leishmaniasis disease. PLoS Pathogens 9 (1): e1003053.

The percentage contributions of authors in the preparation of the final manuscript were as follows: L-I McCall (70%), WW Zhang (10%), G Matlashewski (20%).

Chapter 2:

McCall LI, Matlashewski G (2010) Localization and induction of the A2 virulence factor in Leishmania: evidence that A2 is a stress response protein. Molecular Microbiology 77: 518-530.

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Table of contents

List of tables and figures

List of abbreviations

A2	Amastigote protein 2
A2rel	A2-related gene
AIDS	Acquired immunodeficiency syndrome
AP1	Activating protein 1
ATP	Adenosine triphosphate
AZC	Azetidine-2-carboxylic acid
BCG	Bacillus Calmette-Guérin
BiP	Binding protein
CD	Cluster of differentiation
Cox2	Cyclooxygenase 2
CPB	Cysteine proteinase B
DALYs	Disability-adjusted life years
DCFDA	Dichlorofluorescein diacetate
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FML	Fucose-mannose ligand
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gp	Glycoprotein
GPI	Glycosylphosphatidylinositol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JAK/STAT	Janus kinase / Signal transducer and activator of transcription
kb	Kilobase
kDa	Kilodalton
KMP11	Kinetoplastid membrane protein 11
KSAC	KMP11+SMT+A2+CPB fusion protein
LeIF	*Leishmania* elongation initiation factor
L. d.	*Leishmania donovani*
L. d. – A2	*Leishmania donovani* transfected with the antisense construct
L. d. + C	*Leishmania donovani* plus KSneo control plasmid
LDU	Leishman-Donovan Units
L. m.	*Leishmania major*
L. m. – A2	*Leishmania major* plus KSneo A2 plasmid
L. m. + C	*Leishmania major* plus KSneo control plasmid
LmSTI1	*L. major* stress-inducible 1
LPG	Lipophosphoglycan

LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
MHC	Major histocompatibility complex
MPL-SE	Monophosphoryl lipid A in stable emulsion
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor κB
NRAMP1	Natural resistance-associated macrophage protein 1
NO	Nitric oxide
OD	Optical density
ORF	Open reading frame
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PKDL	Post-kala azar dermal leishmaniasis
PSA	Parasite surface antigen
PTP1B	Protein tyrosine phosphatase 1B
ROS	Reactive oxygen species
RNA	Ribonucleic acid
rpm	Rotations per minute
SAcP	Secreted acid phosphatase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIDER1	Short interspersed degenerated retroposons
SLA	Soluble *Leishmania* antigen
SMT	Sterol 24-*c*-methyltransferase
SNAP	*S*-nitroso-*N*-acetylpenicillamine
SNP	Single nucleotide polymorphism
TCPTP	T cell protein tyrosine phosphatase
TGFβ	Tumor growth factor β
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
SHP1	SH2-containing protein tyrosine phosphatase-1
SLA	Soluble Leishmania antigen
SL-CL	Sri Lankan cutaneous clinical isolate
SL-VL	Sri Lankan visceral clinical isolate
TSA	Thiol-specific antioxidant antigen
UPR	Unfolded protein response
UTR	Untranslated region
WHO	World Health Organization
WT	Wild-type

Chapter 1

Review of literature and objectives of research

1.1 *Leishmania* and leishmaniasis: general considerations

Leishmania (order Kinetoplastida, family Trypanosomatidae) are vector-borne protozoan parasites. Out of the approximately thirty *Leishmania* species, twenty are human pathogens, the causative agents of human leishmaniasis in the Old World (Europe, Asia and Africa) and the New World (Americas).

Leishmania are divided into two subgenera, the subgenus *Leishmania*, found in both Old and New World, and the subgenus *Viannia,* exclusively found in South America. Subgenera are divided into species complexes and these are further divided into species and strains (**Figure 1.1**). Nomenclature is still controversial, with some authors for instance classifying *L. infantum* as a subspecies of *L. donovani.* Parasite strain identification is commonly performed by examining isoenzyme migration patterns via electrophoresis. Parasites with similar migration patterns are classified into the same "zymodeme" [1].

Two theories of *Leishmania* evolution have been proposed, one that suggests an African origin [2] and another one postulating a South American origin for the *Leishmania* ancestor [3]. The separation between Old and New World *Leishmania* species occurred 40-80 million years ago [4] and Old World species such as *L. major* and *L. donovani* then diverged approximately 14-24 million years ago [3]. Overall, Old World species such as *L. donovani* and *L. major* are therefore more closely related to each other than they are to New World

species such as *L. mexicana*, even though both *L. major* and *L. mexicana* cause cutaneous leishmaniasis. A more recent development is the introduction of *L. infantum* into South America 500 years ago by the conquistadors [5]. New World *L. infantum* is often referred to as *L. chagasi.*

1.1.1 The *Leishmania* lifecycle

Leishmania parasites have a dimorphic lifecycle divided between the promastigote stage in the sand fly vector and the amastigote stage in mammalian hosts. The flagellated promastigote stage is transmitted to the mammalian host by the bite of an infected sand fly (genus *Phlebotomus* or *Lutzomyia*) during a bloodmeal [1]. Neutrophils are the first cells recruited to the site of the bite and take up promastigotes by phagocytosis. Parasites are then taken up by dendritic cells and macrophages, either via phagocytosis of free parasites or of infected neutrophils [6]. Macrophages represent the main host cell for *Leishmania* parasites. Within the macrophage phagolysosome, increased temperature and decreased pH induce promastigote to amastigote differentiation. Amastigotes can then infect other macrophages or be taken up by a feeding sand fly. In the sand fly gut, amastigotes differentiate into procyclic (non-infective) promastigotes. Promastigotes mature as they migrate anteriorally in the sand fly gut, leading to metacyclic (infective) promastigote accumulation at the junction of midgut and foregut and in the proboscis, thereby completing the parasite lifecycle (**Figure 1.2**) [1,7]. Transmission can occur either via an anthroponotic cycle (*L. donovani* and *L. tropica*), where humans are the only reservoir, or via a zoonotic cycle (all

other *Leishmania* species), where transmission occurs from an animal reservoir to humans [1].

The *Leishmania* lifecycle can be fully reproduced *in vitro* for most *Leishmania* species: promastigotes can be cultured *in vitro* at 26ºC and pH 7 [8] and promastigote to amastigote differentiation is induced by transferring promastigotes to conditions that mimic the macrophage phagolysosome (33-37ºC and acidic pH around 5.5). In general, the cooler differentiation temperatures are used for cutaneous species to mimic skin temperatures, while 37ºC is used for visceral species. Changes in both temperature and pH are required to induce promastigote to amastigote differentiation: increased temperatures lead to growth arrest, which is released by decreasing the pH [9]. A promastigote to amastigote differentiation system was recently developed for *L. major* by incubating cells for 10-14 days at 33ºC and pH 5.5 and then separating amastigotes from promastigotes by a discontinuous density gradient [10].

Promastigote to amastigote differentiation is especially key to human disease and has therefore been studied in detail in axenic systems. Four phases have been identified, with the complete differentiation process taking 5 days. From 0-5 hours, parasites sense the change in the environment and arrest in G1 phase; no morphological changes occur. From 5-10 hours, promastigotes aggregate and motion ceases. Morphological changes occur from 10-24 hours, leading to amastigote-shaped cells. Finally, cells mature from 24-120 hours [9].

The differentiation process leads to significant changes in parasite metabolism. For instance, promastigotes use glucose as their main carbon source,

while amastigotes use fatty acids and amino acid catabolism as a source of carbon and energy. Amastigotes produce glucose via gluconeogenesis. These changes allow the cells to adapt to the phagolysosome environment where sugars are limiting. Amastigotes also upregulate antioxidant enzymes and downregulate protein translation [9].

Some authors have reported a number of differences between axenic and mammalian-derived amastigotes [11]. However, protein expression was over 92% identical between axenic amastigotes and intracellular amastigotes [9] and they have similar morphology and metabolism. They also share a number of markers such as the A2 protein family [12,13] (see **section 1.4.1**).

1.1.2 Leishmaniasis: spectrum of disease

Leishmaniasis is associated with a spectrum of disease depending on the infecting species of *Leishmania* (**Table 1.1** and **Figure 1.3**). The three main forms of disease are cutaneous, mucocutaneous and visceral leishmaniasis [14]. Cutaneous leishmaniasis is associated with parasite replication and formation of lesions at the site of sand fly bite days to months after the initial parasite inoculation. Lesions usually progress from papules to nodules and then to wet ulcers with a typical "volcano"appearance, but can also be non-ulcerative. Cutaneous leishmaniasis usually self-heals, albeit slowly (3 months to 3 years depending on the causative species). Cutaneous leishmaniasis is mainly caused by *L. major* and *L. tropica* in the Old World and by parasites from the *L. mexicana* and *L. braziliensis* species complexes in the New World. Rarer clinical

manifestations include disseminated cutaneous leishmaniasis (multiple non-contiguous pleomorphic lesions), diffuse cutaneous leishmaniasis (local spread of non-ulcerative lesions) and leishmaniasis recidivans (centrally-healing lesions with lateral spread) [15].

The second main form of leishmaniasis is mucocutaneous disease. Mucocutaneous leishmaniasis develops years after an initial cutaneous infection and is caused by parasite metastasis to facial mucosal tissues [16]. Clinical symptoms include ulceration of the nose and mouth, followed by perforation of the nasal septum, and destruction of mucosal tissues and cartilage in the nose, mouth and throat, leading to severe facial disfigurement [17]. Death is rare and is most often due to secondary bacterial infections [16]. *L. braziliensis* is the main causative agent of mucocutaneous leishmaniasis, but *L. amazonensis* as well as members of the *Viannia* subgenus such as *L. panamensis* and *L. guyanensis* can also cause mucocutaneous leishmaniasis [17]. The capacity of *L. guyanensis* to cause mucocutaneous rather than cutaneous disease has been tied to infection of the parasite by an RNA virus, leading to an exacerbated immune response, but it is still unclear whether this is applicable to other members of the *Viannia* subgenus [18].

Finally, the most severe form of disease is visceral leishmaniasis, also known as kala azar, in which parasites disseminate to the liver, spleen and bone marrow. Symptoms include high fever, hepatosplenomegaly, cachexia, hypergammaglobulinemia and pancytopenia, and the disease is fatal if untreated. Visceral leishmaniasis is caused by *L. donovani, L. infantum* and *L. chagasi*.

Successful treatment and resolution of visceral leishmaniasis may be followed by post-kala azar dermal leishmaniasis (PKDL) occurrences. PKDL is associated with non-ulcerative papules or nodules and is more common in East Africa than in India [1].

1.1.3 Epidemiology

Leishmaniasis is found in both the Old and the New world, mainly in tropical and subtropical regions of the world (**Figure 1.4**) [19], but animal cases of leishmaniasis have been detected as far north as Canada [20] and as far south as Australia [21]. Seventy-five percent of cutaneous leishmaniasis cases occur in Afghanistan, Iran, Syria, Algeria, Ethiopia, Sudan, Brazil, Peru, Colombia, and Costa Rica [22]. Ninety percent of visceral leishmaniasis occurs in India, Nepal, Bangladesh, Sudan, Ethiopia and Brazil [22]. Most mucocutaneous cases are found in Brazil, Bolivia and Peru [16].

Overall leishmaniasis disease burden exceeds two million disability-adjusted life years (DALYs) [23]. Disease prevalence is estimated at 12 million cases, with 350 million people at risk in 88 countries [24]. Incidence is 1.6 million new cases per year, with 0.7 to 1.2 million cutaneous leishmaniasis cases and 200,000 to 400,000 visceral leishmaniasis cases [22]. Mucocutaneous incidence is estimated at 5% of cutaneous leishmaniasis cases in the Americas [25]. Leishmaniasis mortality is approximately 20,000 to 40,000 deaths per year [22], ranking leishmaniasis second in terms of mortality and fourth in terms of morbidity among tropical diseases [23], with the ninth-highest disease burden for

infectious diseases [26]. However, due to significant underreporting, actual incidence and mortality values may be higher [22].

1.1.4 Treatment

1.1.4.1 Standard treatment regimens

Cutaneous leishmaniasis is usually self-healing and may not always require treatment [16]. However, local treatments such intralesional injections of pentavalent antimonials, paromomycin ointments, thermotherapy and cryotherapy as well as systemic treatments (injections of pentavalent antimonials, pentamidine or amphotericin B, or oral doses of miltefosine) can accelerate cure [16]. Mucocutaneous leishmaniasis is treated with injections of pentavalent antimonials or amphotericin B, or with oral courses of miltefosine [16].

For visceral leishmaniasis, treatment recommendations differ depending on location. In India, Nepal and Bangladesh, the preferred treatment for visceral leishmaniasis caused by *L. donovani*, as recommended by the World Health Organization, is liposomal amphotericin B (AmBisome) injections [16]. Single doses of this formulation are sufficient to cure visceral leishmaniasis [27]. Amphotericin B can also be administered in combination with oral miltefosine or intramuscular paromomycin. Combination treatment of miltefosine and paromomycin, as well as treatment with either one individually are also suitable. The last treatment option would be injections of pentavalent antimonials (sodium stibogluconate or meglumine antimoniate), although there is resistance in many regions. Similarly, *L. infantum* visceral leishmaniasis cases in South America and

around the Mediterranean are preferentially treated with liposomal amphotericin B [16]. In contrast, in East Africa, the treatment of choice is still pentavalent antimonials, preferably in combination with paromomycin, although treatment with miltefosine can also be used. PKDL is treated with amphotericin B, pentavalent antimonials or miltefosine [16].

1.1.4.2 Treatment limitations

Many of these treatment regimens are long (pentavalent antimonial or amphotericin B injections courses for up to a month, paromomycin injections for 21 days, miltefosine treatment for 28 days) and associated with significant side effects. The most severe side effects are reported for pentavalent antimonials, pentamidine and amphotericin B. Pentamidine side effects are so severe that they limit its use: diabetes, myotoxicity and nephrotoxicity have been reported. Pentavalent antimonials are also very toxic, leading to nausea, vomiting, muscle and joint pain [16], cardiotoxicity and renal damage [28]. Common amphotericin B side effects include fever, nephrotoxicity and rarely myocarditis. However, these effects are significantly mitigated by the use of lipid formulations which only cause mild reactions. Paromomycin side effects are milder, limited to reversible ear damage, mild pain and rare cases of nephrotoxicity and hepatotoxicity. Miltefosine side effects are also minor (nausea, vomiting, diarrhea), but it is a teratogen and therefore unsuitable for the treatment of pregnant women or women of child-bearing age [16].

Treatments themselves are expensive (as of 2010, $7/patient for paromomycin, $65 for miltefosine [16]). Although preferential pricing agreements exist between the World Health Organization and pharmaceutical companies, and generic versions of existing drugs are being developed [29], long-term treatment regimens are associated with additional costs beyond the drugs themselves, such as food, travel, hospital accommodation and lost wages. For example, the overall cost of a 30-day amphotericin B treatment course in Bihar has been estimated at $372.66 to $719.45 [30]. Even generic pentavalent antimonials can cost $13 per patient [31]. This is significant in an area where daily wages may be lower than $1 [32]. Similarly, in Nepal and Bangladesh, treatment costs were equivalent to almost a year's income [32].

In addition to costs and side effects, the emergence of drug resistance is a significant concern for leishmaniasis treatment. Different *Leishmania* species have different intrinsic susceptibility to miltefosine [33]. Similarly, AmBisome treatment seems to be less effective in Africa [29]. Acquired clinical drug resistance is also a major concern. For instance, resistance to pentavalent antimonials appeared in Bihar (India) in the 1970s and since then, treatment failure has been observed in over 60% of patients there [31], may be spreading to Nepal [34] and has been reported in Sudan [35]. Resistance to pentamidine has increased over time in India [36] and susceptibility to miltefosine may be decreasing [37]. Rare clinical resistance to amphotericin B has also been reported [38]. Finally, many anti-*Leishmania* drugs such as pentavalent antimonials, miltefosine and amphotericin B induce a common mechanism of reactive oxygen

species-associated apoptosis. Cross-tolerance to drug-induced apoptosis was observed in the laboratory [39] and increased resistance to amphotericin B and miltefosine has been observed in regions with high resistance to pentavalent antimonials [40].

Drug combinations, such as the WHO-recommended liposomal amphotericin B plus miltefosine, liposomal amphotericin B plus paromomycin, or miltefosine plus paromomycin [41], can limit the emergence of drug resistance but would increase costs. Single-dose regimens of amphotericin B in India would increase treatment compliance, decrease costs and decrease risks of resistance [27]. However, given the limitations of existing treatments, the severity of this disease, and the risk of drug resistance, the identification of new treatment options is required. A better understanding of the factors that mediate visceral disease may lead to the discovery of novel virulence factors that can be used to develop new drug targets.

1.2 Development of visceral leishmaniasis

1.2.1 Navigating from the skin to the viscera

The sand fly vector lacerates blood vessels during feeding, so parasites are introduced intradermally into a pool of blood [42]. Free amastigotes have been detected in the bloodstream and could be directly delivered to blood-filtering organs [43]. Alternatively, the eventual spread to visceral organs could involve the movement of infected cells. Neutrophils are the earliest cells recruited to the site of the sand fly bite [44,45] and represent the first infected cell population [6].

Infected neutrophils or free parasites are then taken up by dendritic cells and macrophages that migrate away from the site of the bite [6]. The dermal dendritic cell population infected at early time points may differ between *L. major* and *L. donovani* [44] and there is evidence that *L. donovani*-infected macrophages and dendritic cells leave intradermal injection sites in higher numbers than *L. major*-infected cells [46]. However, the route used by infected cells to eventually reach the visceral organs remains poorly understood.

Experimental subcutaneous needle infection with *L. major* in BALB/c mice is associated with parasite proliferation and lesion development at the site of injection, as well as parasite dissemination to visceral organs [47]. Dissemination of *L. major* to the visceral organs does not occur in C57BL/6 mice, indicating an important role for the host immune response in the control of visceralization [47]. In comparison, subcutaneous injection of *L. donovani* in BALB/c mice only causes minimal swelling at the site of injection and no dissemination to the viscera [48]. Therefore, these subcutaneous infection models do not accurately reflect the situation in humans where *L. donovani*, but not *L. major*, metastasizes to the visceral organs.

Intradermal infection models have been developed in BALB/c mice [49] and in hamsters [50], in which parasites are cleared from the skin and disseminate to the visceral organs. However, for both models, the inoculation dose is one hundred- to a thousand-fold higher than the natural sand fly inoculum [51]. Nevertheless, these models more closely mimic natural infection routes for

visceral disease and could provide better insight into the cells and pathways involved in reaching the visceral organs.

1.2.2 Surviving stress: parasite proliferation in the viscera

Experimental intravenous injection of *Leishmania* in mice bypasses the normal requirement for the parasite to transit from skin to viscera and therefore focuses exclusively on parasite survival within the liver and spleen. However, although intravenous infection models of BALB/c mice are commonly used to study visceral leishmaniasis, these do not fully reflect human visceral leishmaniasis progression. In mice, infection in the liver is self-resolving while spleen infection is progressive and overall infection is asymptomatic [52]. In contrast, infection of hamsters is associated with symptomatic disease and can be fatal [53].

Nevertheless, intravenous infection with cutaneous species such as *L. major* is associated with limited liver and spleen parasite burden while intravenous infection with viscerotropic *L. donovani* and *L. infantum* results in high levels of visceral infection [54]. Therefore, regardless of the mechanism to exit the skin, cutaneous and visceral species differ dramatically: visceral species are much better adapted to survive and proliferate in visceral organs than cutaneous species.

Temperatures within infected mouse footpad dermis vary between 28 and 32°C [55], whereas fever in visceral leishmaniasis exceeds 40°C [56]. Viscerotropic parasites must therefore withstand higher temperatures than

cutaneous ones, and indeed, promastigotes from cutaneous species are considerably more sensitive to heat shock than promastigotes of visceral species [57,58]. In addition, transfection of some *L. donovani* genes into *L. major* enhanced *L. major* survival at higher temperatures and visceralization [54,57,59].

Fever itself can also augment the immune response by increasing dendritic cell and neutrophil migration, pro-inflammatory cytokine production and Th1 cell activity [60]. Given that fever increases oxidant production by phagocytic cells [60], viscerotropic species are expected to be more resistant to oxidants than cutaneous species: *L. donovani* is more resistant to nitric oxide (NO) and hydrogen peroxide than *L. major* [61].

The host macrophage population targeted by *Leishmania* also differs between cutaneous and visceral species: cutaneous species infect inflammatory monocyte-derived macrophages and dendritic cells [6,62] while visceral species infect Kupffer cells, spleen macrophages and bone marrow macrophages [63]. These different macrophage populations express different levels of cell surface molecules [64] and of NRAMP1, a cation transporter associated with resistance to *Leishmania* [65]. They also differ in their response to IFNγ stimulation, and in their capacity to produce cytokines, activate T lymphocytes and kill pathogens [66,67,68]. Therefore, cutaneous and visceral species have adapted to replicate in distinct host macrophage environments. However, no direct comparisons of the susceptibility and killing potential of these different macrophage populations have been performed during *Leishmania* infection.

1.3 Vector determinants of visceral leishmaniasis

Leishmaniasis results from the interplay of vector, parasite and host. Therefore, sand fly characteristics have been postulated to play a role in the development of visceral disease. *L. chagasi* transmitted by *Lutzomyia longipalpis* sand flies can cause cutaneous or visceral leishmaniasis in parts of South America. Saliva of sand flies from a cutaneous region caused low levels of vasodilatation and promoted footpad swelling following subcutaneous infection in mice, whereas saliva of sand flies collected in a visceral region caused higher vasodilatation but did not enhance cutaneous lesion formation [69]. This suggested that higher vasodilatation promotes better parasite access to the visceral organs and that vector species may influence disease development. However, this may not be a general difference between vectors of cutaneous and visceral *Leishmania* species: sequence analysis of salivary gland proteins from a *Phlebotomus* species associated with cutaneous *L. infantum* cases in the Old World showed that its salivary proteins were more closely related to those of vectors that transmit visceral *L. donovani* and *L. infantum* than to vectors of cutaneous *L. major* and *L. tropica* [70]. Finally, the number of parasites transmitted by sand flies could also influence disease outcome. A higher infective dose may promote a stronger local immune response that limits the parasite spread, thereby preventing dissemination to the visceral organs [51]. However, many of the associations between sand fly characteristics and visceral *versus* cutaneous disease are still unconfirmed, and the role of the sand fly in disease pathology is therefore an area that requires more attention.

1.4 Parasite determinants of visceral leishmaniasis

Parasite characteristics are a major determinant of visceral disease (**Figure 1.5**). Indeed, although *L. infantum* has been associated with both cutaneous and visceral leishmaniasis, the strain involved differ between cutaneous and visceral cases [71]. The maintenance of species-specific differences following needle injection into inbred mice also indicates that parasite characteristics are important determinants of visceral disease development: *L. infantum* strains from cutaneous patients were unable to establish visceral infection following intravenous injection into BALB/c mice, unlike *L. infantum* strains from visceral patients [72].

A number of parasite characteristics may be involved in disease development, including parasite growth rates, species-specific genes and virulence factors. The impact of promastigote differentiation into the infective metacyclic stage is still controversial: some groups found no differences between cutaneous and visceral strains [73], whereas other groups found a positive correlation between the levels of metacyclics and *in vivo* virulence [74]. Similarly, increased *in vitro* infectivity to macrophages has been tied to increased *in vivo* virulence in some studies [75] but not in others [73,76]. Visceral strains have also been postulated to have a faster growth rate than cutaneous strains [1], but other studies and our own work have not validated this link [74,75].

Although visceral and cutaneous species may be equally infective to macrophages, they had different impacts on macrophage gene expression. In particular, *L. donovani* induces higher levels of Cox2 and prostaglandin E

synthase than *L. major* [77]. Both of these enzymes are part of the PGE2 biosynthetic pathway which has been associated with visceralization [78]. Spermine/spermidine N1-acyl transferase 1, a rate-limiting enzyme of polyamine metabolism, was also higher following *L. donovani* infection [77]. This may increase polyamine production and promote parasite growth [79]. Finally, several of the cellular genes induced more strongly by *L. donovani* than by *L. major* are stress proteins (HSP70, HSP72, T cell death-associated gene 51, Gadd45β), suggesting that infection with visceral disease species may be more stressful for the host macrophage [77].

L. major induced higher leukocyte recruitment than *L. donovani* in an air pouch model of infection, with increased chemokine and chemokine receptor expression in the air pouch exudate, in association with higher pro-inflammatory cytokine production [80]. *L. major* also induced higher IL12 production by dendritic cells than *L. donovani* or *L. tropica.* Higher IL12 production would promote increased T helper cell activation and parasite killing [81]. Similarly, TNFα production by infected monocytes is higher following infection with *L. major* than with *L. infantum* [82]. The higher pro-inflammatory response to *L. major* may restrict it to cutaneous sites and decrease its spread to visceral organs. In contrast, *L. donovani*-infected macrophages and dendritic cells leave the mouse dermis in higher amounts than *L. major*-infected cells, thereby promoting spread to the viscera [46].

1.4.1 *L. donovani* A2 protein and visceral disease

1.4.1.1 A2 genomic organization

The *L. donovani* A2 proteins are a major determinant of visceralization and the prototypical amastigote-specific gene [83]. A2 genes are arranged in tandem arrays on chromosome 22 [84], with up to four such arrays per diploid genome (**Figure 1.6**) [54]. A2 proteins in *L. donovani* range from 42 to 100 kDa and are made up almost entirely of 40 to 90 copies of a repetitive ten amino acid sequence (val-gly-pro-leu-ser-val-gly-pro-gln-ser VGPLSVGPQS), preceded by an N-terminal secretory leader sequence [83,85]. The number of isoforms varies between strains, with up to nine different-sized A2 proteins detected in the *L. donovani* 1S/Cl2D strain. In addition, sequencing of seventeen *L. donovani* clinical isolates from India and Nepal showed significant sequence and structural variation at the A2 locus [86]. A2 is expressed in *L. donovani* [87] and *L. mexicana* species complexes, but not in *L. tropica* or *L. braziliensis* species complexes [88].

A2 genes alternate with A2rel genes and each A2-A2rel cluster is flanked by 5' and 3' A2rel genes that have partial sequence identity with internal A2rel genes. Unlike A2, A2rel is present in *L. donovani, L. mexicana, L. tropica* and *L. braziliensis* species complexes [89]. The function of A2rel genes is unknown, but they may play a role in promastigote survival [54]. They encode a putative protein of 48 kDa with a signal peptide and two transmembrane domains [89]. A2rel mRNA levels are equal in promastigotes and amastigotes [84,89]. 3'A2rel mRNA levels are increased by the combination of increased temperature and

decreased pH as well as by increased temperature alone [90] and are higher in amastigotes than in promastigotes [91]. In contrast, 5' A2rel mRNA levels are higher in stationary phase promastigotes than in amastigotes [91].

1.4.1.2 Regulation of A2 expression

A2 protein expression is induced by promastigote to amastigote differentiation [87] and by a variety of stresses, including unfolded protein stress (UPR) [92] and misfolded protein stress [93,94], but not by thapsigargin-induced calcium depletion stress [95]. Regulation of A2 protein expression is complex and involves mainly the 3' UTR of the A2 mRNA. Regulation is via increased mRNA stability rather than by increased mRNA synthesis, with higher A2 mRNA levels in amastigotes compared to promastigotes [89], and this requires a combination of elevated temperature and decreased pH [84]. However, our data (see chapter 3) indicate that heat shock alone is sufficient to induce A2 protein expression. Other mechanisms are therefore involved in the regulation of A2 protein levels when temperature alone is changed.

The A2 3' UTR contains a 309 nucleotide regulatory element [96], subsequently identified as a SIDER1 retrotransposon [97]. However, it cannot account for the full temperature regulation [96]. SIDER1 retrotransposons promote increased translation in response to heat shock [98] without affecting mRNA stability [96]. This is supported by microarray data showing no changes in A2 mRNA levels following heat shock alone [91]. Indeed, increased A2 translation with more mRNA association with polysomes was observed following

increased temperature alone or following a combination of increased temperature and decreased pH. Acidic pH alone was not sufficient to induce increased A2 translation and protein expression [95]. Decreased A2 mRNA levels and decreased mRNA association with polysomes are observed when amastigotes are switched back to promastigote culture conditions. Finally, decreased protein levels are observed prior to any changes in A2 mRNA levels, indicating that translation control is the main and fastest mechanism of regulation of A2 protein levels [95]. These observations therefore support a dual mechanism to control A2 protein expression, via increased translation and via increased mRNA stability, with the mechanism involved dependant on the induction conditions.

1.4.1.3 Role of A2 in parasite virulence

Antisense RNA downregulation of A2 [99] or partial knockout of A2 genes [54] decreased liver parasite burden during *L. donovani* infection. Conversely, introducing A2 genes into *L. major* enhanced the ability of *L. major*-infected cells to migrate out of the dermis and increased parasite survival in mouse visceral organs [46]. Likewise, A2 is absent in *L. tarentolae* (a lizard *Leishmania* species) [100] and expression of *L. donovani* A2 in *L. tarentolae* enhanced the ability of *L. tarentolae* to survive in mouse visceral organs [101] (**Table 1.2**). Overall, these results indicate that A2 plays a key role in parasite survival in the visceral organs in animal models. Since A2 is expressed during human visceral infection [88], it may play a similar role in the human host.

In contrast, A2 inhibits cutaneous disease: expressing A2 in *L. major* was associated with decreased footpad parasite burden and swelling [46] and A2 was downregulated in human PKDL cases [102]. A2 may therefore contribute to the inability of *L. donovani* to cause cutaneous leishmaniasis. A2 has some sequence homology with the repetitive S-antigen of *Plasmodium falciparum* [83], but the function of this antigen is unknown [103]. Similarly, prior to the work presented in this thesis, the function of A2 was unknown.

1.4.1.4 A2 as a vaccine candidate

A2 is also an important vaccine candidate. A2 encodes MHC I - and MHC II-binding sequences as well as a B cell epitope [104]. A number of A2-based vaccination protocols were protective in experimental animals against visceral leishmaniasis, including immunization with recombinant A2 protein [105], plasmids encoding A2 [106,107], *L. tarentolae* expressing A2 [108] and an adenovirus expressing A2 [104] in mice; recombinant A2 in dogs [109], and a combination of recombinant A2 and adenovirus-expressed A2 in Rhesus monkeys [110]. A2 is also being developed as part of the polyprotein vaccine candidate KSAC (fusion of kinetoplastid membrane protein 11 (KMP11), sterol 24-*c*-methyltransferase (SMT), A2, and cysteine proteinase B (CPB)) [111]. Importantly, this vaccine was also protective against vector-transmitted parasites [112]. Phase III clinical trials in dogs of a recombinant A2 formulation have been completed and the vaccine is now licensed for veterinary use in Brazil under the name Leish-Tec [110]. Finally, recombinant A2 in combination with recombinant

IL12 [113], and A2 DNA vaccination [107] were also protective against cutaneous leishmaniasis caused by *L. amazonensis*.

1.4.1.5 Other applications of A2

A2 was originally identified as an amastigote-specific protein [83]. It has therefore been used in a number of studies as a marker of promastigote to amastigote differentiation (see for instance [12,93,114,115,116,117]). The A2 3' UTR has also been used to direct the expression of suicide genes such as a truncated 3' nucleotidase/nuclease. Similarly, the herpes thymidine kinase was targeted to the A2 chromosomal locus. These were expressed specifically in amastigotes and lead to decreased amastigote viability under drug pressure [118]. Such suicide genes may be useful for the generation of live-attenuated vaccines (see **section 1.6.3.2**).

Finally, A2 has also been investigated for diagnostic purposes, by detecting anti-A2 antibodies by Western blot, ELISA and immunoprecipitation [88,119,120,121]. These could be used to diagnose *L. donovani* and *L. mexicana* infections in humans and were up to 92% sensitive. Anti-A2 antibodies were also detected post-treatment. *T. cruzi-, L. tropica-,* and *L. braziliensis*-infected patients all gave negative results [88]. An ELISA to detect anti-A2 antibodies in dogs could detect both symptomatic and asymptomatic infections with a sensitivity of 94%. Although sensitivity was high in this test, specificity was lower: positive results were also obtained in a significant number of

mucocutaneous and cutaneous leishmaniasis cases in humans, as well as in 10% of samples from leprosy patients [121].

1.4.2 Other visceral disease-promoting factors

A2 is the prototypical visceral disease-promoting gene. However, completion of the *L. major, L. donovani, L. braziliensis,* and more recently *L. mexicana* genomes [122] has highlighted a number of other *L. donovani*-specific genes that are absent or found as pseudogenes in cutaneous species (**Figure 1.7**) [123]. The ability of some of these genes to promote parasite survival in visceral organs has been investigated by ectopically expressing *L. donovani*-specific genes in *L. major* and monitoring spleen and liver parasite burden as well as parasite recovery from the spleen in BALB/c mice [124,125,126]. Interestingly, three *L. donovani*-specific genes could promote *L. major* survival in the viscera, including the orthologs of LinJ.28.0340, LinJ.15.0900 and LinJ.36.2480 [125] (**Table 1.3**). Knocking out LinJ.28.0340 and LinJ.36.2480 in *L. donovani* also decreased parasite survival in the visceral organs. LinJ.28.0340 is a cytosolic protein of unknown function [125], LinJ.15.0900 a nucleotide sugar transporter localized in the Golgi apparatus [126], and LinJ.36.2480 a cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is a rate-limiting enzyme involved in glucose metabolism and ATP production [127]. The impact of LinJ.36.2480 on parasite survival in the visceral organs suggests that energy production may be an important difference between cutaneous and visceral species [127]. However, apart from LinJ.28.0340, these genes are present in *L.*

mexicana [125,126] and several of them also promote increased footpad swelling, making them general virulence factors, rather than visceralization-promoting factors.

1.4.3 Other virulence factors not specific to visceral leishmaniasis

1.4.3.1 Antioxidants

Antioxidant production constitutes a key host defence against leishmaniasis [128]. Both reactive oxygen and nitrogen species can be produced, although parasites can be cleared even in the absence of reactive oxygen species whereas nitric oxide production is essential [129]. Oxidants damage nucleic acids, proteins and lipids. Superoxide and hydroxyl radicals cause lipid peroxidation, leading to increased membrane fluidity and permeability. Hydroxyl radicals cause DNA damage with base oxidation and can lead to major chromosomal rearrangements [130]. Iron-sulfur clusters of proteins are very sensitive to oxidative stress. Oxidants can also lead to peptide bond cleavage and amino acid modifications of sulfur-containing amino acids and cyclic amino acids. Arginine, proline, and lysine can also become carbonylated. These modifications inhibit enzyme function, target proteins for proteosomal degradation and can also induce the formation of cytoxic protein aggregates [130].

Leishmania in turn have evolved a number of defences against these oxidants, including non-enzymatic scavengers such as thiols [131] and lipophosphoglycan [132], and enzymatic systems. In these, electrons are

transferred from NADPH to trypanothione reductase, which reduces trypanothione. In turn, electrons are then transferred to a redox shuttle such as tryparedoxin, ascorbate or glutathione. Peroxidases perform the final electron transfer reaction to reduce oxidants. Peroxidases in *Leishmania* include 2-cysteine peroxiredoxins, non-selenium glutathione peroxidases and ascorbate peroxidases (**Figure 1.8**). *Leishmania* lack catalases but express iron superoxide dismutases. While mammalian cells also use glutathione for antioxidant defenses, the trypanothione-tryparedoxin system is the main antioxidant pathway in Kinetoplastida and is specific to this order [133,134]. Finally, *Leishmania* also target macrophage signaling cascades to inhibit oxidant production [135].

1.4.3.2 Heat shock proteins

Leishmania parasites are exposed to abrupt changes in temperature when transferred from the insect gut to the mammalian host [1] as well as to high fever during visceral leishmaniasis [56]. Heat shock is a non-specific stressor and therefore affects many different subsets of the cell, leading to intracellular ROS production, protein damage and changes in membrane fluidity [136]. With regards to protein synthesis, heat shock leads to protein misfolding and aggregation, oxidative protein damage and translation inhibition. Multiple subcellular structures are affected: the endoplasmic reticulum and the Golgi become fragmented, mitochondria and lysosome numbers decrease, and cytoskeleton organization is affected, with severe stress leading to actin, tubulin and intermediate filament collapse. Changes in mitochondria in particular lead to

decreased ATP levels and alterations in energy metabolism. Nuclear structure is also affected with enlargement of nucleoli. Finally, membrane structure changes and membrane fluidity and permeability are increased, leading to a drop in intracellular pH and changes in ion concentrations. Persistent heat shock leads to cell cycle arrest and eventually cell death [137].

Given the severity and pleiotropic effects of heat shock, many different response pathways have been implicated in the protection against heat shock. Chaperonse in particular play a key role in protein refolding (the classical heat shock proteins (HSP)). However, other pathways such as proteolysis, DNA repair, changes in energy metabolism and mainenance of cellular and membrane structural integrity are also implicated [137].

Classical HSPs have been implicated in *Leishmania* virulence, with different expression patterns of heat shock protein during heat shock, low pH stress [138] or nitric oxide treatment [139] between virulent and avirulent *L. donovani* strains. HSP70 [140] and HSP83 [141] have also been associated with protection against antimonials. Knocking out one of the six HSP70 copies in *L. infantum* was associated with significantly decreased replication within macrophages and BALB/c spleen and liver parasite burden. Similarly, knocking out HSP100 in *L. major* significantly decreased footpad swelling during murine infection [142].

1.4.3.3 Glycoproteins gp63 and gp46

gp63 is a zinc metalloprotease attached to the parasite membrane by a glycosylphosphatidylinositol (GPI) anchor and also secreted by *Leishmania.* It is expressed by both promastigotes and amastigotes, but promastigotes express more isoforms of the protein. *L. major* and *L. amazonensis* gp63-deficient parasites were associated with delayed lesion development in experimental mouse infection, indicating a role of gp63 *in vivo* [143]. It is involved in parasite opsonization [144] and in resistance against host antimicrobial peptides and complement-mediated lysis. Secreted gp63 can transit to the host macrophage cytosol and nucleus to affect numerous signaling pathways, including inactivation of mTOR pathway and protein translation, activation of protein tyrosine phosphatases SHP1, PTP1B and TCPTP leading to altered MAP kinase signaling, inhibition of signaling through the IFNγ receptor and JAK/STAT, and inhibition of NF-κB and AP1 signaling. The net result is inhibition of TNFα, IL12 and nitric oxide production, and increased parasite survival within the host [143]. PSA-2 (gp46) has also been implicated in resistance against complement lysis [145] and is involved in attachment to macrophages via complement receptor 3 [146].

1.4.3.4 Lipophosphoglycan

Lipophosphoglycan (LPG) is a key component of the *Leishmania* surface glycocalyx. LPG is a glycolipid composed of Galβ1,4Man-PO_4 repeats followed

by a glycan core attached to the parasite membrane via a GPI anchor. The repeat units are capped by a variable oligosaccharide. Branching oligosaccharides can be added to the repeat units, depending on the *Leishmania* species. LPG is highly expressed in promastigotes but significantly downregulated in amastigotes [147].

LPG plays a key role in the interaction between the sand fly and *Leishmania.* Parasites attach to the sand fly midgut via LPG, thus preventing parasite excretion from the gut during bloodmeal excretion. Variations of the branching sugars attached to the repeats are the major determinant of vector competence and permissiveness [7].

LPG is also essential for host-parasite interactions in Old World *Leishmania* but not in New World species such as *L. mexicana* [148]. In particular, LPG-deficient *L. major* was significantly less virulent in BALB/c mice and parasite survival within macrophages was impaired [149]. LPG protects against complement-mediated lysis killing by neutrophil extracellular traps (NETs) [150]. LPG also delays phagolysosome maturation by promoting the accumulation of filamentous actin around the phagosome [147] and preventing V-ATPase recruitment to delay phagosome acidification [151]. Finally, LPG downmodulates the production of reactive oxygen species in host macrophages by inhibiting protein kinase C [147] and blocking NADPH oxidase assembly in the phagolysosome membrane [152].

1.4.3.5 Cysteine peptidases

Cysteine peptidases play a key role in *L. mexicana* [153] and *L. infantum* [154] virulence. Parasites express multiple isoforms of these cysteine peptidases, arranged in tandem arrays in the parasite genome. Different isoforms are preferentially expressed at different stages of the parasite lifecycle and the isoforms also have different substrate specificities. Cysteine peptidases mature in the flagellar pocket and then transit to the lysosomes of amastigotes. During infections, they can also be detected in the extracellular environment, the phagolysosome outside of the amastigotes and the cytoplasm of the host macrophage. They induce IL4 and IgE production and TGFβ activation, and cleave the IL2 receptor, MHC II, NF-κB and IκB. The net result is inhibition of antigen presentation, IL12 synthesis and of the Th1 response while promoting non-healing Th2 responses [155].

1.4.4 Atypical leishmaniasis cases: potential to identify novel determinants of visceral disease

Although many virulence factors and species-specific genes have been identified, none of them can fully restore *L. major* virulence to the same level as *L. donovani* [125]. Virulence in visceral organs likely involves a combination of these *L. donovani*-specific genes as well as other factors. Additional mechanisms likely to be implicated include gene amplifications, polymorphisms and differences in post-transcriptional regulation and protein expression patterns.

The study of atypical leishmaniasis cases can lead to the identification of novel determinants of visceral disease.

1.4.4.1 Sri Lanka

Although *L. donovani* normally causes visceral leishmaniasis, in Sri Lanka there have been over 2,000 cases of cutaneous leishmaniasis caused by *L. donovani* in the past ten years, while visceral disease is rare [156]. Imported cases of cutaneous leishmaniasis were first reported in Sri Lanka in 1990 [157] and the first locally-acquired case was detected in 1992 [158]. The first locally-acquired case of visceral leishmaniasis was reported in 2007 [159] and the first case of mucocutaneous disease in 2005 [160]. Since then, there have only been three other cases of visceral leishmaniasis (S. Ranasinghe, personal communication).

Isoenzyme analysis, DNA sequencing and microsatellite analysis identified *L. donovani* zymodeme MON-37 as the causative agent of cutaneous leishmaniasis in Sri Lanka [161,162,163]. MON-37 *L. donovani* is also found in Ethiopia, Israel and India, and is closely related to the MON-2 zymodeme, the most common in India [164,165]. However, the rK39 serodiagnostic test, which is used to diagnose visceral leishmaniasis, gives negative results with cutaneous leishmaniasis patients in Sri Lanka [166].

It is unclear whether leishmaniasis in Sri Lanka is anthroponotic or zoonotic. *L. donovani* in India is anthroponotic and transmitted by *Phlebotomus argentipes* [1]. *Phlebotomus argentipes* is the main *Phlebotomus* species in Sri Lanka and the most likely vector [167,168], with human biting rates comparable

to those in India [169], supporting an anthroponotic mode of transmission. However, *Phlebotomus salehi,* a vector for *L. major,* has also been detected in Sri Lanka [170]. In addition, time outdoors is a risk factor for developing leishmaniasis in Sri Lanka [171], and a few dogs were positive for *Leishmania,* either by direct detection of amastigotes [172] or by serodiagnosis [173]. These observations suggest that an animal reservoir could be involved.

Cases are found throughout Sri Lanka [156], but patient characteristics differ depending on the region. In the North-Central province, patients are mainly male soldiers 20-40 years old [171]. In contrast, patients in the Southern province are mainly male and female adolescents 10-19 years old [174]. Differences in patient characteristics may be due to differences in exposure to the vector and the parasite, as well as to possible reservoirs.

Patients usually present with single lesions [174], mainly dry and scaly, non-itchy, either as papules, nodules or ulcers, some of them surrounded by depigmented areas [175]. Lesions are slow-progressing [175], usually self-heal [156], and are found on exposed skin, mainly on the face and arms [175].

Most patients are of Sinhalese ethnicity [176]. However, a study of host single nucleotide polymorphisms (SNPs) did not detect any SNPs associated with a predisposition to developing cutaneous disease [177,178], suggesting that parasite factors rather than host characteristics may be the key determinant of the atypical cutaneous *L. donovani* cases in Sri Lanka.

1.4.4.2 Other atypical *L. donovani* foci

In addition to the situation in Sri Lanka, a number of other atypical leishmaniasis cases with *L. donovani* or *L. infantum* as the causative agent of cutaneous rather than visceral leishmaniasis have been reported in India [179] and in Bhutan [180], in the Middle East [181,182,183], in Cyprus [165,184], in Africa [185,186,187] and in South America (*L. chagasi*) [188,189,190,191]. Symptoms for these cases may differ from classical cutaneous leishmaniasis, for instance with regards to age of patients, parasite burden and increased frequency of non-ulcerative lesions [189]. The situation is further complicated by the fact that cure of visceral leishmaniasis can be followed by PKDL [192].

Interestingly, *L. donovani* isolated from cutaneous lesions in Turkey is closely related to MON-37 cutaneous *L. donovani* from Cyprus and India [193]. Similarly, certain *L. infantum* zymodemes are associated with specific clinical manifestations: zymodemes MON-11, 29, 33, 78, 111 only cause cutaneous leishmaniasis, zymodemes MON-27, 28, 72, 77, 187 only cause visceral disease, and some zymodemes can cause either cutaneous or visceral disease (MON-1, 24, 34, 80). Finally, the situation is altered in HIV co-infections where zymodemes that usually cause cutaneous disease can cause visceral leishmaniasis instead (MON-29, 33, 78), indicating the importance of host characteristics as well as parasite factors [194,195].

1.4.4.3 Normally cutaneous species causing visceral leishmaniasis

Similarly to situations described above in which normally visceral parasites can cause cutaneous disease, cutaneous parasites such as *L. tropica* can cause visceral infections. There are a number of clinical reports from visceral leishmaniasis patients in which the causative agent was identified as *L. tropica* in India [196], the Middle East [197,198] and Africa [199]. In all cases, patients were either children or teenagers. Canine visceral leishmaniasis caused by *L. tropica* has also been reported in Morocco [200,201] and in Iran [202].

Some soldiers infected with *L. tropica* during the Gulf War developed viscerotropic leishmaniasis. Parasites are found in visceral organs, but some symptoms differ from those of classical visceral leishmaniasis. In particular, there may not be significant hepatosplenomegaly, hypergammaglobulinemia, and cachexia. Other non-specific symptoms such as abdominal pain and diarrhea can be observed [203]. A total of twelve Gulf War veterans were diagnosed with viscerotropic leishmaniasis [204,205].

No specific *L. tropica* genotype has been correlated with visceralization [206], but differences in virulence in animal models were observed between cutaneous, viscerotropic and visceral *L. tropica* clinical isolates. In mice, some but not all visceral isolates and all viscerotropic isolates were unable to establish footpad lesions. However, cutaneous, visceral and viscerotropic isolates were all unable to cause persistent visceral infection following intravenous injection. Following intracardiac injection in hamsters, all strains were able to cause visceral infections. Spleen and liver parasite burden peaked at higher levels for the

visceral strains than for the cutaneous one, causing parasite burdens similar to visceral *L. donovani.* In contrast, viscerotropic strains were cleared faster than either cutaneous or visceral strains. Following footpad infection in hamsters, visceralization to the liver and spleen was observed for visceral isolates, while cutaneous and viscerotropic isolates did not show significant visceralization [76]. These observations suggest that viscerotropic leishmaniasis cases may be due to increased host susceptibility rather than increased virulence of the parasites. However, more studies are required to validate these results and to identify factors that mediate the increased virulence of the visceral *L. tropica* isolates.

In the New World, *L. amazonensis* and *L. colombiensis* have been associated with visceral leishmaniasis in children [207,208,209,210], up to one quarter of all visceral cases in one study [208]. *L. colombiensis* is related to a different genus of Trypanosomatidae, *Endotrypanum* and is distinct from the classical members of the *Leishmania* genus (the section euleishmania). *L. colombiensis* is classified as a member of the section paraleishmania [211]. There is also a case report of visceral leishmaniasis caused by *L. mexicana* in an organ transplant patient with co-infections with bacteria and *T. cruzi* [212].

No differences in age and sex of the patients as well as in antibody titers and clinical manifestations were observed between cases caused by *L. amazonensis* and *L. chagasi.* More deaths occurred in the *L. amazonensis* group and *L. amazonensis* was also associated with PKDL cases [208]. In animal models of leishmaniasis, some differences were observed between cutaneous and visceral *L. amazonensis* isolates, with smaller cutaneous lesions following

infection with visceral isolates in one study [22], while in another study a visceral isolate was able to cause large footpad lesions [207]. However, both cutaneous and visceral isolates caused significant spleen parasitemia, with earlier increases in parasite burden observed for cutaneous isolates [213]. More work in animal models is therefore required to determine whether cutaneous and visceral *L. amazonensis* differ or whether the visceral cases are due to host characteristics leading to increased disease susceptibility. Given that *L. amazonensis* expresses A2 [88], it is tempting to speculate that A2 may play a role in *L. amazonensis* visceralization. However, this awaits experimental confirmation.

Finally, a new species of *Leishmania, L. siamensis* has been proposed to cause visceral leishmaniasis in HIV-positive [214,215,216] and HIV-negative [217] adult patients in Thailand. This species is more closely related to *L. colombiensis* than to other *Leishmania* species [217]. A different phylogenetic analysis confirmed that this species is not closely related to visceral species such as *L. donovani* or *L. infantum,* and showed that it was most closely related to *L. enriettii* [216] (euleishmania [211], usually considered non-pathogenic to humans [1]). However, *L. colombiensis* was not included in this comparison. Autochthonous cutaneous leishmaniasis caused by *L. siamensis* has been reported in the United States [218], Germany and Switzerland [219] in horses and cows.

1.4.4.4 Hybrids of different *Leishmania* species

Hybrids of different *L. infantum* zymodemes [220] and of different *L. donovani* strains [221] have been detected in Africa. Similarly, hybrids of closely

related species such as *L. major* with *L. arabica* [222] and *L. braziliensis* with either *L. peruviana* [223,224], *L. panamensis* [225] and *L. guyanensis* [226,227] have all been reported. Hybrids of species associated with different disease manifestations have also been observed. These include *L. donovani* with *L. aethiopica* [228] and *L. major* with *L. donovani* [229]. Hybrids have also been generated in the laboratory between two different *L. donovani* strains [230] and between two different *L. major* strains [231]. So far however, no report of cross-species hybridization in the laboratory has been published.

Hybrids can be studied *in vivo* to determine their virulence. Genetic analysis could then be performed to compare the hybrids to the parental strain and identify factors that correlate with virulence. Hybrids of parental species that have different disease phenotypes therefore have significant potential to help identify determinants of disease development. Studying the *L. major/L. donovani* and *L. aethiopica/L. donovani* hybrids would be particularly interesting. Only *L. braziliensis/L. peruviana* hybrids and laboratory-generated hybrids of *L. major* strains have been studied *in vivo*. Hybrids either showed higher parasite burdens at late time points than the parental strains [232] or had virulence similar to one of the two parental strains, depending on the hybrid clone [231].

1.4.5 PKDL: an additional complication

The comparison between cutaneous and visceral species is complicated by the fact that cutaneous manifestations known as post kala azar dermal leishmaniasis (PKDL) can occur following treatment of visceral disease [192].

With regards to parasite differences, some polymorphisms at the 28S rRNA locus have been identified between parasites isolated from PKDL cases and visceral leishmaniasis cases [233]. However, the significance of these polymorphisms is unclear. At the mRNA and protein level, a number of surface proteins are overexpressed in parasites isolated from PKDL patients, including gp46, gp63 and amastin, but the difference was minor (two-fold difference in protein levels) [234]. Since gp46 levels are low in *L. tropica* patients, this protein may not be required to establish cutaneous lesions [102].

PKDL has also been associated with downregulation of MAPK, Uba5, A1 and A2 mRNA levels. Low levels of Uba5 and A2 may therefore be associated with cutaneous disease whereas high levels inhibit lesion formation in the skin. In contrast, MAPK and A1 levels are higher in *L. tropica* cutaneous lesions than in PKDL lesions, but lower than in visceral disease [102]. Therefore, MAPK and A1 may not be general determinants of cutaneous disease. More studies are therefore required to identify parasite factors that mediate the switch from visceral leishmaniasis to PKDL and to confirm their importance in pathogenesis.

A number of studies have also looked at host risk factors associated with development of PKDL (reviewed in [235]). Increased IL10 [236,237,238,239], TNFα [240,241] and TGFβ [238], as well as IFNγ receptor polymorphisms [242], have all been reported to be associated with increased risk of PKDL. Treatment choices may also influence whether the patient develops PKDL: treatment with amphotericin B has been associated with fewer subsequent cases of PKDL than

treatment with pentavalent antimonials, possibly due to lower IL10 and TGFβ levels following treatment with amphotericin B [238].

1.5 Host determinants of visceral leishmaniasis

1.5.1 Protective immunity against visceral leishmaniasis

An effective Th1 cellular immune response is required to control infection: IL12 released by antigen presenting cells contributes to the differentiation of Th1 cells. These release IFNγ, resulting in macrophage activation and the production of leishmanicidal NO [243] (**Figure 1.9**). However, an IFNγ-mediated response is not sufficient since new studies indicate that visceral leishmaniasis patients are also able to produce IFNγ in response to *Leishmania* antigen [244]. Disease is strongly correlated with the production of high amounts of IL10 [244,245], a potent immunoregulator that supresses Th1 responses and inhibits leishmanicidal functions [246].

There is however significant differences in disease progression and immune response between organs in visceral leishmaniasis in the BALB/c intravenous model of infection. Infection in the liver is self-resolving and involves granuloma formation, while infection in the spleen and bone marrow is persistent and progressive [243,247].

Infected Kupffer cells in the liver secrete chemokines such as CCL2, CCL3 and CXCL10 that lead to early macrophage and neutrophil recruitment. CD4+ and CD8+ cells are recruited later to the granuloma. IFNγ and TNFα activate macrophages to produce reactive oxygen and nitrogen species, leading to

parasite killing. IL4 is involved in granuloma resolution and priming CD8+ cells, indicating that both Th1 and Th2 responses are required for resolution of visceral leishmaniasis [243].

In the spleen, red pulp macrophages, marginal zone macrophages and marginal metallophilic macrophages phagocytose parasites. Chemokine production by spleen stromal cells induces dendritic cell and T cell recruitment. Dendritic cells then present parasite antigens to T cells and produce IL12 to induce Th1 differentiation. However, past day 14 post-infection, heavily infected macrophages produce high amounts of TNFα, leading to destruction of the spleen architecture, and subsequently to decreased T cell and dendritic cell recruitment to the spleen [243].

T cells are primed in the spleen and then migrate to the liver to promote healing. Therefore, the inability to resolve infections in the spleen is not due to failure to activate T cells, but may rather be due to an inability of T cells to reach infection foci due to the disruption of the spleen microarchitecture [248].

1.5.2 Genetic background

The ratio of subclinical to symptomatic visceral leishmaniasis is estimated at up to 18 to 1 [249], demonstrating that many people infected with visceral *Leishmania* species develop an effective immune response and do not manifest clinical disease. The host genetic background influences the development of disease [250,251] (reviewed in [252,253,254]). In particular, NRAMP1 plays a key role in susceptibility to visceral disease [255]. A number of cytokines,

chemokines and their receptors (TNFα, [256]; IL4 [257]; TGFβ [258]; IL2 receptor [259]; CXCR2 [260]) as well as mannan-binding lectin [261] and the Delta-like 1 ligand for Notch 3 (DLL1) [262] have also been associated with symptomatic *versus* asymptomatic disease.

1.5.3 Poverty and susceptibility to visceral leishmaniasis

Malnutrition increases the risk of developing symptomatic visceral leishmaniasis in humans [263,264] (recently reviewed in [265]) and in experimental mice models [78]. Malnutrition is associated with decreased NO and TNF levels in the visceral organs, and increased prostaglandin E2 (PGE2) production [78]. These observations could partially explain why visceral leishmaniasis is closely associated with poverty [32]. Similarly, poverty is associated with poor housing conditions and lack of bed nets that increase exposure to sand flies and therefore to the parasite [32,266]. Conversely, the human and financial cost of leishmaniasis reinforces poverty, generating a vicious cycle [32].

1.5.4 Co-infections and susceptibility to visceral leishmaniasis

AIDS is associated with significantly increased risk of visceral disease (reviewed in [267]). Visceral leishmaniasis in HIV-co-infected individuals has atypical disease manifestations, such as the presence of cutaneous lesions as well as visceral parasitemia [268], and detection of parasite in the skin, lungs and

gastrointestinal tract [269]. Visceral leishmaniasis in these patients can also be caused by strains and species that normally cause cutaneous disease [270].

Although co-infection with HIV has generated a lot of attention, many other pathogens are found in *Leishmania*-endemic regions. This is the case for example in Africa where *L. donovani* and schistosomes can both be found. In experimental C57BL/6 mouse infections, prior infection with *Schistosoma mansoni* (a causative agent of schistosomiasis) was associated with increased spleen and liver parasite burden at later time points, possibly by generating a microenvironment favourable to *L. donovani* in the schistosome egg granuloma [271]. Similarly, prior infection with *Plasmodium chabaudi chabaudi* AS was also associated with higher *Leishmania infantum* spleen parasite burden [272]. Simultaneous infection with *L. infantum* and *Toxoplasma gondii* also increased the spleen parasite burden [273].

In contrast, prior infection with *Trichinella spiralis* [274] or with L3 larvae of *Brugia malayi* (a causative agent of lymphatic filariasis), as well as simultaneous infection with adult *Brugia malayi* and *L. donovani* was associated with decreased spleen parasite burden, most likely due to induction of Th1 responses in the case of L3 larvae [275]. Therefore, the outcome of co-infections depends on the parasite species involved and the immune response generated.

1.5.5 Sand fly saliva and immunity to visceral leishmaniasis

During a bloodmeal, sand flies inject salivary components in addition to parasites. Salivary proteins inhibit Th1 responses and favour Th2 non-protective

responses [276,277,278]. For example, maxadilan from *Lutzomyia longipalpis* saliva decreased IFNγ, IL-12, TNFα and NO production [278,279] and promoted IL-10, TGF-β and PGE2 synthesis [279,280]. *Lutzomyia longipalpis* saliva also decreased dendritic cell maturation [281]. Sand fly salivary components can therefore exacerbate infection [282] and vaccines that were protective against needle challenge were unsuccessful against sand fly challenge or challenge in the presence of salivary gland homogenate [45].

In contrast, exposure to sand fly salivary components prior to *Leishmania* infection can be protective: immunization with sand fly salivary proteins was protective against visceral leishmaniasis in hamsters [50]. In human volunteers, decreased macrophage infection levels were observed in an *in vitro* restimulation assay one year following exposure to sand fly bites [283]. Evidence in the field is more limited. Higher antibody levels against sand fly salivary proteins were associated with decreased seropositivity to *Leishmania* antigens in dogs, suggesting that exposure to sand flies and development of an immune response to sand fly components leads to decreased infection rates [284]. Conversion from a negative to a positive anti-*L. chagasi* delayed-type hypersensitivity test, indicative of exposure and protective responses to the parasite, also coincided with the production of antibodies to *Lutzomyia longipalpis* [285,286]. However, multiple sand fly bites over 15 weeks in BALB/c mice was associated with decreased responses to saliva components [287], suggesting that protection may wane over time in endemic areas [288].

1.6 Vaccines against visceral leishmaniasis

Although drug treatments do exist against visceral leishmaniasis, there are still important toxicity and resistance concerns. A prophylactic vaccine is therefore desirable, and computer simulations show that such a vaccine could be cost-effective [30]. Clearance of an infection is associated with protection from re-infection [289], so vaccination against visceral leishmaniasis should be possible.

1.6.1 Desirable characteristics of a vaccine against visceral leishmaniasis

The contrast between Th1 healing responses and Th2 non-healing responses are a key paradigm of immunity to cutaneous *L. major*. However, protective immunity to visceral species does not follow a clear Th1/Th2 dichotomy. Under experimental conditions, many protective vaccine formulations for visceral leishmaniasis are associated with a mixed Th1/Th2 response rather than a bias towards Th1 (see for instance [105,290,291,292]). The levels of pre-challenge IL4, IFNγ and IL12 have been correlated with the level of protection [293] and a HASPB-1 (hydrophilic acylated surface protein B1)-based vaccine which successfully protected wild-type mice from *L. donovani* was ineffective in IL4- and IL4 receptor-deficient mice [294]. Induction of a Th1 response alone is also not sufficient for protection: DNA immunization with LACK induced strong Th1 responses but was non-protective [295].

The IFNγ to IL10 ratio may be a key determinant of vaccine-induced protection [291], suggesting that Th1 responses are downregulated by IL10

production rather than by IL4 responses [296]. This is supported by the observation that *ex vivo* stimulation of whole blood samples from active visceral leishmaniasis in humans was associated with high IFNγ and TNFα production, comparable to the production from samples from cured visceral leishmaniasis patients, while baseline and post-stimulation IL10 levels were higher in active visceral leishmaniasis patients than in cured patients [244]. A good vaccine should therefore not induce high IL10 production.

In the case of cutaneous leishmaniasis, the frequency of multifunctional T cells before challenge is a predictor of vaccine efficacy [297]. Their role in vaccine-induced protection against visceral infection has not yet been as clearly demonstrated, but a number of successful experimental vaccines were able to induce multifunctional CD4+ cells producing IL2, TNFα and IFNγ [298,299].

CD4+ T cell responses therefore play a key role in protection against visceral leishmaniasis. CD8+ T cells can also be a source of IFNγ and TNFα to promote parasite killing and are also involved in granuloma formation. Importantly for vaccine design, these cells also play an important role in protection against reinfection [300]. CD8+ T cell levels are also enhanced in asymptomatic patients, supporting observations from experimental models [301]. Therefore, vaccine antigens that promote CD8+ T cell activation would be desirable. These may include proteins such as A2 that contain MHC I epitopes [104] or vaccination with DNA constructs. The latter have been shown to promote CD8+ T cell activation better than immunization with proteins [302].

Cross-protection by alum-precipitated autoclaved *L. major* plus BCG against *L. donovani* was observed in canine [303] and primate models of visceral leishmaniasis [304,305] and conversely there is some evidence that immunization with *L. donovani* is protective against cutaneous leishmaniasis [306]. Similarly, immunization with suicide mutants of *L. amazonensis* was protective against visceral disease in hamsters [307]. A direct comparison of autoclaved *L. major* plus BCG to autoclaved *L. donovani* plus BCG in hamsters even suggested that a *L. major*-based vaccine formulation was actually more protective than a vaccine based on *L. donovani*. The differences were however non-significant when BCG was omitted [308]. In contrast, immunization with *L. major* was unsuccessful against visceral leishmaniasis and even exacerbated subsequent infections in mouse models [309,310] and was non-protective in human trials [311]. Therefore, for a greater chance of vaccine success, immunization with antigens closely related to challenge species may be desirable. This can be achieved either by using different strains of the same species in the case of live-attenuated or killed vaccines, or by choosing vaccine antigens that are conserved between the different *Leishmania* species.

Given the differences in the progression of the infection between the spleen and liver, vaccine formulations do not necessarily generate the same level of protection in both organs. Indeed, many live vaccines lead to protection only in the liver and not the spleen (**Table 1.4**). The best candidate would therefore be a vaccine leading to protection in both spleen and liver.

Parasite persistence is required for protection against reinfection with *L. major* [312,313]. However, repeated exposure to the antigen can substitute for parasite persistence [314], suggesting that antigen persistence rather than parasite replication is required. The role of antigen persistence in resistance against visceral infection is less clear. Complete parasite clearance does not occur in the liver and yet the liver is resistant to reinfection, implying a role for parasite persistence in protection against visceral disease there [243]. However, in contrast, chronic spleen infection is associated with CD8+ cell exhaustion, and given the importance of CD8+ cells in protection against reinfection, parasite persistence in the spleen may not be desirable [315].

Finally, a vaccine against visceral leishmaniasis should be cost-effective, especially given that most leishmaniasis cases occur in some of the poorest regions of the world [316]. Computer simulation models indicate that a vaccine costing less than 5$ would be cost-effective regardless of efficacy. Compared to single-dose AmBisome treatment, a 30$ vaccine would be cheaper than treatment only at an efficacy of 75% [30].

1.6.2 Factors influencing vaccine efficacy

A number of characteristics influence vaccine efficacy. In particular, parasite dose can determine whether a protective or non-protective response is induced. Low *L. major* doses were protective against cutaneous leishmaniasis (as low as 33 promastigotes [317]), while intermediate or high *L. infantum* doses

protected against visceral disease (10^4 to 10^5 in the spleen, 10^4 to 10^7 in the liver depending on the study and the immunization route [290,310], see below). These observations highlight the differences between vaccines for cutaneous and visceral disease and the dangers of generalizing the observations from one disease model to the other.

The required dose also differs depending on the immunization site: in the case of visceral leishmaniasis, 4 weeks subcutaneous immunization with 10^7 *L. chagasi* was protective in the liver, while lower doses (10^2, 10^4) were not. Spleen parasite burden was not determined [310]. In contrast, intravenous immunization with 10^4 to 10^7 *L. infantum* for 4 weeks was protective in the liver, even with a three-fold higher challenge dose. Only immunization doses of 10^4 and 10^5 were protective in the spleen [290] (**Table 1.4**). Although one study was performed with *L. chagasi* and the other with *L. infantum,* these two parasites are considered close enough to represent the same species [5].

Vaccine efficacy is also dependent on immunization site: immunization with *L. tarentolae* was more effective against visceral leishmaniasis when immunizing parasites were injected intraperitoneally rather than subcutaneously [318]. Such an impact of immunization site was also observed with immunization with parasite fractions rather than whole parasites: intraperitoneal or intravenous immunization with *L. donovani* membrane antigens were protective against visceral leishmaniasis, while subcutaneous and intramuscular immunization routes were unsuccessful [319]. This may be due to differences in the strength and characteristics of the immune response generated depending on the

immunization site: intraperitoneal and intravenous immunization was associated with stronger cellular immune responses and lower TGFβ production [319]. However, the successful route is dependent on the immunization protocol: in contrast with the previous study, others have shown that subcutaneous infection with *L. donovani* rather than intraperitoneal or intracardiac infections induced the strongest Th1 immune responses [320].

The interval between immunization and challenge also influences the outcome of the vaccination: in the case of immunization with live *Leishmania* parasites, at least a 4 week delay between immunization and challenge was required. Vaccines that were successful with a 4 week delay were non-protective when 1-2 week delays were tested [310,321] (**Table 1.4**).

To generate the desired type of immune response leading to protection, adjuvants can be added to the vaccine formulations. First-generation vaccine trials used BCG, sometimes in combination with alum precipitation of the antigen [322]. Current licensed canine vaccines use saponin [323] and human trials are using MPL-SE [322]. BCG is still used for immunization against tuberculosis and promotes cellular immune responses [322]. Alum (aluminum salts) promotes Th2 responses with eosinophil recruitment and enhanced IgE and IgG1 production [324]. Saponin is produced from tree bark and promotes cell-mediated immune responses [322]. Monophosphoryl lipid A (MPL) is a chemical modification of the lipid A portion of LPS, a TLR4 agonist. MPL promotes strong Th1 responses with low levels of IL4 and IL10. MPL can be formulated with squalene (SE) as an oil-in-water emulsion, MPL-SE. Alum, MPL and AS04,

a combination of alum and MPL, are licensed for use in the United States. An adjuvant formulation based on glucopyranosyl lipid adjuvant in an oil-in-water emulsion (GLA-SE) induces similar but more potent responses than MPL-SE. Finally, CpG oligodeoxynucleotides activate cells via TLR9 and induce IL-12, TNF-α, and IFN-γ production [324].

Delivery systems can also be used to promote antigen uptake by antigen presenting cells. Formulation of the vaccine as an oil-in-water emulsion, for instance by the addition of squalene, promotes antigen maintenance at the site of injection, leading to gradual release of the antigen and continued stimulation. However, this is associated with a number of side effects that may be undesirable for human use. Formulations involving liposomes can promote antigen uptake and presentation by antigen presenting cells. They have been shown to promote humoral immune responses and may also activate CD8+ T cells [322].

1.6.3 First-generation vaccines against visceral leishmaniasis

1.6.3.1 First-generation vaccine trials

First-generation vaccines include killed or live-attenuated parasites. These vaccines were based on the practise of leishmanization whereby individuals are inoculated with parasites in a hidden body location, to protect against the formation of cutaneous lesions in visible locations. Leishmanization was performed in Israel in the 1970s and Iran in the 1980s, but has since then been discontinued [325].

Clinical trials of first-generation vaccines were conducted in humans, focusing mainly on cutaneous leishmaniasis [326]. A meta-analysis of all phase 3 clinical trials using first-generation killed whole parasite vaccines against cutaneous leishmaniasis indicates that all but one were unsuccessful in protecting against disease. The only successful study involved a combination of *L. amazonensis, L. guyanensis* and *L. braziliensis* inactivated by merthiolate and adjuvanted with BCG [326].

Evidence that leishmanization may protect against visceral leishmaniasis comes from a study in Sudan where it was observed that only individuals unresponsive to the leishmanin skin test (not previously exposed to *Leishmania*) developed visceral disease [327]. Immunization with live *Leishmania* parasites was found to be protective in mice, hamsters and dogs against visceral leishmaniasis [328] (**Table 1.4**). Heat-killed and autoclaved *L. donovani* were protective in mice against visceral leishmaniasis [329]. The only human study that investigated protection against visceral disease used autoclaved *L. major* in combination with BCG and did not detect any vaccine-specific protection against visceral leishmaniasis compared to BCG alone [311].

1.6.3.2 Advantages and disadvantages of first-generation vaccines

Although live-attenuated vaccines are often considered less desirable than killed or recombinant vaccines, live-attenuated vaccines are still used for many diseases, including measles, mumps, rubella and chickenpox (**Table 1.5**) [330]. Live-attenuated or killed whole parasite vaccines include many different

immunogenic antigens, unlike defined protein or DNA vaccines. Live-attenuated vaccines may also persist longer in the host [289]. This is especially important in the case of leishmaniasis since antigen persistence is involved in vaccine efficacy (see **section 1.6.1**). Therefore, live-attenuated vaccines for leishmaniasis should not be ruled out.

Leishmanization for cutaneous leishmaniasis was discontinued due to concerns regarding non-healing lesions at the site of immunization [325]. In the case of cutaneous leishmaniasis, side effects for vaccines are especially significant since the disease is usually self-healing. However, given the severity of visceral leishmaniasis, such side effects may not be as much of an obstacle. In addition, some new advances have been developed to mitigate these effects. In particular, mixing killed *L. major* with live *L. major* was associated with delayed lesion development. Adding CpG oligodeoxynucleotides to live *L. major* was also associated with significantly smaller lesion size and lower parasite burden, most likely due to increased IFNγ production. These vaccine formulations were all protective. Given that one of the arguments against leishmanization is the formation of large lesions, in some cases non-healing, these observations suggest that the addition of CpG or CpG plus killed parasites may mitigate this effect and make leishmanization more acceptable [331].

Another concern regarding first-generation vaccines is the difficulty of standardizing vaccine preparations [323]. In addition, parasite virulence and vaccine efficacy tend to wane with continued parasite cultures. Attenuated parasites may also revert to a virulent phenotype [323]. Although this is less

likely to occur in parasites where attenuation occurred in a defined manner, for example by knockout of all alleles of a virulence factor, compensatory mutations could occur over time to increase virulence. This was observed in the case of LPG2 knockout parasites [332] and HSP100 knockouts [333], indicating that such a reversion is a real possibility.

The most significant argument against live-attenuated vaccines is the fact that they could not be used to immunize HIV-positive patients since persistent parasites could lead to disease [323]. HIV-positive patients represent 2-9% of all visceral leishmaniasis cases, but this figure can reach up to 30% in certain regions, a non-negligible proportion of leishmaniasis cases [316]. The use of parasites with suicide cassettes has been proposed to ensure parasite clearance [118,307]. However, defined protein or DNA vaccines may represent the only suitable option for these patients.

1.6.4 Second- and third-generation vaccines

1.6.4.1 Second- and third-generation vaccine trials for *Leishmania*

Second- and third-generation vaccines are composed of parasite fractions or recombinant antigens, either delivered directly or by DNA vaccination. A large number of antigens have been investigated mainly in mice models, either singly or in combination. Defined antigens used for protein or DNA immunization include for instance gp29 [334], A2, gp63, LACK, cysteine peptidases, histone proteins (see **Table 1.6**)... DNA immunization has the advantage that the plasmid backbone contains unmethylated CpG dinucleotides,

leading to an adjuvant effect and inducing both humoral and cellular immune responses [322]. DNA vaccines are often very immunogenic and they are stable, making them suitable for tropical regions [328].

Two second-generation vaccines are currently in clinical development. The first is Leish-F1 (previously known as Leish 111f), a fusion of the thiol-specific-antioxidant antigen (TSA), *L. major* stress-inducible 1 (LmSTI1), and *Leishmania* elongation initiation factor (LeIF) adjuvanted with MPL-SE [335]. This vaccine has been shown to be safe and immunogenic in a phase 1 trial in India for visceral leishmaniasis [336] and in phase 1 and 2 trials for cutaneous leishmaniasis in South America [337,338]. It was however unsuccessful in a phase 3 trial in dogs against *L. infantum* [339]. The second, known as Leish-F3, is a fusion peptide of nonspecific nucleoside hydrolase (NH) and sterol 24-c-methyltransferase adjuvanted with GLA-SE [340] and is beginning phase 1 trials in the United States and India [338].

Preclinical trials are taking place for three other vaccines. The first, called Rapsodi, is a protein-based vaccine using the promastigote surface antigen (PSA). The second uses a viral vector and is being developed at York University, and the third is a DNA construct under the name Leishdnavax [335].

1.6.4.2 Advantages and disadvantages of second and third-generation vaccines

A major advantage of second- and third-generation vaccines is their safety compared to live-attenuated vaccines [19]. Second-generation vaccines are also

easier to standardize than live or killed *Leishmania* preparations [19]. Protein-based vaccines may not be as immunogenic as whole cell vaccines, but this can be remedied by the addition of adjuvants [19,289]. Given the role of antigen persistence in protection against leishmaniasis, the protection induced by second-generation vaccines may also not be as durable as the protection generated by live-attenuated parasites [312,313]. In addition, although DNA vaccines are stable [328], protein-based formulations may not be stable under tropical conditions [19]. Finally, second- and third-generation vaccines are more complicated to produce and require more sophisticated equipment to generate them than first-generation vaccines, making them a more expensive option [289].

1.6.5 Licensed *Leishmania* vaccines

Three vaccines are licensed for canine leishmaniasis. The first, Leishmune, is composed of the fucose-mannose ligand (FML) fraction of the parasite. In addition to protecting immunized dogs from disease, it also prevents vector transmission. This is especially important given that dogs are an important reservoir of disease. The second vaccine, Leish-Tec, is based on recombinant A2 [323]. Both are licensed in Brazil. Finally, a vaccine composed of the purified excreted-secreted proteins of *Leishmania infantum* is licensed in Europe under the name CaniLeish [341]. All of these vaccines are adjuvanted with saponin.

1.7 Rationale and objectives of research

Visceral leishmaniasis is one of the most fatal tropical diseases, while cutaneous leishmaniasis is self-healing. A key question of leishmaniasis research is therefore the identification of factors that determine whether infection will lead to a limited, contained cutaneous lesion, or to fatal dissemination to the visceral organs.

Introducing A2 proteins into *L. major* allows this cutaneous parasite to establish a visceral infection, indicating that A2 is a key determinant of visceralization. However, the mechanisms by which A2 promotes visceralization are still unknown. Environmental stressors differ significantly between cutaneous and visceral species (see **section 1.2.2**). Therefore the first objective of this thesis was to investigate the relationship between A2, the parasite stress response and parasite survival, with the goal of providing a mechanism to explain the role of A2 in visceralization.

Comparing *L. major* and *L. donovani* has been very helpful in identifying a number of factors that contribute to parasite virulence and the development of visceral disease. However, the evolutionary distance between these two species makes further comparisons difficult. Studying closely related strains of the same species that cause different disease manifestations can allow us to identify the most important determinants of the switch between cutaneous and visceral disease. Using two clinical *L. donovani* isolates from Sri Lanka, one from a cutaneous leishmaniasis patient and one from a visceral leishmaniasis patient, the second objective of this thesis was to identify factors that mediate the attenuation

of the cutaneous *L. donovani* strain. Given the importance of A2 in *L. donovani* virulence, we focused first on comparing A2 levels between these strains and on examining the role of A2 in the virulence of these two isolates.

Finally, although parasite characteristics play an important role in determining disease phenotype, host genetic background and history also influence the development of symptomatic disease. Given the low rates of visceral leishmaniasis in Sri Lanka and the high incidence of cutaneous disease, we hypothesized that prior exposure to the cutaneous strain could protect from visceral leishmaniasis. Studying this question would allow us to guide disease control efforts in Sri Lanka. In addition, there is no human vaccine against leishmaniasis. A successful natural model of immunization would help vaccine development. Therefore, the final objective of this thesis was to investigate whether immunization with a cutaneous isolate from Sri Lanka protects against visceral disease caused by a visceral clinical isolate from Sri Lanka, in a mouse model of leishmaniasis.

1.8 Tables

Table 1.1 Leishmania species and disease phenotype.

Visceral leishmaniasis	Common	*L. donovani* *L. infantum* *L. chagasi* (=*L. infantum*)
	Rare	*L. tropica* *L. amazonensis* *L. mexicana* *L. colombiensis* *L. siamensis*
Mucocutaneous leishmaniasis	Common	*L. braziliensis*
	Rare	*L. panamensis* *L. amazonensis* *L. guyanensis*
Cutaneous leishmaniasis	Common	*L. major* *L. tropica* *L. aethiopica* *L. amazonensis* *L. mexicana* *L. braziliensis* *L. peruviana* *L. guyanensis*
	Rare	*L. infantum* *L. donovani*

Adapted from [14].

Table 1.2 A2 studies.

Parasite species	Method	Impact on A2 protein levels	Axenic culture		*In vitro* macrophage infection		*In vivo* BALB/c infection			Ref
			pro	ama	% infected	ama / macrophage	Footpad infection	Spleen parasite burden	Liver parasite burden	
L. donovani	antisense plasmid	loss of all A2 isoforms	no effect	no effect	no effect	2.6x decrease			25x decrease	[99]
L. donovani	knockout type I	loss of 8 A2 isoforms	slight decrease	slight decrease					3x decrease	[54]
L. donovani	knockout type II	loss of one A2 isoform	slight decrease	slight decrease					2x decrease	[54]
L. donovani	knockout 5'BleT	loss of 8 A2 isoforms; downregulation of one isoform	large decrease	large decrease					3x decrease	[54]
L. major	A2 expression plasmid	One additonal A2 isoform	no effect		no effect	no effect	decreased swelling; 6-8x decreased parasite burden	3x increase	similar to spleen	[54] [46]
L. major	A2 cosmid	three A2 isoforms	no effect		no effect	no effect	no swelling			[46]

pro, promastigote. ama, amastigote

Table 1.3 *Leishmania* genes implicated in the development of visceral disease.

L. infantum	***L. major***	***L. mexicana***	***L. braziliensis***	**Name and localization**	**Effect of introduction into *L. major***			**Effect of KO in *L. donovani***	**Ref**
					Spleen parasite burden	**Liver parasite burden**	**Footpad**		
LinJ.15.0900	pseudogene LmjF.15.0840	LmxM.15.0840	absent	nucleotide sugar transporter Golgi	20x increase	18x increase	increased swelling	no change	[126]
LinJ.22.0670	pseudogene	LmxM.22.0691 LmxM.22.0692	absent	A2 Stress response Endoplasmic reticulum	3x increase		6-8x decrease in parasite burden	LDU: 25x decrease	[46] [54] [57] [99] [342]
LinJ.28.0340	pseudogene LmjF.28.0420	pseudogene LmxM.28.0420	pseudogene LbrM.28.0430	Hypothetical protein Cytosol	11x increase	14x increase	2x increase in swelling	LDU and spleen: 200x decrease	[125]
LinJ.36.2480	pseudogene LmjF.36.2350	LmxM.36.2350	absent	GAPDH Cytosol	8x increase	7x increase		Liver: 4x decrease Spleen: 10x decrease	[127]

Table 1.4 Metabolically active *Leishmania* vaccine studies.

	Immunization							**Challenge**		**Protection**		
Animal model	**Parasite species**	**Dose**	**IM**	**SC**	**IP**	**IV**	**Time before challenge (weeks)**	**Parasite species**	**Dose**	**Spleen**	**Liver**	**Ref.**
BALB/c	*L. tarentolae*	$5x10^6$		✓	✓		6	*L. donovani*	$5x10^7$	✓	✓	[318]
BALB/c	*L. infantum*	10^4				✓	4	*L. infantum*	$3x10^7$	✓	✓	[290]
		10^5				✓	4			✓	✓	
		10^6				✓	4			X	✓	
		10^7				✓	4			X	✓	
		10^3				✓	20			✓	✓	
		10^4				✓	20			✓	✓	
		10^5				✓	20			✓	✓	
BALB/c	*L. infantum*	10^8				✓	1	*L. infantum*	10^8	X	X	[321]
		10^8				✓	4			X	✓	
		10^8				✓	52			X	✓	

	Immunization							Challenge		Protection		
Animal model	**Parasite species**	**Dose**	**IM**	**SC**	**IP**	**IV**	**Time before challenge (weeks)**	**Parasite species**	**Dose**	**Spleen**	**Liver**	**Ref**
BALB/c	*L. chagasi*	10^2		✓			2	*L. chagasi*	10^7	N.D.	X	[310]
		10^4		✓			2			N.D.	X	
		10^7		✓			2			N.D.	X	
		10^2		✓			4			N.D.	X	
		10^4		✓			4			N.D.	N.S.	
		10^7		✓			4			N.D.	✓	
	attenuated *L. chagasi*	10^7		✓			4			N.D.	X	
	L. chagasi	10^2		✓			6			N.D.	X	
		10^4		✓			6			N.D.	N.S.	
		10^7		✓			6			N.D.	✓	
		10^7		✓			8			N.D.	✓	
	L. major	10^2		✓			4			N.D.	X	
		10^7		✓			4			N.D.	X	
BALB/c	*L. major*	10^5		✓			12	*L. infantum*	10^7	X	N.D.	[309]

	Immunization							**Challenge**		**Protection**		
Animal model	**Parasite species**	**Dose**	**IM**	**SC**	**IP**	**IV**	**Time before challenge (weeks)**	**Parasite species**	**Dose**	**Spleen**	**Liver**	**Ref**
BALB/c	*L. donovani* long-term culture (>7 passages)	10^7		✓			4	*L. donovani*	10^7	✓	✓	[343]
dogs	*L. infantum* + gentamicin	10^8		✓			52	*L. infantum*	10^8	✓	✓	[344]
BALB/c	γ-irradiated *L. donovani*	$2x10^6$	✓				4	*L. donovani*	$2x10^6$	✓	✓	[345]
BALB/c	*L. infantum chagasi*	10^7		✓			2	*L. infantum*	10^7	N.D.	✓	[292]
	L. infantum chagasi + DNA cross-linking ("killed but metabolically active")	10^7		✓			2			N.D.	✓	
	L. infantum chagasi	10^7		✓			8			N.D.	✓	
	L. infantum chagasi + DNA cross-linking ("killed but metabolically active")	10^7		✓			8			N.D.	✓	

✓ Protection X No protection N.D. Not determined N.S. Not significant

Table 1.5 FDA-approved live-attenuated vaccines.

Bacterial diseases
Tuberculosis
Typhoid fever
Plague
Viral diseases
Adenovirus
Chicken pox (varicella)
Herpes Zoster
Influenza (some formulations)
Measles
Mumps
Rotavirus
Rubella
Small pox
Yellow fever

Based on information from [330].

Table 1.6 Defined second- and third-generation vaccines.

Antigen	Animal model			Delivery system		
	Mouse	Hamster	Dog	Protein	DNA	Other
A2	✓		✓	✓	✓	
Cysteine peptidases	✓		✓	✓	✓	
dp72	✓			✓		
F14		✓		✓		
γ-GCS	✓				✓	
gp29	✓			✓		
gp63	✓			✓		Salmonella
H2A, H2B, H3, H4 and LACK			✓		✓	
HASPB1	✓			✓		
KMP11	✓	✓			✓	
LACK	✓		✓		✓	Vaccinia virus
LCR1	✓			✓		BCG
LD9, LD72, LD51, LD31	✓			✓		
Leish-111f	✓		✓	✓		
NH36	✓				✓	
ORFF	✓			✓	✓	
PapLe22		✓			✓	
Q protein	✓		✓	✓		

Q protein is a fusion of Lip2a, Lip2b, P0, H2A. Adapted from [323], with additional information from [334].

1.9 Figures

Figure 1.1 *Leishmania* taxonomy.

Leishmania are protozoan parasites from the order Kinetoplastida, family Trypanosomatidae. They are divided into the *Leishmania* and *Viannia* subgenera, which are further divided into species complexes and species. This figure only shows species from the section euleishmania since the classification of species from the section paraleishmania is still under debate. Adapted from [1] and [211].

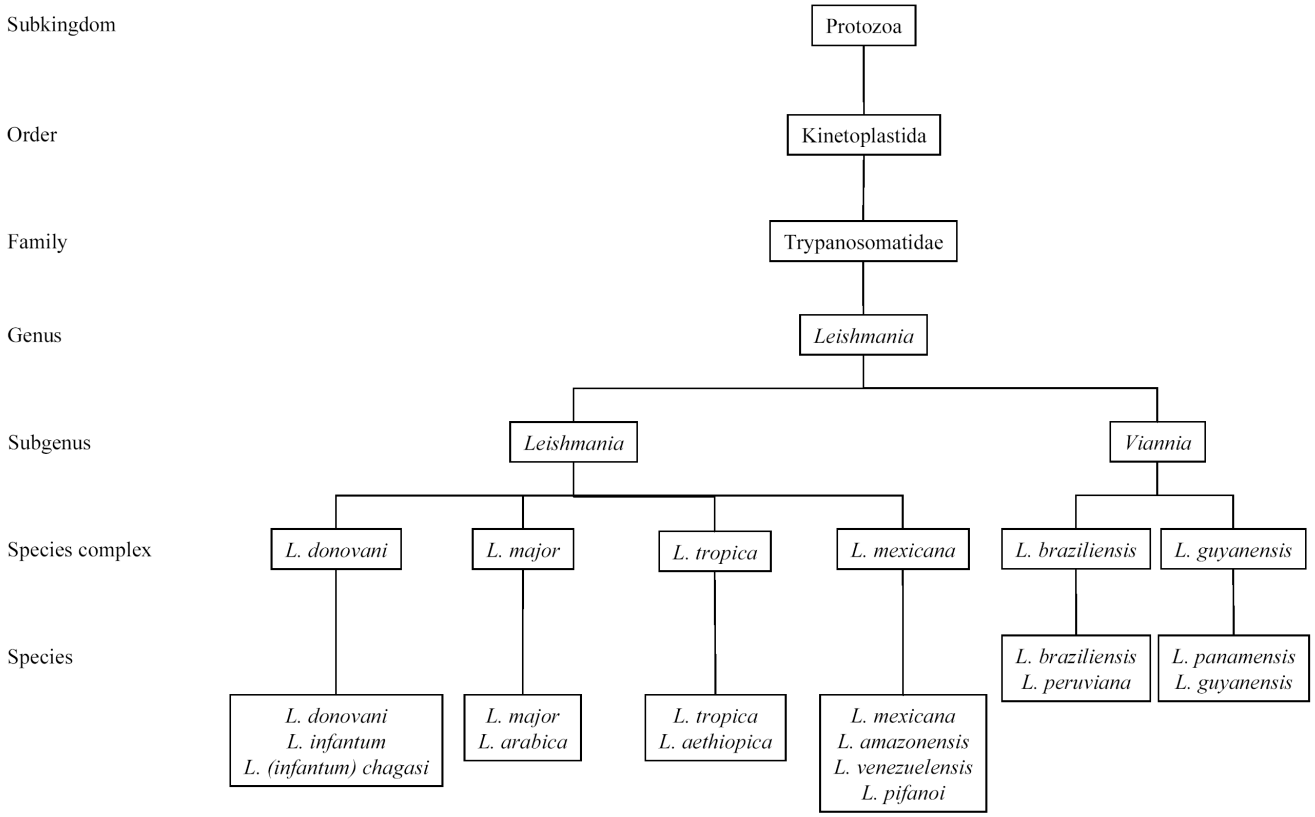

Subkingdom
Order
Family
Genus
Subgenus
Species complex
Species
Protozoa
Kinetoplastida
Trypanosomatidae
Leishmania
Leishmania
Viannia
L. donovani
L. major
L. tropica
L. mexicana
L. braziliensis
L. guyanensis
L. donovani
L. infantum
L. (infantum) chagasi
L. major
L. arabica
L. tropica
L. aethiopica
L. mexicana
L. amazonensis
L. venezuelensis
L. pifanoi
L. braziliensis
L. peruviana
L. panamensis
L. guyanensis

Figure 1.2 *Leishmania* lifecycle.

Leishmania promastigotes are transmitted to the mammalian host by a sand fly vector. Although several cell types can harbour parasites, the main host cell is the macrophage. Within the macrophage phagolysosome, promastigotes differentiate into amastigotes. Amastigotes can then be taken up by sand flies during a bloodmeal. Within the sand fly gut, amastigotes differentiate into promastigotes, thereby completing the parasite lifecycle. From [346].

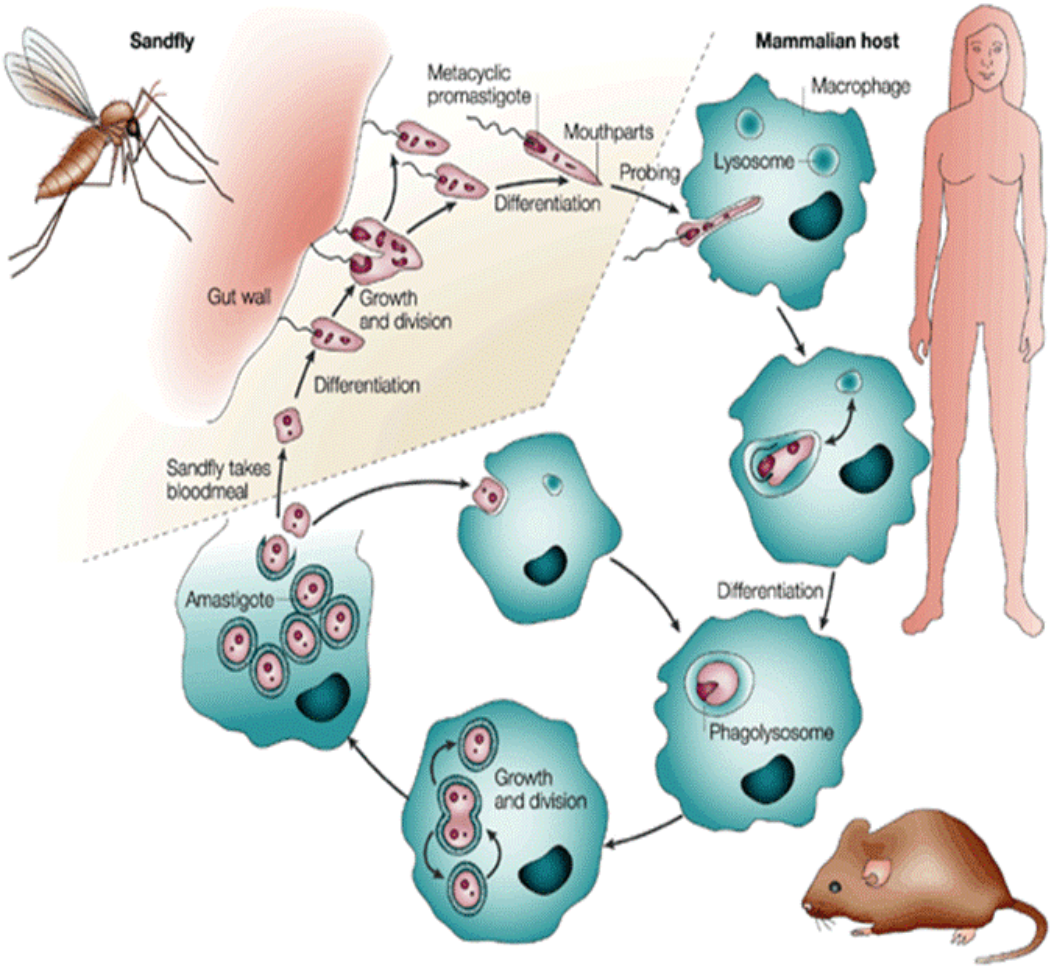


Figure 1.3 *Leishmania* disease manifestations.

There are three main forms of leishmaniasis: cutaneous leishmaniasis where parasites remain at the site of the sand fly bite (**A**), mucocutaneous leishmaniasis with parasite metastasis to the mucosal tissues of the nose, mouth and throat (**B**), and visceral disease in which parasites disseminate to the bone marrow, liver and spleen, leading to high fever and hepatosplenomegaly (**C**). Post-kala azar dermal leishmaniasis (PKDL) may occur following successful treatment of visceral leishmaniasis (**D**). **A-C** are from [1], **D** is from WHO.

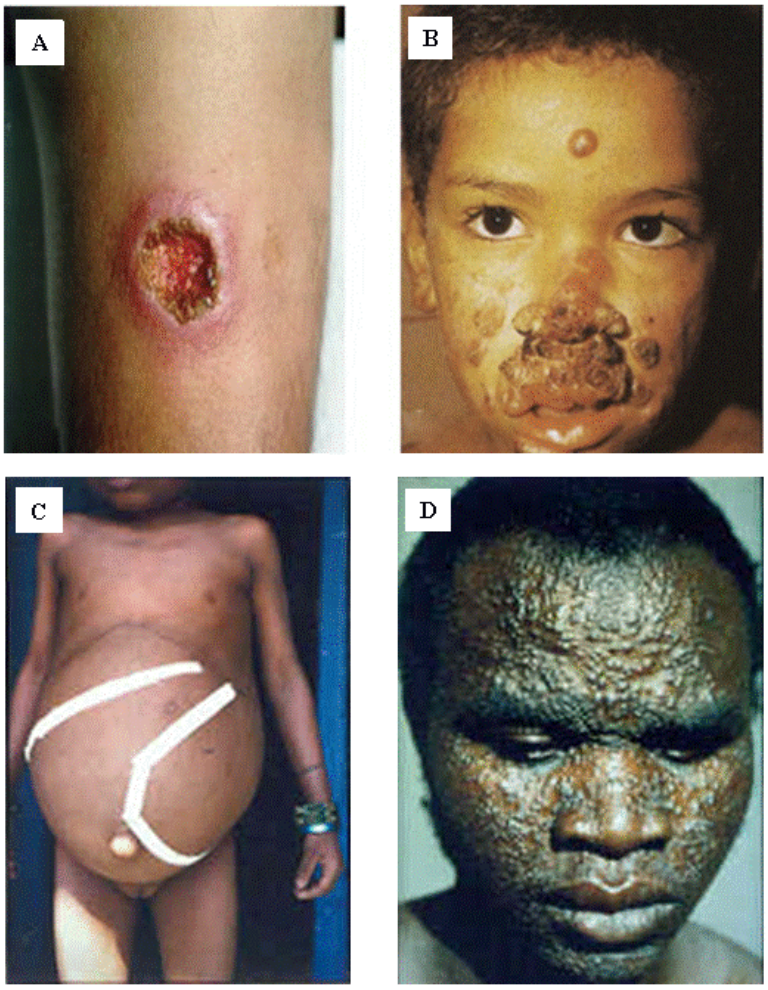


Figure 1.4 Geographic distribution of leishmaniasis.

Leishmaniasis is found mainly in tropical and subtropical regions. Most cutaneous and mucocutaneous cases occur in South America, Africa, the Middle East and South East Asia (**A**). Visceral leishmaniasis has a similar distribution (**B**), with the majority of cases in India, Nepal, Bangladesh, Sudan, Ethiopia and Brazil. Maps from [19].

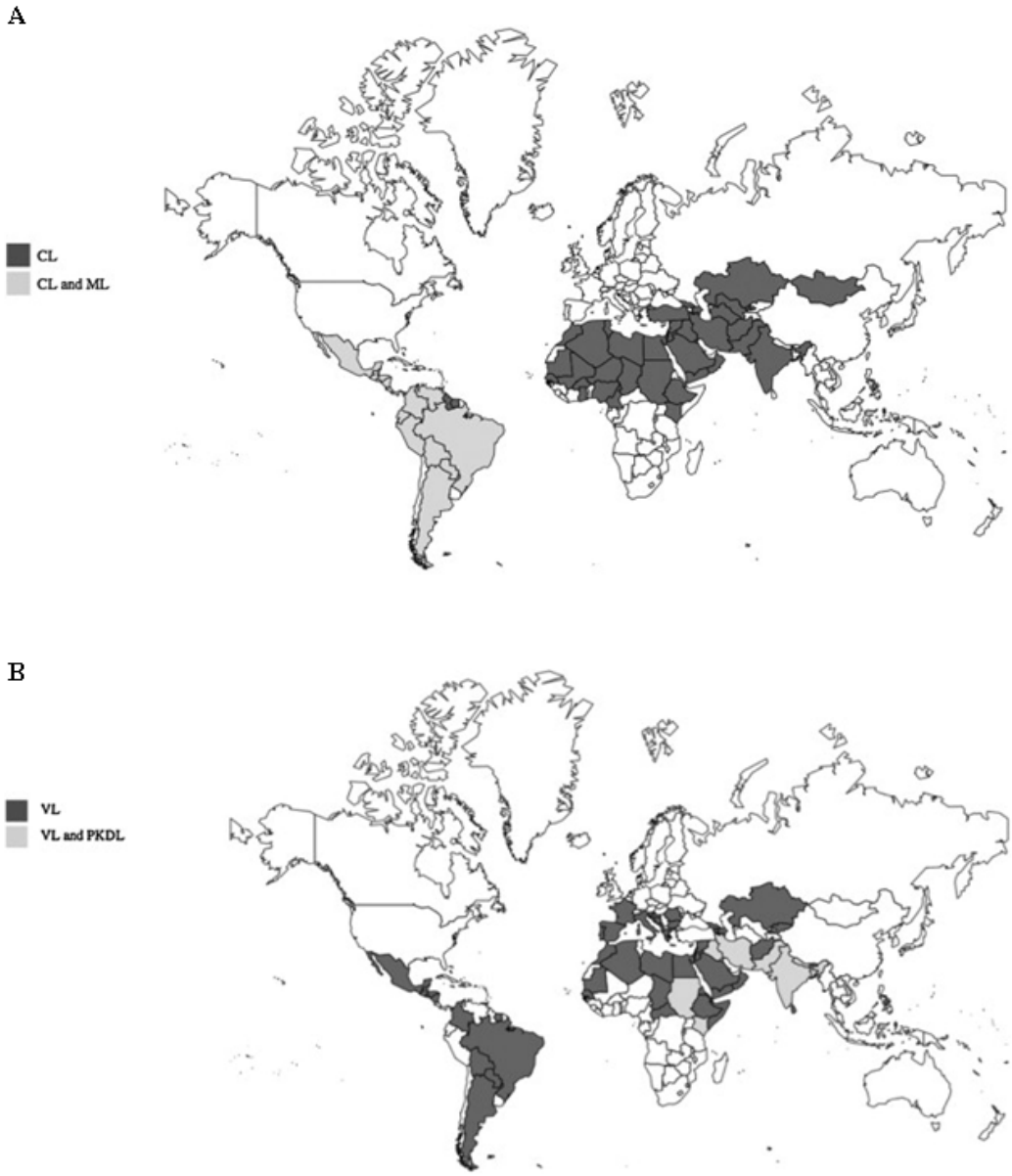


Figure 1.5 Factors influencing the development of symptomatic visceral leishmaniasis.

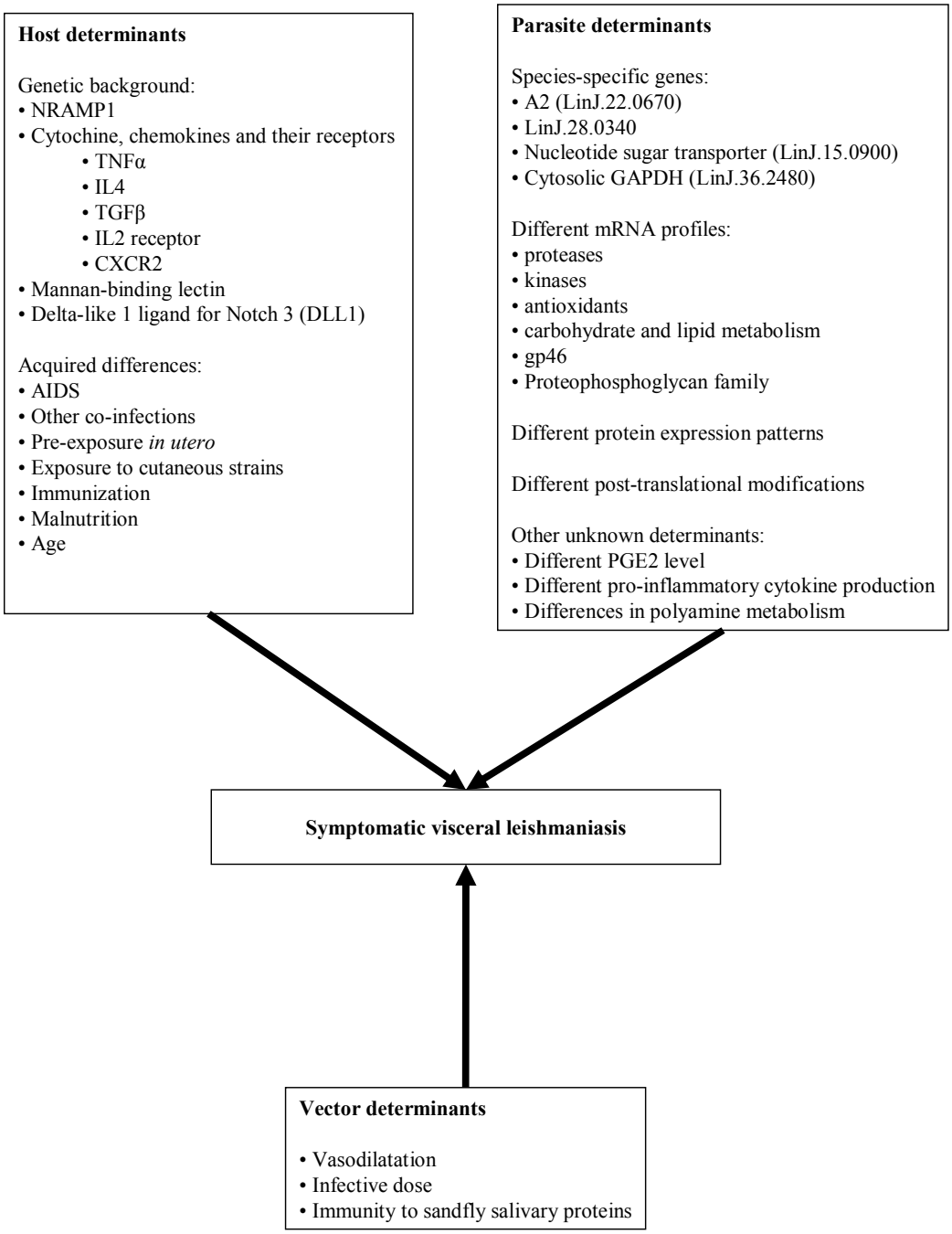


Figure 1.6 A2 genetic organization.

A2 genes are arranged in tandem arrays with internal A2rel genes in the *L. donovani* genome. 5'A2rel genes are located at the 5' end of the array, and 3'A2rel genes at the 3'end (**A**). A2 genes in *L. donovani* are composed of a leader sequence followed by 40-90 copies of a ten amino acid repeat sequence. All but one of these repetitive units are absent in *L. major* (**B**). Figure from [46].

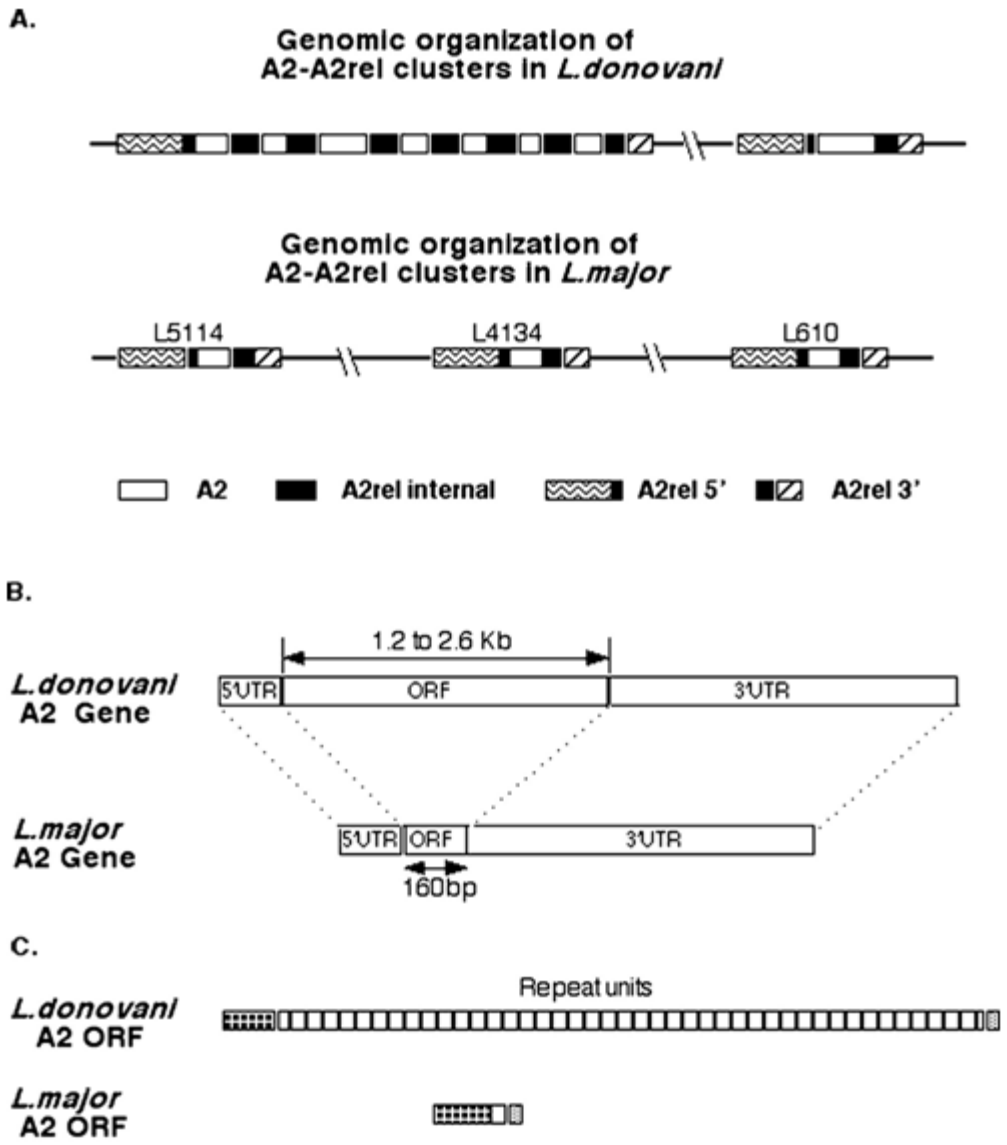


Figure 1.7 Species-specific genes.

Completion of the *L. major, L. mexicana, L. braziliensis* and *L. donovani* genomes has highlighted a number of species-specific genes present only in some *Leishmania* species but absent in the others. From [122].

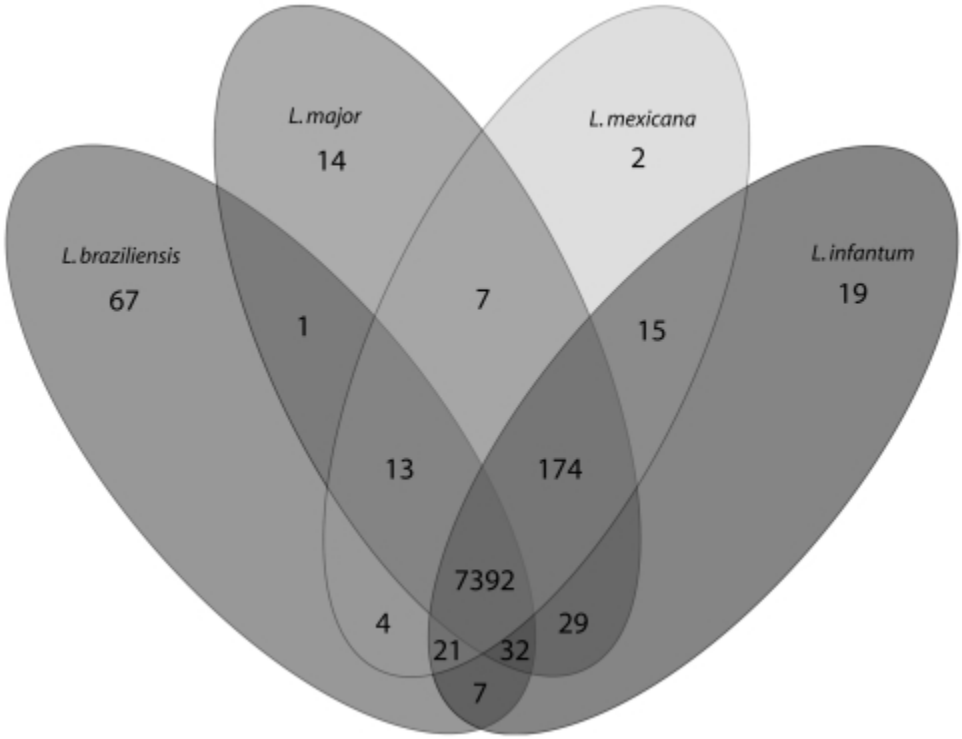


Figure 1.8 *Leishmania* antioxidant systems.

Electrons from NADPH are transferred to trypanothione reductase, which reduces trypanothione. In turn, electrons are then transferred to a redox shuttle such as tryparedoxin, ascorbate or glutathione. Peroxidases perform the final electron transfer reaction to reduce oxidants. Peroxidases in Leishmania include 2-cysteine peroxiredoxins, non-selenium glutathione peroxidases and ascorbate peroxidases. From [134].

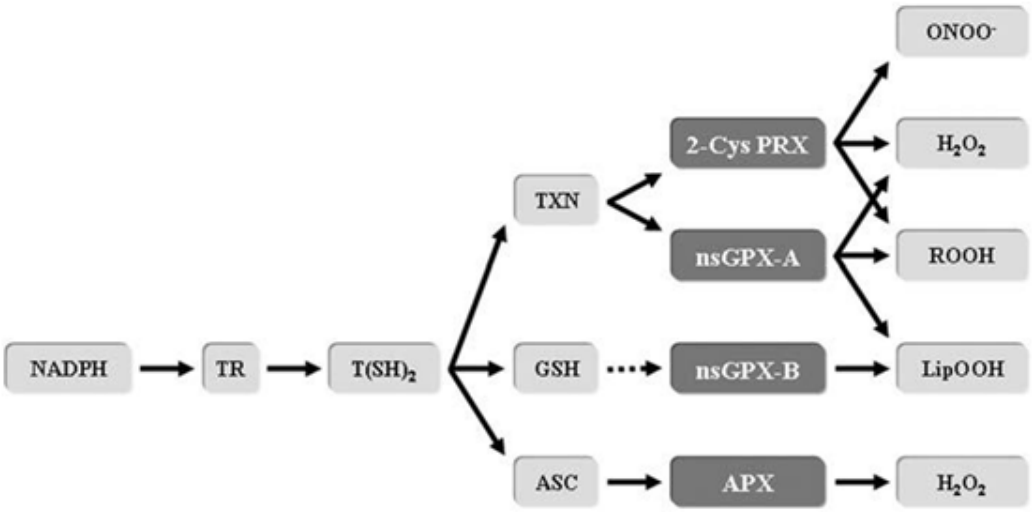
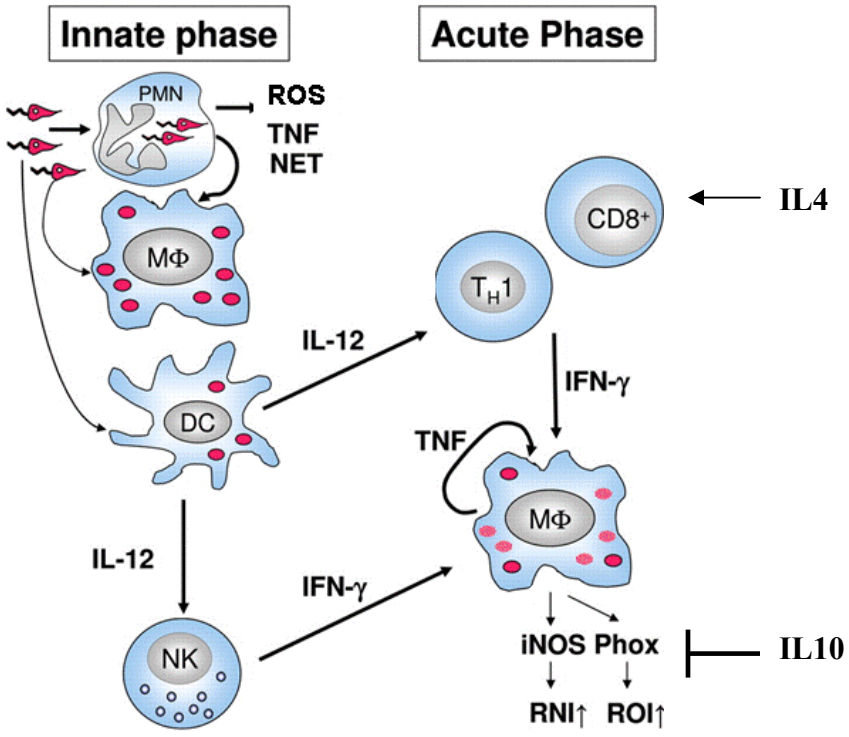


Figure 1.9 Immune response to *Leishmania.*

Following a sand fly bloodmeal, parasites can be taken up by neutrophils, macrophages and dendritic cells. Innate defenses against *Leishmania* include the generation of reactive oxygen species (ROS) and neutrophil extracellular traps (NETs). Infected dendritic cells are the main source of IL12, which stimulates the production of IFNγ by Th1 cells, CD8+ T cells and natural killer cells. IFNγ induces a leishmanicidal response in infected macrophages with autocrine TNFα secretion and production of reactive oxygen species by NADPH oxidase (Phox) and nitric oxide by the inducible nitric oxide synthase (iNOS), leading to parasite clearance. IL4 is also involved in the induction of a protective response. IL10 promotes parasite persistence by downregulating nitric oxide and ROS production and TNFα secretion. Adapted from [347].



Chapter 2

Localization and induction of the A2 virulence factor in Leishmania: evidence that A2 is a stress response protein.

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2.1 Preface

A2 is a key determinant of visceral leishmaniasis [54,99] (see **section 1.4.1**). However, the mechanism by which it increases parasite survival in visceral organs is unknown. To gain further insight into the function of A2, we investigated its subcellular localization by immunoprecipitation and immunofluorescence. A key difference between cutaneous and visceral parasites is the fact that visceral species must be able to survive at the high temperatures caused by the fever observed in visceral leishmaniasis (see **section 1.2.2**). We

compared the sensitivity of *L. major* and *L. donovani* to heat shock and assessed the impact of ectopic A2 expression on the survival of *L. major*. These observations provide the first insight into the function of A2 and the mechanisms by which it promotes visceral leishmaniasis.

2.2 Abstract

Leishmaniasis is a vector-borne infectious disease with a wide range of pathologies depending on the species of *Leishmania.* *Leishmania* parasites are transmitted by the sand fly vector as promastigotes; within the mammalian host, *Leishmania* parasites differentiate into amastigotes and replicate in macrophages. The A2 protein from *L. donovani* is expressed predominantly in amastigotes and therefore likely plays a role in survival in the mammalian host. In the present study, we have determined that the A2 protein colocalized with the *Leishmania* endoplasmic reticulum (ER) Binding Protein, BiP, was induced by stress and complexed with BiP following heat shock. The A2 gene in *L. major* is a non-expressed pseudogene and we present evidence that ectopic expression of a transfected A2 gene in *L. major* enhanced its viability following heat shock. A2 may therefore play a role in protecting *L. donovani* from stress associated with infection in visceral organs, including the fever typically associated with visceral leishmaniasis. Interestingly, when comparing A2 protein localization, we also observed that the *Leishmania* Secreted Acid Phosphatase SAcP protein was transported out of the parasite-containing phagolysosome and was located throughout the macrophage cytoplasm in vesicles, providing the first example of a

secreted *Leishmania*-derived protein exiting the parasite-containing phagolysosome.

2.3 Introduction

Leishmaniasis is an infectious disease transmitted by a sand fly vector, affecting more than twelve million people worldwide, with two million new cases each year and over 300 million people at risk. The pathologies of human leishmaniasis range from fatal infection of the visceral organs to relatively mild cutaneous infection and this is largely dependent on the species of *Leishmania* [192]. With warming temperatures, the range of sand fly transmission of leishmaniasis is predicted to expand, and with it, the population at risk of infection. Co-infections with the Human Immunodeficiency Virus (HIV) are also increasing the risk of developing visceral leishmaniasis [348]. A better understanding of the mechanisms of *Leishmania* pathogenesis may help develop improved interventions that could limit the spread of the disease.

Infection is initiated by injection of the promastigote form of the parasite from the sand fly bite, which then enters and replicates in the macrophage phagolysosome compartment. Once in the phagolysosome, the promastigotes differentiate into amastigotes, which can subsequently infect other macrophages or be ingested by a feeding sand fly. In the gut of the sand fly, amastigotes differentiate back into promastigotes, thereby completing the parasite life cycle [192]. Macrophages represent the main host cell for parasite replication during mammalian infection, and consequently, intracellular *Leishmania* amastigotes are

able to affect the macrophage metabolism in multiple ways [135]. However, the details of how amastigotes survive inside the macrophage and alter its function remain poorly understood.

The A2 protein from *L. donovani* is expressed at high levels in axenic amastigote cultures and at low levels in promastigote cultures, and has been shown to be required for *L. donovani* amastigote survival in mice [99]. Moreover, the A2 gene is a pseudogene in *L. major* and introduction of the *L. donovani* A2 gene into *L. major* enhanced the ability of *L. major* to survive in visceral organs of BALB/c mice [46,54]. These observations argue that A2 plays an important role in *L. donovani* amastigote survival in visceral organs.

To provide insight into the function of A2, we have examined its location in axenic amastigotes and in infected macrophages through immunofluorescence and immunoprecipitation analysis in comparison to other *Leishmania* proteins with known function and cellular location. Since A2 does have a leader sequence [83], it was important to determine whether it remains associated with amastigotes or is secreted into the macrophage during infection. Control Leishmania proteins used in this study included the secreted acid phosphatases (SAcP [349]), the cytoplasmic heat shock protein 83 (HSP83 [350]) and the endoplasmic reticulum binding protein (BiP [351]). In comparison to the other *Leishmania* proteins examined, A2 co-localized closely with BiP in cultures of axenic amastigotes and in infected macrophages, suggesting that A2 is present in the parasite endoplasmic reticulum (ER). Moreover, A2 interacted with the chaperone BiP following heat shock. Finally, ectopic expression of A2 protein in *L. major* increased parasite

survival following heat shock. These results suggest that A2 plays a role in the ER stress response that could help the parasite survive the higher temperatures associated with fever and parasitisation of the visceral organs.

2.4 Materials and methods

2.4.1 Parasite culture

L. donovani 1S/Cl2D strain promastigotes were maintained at 26°C in M199 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, streptomycin, penicillin, adenosine, glutamine and folic acid, at pH 7.2. *L. major* Friedlin V9 strain and *L. mexicana* MNYC/BZ/62/M379 strain were maintained at 26°C in M199 medium supplemented with 10% FBS, 40 mM HEPES, streptomycin, penicillin, adenine, hemin, biotin, biopterin and folic acid, at pH 7.2. To induce differentiation into axenic amastigotes, promastigotes were resuspended at a cell density of $5x10^7$ parasites/mL in amastigote media (M199 medium supplemented with 25% FBS, streptomycin, penicillin, succinic acid, adenine, glycerol, L-proline and folic acid, at pH 5.5) and transferred to 37°C, 5% CO_2.

2.4.2 Cell culture

B10R macrophage cells were maintained in Dulbecco's Modified Essential Medium (DMEM, Invitrogen), supplemented with 10% FBS, 100 units/mL of penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2.

2.4.3 Immunofluorescence

For immunofluorescence in infected macrophages, B10R cells ($1x10^5$) were grown on coverslips in DMEM supplemented with 10% FBS, 100 units/mL of penicillin and 100 μg/mL streptomycin for one day prior to infection. The cells were infected at a 20:1 parasite to macrophage ratio with *L. major* or *L. donovani* axenic promastigotes for 24 h or left uninfected, following which non-internalized parasites were detached by extensive washing in Phosphate-Buffered Saline (PBS). Cells were fixed for 30 min in 4% paraformaldehyde in PBS and permeabilized for 20 min in 1% Triton-X in PBS. Antibody incubations were 50 min in duration and were followed by five washes in PBS.

For immunofluorescence in heat shocked promastigotes, promastigotes ($1x10^7$ cells) were resuspended in fresh promastigote (neutral pH) media at $5x10^6$ cells/mL and incubated at 26°C or 40°C for 4 h. Promastigotes were also grown for 24 h under amastigote culture conditions (37°C pH 5.5) to initiate differentiation into amastigotes. Parasites were then collected by centrifugation, washed once with PBS and air-dried on poly-L-lysine (Cedarlane)-coated coverslips. Cells were fixed for 10 min in 4% paraformaldehyde in PBS and permeabilized for 25 min in 0.25% Triton X-100/3% BSA/50 mM Tris-HCl in PBS. Antibody incubations were 1 h in duration and were followed by four washes in PBS.

The primary antibody was a 1:100 dilution of mouse anti-A2 antibody combined with either a 1:1,000 dilution of rabbit anti-SAcP (a gift from Dr. Dwyer), a 1:1,000 dilution of rabbit anti-BiP (a gift from Dr. J. Bangs), or a

1:5,000 dilution of rabbit anti-HSP83 (a gift from Dr. Zilberstein).The secondary antibody was a combination of 1:500 fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma) and 1:500 Texas red-conjugated anti-rabbit IgG (Santa Cruz). Antibodies were diluted in 4% Bovine Serum Albumin (BSA) in PBS. The DNA dye DRAQ5 (Biostatus, 1:1,000) was added for 30 min, and the coverslips were then mounted in Permafluor mounting medium (Thermo). Slides were viewed using an Olympus Fluoview FV1000 confocal microscope, with images generated by sequential scanning.

2.4.4 Immunoprecipitation

The cells and the culture supernatant were collected by centrifugation. The parasites were washed once with PBS, lysed on ice for 20 min in *Leishmania* lysis buffer (1% Triton X-100, 50 mM Tris pH 8, 137 mM NaCl, complete protease inhibitor cocktail (Roche)), and clarified by centrifugation at 16,000 rpm for 10 min at 4°C. The culture supernatant was neutralized with 5N NaOH, and concentrated using an Amicon ultra-15 centrifugal filter unit (Millipore). Samples were incubated overnight at 4ºC with anti-A2, anti-BiP, anti-HSP83 or anti-SAcP antibodies. Protein A-sepharose beads (Sigma) were then added to each sample for 30 min, following which the beads were washed three times with *Leishmania* lysis buffer without detergent, transferring to a fresh tube for the final wash. Immunoprecipitated proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected by Western blot.

For cell pellet preparation, promastigotes were collected by centrifugation, washed once with PBS and lysed in SDS-PAGE loading buffer (0.0625 M Tris pH 6.8, 10% glycerol, 2% Sodium Dodecyl Sulfate (SDS), 10% β-mercaptoethanol).

2.4.5 Cross-linking

Paraformaldehyde cross-linking was performed as described in [352]. Briefly, cells were collected, washed with PBS and resuspended in 1% paraformaldehyde for 20 min at 37°C. Glycine (125 mM final concentration) was added for 5 min, and cells were then washed twice in PBS and lysed as above. The cross-linking was reversed by boiling the samples for 20 min in SDS-PAGE loading buffer.

2.4.6 SDS-PAGE and Western blotting

Samples were boiled 5 min in SDS-PAGE loading buffer and were separated by SDS-PAGE using a 10% acrylamide gel, except for SAcP where a 7% gel was used. Proteins were transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked 1 h in 5% skim milk in PBS. The primary and secondary antibody incubations were 1 h in duration and were followed by five 5 min washes in PBS with 0.1% Tween (PBS-T). All antibodies were diluted in 5% skim milk in PBS-T. Immunoprecipitated A2 was detected with a 1 in 5 dilution of mouse monoclonal anti-A2 antibody (hybridoma supernatant); the secondary antibody was a 1:1,000 dilution of peroxidase-conjugated Trueblot anti-mouse

IgG antibody (eBioscience), which only detects non-reduced forms of mouse antibodies. In the A2 thermotolerance assay, A2 was detected with a 1:4,000 dilution of mouse monoclonal anti-A2 antibody (ascites), followed by a 1:2,500 dilution of peroxidase-conjugated anti-mouse IgG antibody (Rockland). *Leishmania* Binding Protein (BiP) was detected with a 1:4,000 dilution of rabbit anti-BiP antibody (a kind gift from Dr. J. Bangs), *Leishmania* HSP83 with a 1:5,000 dilution of rabbit anti-HSP83 antibody (a kind gift from Dr. Zilberstein) and *Leishmania* Secretory Acid Phosphatase (SAcP) with a 1:1,000 dilution of rabbit anti-SAcP antibody (a kind gift from Dr. Dwyer). The secondary antibody was a 1:3,000 dilution of peroxidase-conjugated anti-rabbit IgG (GE Healthcare). Tubulin was detected with a 1:2,000 dilution of mouse monoclonal anti-tubulin antibody (Oncogene). The secondary antibody was a 1:2,500 dilution of peroxidase-conjugated anti-mouse IgG antibody (Rockland). All proteins were visualized using ECL Western Blot Detection Reagents (GE Healthcare).

2.4.7 Alamar blue viability assay

Following heat shock, parasites were diluted to a cell density of $1x10^6$ cells/mL and incubated at 26°C in the presence of Alamar blue reagent (Invitrogen) for 72 h. Absorbance was determined at 570 and 600 nm as per manufacturer's instructions.

2.5 Results

2.5.1 Localization of A2 during macrophage infection with *Leishmania*

To determine the localization of A2 during macrophage infection, we compared A2 by immunofluorescence to other Leishmania proteins including: a secreted protein, SAcP [349]; a cytoplasmic protein, HSP83 [350] and the ER chaperone protein, BiP [351]. See table 2.1 for accession numbers in Methods. For these analyses we used *L. major* and *L. donovani* -infected macrophages. *L. major* served as a negative control since it does not express A2 proteins, whereas *L. donovani* expresses A2 [88]. All of the control proteins were detected as expected in these analyses, although most interestingly SAcP was detected in small foci throughout the infected macrophage and was not associated with parasite nuclei staining, indicating that SAcP is secreted outside of the *Leishmania*-containing phagolysosome (**Figure 2.1A**). Note also that SAcP was expressed at higher levels in *L. donovani* than in *L. major* as previously reported in axenic cultures [353]. HSP83 was expressed by all *Leishmania* and was found distributed throughout the parasite as expected (**Figure 2.1B**) and BiP was found in small foci in all parasites (**Figure 2.1C**).

In comparison, A2 was observed in discrete foci closely associated with the parasite nuclei during infection with *L. donovani* but not *L. major*, as expected since *L. major* does not express A2 [46]. The A2 staining foci did not colocalize with SAcP (**Figure 2.1A**), partly colocalized with HSP83 (**Figure 2.1B**) and colocalized very similarly with BiP (**Figure 2.1C**), suggesting an ER localization

for A2. No fluorescence was detected when the primary antibody was omitted and only secondary antibody was used (data not shown).

Interestingly, not all of the parasite nuclei were surrounded by the A2 foci (see *L. donovani* in **Figure 2.1A** for example), and those parasites that expressed A2 did not all do so to the same extent (for example, *L. donovani* **Figure 2.1B** and **2.1C**). Overall, this suggests that the levels of expression of A2 vary within a population of *L. donovani* parasites when inside the macrophages.

2.5.2 Secretory patterns of A2, SAcP, HSP83 and BiP in parasite axenic culture

The immunofluorescence data suggested that although A2 has a leader sequence [83], it is not secreted from the parasite-containing phagolysosome as was evident with SAcP. We wanted to further explore this observation by comparing protein secretion from axenic parasite cultures. *Leishmania* proteins A2, SAcP, HSP83 and BiP were first collected by immunoprecipitation from live axenic parasite culture supernatants and from the parasite cell lysates. The immunoprecipitated proteins were then detected by Western blotting. As shown in **Figure 2.2**, A2 was only detected in *L. donovani* amastigote lysates (**Figure 2.2B**) and could not be detected from the parasite culture supernatants (**Figure 2.2A**), confirming that A2 is induced in amastigote cultures and is not secreted. Note that A2 is a multi-gene family expressing different sizes of a 10 amino-acid repeat motif and therefore typically migrates in multiple bands on SDS-PAGE [85]. As expected, no A2 was detected in *L. major* cultures since *L. major* does not express

A2 [46]. In contrast, the secreted SAcP was found in high amounts in the *L. donovani* promastigote and amastigote culture supernatants (**Figure 2.2C**). SAcP was found at a higher molecular mass when derived from amastigotes when compared to promastigotes, as previously shown, suggesting that it is more highly glycosylated in amastigotes than promastigotes [117]. Only very little SAcP was detected in *L. major* culture supernatants, in accordance with the observation that *L. major* express less SAcP than other *Leishmania* species [353] and also as shown in the above immunofluorescence data (**Figure 2.1A**). HSP83 and BiP were associated predominantly with the parasites lysates (**Figure 2.2F** and **2.2H** respectively) although small amounts were also detected in the culture supernatant (**Figure 2.2E** and **2.2G** respectively). This may be due to protein release following parasite death in culture, although under these circumstances no A2 was detected in the supernatant. Taken together, these data support the immunofluorescence data in showing that A2 is not secreted, in contrast to SAcP that is secreted from *L. donovani*.

2.5.3 A2 is induced at higher levels following heat shock than after 24 h differentiation of promastigotes into amastigotes

The immunofluorescence data in infected macrophages showed that A2 gave a similar staining pattern as BiP, suggesting that A2 may localize to the ER. BiP has been shown to play a role in the ER stress response [354] and A2 is induced by several different stresses, including heat shock and treatment with the proline analog azetidine-2-carboxylic acid (AZC) that induces an unfolded protein

response [93]. It was also interesting that A2 appeared to be unequally expressed by *L. donovani* amastigotes in infected macrophages (see example in **Figure 2.1**). These results are consistent with A2 playing a role in the stress response and therefore not being directly involved in differentiation from promastigotes to amastigotes. It was therefore of interest to compare A2 immunofluorescence levels in promastigotes following heat shock (4 h 40°C pH 7) compared to promastigotes grown under *in vitro* differentiation conditions (24 h at 37°C pH 5.5). As shown in **Figure 2.3**, there was much stronger induction of A2 following 4 h heat shock than after 24 h under conditions that induce differentiation: more *L. donovani* parasites expressed A2 and A2 expression levels were higher in the heat shocked parasites. Moreover, as shown in **Figure 2.1**, not all parasites induced to differentiate expressed the A2 protein compared to the heat shock response where all parasites appear to express A2.

2.5.4 A2 co-localizes with BiP following heat shock

To determine the subcellular localization of induced A2 following heat shock in axenic promastigotes, A2, HSP83 and BiP were located by immunofluorescence (**Figure 2.4** and **2.5**). Similar to observations during macrophage infection (**Figure 2.1B**), HSP83 was detected throughout the parasites (**Figure 2.4**) and BiP was again found in small foci in all parasites (**Figure 2.5**). A2 localized in small foci in heat shocked *L. donovani* (but not *L. major*) and was colocalized very closely with BiP (**Figure 2.5**), similar to what was observed in the infected macrophages (**Figure 2.1C**).

2.5.5 A2 interacts with the ER chaperone BiP

The immunofluorescence results from **Figures 2.1** and **2.5** suggest that A2 and BiP colocalize. It has been argued that proteins capable of forming beta strands can interact with unfolded proteins in the ER [355] and A2 has been predicted to fold into beta-sheets [104,356]. We therefore investigated the possibility that A2 may bind ER proteins following heat shock and determined whether A2 can interact with BiP. As shown in **Figure 2.6A**, immunoprecipitation of A2 following heat shock and Western blot analysis of co-immunoprecipitated BiP revealed that BiP was specifically associated with A2 following heat shock of *L. donovani*. A2 did not interact with another *Leishmania* abundant protein, HSP83 (**Figure 2.6B**), or with gp63 (**Figure 2.6C**) and SAcP (**Figure 2.6D**), both of which are secreted through the ER [357,358,359,360]. These results argue that the interaction with BiP is specific and further confirm the localization of A2 in the ER.

2.5.6 A2 promotes parasite viability following heat shock

L. major, being a cutaneous parasite, has lost the ability to express the A2 protein family, and introducing the A2 gene into *L. major* favors parasite survival during visceral infection in BALB/c mice [54]. However, the mechanisms by which A2 mediates this increased survival in visceral organs are unknown. Since we observed induction of A2 and association with BiP in the ER following heat shock, and since *L. donovani* infection is associated with fever whereas *L. major*

infection is not, we determined whether expression of A2 helped parasites survive heat shock. For these analyses, *L. major* transfected with the pKSneo-A2 and the control pKSneo plasmid [54] were heat shocked at 40°C pH 7 for 2 or 4 hours. Expression of the A2 transgene and cell viability were then compared in the A2-expressing *L. major*, in the control plasmid-containing *L. major* and in wild-type *L. donovani* and *L. major*. As shown in **Figure 2.7**, A2 expression was induced following heat shock in the transgenic *L. major* containing the A2 gene and, as expected, in *L. donovani*. No A2 expression was detected in *L. major* transfected with the control plasmid either at 26°C or 40°C, or in the wild-type L. donovani at 26°C. Only minimal A2 expression was observed for the A2-transfected *L. major* at 26°C.

Parasite viability was determined by the Alamar blue viability assay (**Figure 2.7E**). Wild-type *L. major* is considerably more sensitive to heat shock than wild-type *L. donovani* ($p<0.0001$ for 2 and 4 h heat shocks) consistent with *L. donovani* parasitizing the visceral organs and surviving fever. Introducing A2 into *L. major* significantly increased viability following heat shock compared to wild-type *L. major* ($p<0.0001$ and $p<0.005$ for 2 h and 4 h heat shock respectively), as well as compared to *L. major* transfected with the control plasmid ($p<0.0001$ and $p<0.005$ for 2 h and 4 h heat shock respectively). Similar results were also obtained when parasite viability was measured by limiting dilution (data not shown).

2.6 Discussion

This study provides novel evidence that A2 is a stress response protein that may play a role in enabling *L. donovani* to survive the higher temperatures associated with visceral organ infection and the fever typical of kala azar. This study further provides evidence that A2 is located in the parasite ER and is capable of complexing with the ER chaperone BiP under conditions of stress. These results argue that A2 does not play a direct role in differentiation, as previously assumed [85,361] but does play a role in amastigote survival following differentiation.

The localization of A2 was compared to three different types of *Leishmania* proteins: secreted proteins (SAcP), cytoplasmic proteins (HSP83) and ER proteins (BiP). The Signal P 3.0 server [362,363] predicts a signal peptide at the N terminus of A2, BiP and SAcP and the absence of a secretion signal in the cytoplasmic HSP83 (data not shown). Moreover, A2 has previously been shown to have a leader sequence that can direct import of A2 into canine pancreatic microsomes, where it is then removed [83]. However, we were unable to detect A2 in axenic culture supernatants, or in macrophage cells independent from the parasite. A2 colocalized closely with BiP in infected macrophages and in heat shocked promastigotes, indicating that it is located in the ER. The co-localization with BiP was further supported by the observation that A2 interacts with BiP following heat shock, arguing that A2 is directed to the ER following stress and during macrophage infection which can be considered a form of stress to the promastigote. Since BiP also possesses a secretory leader as predicted by Signal

P 3.0 [362,363], our data suggest that the A2 leader sequence directs A2 to the ER. However, A2 does not have any known ER retention signals, so it may be maintained there by a novel retention sequence or by interaction with ER proteins.

Not all *L. donovani* parasites expressed A2 to the same extent inside the infected macrophages and interestingly, some parasites had no detectable A2 expression (**Figure 2.1**). We believe this is an important observation because it argues that A2 is not needed to survive as an amastigote inside macrophages. In contrast, it was observed that all promastigotes placed at 40°C expressed A2 but only a subset expressed A2 when induced to differentiate into axenic amastigotes (**Figure 2.3**). This suggests that the variations in A2 expression levels within an amastigote population may represent different stress levels within the infected macrophage.

Ectopic expression of A2 in *L. major* increased survival following 40°C heat shock (**Figure 2.7**). This result suggests a possible explanation for the increased visceralization of A2-expressing *L. major* [46] and the role of A2 in *L. donovani* virulence in general [99]: A2 may help visceralizing parasites survive the higher temperatures associated with the internal organs. Moreover, visceral leishmaniasis is characterized by chronic fever, so that *L. donovani* parasites must be able to survive temperatures above 37°C in the visceral organs and this may be due in part to the presence of A2. The lack of A2 in *L. major* may not be detrimental to this organism because the skin temperatures are lower than those within the visceral organs and cutaneous leishmaniasis is not associated with fever. This hypothesis is consistent with a recent report showing that high levels

of A2 mRNA is detected in bone marrow-derived samples, but not in post kala azar dermal leishmaniasis (PKDL)- derived samples from *L. donovani*-infected patients [102]. Therefore, in patients with *L. donovani* infection, A2 is not expressed in the skin (PKDL-derived) but is expressed in visceral organs (bone marrow) where the temperature is higher than in the skin. The loss of A2 expression could be an important factor in allowing *L. donovani* to survive in the skin in PKDL lesions and this is consistent with the experimental observations in our study.

Although A2 significantly increased *L. major* viability following heat shock compared to control-transfected parasites, the survival of A2-expressing *L. major* was still lower than for wild-type *L. donovani.* This could be due to a requirement for other *L. donovani*-specific genes that play an additional role in *L. donovani* survival. Alternatively, A2-transfected *L. major* only express two different sizes of A2. It is therefore possible that additional different-sized A2 proteins are required to restore *L. major* survival up to the levels observed for *L. donovani.* This is supported by the observation during A2 knockout experiments in *L. donovani* that at least three different sizes of A2 always remain [54]. Finally, the difference in viability between A2-expressing *L. major* and wild-type *L. donovani* could be due to differences in total A2 protein levels.

The mechanism by which A2 may help Old World parasites survive these ER stresses is currently under investigation. However, the interaction between A2 and BiP suggests that A2 may moderate the function of BiP. Alternatively, it has been argued that beta sheet strands can interact with unfolded or misfolded

proteins in the ER [355]. Since A2 has been predicted to fold into multiple beta-sheets [104,356], it too may bind misfolded proteins in the ER and stabilize or refold them, or mediate their degradation. The direct interaction of A2 and BiP or indirect interaction through other unfolded proteins would explain the co-immunoprecipitation of A2 and BiP observed within. A role for A2 in responding to misfolded proteins is supported by the observation that A2 is induced in promastigotes deficient in a flagellar protein [94]. The induction of A2 is postulated to be due to the accumulation of flagellar components inside the cell and the induction of an unfolded protein response.

Another interesting outcome of this study was the observation that *Leishmania* SAcP was secreted out of the parasitized phagolysosome and into discrete vesicles throughout the macrophage cytoplasm. SAcP had previously been reported to leave the parasite during infection in macrophage U937 cells but it was unclear whether it was able to escape the parasite-containing phagolysosome [364]. SAcP may therefore have far-reaching effects on macrophage protein phosphorylation and on macrophage activity. We are currently performing knockouts of the *L. donovani* SAcP gene to determine what effect this has on parasite survival in macrophages and infected mice.

In summary, the observations made in this study suggest that A2 localizes in the ER under conditions of stress where it is able to complex with the ER chaperone, BiP. Ectopic expression of A2 in *L. major* significantly improved parasite survival following heat shock and may therefore help *L. donovani* survive stresses associated with the visceral organs and with fever. Moreover, A2 also

interacted with BiP following heat shock, providing a possible biochemical role of A2 in *L. donovani*. These observations may guide us to a better understanding of host-parasite interactions during infection with different *Leishmania* species and of how this may affect the different pathologies associated with leishmaniasis.

2.7 Acknowledgements

We wish to thank Professor Dan Zilberstein for insightful discussion throughout the course of these experiments and for the anti-HSP83 antibodies. We also wish to thank Dr. J. Bangs for the anti-BiP antibodies and Dr Dennis Dwyer for the anti-SAcP antibodies. This work was supported by a research grant from the Canadian Institute of Health Research (CIHR) and a scholarship from the Fonds de la Recherche en Santé du Québec (FRSQ) to LIM.

2.8 Tables

Table 2.1 Protein accession numbers.

Protein	*Leishmania* species	Accession number
A2	*L. donovani*	LinJ22_V3.0670
	L. major	None
HSP83	*L. donovani*	LinJ33_V3.0350
		LinJ33_V3.0360
		LinJ33_V3.0370
	L. major	LmjF33.0312
		LmjF33.0314
		LmjF33.0316
		LmjF33.0318
		LmjF33.0320
		LmjF33.0323
		LmjF33.0326
		LmjF33.0330
		LmjF33.0333
		LmjF33.0336
		LmjF33.0340
		LmjF33.0343
		LmjF33.0346
		LmjF33.0350
		LmjF33.0355
		LmjF33.0360
		LmjF33.0365
BiP	*L. donovani*	LinJ28_V3.1310
	L. major	LmjF28.1200
SAcP	*L. donovani*	LinJ36_V3.6740
		LinJ36_V3.6770

	L. major	LmjF36.6460
		LmjF36.6480
gp63	*L. donovani*	LinJ10_V3.0500
		LinJ10_V3.0490
		LinJ10_V3.0510
		LinJ10_V3.0520
		LinJ10_V3.0530
		LinJ28_V3.0600
		LinJ28_V3.0610
	L. major	LmjF10.0460
		LmjF10.0465
		LmjF10.0470
		LmjF10.0480
		LmjF28.0570

2.9 Figures

Figure 2.1 Comparison of A2 and SAcP, HSP83 and BiP localization in infected macrophages.

B10R murine macrophages were infected with *L. major* or *L. donovani* promastigotes for 24 h, following which non-internalized parasites were washed away. **Panel A** A2 (green) and SAcP (red). **Panel B** A2 (green) and HSP83 (red). **Panel C** A2 (green) and BiP (red). Nuclei from the macrophages and *Leishmania* were stained with DRAQ5 (grey). Yellow indicates colocalization. The immunofluorescence images were generated by confocal microscopy with sequential scanning as detailed in Methods. Arrowheads indicate the localization of some parasites. Scale bars represent 5 μm. **Note**: The A2 gene in *L. major* is a pseudogene and therefore *L. major*-infected cells serve as a negative control for A2 expression. In the case of *L. donovani*-infected cells, not all HSP83-expressing parasites co-express detectable A2. **Note** also the similar staining pattern between BiP and A2.

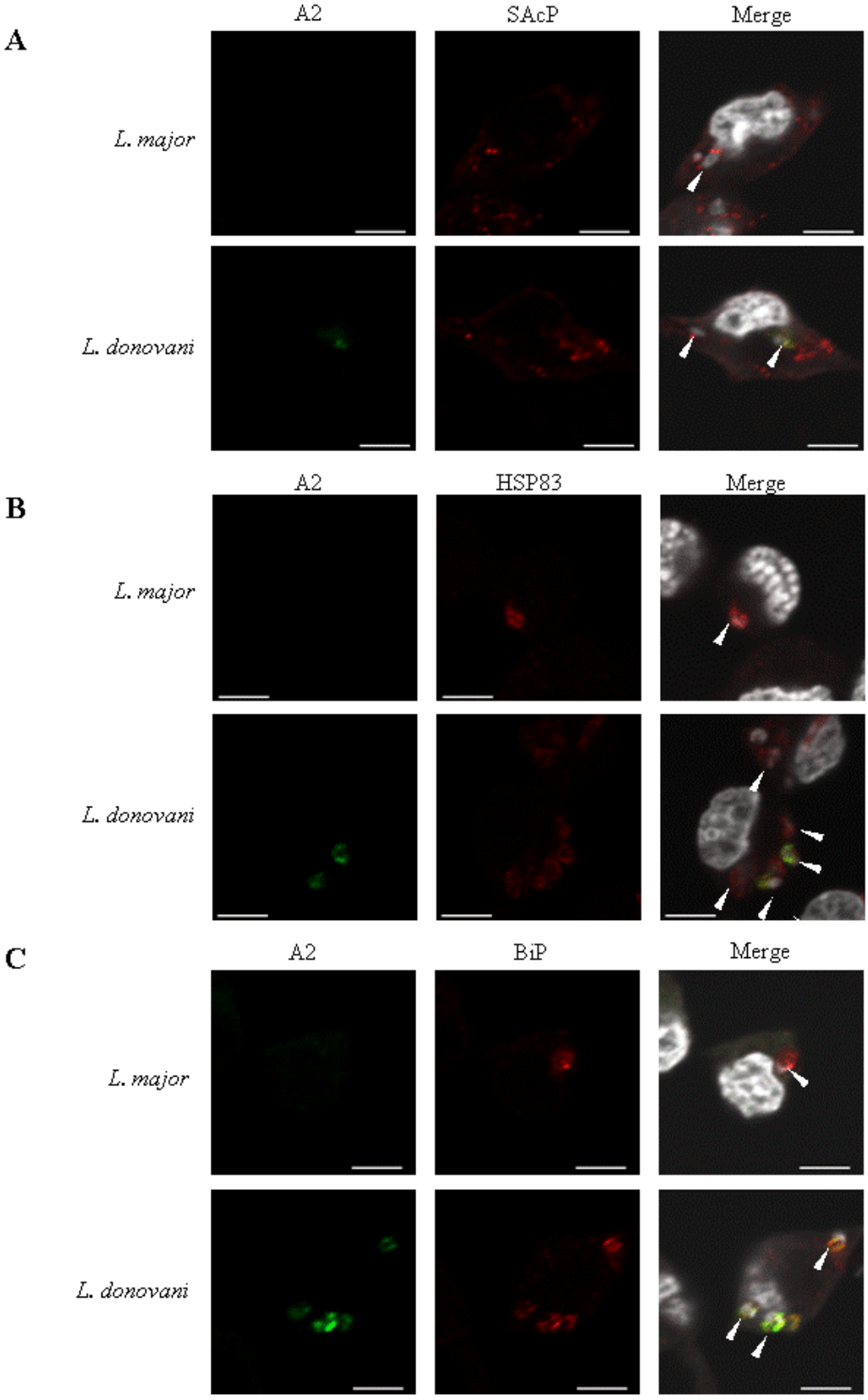

A
A2
SAcP
Merge
L. major
L. donovani
B
A2
HSP83
Merge
L. major
L. donovani
C
A2
BiP
Merge
L. major
L. donovani

Figure 2.2 Comparison of the secretion patterns of A2, BiP and SAcP from *Leishmania* in axenic culture.

A, Immunoprecipitation of secreted A2 from culture supernatants. **B,** Immunoprecipitation of intracellular A2 from parasite lysates. **C**, Immunoprecipitation of secreted SAcP from culture supernatants. **D**, Immunoprecipitation of intracellular SAcP from parasite lysates. **E**, Immunoprecipitation of secreted HSP83 from culture supernatants. **F**, Immunoprecipitation of intracellular HSP83 from parasite lysates. **G**, Immunoprecipitation of secreted BiP from culture supernatants. **H**, Immunoprecipitation of intracellular BiP from parasite lysates. Promastigotes ($300x10^6$) were grown in fresh media under promastigote (pro) or amastigote (ama) culture conditions for 24 h, then A2, SAcP, HSP83 and BiP were immunoprecipitated and detected by Western blot as detailed in Methods. *L.m*., *L. major*. *L.d*., *L. donovani*.

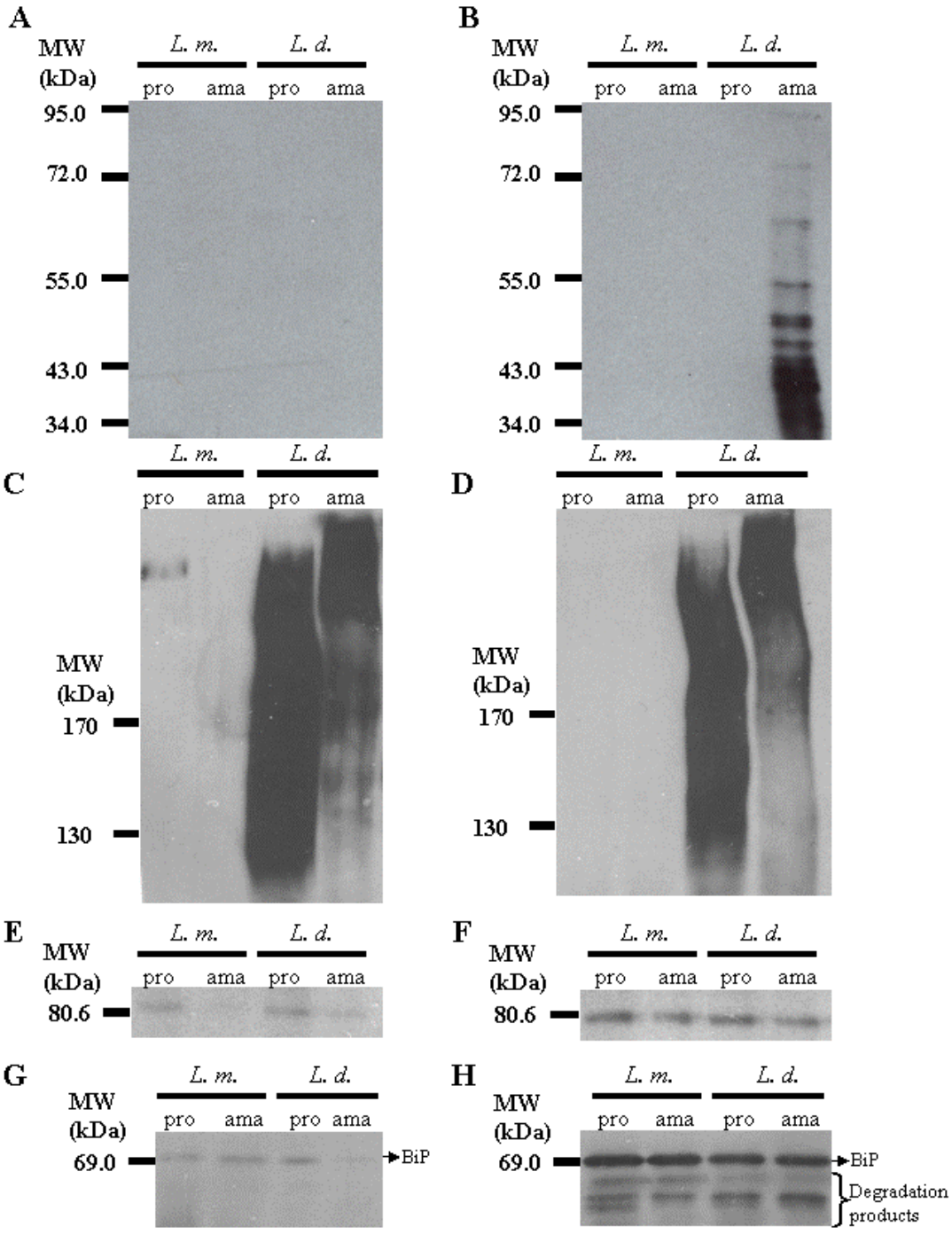

A
MW
(kDa)
L. m.
L. d.
pro
ama
pro
ama
95.0
72.0
55.0
43.0
34.0
B
MW
(kDa)
L. m.
L. d.
pro
ama
pro
ama
95.0
72.0
55.0
43.0
34.0
C
L. m.
L. d.
pro
ama
pro
ama
MW
(kDa)
170
130
D
L. m.
L. d.
pro
ama
pro
ama
MW
(kDa)
170
130
E
MW
(kDa)
L. m.
L. d.
pro
ama
pro
ama
80.6
F
MW
(kDa)
L. m.
L. d.
pro
ama
pro
ama
80.6
G
MW
(kDa)
L. m.
L. d.
pro
ama
pro
ama
69.0
BiP
H
MW
(kDa)
L. m.
L. d.
pro
ama
pro
ama
69.0
BiP
Degradation
products

Figure 2.3 Comparison of A2 expression by immunofluorescence following 40°C heat shock or following 24 h promastigote to amastigote differentiation. *L. major* or *L. donovani* promastigotes were heat shocked for 4 h at 40°C and pH 7, or grown under amastigote culture conditions (37°C pH 5.5) for 24 h to induce differentiation. A2 (green) was detected by immunofluorescence. Scale bars represent 5 μm. **Note** the higher proportion of parasites expressing A2 following heat shock compared to A2 expression under differentiation conditions.

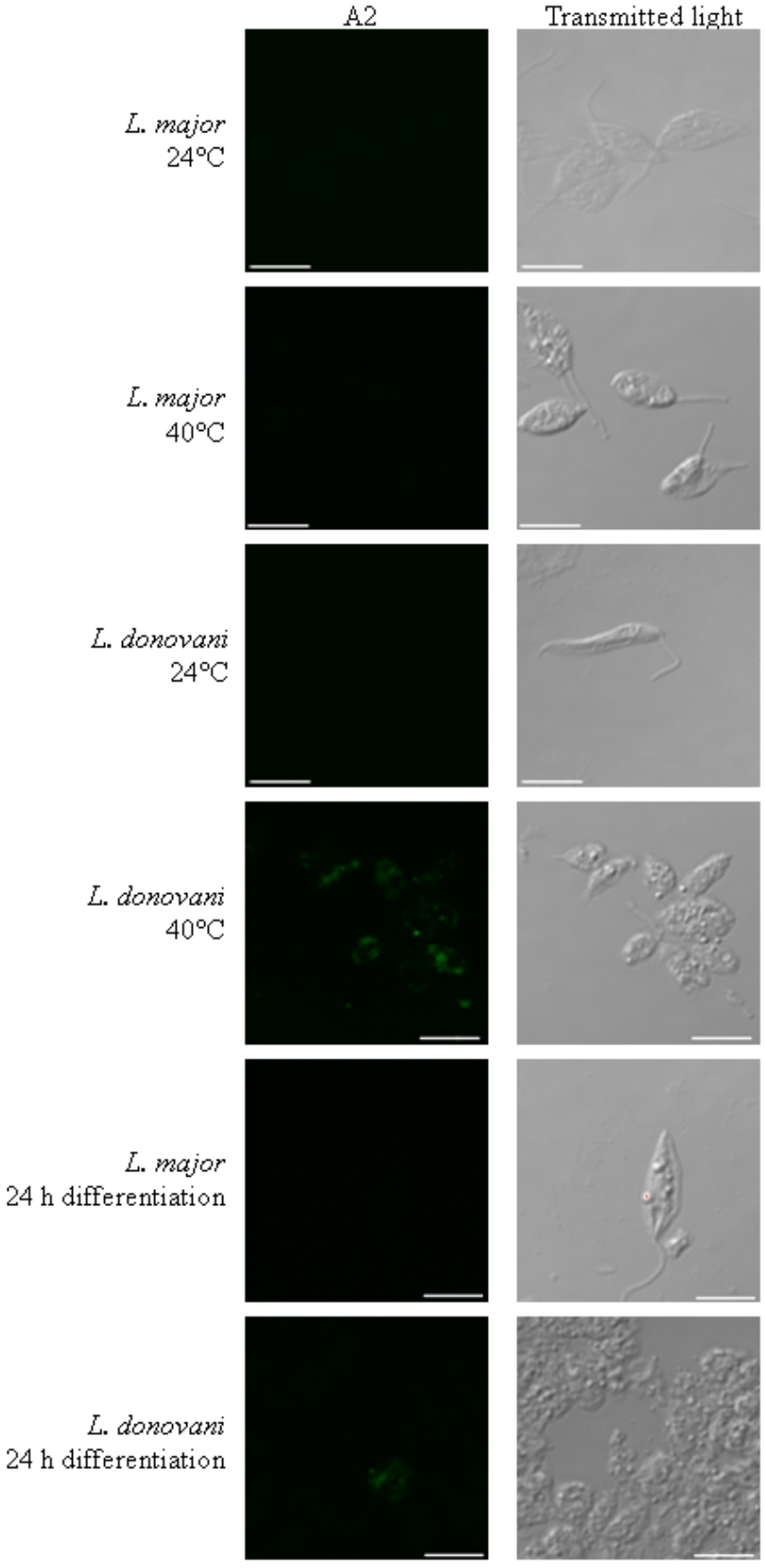

A2
Transmitted light
L. major
24°C
L. major
40°C
L. donovani
24°C
L. donovani
40°C
L. major
24 h differentiation
L. donovani
24 h differentiation

Figure 2.4 Comparison of A2 and HSP83 localization in heat shocked parasites.

L. major or *L. donovani* promastigotes were heat shocked for 4 h at 40°C pH 7. A2 (green) and HSP83 (red) were detected by immunofluorescence, and parasite nuclei were stained with DRAQ5 (grey). Yellow indicates colocalization. Arrowheads indicate parasites. Scale bars represent 5 μm.

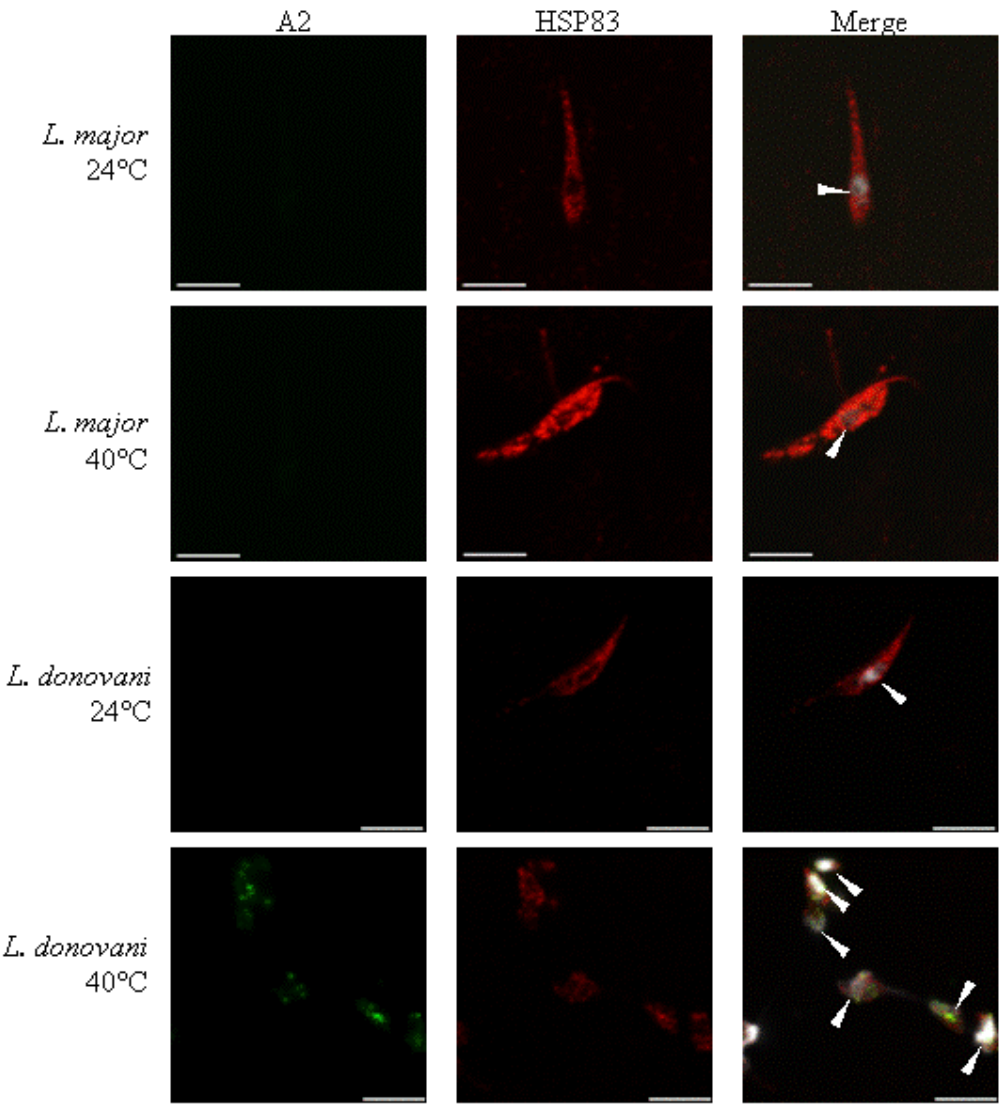


Figure 2.5 Comparison of A2 and BiP localization in heat shocked parasites. *L. major* or *L. donovani* promastigotes were heat shocked for 4 h at 40°C and pH 7. A2 (green) and BiP (red) were detected by immunofluorescence, and parasite nuclei were stained with DRAQ5 (grey). Two pictures are shown for *L. donovani* at 40°C. Yellow indicates colocalization. Arrowheads indicate parasites. Scale bars represent 5 μm. **Note** the colocalization of BiP and A2.

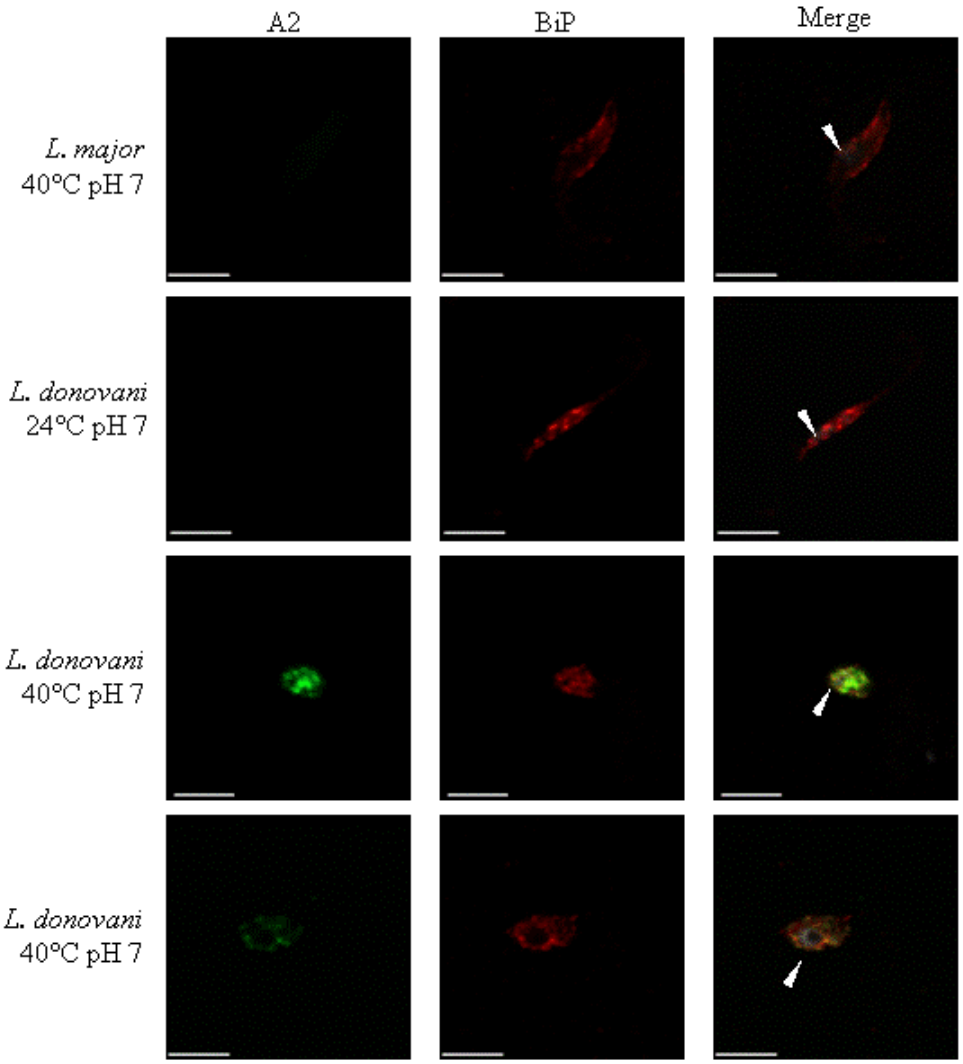


Figure 2.6 Co-immunoprecipitation of A2 and BiP.

Wild-type *L. major* (*L. m.*) and *L. donovani* (*L. d.*) promastigotes were heat shocked at 40°C for 4 h. Proteins were cross-linked with 1% paraformaldehyde and cells were lysed. Immunoprecipitation with either anti-A2 or anti-BiP antibodies was performed followed detection by Western blot of BiP (**A**), HSP83 (**B**), gp63 (**C**) or SAcP (**D**). HSP83, gp63 and SAcP were also detected in the *Leishmania* cell pellet as a control for the immunoprecipitation (lanes 5-8). **Note** the specific co-immunoprecipitation of A2 and BiP after 40°C heat shock in *L. donovani* (**A**, lane 4) and the absence of co-immunoprecipitation of A2 and HSP83 (**B**, lanes 1-4), A2 and gp63 (**C**, lanes 1-4) and A2 and SAcP (**D**, lanes 1-4).

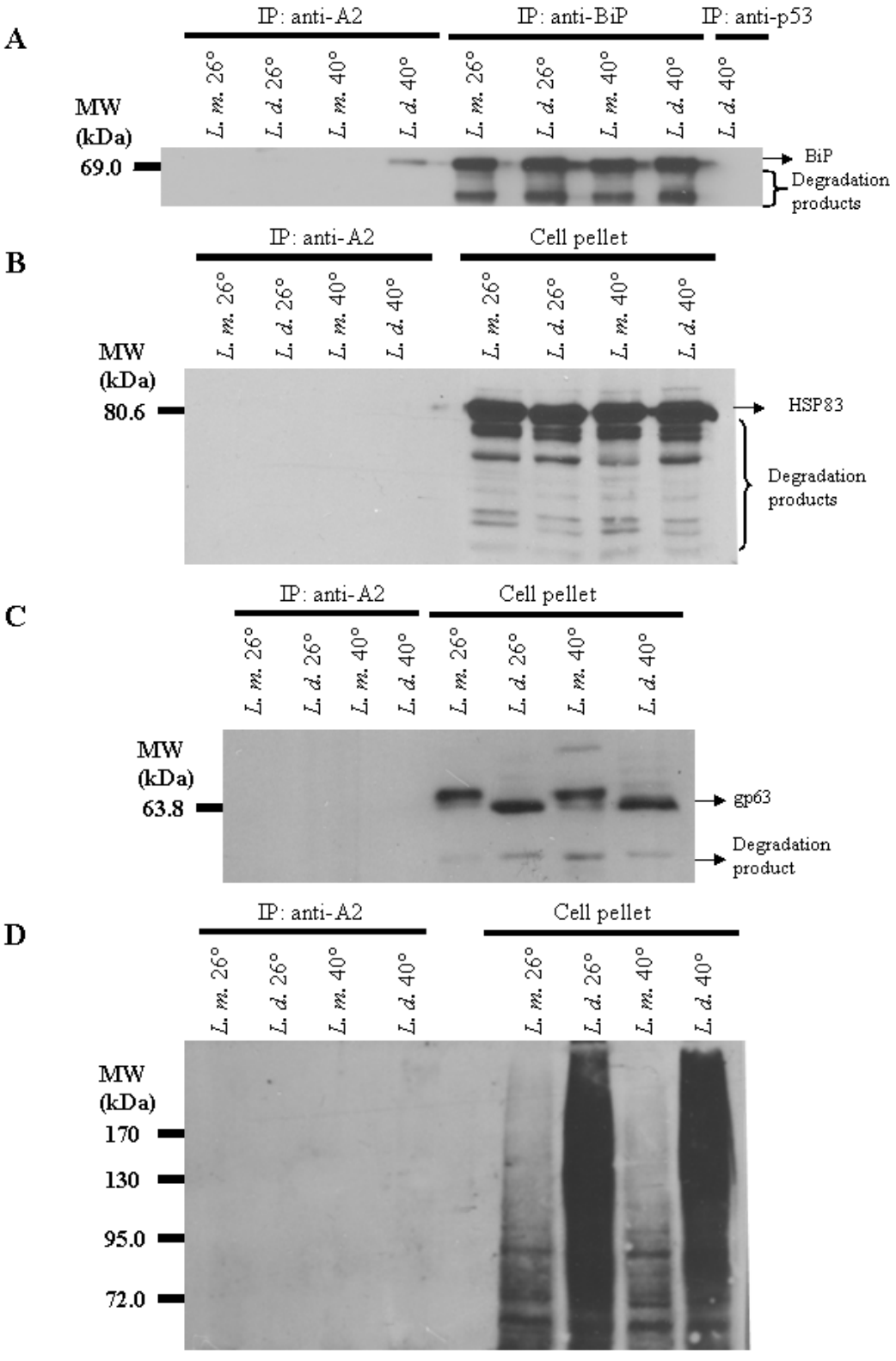
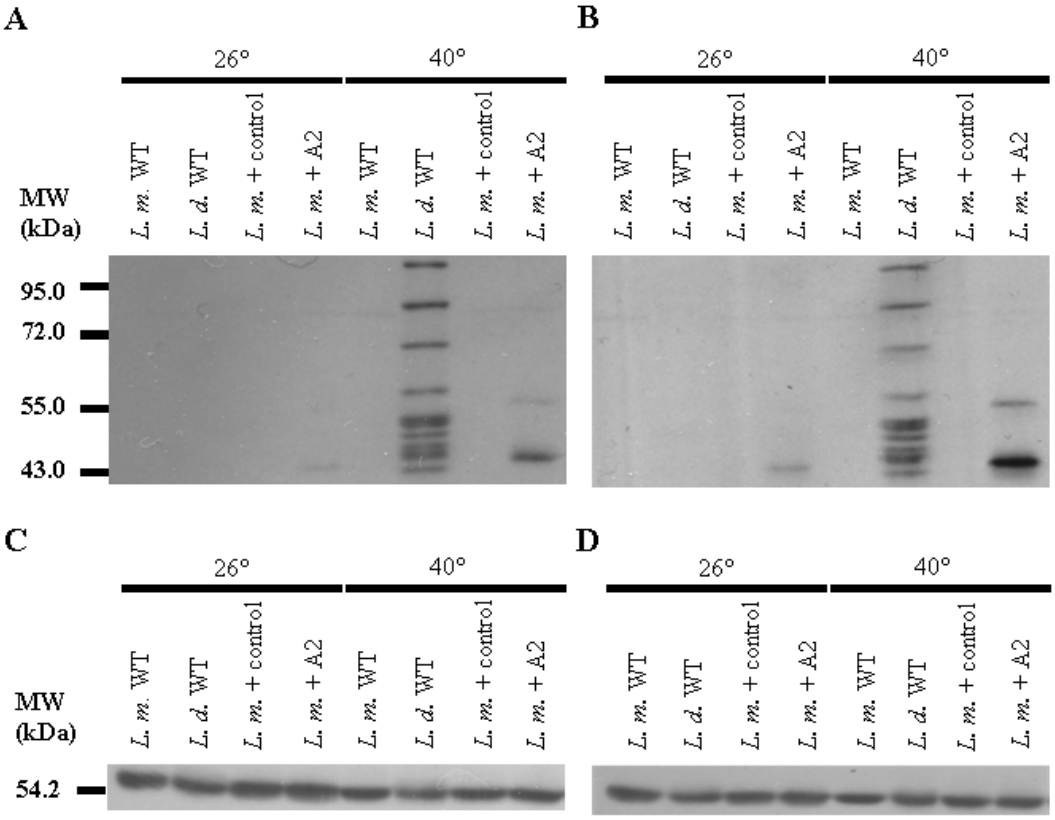
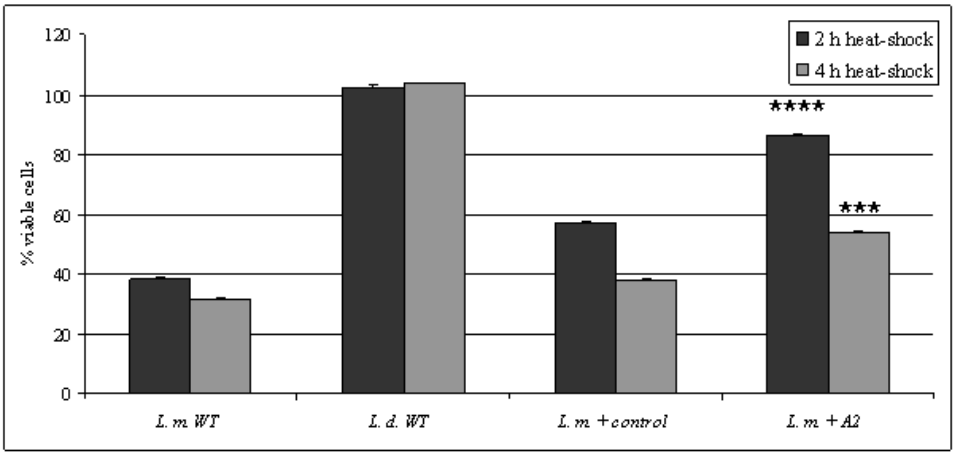

A
IP: anti-A2
IP: anti-BiP
IP: anti-p53
L. m. 26°
L. d. 26°
L. m. 40°
L. d. 40°
MW (kDa)
69.0
BiP
Degradation products
B
IP: anti-A2
Cell pellet
80.6
HSP83
Degradation products
C
63.8
gp63
Degradation product
D
170
130
95.0
72.0

Figure 2.7 A2 increases parasite survival following heat shock.

Wild-type *L. major* (*L. m.* WT) and *L. donovani* (*L. d.* WT) promastigotes, *L. major* transfected with the KSneo plasmid encoding A2 (*L. m.* + A2), and *L. major* transfected with the empty KSneo plasmid (*L. m.* + control) were heat shocked at 40°C for 2 h or 4 h. **A,** A2 expression following 2 h heat shock. **B**, A2 expression following 4 h heat shock. **C and D,** Anti-tubulin loading controls following 2 h (**C**) and 4 h (**D**) heat shock. **E,** Parasite survival following heat shock. Viability was assessed by the Alamar blue assay 72 h following heat shock, as detailed in Methods. Values + standard deviation are shown.


A
B
26°
40°
L. m. WT
L. d. WT
L. m. + control
L. m. + A2
MW (kDa)
95.0
72.0
55.0
43.0
C
D
54.2

E


2 h heat-shock
4 h heat-shock
% viable cells
120
100
80
60
40
20
0
****
***
L. m WT
L. d. WT
L. m +control
L. m +A2

Chapter 3

Involvement of the *Leishmania donovani* virulence factor A2 in protection against heat and oxidative stress

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3.1 Preface

Ectopic expression of A2 in *L. major* promastigotes protects *L. major* against heat shock (**chapter 2**). However, promastigotes rapidly differentiate into amastigotes in the mammalian host, so amastigotes rather than promastigotes are the life stage exposed to fever. Until recently, *in vitro* culture of axenic amastigotes could not be performed for *L. major*. Therefore, to study the role of A2 in amastigote survival, we needed to use *L. donovani* instead of *L. major*. Using *L. donovani* transfected with an antisense construct previously shown to

significantly downregulate A2 expression [99], we investigated the role of A2 in *L. donovani* axenic amastigote survival during heat shock.

In addition to fever, reactive oxygen and nitrogen species represent a key host defense against leishmaniasis. We therefore compared the levels of intracellular reactive oxygen species and parasite survival during oxidative stress between A2-deficient and A2-expressing amastigotes.

Finally, A2 associates with BiP (**chapter 2**), a key component of the unfolded protein response, so we determined whether A2 can protect against unfolded protein stress caused by dithiothreitol.

3.2 Abstract

Leishmania is an obligate intracellular protozoan parasite that infects cells of the reticulo-endothelial system. Host defences against *Leishmania* include fever and oxidant production, and the parasite has developed a number of defence mechanisms to neutralize the host response. The *L. donovani* A2 family of proteins has been shown to be essential for survival in mammalian visceral organs. Here we provide evidence that A2 proteins protect the parasite against host defences, namely heat stress (fever) and oxidative stress. A2 is however unable to protect the cells from endoplasmic reticulum stress induced by dithiothreitol. To downregulate A2 protein expression, *L. donovani* was transfected with an A2 antisense RNA expressing-vector, resulting in significant reduction of A2 levels. The resulting A2-deficient cells were more sensitive to heat shock and this was associated with increased production of internal oxidants

during heat shock. Moreover, axenic amastigotes with downregulated A2 expression had increased internal oxidants and decreased viability following treatment with hydrogen peroxide or a nitric oxide donor when compared to control cells. Overall, these results suggest that A2 protects *L. donovani* from a variety of stresses, thereby allowing it to survive in the internal organs of the mammalian host and to cause visceral disease.

3.3 Introduction

Leishmania are protozoan parasites that have a dimorphic lifecycle where the promastigote stage proliferates in the sand fly vector and is transmitted to its mammalian host during a blood meal. Within the mammalian host, the promastigotes differentiate into amastigotes and multiply predominantly inside macrophages. Amastigotes can then infect other macrophages or be taken up by feeding sand flies. Human infection is associated with a variety of disease manifestations depending mainly on the infecting *Leishmania* species. In particular, *Leishmania donovani* causes visceral infections and *Leishmania major* cutaneous disease [192].

Since *Leishmania* specifically multiply inside macrophages, they are exposed to a battery of host defences, including reactive oxygen and nitrogen species [128]. Nitric oxide in particular is especially important for parasite clearance [365]. Infection with visceralizing species such as *L. donovani* is also associated with high fever [8]. The parasite must therefore be able to withstand oxidative stress and heat stress. *Leishmania* has evolved a broad spectrum of

mechanisms to protect itself against these host defences, including enzymes such as superoxide dismutase [131], 2-cys peroxiredoxins, ascorbate peroxidases and glutathione peroxidase-like enzymes [134]. These enzymes detoxify reactive oxygen and nitrogen species using electrons from NADPH transferred via trypanothione and tryparedoxin, ascorbate and glutathione [134]. Non-enzymatic scavengers such as thiols also play a key role in antioxidant defenses [131]. Finally, *Leishmania* can also inhibit host intracellular pathways, thereby leading to downregulation of oxidant production by the macrophage [135].

A2 genes are arranged in tandem arrays on chromosome 22 (LinJ.22.0670) [84], with up to four such arrays per diploid genome [54]. A2 proteins in *L. donovani* have 40 to 90 copies of a repetitive ten amino acid sequence, leading to a family of proteins with sizes ranging from 42 to 100 kDa [85]. The number of proteins varies between strains, with up to nine different-sized A2 proteins detected in the *L. donovani* 1S/Cl2D strain. Although A2 was originally considered amastigote-specific and expressed at low levels in promastigotes [87], A2 has since been shown to be induced in promastigotes by a variety of stresses, including heat shock, protein misfolding and induction of the unfolded protein response [57,92,93]. A2 is localized to the endoplasmic reticulum (ER) during mammalian infection and in heat shocked promastigotes [57].

A2 proteins are expressed by the visceral disease-causing *L. donovani*, but not by *L. major* [46]. Downregulation of A2 is associated with decreased macrophage infection levels *in vitro* and decreased parasite levels in the spleen and liver during experimental mouse infection [54,99]. In contrast, experimental

introduction of an ectopic A2 gene into *L. major* leads to increased survival in visceral organs [46]. More recently, it has been demonstrated that experimental introduction of A2 into *L. major* resulted in increased protection of *L. major* from heat shock [57]. Based on these observations involving ectopic A2 expression in *L. major,* it was necessary to confirm the role of endogenous A2 in *L. donovani* as described within. Since there are too many A2 gene families to perform gene deletions in *L. donovani* [54], we have used antisense A2 RNA to downregulate A2 protein expression as previously described [99]. This approach leads to stable downregulation of all sizes of A2 in *L. donovani* [99].

Downregulation of A2 expression in *L. donovani* was associated with decreased resistance to heat shock. Interestingly, it was further observed that downregulation of A2 resulted in increased internal oxidant levels following heat shock and decreased resistance against reactive oxygen species (ROS) and nitric oxide. A2 could not however protect from unfolded protein stress in response to dithiothreitol (DTT). Given that heat shock is associated with the generation of internal ROS [366] and changes in membrane fluidity [136], this may be one way in which A2 promotes survival at higher temperatures. Overall, these results provide a possible process to explain the role of A2 in supporting *L. donovani* survival within visceral organs and indicate that A2 may be a key determinant of the *L. donovani*-*L. major* dichotomy.

3.4 Materials and methods

3.4.1 Parasite culture and transfections.

L. donovani 1S/Cl2D strain promastigotes were maintained at 26°C pH 7 as previously described [57]. Briefly, cells were grown in M199 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, streptomycin, penicillin, adenosine, glutamine and folic acid, at pH 7.2.

Promastigotes were transfected as described in [367] with the KSneo-control plasmid and KSneo-A2 (R) antisense plasmid generated by Zhang and Matlashewski [99]. Briefly, the KSneo-A2 (R) plasmid contains a 1.6-kb A2 coding sequence and part of the A2 3′ untranslated region (UTR), inserted in the antisense orientation in between the A2 5' UTR and 3' UTR. The KSneo-control plasmid contains only the A2 5' and 3'UTR in the sense orientation. Drug selection is provided by a neomycin resistance gene. Transfected parasites were maintained in media supplemented with 200 μg/mL G418 (Wisent), leading to stable maintenance of the plasmid in transfected parasites.

To induce promastigote to amastigote differentiation, promastigotes were resuspended in amastigote media (M199 medium supplemented with 25% FBS, streptomycin, penicillin, succinic acid, adenine, glycerol, L-proline and folic acid, at pH 5.5) at a cell density of $5x10^6$ cells/mL and transferred to 37°C, 5% CO_2.

3.4.2 Heat shock and promastigote viability assay.

Promastigotes ($1x10^7$ cells) were resuspended in fresh promastigote media at $5x10^6$ cells/mL and incubated at 26°C or 40°C for 12 h. Following heat shock,

promastigotes were diluted to a cell density of $1x10^6$ cells/mL, incubated at 26°C in the presence of Alamar blue reagent (Invitrogen) for 72 h and absorbance was determined at 570 and 600 nm as previously described [57,368].

3.4.3 Amastigote viability assays.

For heat shock, amastigotes were incubated at 37°C or 40°C for 12 h and then diluted into fresh amastigote media. For oxidative stress assays, amastigotes were washed twice in HEPES-NaCl buffer [369], resuspended in HEPES-NaCl buffer and treated with H_2O_2 (BioShop) or *S*-nitroso-*N*-acetylpenicillamine (SNAP, Sigma) for 4 h at 37°C or 40°C. The cells were then resuspended in amastigote media. OD600 was measured 48 h after stress treatments, except for DTT treatment where amastigotes were treated for 72 h with dithiothreitol (DTT, Fisher Scientific) dissolved in water. To account for any possible discrepancies in growth rates between A2-expressing and A2-deficient cells, the % survival was calculated for each strain as: (OD600 value of stressed sample/OD600 value of unstressed sample)×100 as described in [370]. For DTT treatments, the OD600 of the treated samples were divided by the OD600 of water-treated cells. We used OD600 measurements for amastigote viability assays rather than Alamar blue because the optimal pH for Alamar blue assays is between pH 6.8 and 7.4 as per manufacturer's instructions.

3.4.4 Dichlorofluorescein diacetate (DCFDA) assay.

DCFDA assays were performed as described in [370]. Briefly, cells were collected, washed twice with HEPES-NaCl buffer, and incubated in HEPES-NaCl buffer containing 5 μM CM-H_2DCFDA (Invitrogen) for 45 min. Promastigotes were incubated in DCFDA-containing buffer at 26°C and amastigotes at 37°C. The cells were then washed and resuspended in HEPES-NaCl buffer. Hydrogen peroxide or SNAP were added immediately prior to measuring fluorescence at 485 nm excitation and 535 nm emission wavelenghts on a Spectramax M5 fluorometer (Molecular Devices). Values were standardized to the measurements at t=0.

3.4.5 SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting were performed as previously described [57]. Briefly, samples were boiled 5 min in SDS-PAGE loading buffer and were separated by SDS-PAGE using a 10% acrylamide gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked 1 h in 5% skim milk in PBS. The primary and secondary antibody incubations were 1 h in duration and were followed by five 5 min washes in PBS with 0.1% Tween (PBS-T). All antibodies were diluted in 5% skim milk in PBS-T. A2 was detected with a 1:8,000 dilution of mouse monoclonal anti-A2 antibody (ascites) and tubulin with a 1:2,000 dilution of mouse monoclonal anti-tubulin antibody (Oncogene), followed by a 1:2,500 dilution of peroxidase-conjugated anti-mouse IgG antibody

(Rockland). All proteins were visualized using ECL Western Blot Detection Reagents (GE Healthcare).

3.5 Results

3.5.1 A2 protects *L. donovani* from heat shock.

Viscerotropic parasites are exposed to higher temperatures within the visceral organs than cutaneous species that are restricted to the skin. In addition, visceral leishmaniasis is associated with chronic high fever [8]. Therefore, viscerotropic parasites must be able to withstand higher temperatures than cutaneous species. Previous work showed that the experimental introduction of an ectopic A2 gene into *L. major* protects *L. major* promastigotes from heat shock [57]. It was however necessary to determine whether this can be generalized to *L. donovani,* the species where A2 is normally expressed. A2 expression in *L. donovani* was downregulated by transfecting *L. donovani* with an A2 antisense RNA-expressing vector or the control empty KSneo plasmid as previously described [99]. Cells expressing the antisense vector have significantly decreased A2 protein levels following heat shock at 40°C, whereas A2 expression levels are similar in wild-type *L. donovani* and control-transfected *L. donovani*. However, antisense mRNA transfection does not fully abrogate A2 expression since low levels of residual A2 proteins can be detected in these cells (**Figure 3.1A**). These cells are described from here on as "A2-deficient cells". *L. donovani* promastigotes with antisense A2 mRNA expression were then subjected to heat shock and this resulted in reduced viability of the A2-deficient cells following a

12 h heat shock compared to the control-transfected cells expressing wildtype levels of A2 (**Figure 3.1B**) ($p<0.001$). For these analyses, all viability assays compared *L. donovani* transfected with an A2 antisense RNA-expressing vector (L. d. - A2) to control-transfected cells rather than to wild-type *L. donovani* to account for any potential effects that transfection and selection could have on *L. donovani.*

Amastigotes are the life stage of the parasite in the mammalian host and therefore the main stage exposed to fever during visceral leishmaniasis. Accordingly, we investigated whether A2 also protects axenic amastigotes from heat shock. *L. donovani* transfected with the A2 antisense RNA-expressing vector or the control vector were induced to differentiate into axenic amastigotes. Promastigote to amastigote differentiation is associated with induction of A2 [87]. A2 expression was detected in control-transfected amastigotes, whereas A2 was significantly downregulated in cells expressing the antisense construct (**Figure 3.1C**). Amastigotes are more resistant than promastigotes to heat shock, and we did not observe a significant decrease in viability in amastigotes following 6 and 8 h heat shock. However, when the heat shock duration was increased to 12 h, the viability of A2-deficient amastigotes was significantly reduced (**Figure 3.1D**) ($p<0.001$), indicating that A2 protects both promastigotes and amastigotes from heat shock.

3.5.2 Decreased internal reactive oxygen species during heat shock in cells expressing A2.

Cellular damage during heat shock is associated with protein misfolding and with the production of ROS [366]. Therefore, to investigate the mechanism by which A2 protects against heat shock, it was of interest to measure internal ROS during heat shock in control and A2-deficient *L. donovani* promastigotes. There was a significantly higher ($p<0.05$) amounts of ROS detected in *L. donovani* promastigotes with reduced A2 protein levels compared to the control promastigotes (**Figure 3.2**). ROS levels are also higher in A2-deficient promastigotes in the absence of heat shock, but the difference is non-significant (data not shown).

3.5.3 A2 does not protect *L. donovani* from unfolded protein stress induced by DTT.

Heat shock is also associated with protein damage and protein misfolding [366], and there is some overlap between the response to misfolded proteins caused by heat stress and the response to unfolded proteins in the ER. Heat shock can induce an unfolded protein response (UPR) in yeast [371] and conversely unfolded protein stress can partially induce a heat shock response in cells deficient in the activation of the UPR pathway [372].

Previous work revealed that A2 interacts with BiP [57], a key regulator of the UPR [373]. Treatment with DTT in *L. donovani* has been shown to induce an UPR, as characterized by eukaryotic initiation factor 2 alpha (eIF2α)

phosphorylation [92]. In addition, DTT treatment has previously been shown to induce A2 expression [92]. We therefore examined whether A2 could protect *L. donovani* against unfolded protein stress. Control-transfected and A2-deficient amastigotes were equally susceptible to DTT (**Figure 3.3**), indicating that, interaction with BiP notwithstanding, A2 does not protect *Leishmania* against unfolded proteins in the ER or against any other toxic effects caused by treatment with DTT. Similar results were obtained when viability was measured 48 h after treatment (data not shown). Although tunicamycin is another classical activator of the UPR [373], it is unable to induce significant amounts of stress or eIF2α phosphorylation in *L. donovani* [92] and was therefore not tested.

3.5.4 A2 protects *L. donovani* from oxidative stress.

In addition to fever, another key host defence against invading microorganisms is the production of reactive oxygen and nitrogen species, in particular in the phagolysosome of macrophages. To examine the involvement of A2 in the *L. donovani* response to oxidative stress, A2-expressing and A2-deficient amastigotes were treated with hydrogen peroxide or *S*-nitroso-*N*-acetylpenicillamine (SNAP), a nitric oxide donor, and the increase in internal ROS was monitored over time. The DCFDA probe used in this assay can be oxidized by both nitric oxide and ROS. Therefore the oxidant levels measured include both direct oxidation of the probe by hydrogen peroxide treatment or by NO release from SNAP, as well as the ROS produced by the cell in response to these stresses. This allows us to compare total oxidant levels in cells with normal

A2 expression to levels in cells with decreased A2 expression. Internal ROS levels were significantly increased ($p<0.05$) in amastigotes in which A2 expression was downregulated compared to the control amastigotes (**Figure 3.4**). Similar results were observed in promastigotes treated with hydrogen peroxide under heat shock conditions to induce A2 expression (data not shown).

We next determined whether there was a decrease in *L. donovani* viability associated with increased internal oxidant levels. As shown in **Figure 3.5**, the increased internal oxidant levels in A2-deficient *L. donovani* amastigotes was associated with decreased viability following treatment with hydrogen peroxide or SNAP. Since the parasite is exposed to a combination of stresses in the mammalian host, hydrogen peroxide and SNAP treatment were performed at 37°C and under heat shock (fever) conditions at 40°C. For SNAP treatment, viability was lower at 40°C than at 37°C, indicating a cumulative effect of heat shock and SNAP treatment. In contrast, parasite viability was similar at 37°C and 40°C for H_2O_2-treated parasites, indicating that heat shock and H_2O_2 treatment did not have cumulative effects. It may be that hydrogen peroxide is so toxic to the cells that oxidant-induced toxicity masks any additional effect of the heat shock. Overall, these observations indicate that A2 protects *L. donovani* from oxidative stress under normal amastigote culture conditions and during heat shock.

3.6 Discussion

Virulence is associated with an increased capacity to withstand host defences, such as reactive oxygen and nitrogen species and increased

temperatures. The ability to survive higher temperatures and fever may be a key determinant of the ability to parasitize visceral organs [57]. Since the *L. donovani* virulence factor A2 promotes parasite survival in mammalian infections [54,99], we investigated whether A2 protects *L. donovani* from heat shock and oxidative stress.

A2 downregulation was achieved by transfection of *L. donovani* with an antisense mRNA-expressing construct as described by Zhang and Matlashewski [99]. This construct contains an A2 open reading frame (ORF) and part of the A2 3'UTR in the antisense orientation. However, this 3' UTR fragment in the antisense orientation is not required for the downregulation of A2 expression, indicating that the downregulation of A2 expression is mediated by the antisense A2 open reading frame and not the 3' UTR [374]. Overall, these results argue that the effect of the A2 antisense mRNA is specific to A2 and this could be due to the repetitive A2 sequence providing a lower C_0t value for hybridization. Moreover, antisense RNA does not appear to work for other target mRNAs in *L. donovani* [375]. Taken together, we believe it is therefore unlikely that there are off-target effects of the antisense A2 RNA.

The impact of A2 on parasite survival was monitored in axenic amastigotes. Although these may differ from mammalian-derived amastigotes in some aspects [11], axenic and intracellular amastigotes share many common characteristics, including morphology, metabolism and amastigote-specific protein expression patterns [12]. In addition, A2 is expressed in both intracellular and axenic amastigotes [13] and A2 protein expression patterns are similar

between axenic and intracellular amastigotes (our personal observations), making axenic amastigotes a good surrogate for intracellular amastigotes in our assays.

Overall, the results presented in this study indicate that downregulation of A2 is associated with increased sensitivity to heat and oxidants and therefore that A2 plays a key role in the *L. donovani* stress response. Such a pleiotropic effect has also been observed for other *Leishmania* virulence factors. For instance, calcineurin is essential for *L. major* virulence and can protect the parasites against heat shock and stress induced by tunicamycin [376]. Similarly, *L. mexicana* lacking a subunit of the adaptor protein 1 is avirulent [377] and is more susceptible to heat shock [378].

The ability to protect against diverse stresses may be due to common mechanisms of cellular damage induced by these different stresses and to common response pathways. Indeed, many stresses are associated with the production of ROS, such as heat shock and treatment with exogenous oxidants (see **Figures 3.2 and 3.4**). In addition, many stress response pathways are intimately linked: antioxidants can protect against UPR stress [379] and conversely, the UPR can protect against oxidative stress [380].

A2 is localized to the ER [57] and this organelle plays a key role in the response to many different stresses. The link between heat shock, ER stress and oxidative stress may explain the capacity of A2 to protect against a variety of stresses that lead to the generation of ROS. The ER is also a source of ROS during unfolded protein stress [381].

Heat shock is associated with internal ROS production, protein damage and changes in membrane fluidity [136]. The ability to A2 to protect against heat shock may be linked to the fact that control-transfected promastigotes produce fewer internal ROS during heat shock than A2-deficient cells (**Figure 3.2**). However, A2 could not protect against unfolded protein damage caused by DTT (**Figure 3.3**), even though A2 interacts with the ER chaperone BiP [57], an important regulator of the UPR in yeast and higher eukaryotes [373] and even though A2 is induced by DTT treatment [92].

There are several possible explanations for the lack of protection by A2 against DTT. First, although DTT can induce inhibition of protein translation via induction of eIF2α phosphorylation, it does not alter BiP expression levels in *L. donovani* [92], indicating that UPR induction by DTT in *L. donovani* is non-classical. It is therefore possible that BiP does not play a role in the UPR induced by DTT in *Leishmania* and so the interaction of A2 with BiP would not affect the UPR. Alternatively, the interaction of A2 and BiP may not modify the function of BiP. BiP is involved in the import of proteins into the ER and in protein folding [382] and this may be the cause of the A2-BiP interaction. The mechanism of cellular damage by DTT also differs from stresses such as heat shock in that DTT has antioxidant properties and has been shown to block endogenous ROS production in UPR-deficient cells [380]. In addition, DTT can inhibit the heat shock response [383], suggesting that the ability of A2 to protect against stress requires an active heat shock response. Finally, we cannot rule out that other

negative effects of DTT in addition to UPR activation also contributed to loss of cell viability and that A2 does not protect against these other negative effects.

In addition to its ability to protect against heat shock, A2 can also protect against exogenous oxidative stress. Heat shock has been reported to decrease susceptibility of *L. donovani* promastigotes to hydrogen peroxide [384,385], and heat shock rapidly induces A2 expression in this parasite [57]. Similarly, amastigotes are less sensitive to oxidative stress than promastigotes [386,387], even though promastigote to amastigote differentiation is associated with the downregulation of lipophosphoglycan [388], an oxidant scavenger [132]. In addition, *L. major* is more sensitive to hydrogen peroxide [389] and nitric oxide [61] than *L. donovani*. Our results suggest that one of the factors mediating the increased resistance of amastigotes or heat shocked promastigotes to oxidative stress is the induction of A2.

In addition to its ability to protect the cells from lethal stresses, A2 actually promotes increased parasite growth during stress: control-transfected amastigotes grew better at 40°C than at 37°C (**Figure 3.1**). The ability of A2-expressing *L. donovani* to grow better in the presence of stress may represent an adaptation of the parasite to grow under stressful conditions in the mammalian host. Indeed, the number of amastigotes per infected macrophage are lower following infection with A2-deficient cells compared to control-transfected parasites, both in unstimulated macrophages [99] and following macrophage activation with lipopolysaccharide (data not shown). This may provide A2-

expressing cells with a selective advantage during *in vivo* infection and may explain the importance of A2 during mammalian infection.

The biochemical mechanism by which A2 protects against these different stresses is unclear. A2 has no sequence homology with proteins of known function, including antioxidants. A2 is predicted to have a structure similar to type I collagen by the CPH models 3.2 protein homology modeling server (**Figure 6.1**) [390]. Overall, this suggests that A2 may have a structural rather than enzymatic role, indicating that A2 is unlikely to directly detoxify reactive oxygen and nitrogen species. However, modulation of A2 expression may alter the levels of antioxidant enzymes, thereby leading to the observed changes in intracellular ROS levels and in sensitivity to oxidants. Indeed, modulation of one antioxidant enzyme, ascorbate peroxidase, has been associated with changes in the expression levels of other antioxidants such as non-selenium glutathione peroxidase [391]. Antioxidant pathways such as the cytosolic tryparedoxin peroxidase [392], and glutathione and trypanothione production play a key role in virulence in *Leishmania* [369].

Alternatively, a number of structural proteins such as intermediate filaments have been implicated in the stress response. Several mechanisms have been proposed, including mechanical support and scaffolding functions to promote protein-protein interactions [393]. Therefore, A2 may represent a binding scaffold, interacting with other proteins that are in turn responsible for the detoxification of reactive oxygen and nitrogen species. Indeed, A2 interacts with BiP [57], a member of the HSP70 family of proteins [351], which are induced by

oxidants and have been associated with protection of *L. chagasi* from oxidative stress [385].

Overall, these results provide important insight into the function of the *L. donovani* virulence factor A2 and its role in pathogenesis. Specifically, A2 protects the parasite from host defence mechanisms, including reactive oxygen and nitrogen species and fever. Downregulation of A2 is conversely associated with increased susceptibility to these factors and as such may represent a key distinction between cutaneous and visceral Old World *Leishmania* species.

3.7 Acknowledgments

This work was supported by a research grant from the Canadian Institute of Health Research (CIHR), and scholarships from the Fonds de la Recherche en Santé du Québec (FRSQ) and from CIHR to LIM.

3.8 Figures

Figure 3.1 A2 protects L. donovani from heat shock.

A, Antisense downregulation of A2 during heat shock. Wild-type *L. donovani* (*L. d.* WT) promastigotes, *L. donovani* transfected with the control empty KSneo plasmid (*L. d.* + control), and *L. donovani* transfected with the A2 antisense RNA expressing plasmid (*L. d.* – A2) were heat shocked at 40°C for 12 h. The A2 proteins and tubulin were detected by Western blot. **Top,** A2 expression levels. Expression of A2 is substantially but not fully abrogated in *L. donovani* transfected with the antisense construct (*L. d.* –A2). **Bottom,** Anti-tubulin loading control. **B,** Alamar blue viability assay. Promastigotes were heat shocked for 12 h and viability was measured using the Alamar blue assay. ***, $p<0.001$. Values + standard error are shown. Representative of three independent experiments performed in triplicate. **C,** Promastigote to amastigote differentiation was induced in *L. donovani* transfected with the control empty KSneo plasmid (*L. d.* + control), and *L. donovani* transfected with the antisense A2 plasmid (*L. d.* – A2). A2 and tubulin were detected by Western blot in amastigotes. **Top,** A2 expression levels. Expression of A2 is substantially but not fully abrogated in amastigotes expressing the antisense construct (*L. d.* - A2). **Bottom,** Anti-tubulin loading control. **D**, Amastigotes of *L. donovani* transfected with the control empty KSneo plasmid (*L. d.* + control) or the antisense A2 plasmid (*L. d.* – A2) were heat shocked for 12 h. OD600 measurements were performed 48 h later and % survival was calculated as described in methods. ***, $p<0.001$. Values +

standard error are shown and represent the average of four independent experiments.

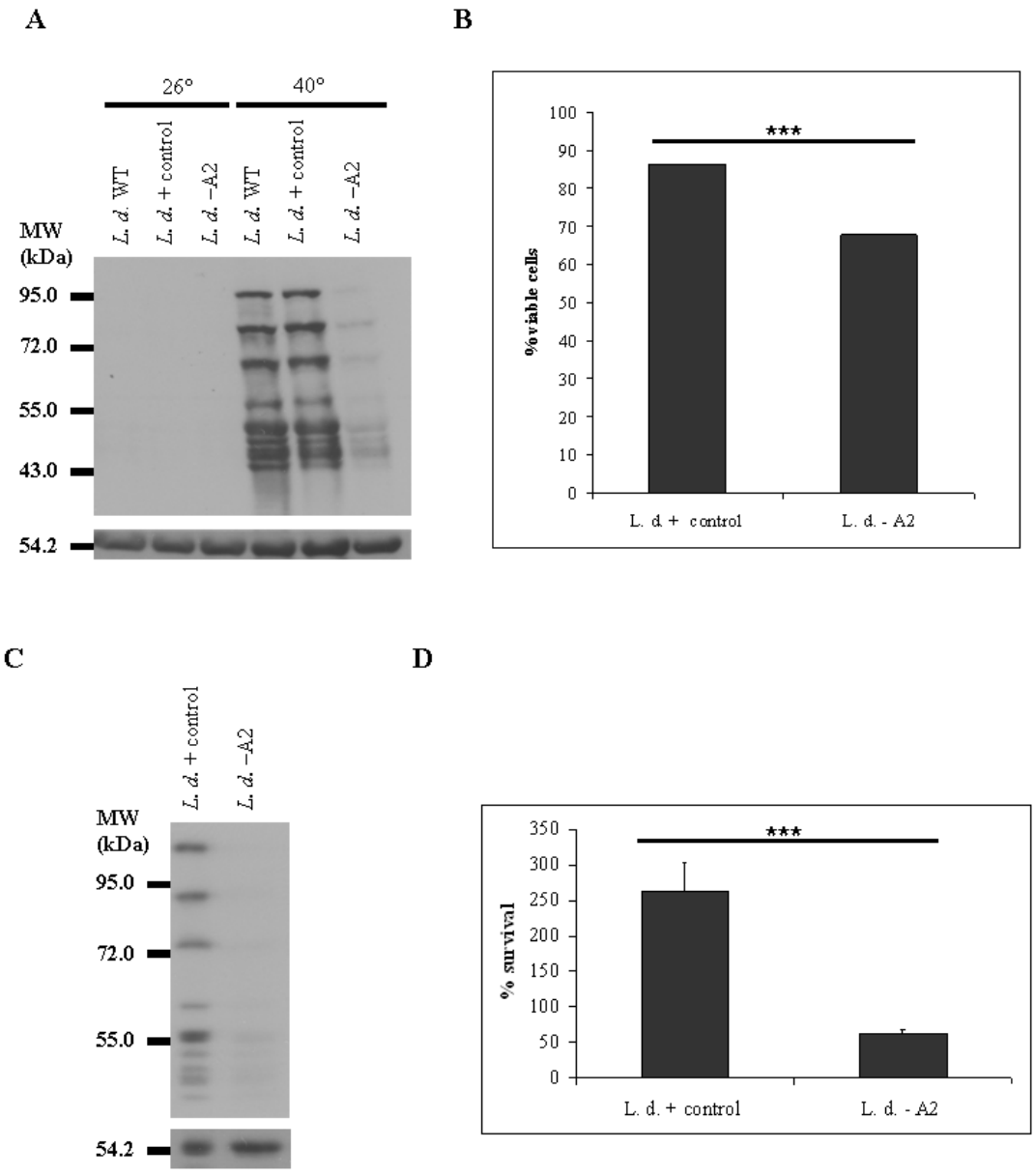


Figure 3.2 Internal reactive oxygen species during heat shock in *L. donovani*. *L. donovani* transfected with the control KSneo plasmid (*L. d.* + control), and *L. donovani* transfected with the A2 antisense RNA expressing vector (*L. d.* – A2) promastigotes were heat shocked at 40°C for 4 h. Internal ROS were monitored during the heat shock using the oxidant-sensitive fluorescent dye DCFDA. Values were standardized to t=0 measurements. Values + standard error are shown and represent the average of three independent experiments. $p<0.05$ after 30 min.

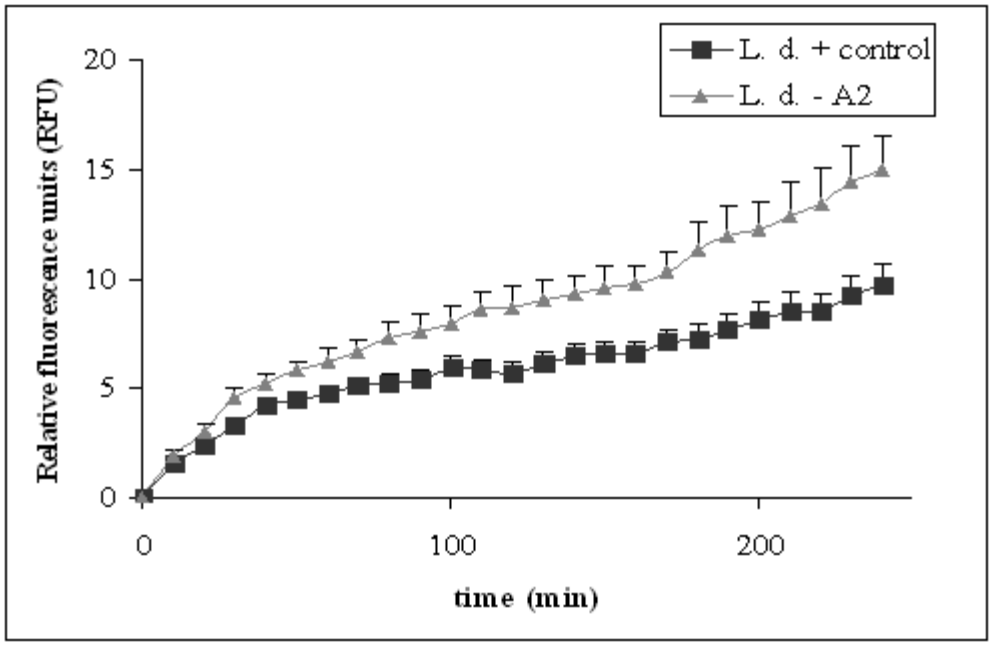


Figure 3.3 A2 does not protect *L. donovani* from unfolded protein stress. Amastigotes of *L. donovani* transfected with the control KSneo plasmid (*L. d.* + control) or the antisense A2 plasmid (*L. d.* – A2) were treated for 72 h with DTT. OD600 measurements were performed and % survival was calculated as described in methods. Values + standard error are shown and represent the average at least three independent experiments.

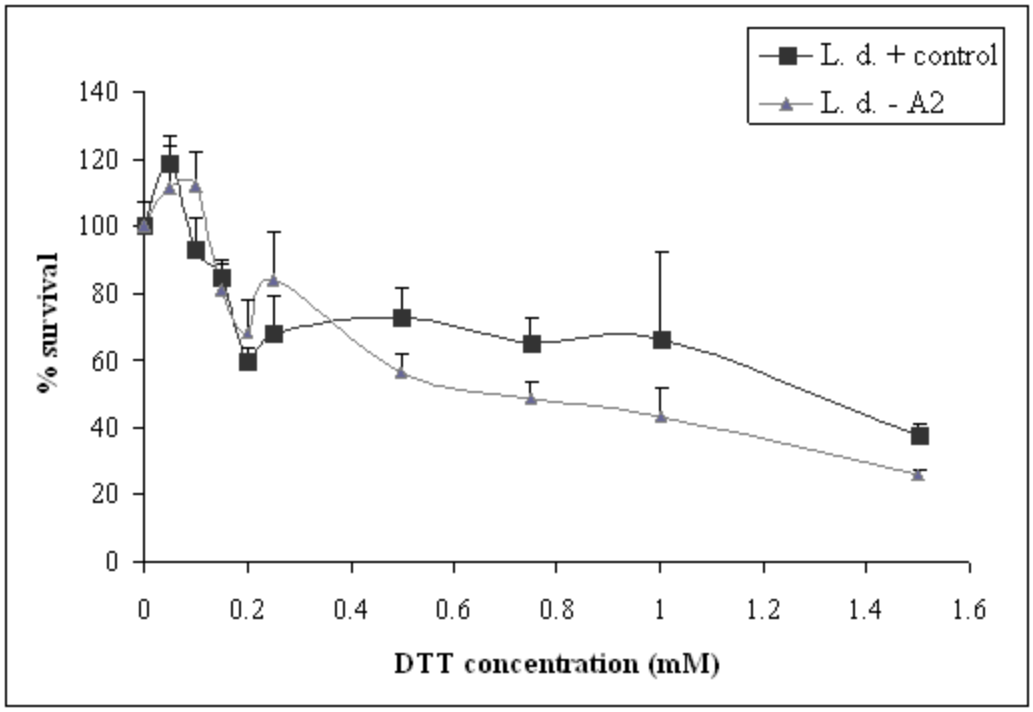
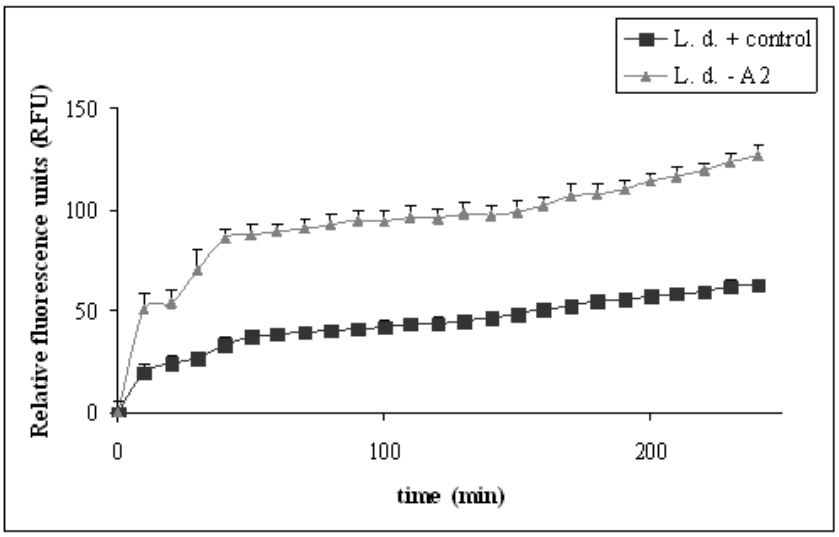


Figure 3.4 Internal oxidant levels during hydrogen peroxide or SNAP treatment.

A, axenic amastigotes were labelled with the DCFDA dye and exposed to 800 μM H_2O_2. Values were standardized to t=0 measurements. Values + standard error are shown and are representative of three independent experiments. $p<0.05$. **B,** Treatment with 100 μM SNAP. Values + standard error are shown and represent the average of three independent experiments. $p<0.05$ after 60 min treatment.

A


L. d. + control
L. d. - A2
Relative fluorescence units (RFU)
150
100
50
0
0
100
200
time (min)

B

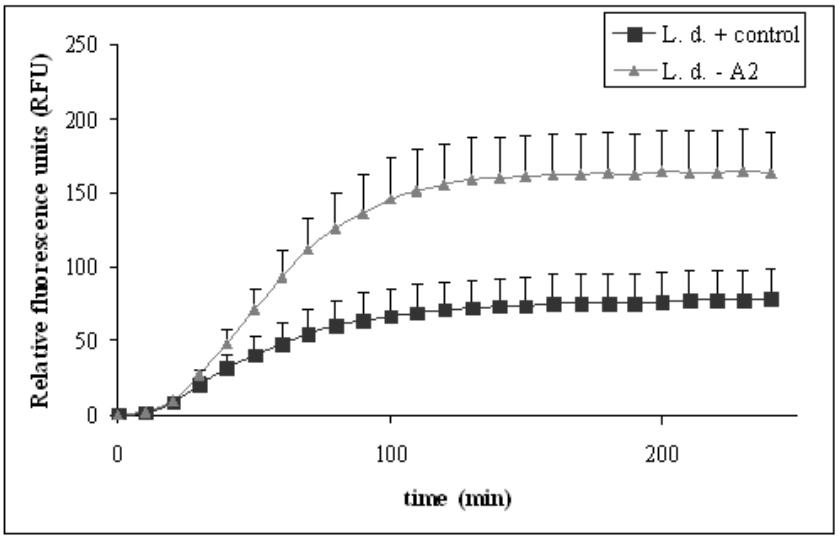
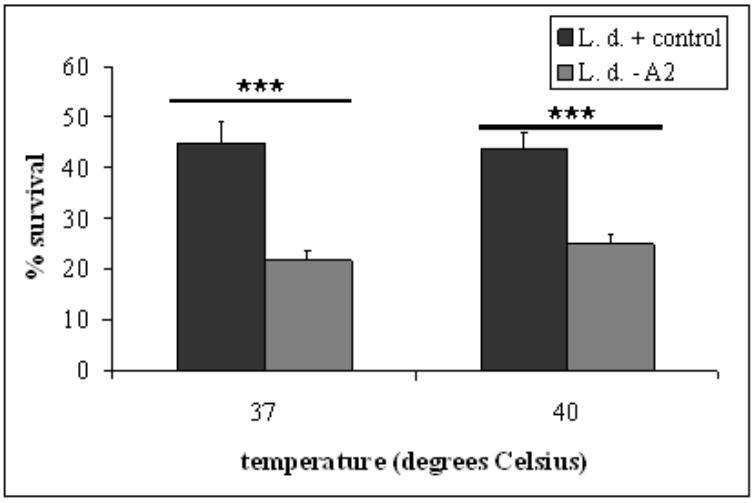

L. d. + control
L. d. - A2
Relative fluorescence units (RFU)
250
200
150
100
50
0
0
100
200
time (min)

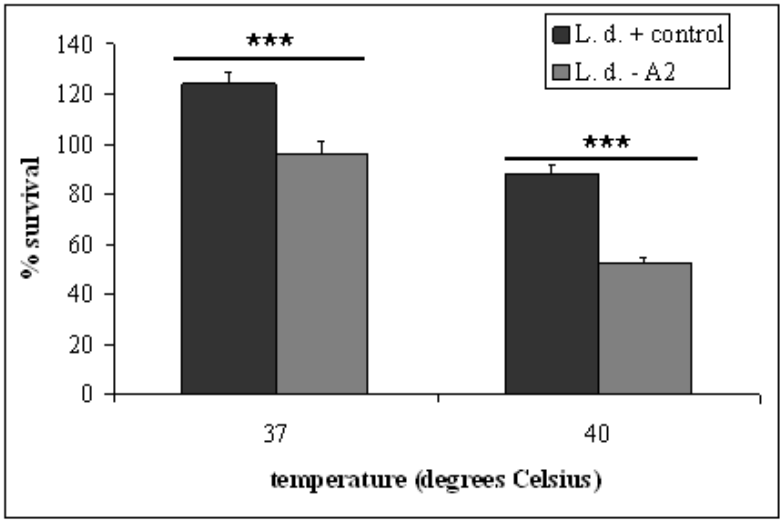
Figure 3.5 A2 protects *L. donovani* from oxidative stress.

Amastigotes of *L. donovani* transfected with the control KSneo plasmid (*L. d.* + control) or the antisense A2 plasmid (*L. d.* – A2) were treated for 4 h with 800 μM H_2O_2 (**A**) or 100 μM SNAP (**B**). OD600 measurements were performed 48 h later and % survival was calculated as described in methods. ***, $p<0.001$. Values + standard error are shown and represent the average of four independent experiments.

A


L. d. + control
L. d. - A2
***
***
% survival
60
50
40
30
20
10
0
37
40
temperature (degrees Celsius)

B


L. d. + control
L. d. - A2
***
***
% survival
140
120
100
80
60
40
20
0
37
40
temperature (degrees Celsius)

Chapter 4

Isolation of distinct *L. donovani* isolates that cause cutaneous and visceral leishmaniasis in Sri Lanka: *in vivo* and *in vitro* analysis of clinical disease phenotype determinants

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4.1 Preface

Comparison of the *L. major* and *L. donovani* genomes has highlighted a number of species-specific genes that play a role in parasite virulence [124,125,126]. However, additional factors may be involved in determining visceral pathology. Although both *L. major* and *L. donovani* are from the Old World, the last common ancestor of *L. major* and *L. donovani* was 14-24 million years ago [3]. Many differences between these species may not be related to their different tropisms. Studying closely related strains of the same species that cause

different disease manifestations could enable us to identify the most important determinants of visceral leishmaniasis.

L. donovani is a causative agent of visceral leishmaniasis in East Africa and South East Asia, including the main focus of visceral disease found in India, Nepal and Bangladesh [192]. However, in Sri Lanka, *L. donovani* causes mainly cutaneous leishmaniasis, while visceral disease is rare [156]. Studying the factors that mediate the attenuation of *L. donovani* in Sri Lanka could help us identify key determinants of visceral disease, presumably absent or expressed at lower levels in cutaneous *L. donovani* compared to visceral *L. donovani.*

We therefore obtained two clinical isolates from Sri Lanka, one from a cutaneous leishmaniasis lesion and one from a visceral leishmaniasis patient. We compared the virulence of these two isolates *in vitro* in B10R macrophages and *in vivo* in our BALB/c subcutaneous and intravenous infection models. In addition, given the key role of A2 in visceral leishmaniasis, we compared A2 expression between these isolates and established the importance of A2 in the attenuation of the Sri Lankan cutaneous *L. donovani*.

4.2 Abstract

Leishmania donovani normally causes visceral disease. However, in Sri Lanka *L. donovani* has caused numerous cutaneous leishmaniasis cases, while visceral leishmaniasis is rare. Clinical isolates from cutaneous and visceral leishmaniasis patients were obtained from Sri Lanka. Experimental intravenous and subcutaneous mouse infections showed that disease phenotype was

maintained in our experimental model. The molecular basis of this differential virulence was also investigated and we showed that decreased A2 protein levels are a key determinant of the attenuated virulence of the cutaneous parasite. Overall, these results indicate that the clinical situation in Sri Lanka is due to the presence of different parasite strains rather than to host background and highlight the importance of the A2 virulence factor in a clinical setting. This work provides the basis for further investigation of the determinants of visceral disease and for disease control efforts in Sri Lanka.

4.3 Introduction

Leishmaniasis is a parasitic disease caused by *Leishmania* protozoa. *Leishmania* promastigotes are transmitted to mammalian hosts by the bite of a sand fly. Within the mammalian host, promastigotes are phagocytosed by macrophages, where promastigotes differentiate into amastigotes. Infected macrophages or free amastigotes can then be taken up by sand flies during a blood meal. Within the sand fly gut, amastigotes differentiate into promastigotes, thereby completing the parasite lifecycle [192].

Leishmaniasis is associated with a spectrum of clinical manifestations, from cutaneous leishmaniasis to visceral disease. Cutaneous leishmaniasis is associated with parasite proliferation and lesion development at the site of the sand fly bite. These lesions are self-healing but scarring. In contrast, parasites disseminate to the bone marrow, liver and spleen in visceral leishmaniasis, and clinically manifest disease is fatal without treatment. A key question in

leishmaniasis research is what determines whether a patient develops cutaneous or visceral leishmaniasis. Disease phenotype is strongly influenced by parasite factors and in particular *Leishmania* species. Parasites from the *Leishmania donovani* species complex are the main causative agents of visceral leishmaniasis [192]. However, a number of atypical cases have been reported associating this species with cutaneous rather than visceral cases in India [179], in the Middle East [181,182] and in Cyprus [165].

One of the major foci of cutaneous leishmaniasis caused by *L. donovani* is Sri Lanka. There have been over 2000 cases of cutaneous leishmaniasis caused by *L. donovani* in Sri Lanka in the past ten years, whereas visceral disease is rare [156]. Parasites from cutaneous leishmaniasis patients have been typed to the *L. donovani* zymodeme MON-37 [161,162], but so far the basis for these atypical disease manifestations has not been identified. This is investigated here by studying two clinical isolates from Sri Lanka.

The first clinical isolate, referred to as SL-CL, comes from a cutaneous lesion on the nose of a 28 year old male. The second clinical isolate, SL-VL, was obtained from a bone marrow aspirate of a 53 year old visceral leishmaniasis patient with chronic fever, hepatosplenomegaly, lymphadenopathy, low haemoglobin and seropositivity for rK39. To determine whether these different tropisms were due to host or parasite factors, we performed subcutaneous and visceral infections in BALB/c mice. Disease phenotype was maintained in our animal model: only the SL-CL isolate caused footpad swelling, while the SL-VL isolate caused significant liver and spleen parasitemia. We then investigated the

biological basis of this differential virulence by comparing the expression levels of the known *L. donovani* virulence factor A2 [54,99]. SL-CL expressed lower A2 expression levels and fewer A2 isoforms than SL-VL. The impact of this on SL-CL virulence was assessed by *in vivo* infection with SL-CL ectopically expressing an additional A2 gene as well as by downregulating A2 expression in SL-VL. Results demonstrated that the inability of the SL-CL isolate to cause visceral infection was due at least in part to decreased A2 expression. Overall, this work demonstrates for the first time that the cutaneous and visceral cases in Sri Lanka are caused by different parasites rather than by differences in host characteristics, and indicates that decreased A2 expression is one mechanism that allows *L. donovani* to switch from visceral to cutaneous tropism in a clinical setting.

4.4 Materials and methods

4.4.1 Sri Lanka *L. donovani* isolates and culture conditions

The SL-CL isolate was obtained from a cutaneous lesion on the nose from a 28 year old male. The SL-VL isolate was obtained from a bone marrow aspirate of a 57 year old visceral leishmaniasis patient with chronic fever, hepatosplenomegaly, lymphadenopathy, low haemoglobin and seropositivity for rK39.

Biopsy samples were directly inoculated into Leishmania promastigote culture medium: M199 medium (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum, 40 mM HEPES (pH 7.4), 0.1 mM adenine, 5 mg/L hemin,

1 mg/L biotin, 1 mg/L biopterin, 100 U/mL penicillin and 100 μg/mL streptomycin at room temperature and were maintained in this medium at 26°C after arriving in the lab. For axenic amastigote culture, *Leishmania* promastigotes were shifted to 37°C, pH 5.5 culture media to mimic the macrophage phagolysosome environment associated with the amastigote stage [93,117].

4.4.2 Transfections

Promastigotes were transfected as described by Robinson and Beverley [367] with the KSneo-control plasmid, the KSneo-A2 plasmid [85] or the KSneo-A2 (R) antisense plasmid [99]. Transfected parasites were maintained in media supplemented with 200 μg/mL G418 (Wisent).

4.4.3 Macrophage infections

B10R macrophages were maintained in Dulbecco's Modified Essential Medium (DMEM, Invitrogen), supplemented with 10% FBS, 100 units/mL of penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2. Macrophages were infected with stationary phase promastigotes at a 2 to 1 parasite to macrophage ratio. 24 h post-infection, cells were collected by cytospin and stained with DiffQuick (Dade Behring).

4.4.4 Mouse infections

Female BALB/c mice (17-20 g) were purchased from Charles River Laboratories and maintained in the animal care facility under pathogen-free

conditions. For studies of cutaneous infection, mice were injected subcutaneously with $5x10^6$ SL-CL or SL-VL stationary phase promastigotes in their hind footpads. Footpad swelling was monitored by weekly caliper measurements for 11 weeks. For studies of visceral infection, mice were infected intravenously via the tail vein with $5x10^7$ (SL-CL, SL-VL, SL-VL + control or SL-VL – A2) or $1x10^8$ (SL-CL + control or SL-CL + A2) stationary phase promastigotes in 100 μL PBS. Mice were sacrificed 28 days following infection. Liver parasite burden was determined by direct counting of amastigotes on Diff-Quick stained liver impressions and expressed as Leishman-Donovan Units (LDU): number of amastigotes per 1000 cell nuclei × liver weight (g) [99]. Spleen parasite burden was determined by limiting dilution of spleen homogenates.

4.4.5 SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed as previously described [57]. Briefly, proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). A2 was detected with a 1:8,000 dilution of mouse monoclonal anti-A2 antibody (ascites) and tubulin with a 1:2,000 dilution of mouse monoclonal anti-tubulin antibody (Oncogene), followed by a 1:2,500 dilution of peroxidase-conjugated anti-mouse IgG antibody (Rockland). All antibodies were diluted in 5% skim milk in PBS-T and all antibody incubations were 1 h in duration. Proteins were visualized using ECL Western Blot Detection Reagents (GE Healthcare).

4.5 Results

Parasites were successfully isolated and grown in culture from a cutaneous lesion (SL-CL) and from a bone marrow aspirate of a visceral leishmaniasis patient (SL-VL). Sri Lanka *L. donovani* have previously been shown to belong to zymodeme MON-37, which can be distinguished from Indian *L. donovani* strains by a single amino acid change in the 6-phosphogluconate dehydrogenase (6PGDH) gene [164]. The isolates used in this study are indeed MON-37 *L. donovani* from Sri Lanka, as confirmed by sequencing of the 6PGDH gene in both the visceral and cutaneous-derived isolates [394].

4.5.1 Disease phenotype is maintained during BALB/c mouse infection

The atypical cutaneous leishmaniasis cases in Sri Lanka have been postulated to be due to differences in host genetic background, although no significant genetic differences were identified [176,177]. To investigate whether cutaneous cases in Sri Lanka are due to host factors or parasite factors, we infected inbred BALB/c mice (same genetic background) with each isolate. Intravenous visceral infections showed that only the isolate from the visceral patient (SL-VL) could cause significant liver and spleen parasitemia (**Figure 4.1**). Similarly, only the isolate from the cutaneous patient (SL-CL) was able to cause significant footpad swelling (**Figure 4.2**). These results indicate that parasite-intrinsic factors are the main determinants of disease phenotype in Sri Lanka and form the basis for the identification of the factors that lead to *L. donovani*

attenuation in Sri Lanka. These observations also validate the use of these BALB/c mouse infection models for the study of visceral disease determinants.

4.5.2 SL-CL and SL-VL are equally infective to macrophages *in vitro*

The SL-CL and SL-VL isolates therefore differ significantly with regards to their ability to proliferate in the mouse footpad and visceral organs. *In vivo* virulence has been tied to differential infectivity to macrophages *in vitro* in some studies [74,75] but not in others [73,76]. We therefore investigated whether SL-CL and SL-VL differ in their infectivity to macrophages. Infections were performed at 34°C to mimic footpad temperatures and 37°C to model visceral organ infection. At both temperatures, there was no significant difference in macrophage infection levels or in the number of amastigotes per infected macrophage (**Figure 4.3**). Overall, this suggests that other factors than infectivity to macrophages mediate the differential virulence of these two clinical isolates.

4.5.3 Impact of the A2 virulence factor on SL-CL and SL-VL virulence

The A2 family of proteins are a key determinant of visceral disease. Decreased A2 expression has been tied to decreased parasite viability in mouse visceral organs and increased A2 levels to increased virulence [54,99]. A2 is a multigene family of proteins and different parasite strains express different numbers and sizes of A2 [83]. A2 protein expression levels were therefore assessed in SL-CL and SL-VL by Western blot. As shown in **Figure 4.4**, A2

protein levels were overall lower in SL-CL than in SL-VL, and SL-CL expressed fewer isoforms of A2 than SL-VL.

Given the importance of A2 in *L. donovani* virulence, we investigated whether these observed differences in A2 expression were involved in their differential virulence. A2 protein expression was increased in SL-CL by transfection with the KSneo-A2 plasmid [85] (SL-CL + A2) and downmodulated in SL-VL by transfection with the KSneo-A2 (R) antisense plasmid [99] (SL-VL – A2). The KSneo-A2 plasmid leads to ectopic expression of an additional size of A2 (**Figure 4.5A**, arrow), restoring A2 levels in SL-CL to levels comparable to those found in SL-VL. A2 expression is almost completely abrogated in parasites transfected with the antisense KSneo-A2 (R) plasmid (**Figure 4.6A**).

Virulence of transfectants was assessed by intravenous infection in BALB/c mice. In both cases, virulence was compared to parasites transfected with the empty control vector (SL-CL + control and SL-VL + control). Ectopic expression of A2 in SL-CL significantly increased SL-CL virulence (**Figure 4.5B**). Addition of a single copy of A2 was associated with over a seventy-fold increase in spleen parasite burden. This indicates that loss of A2 is a key determinant of the attenuated virulence of the SL-CL isolate. However, virulence was not fully restored to levels comparable to those of the SL-VL isolate, indicating that factors other than A2 also play a role.

Downregulation of A2 expression in the SL-VL isolate significantly decreased the infectivity of the parasite in the visceral organs (**Figure 4.6B**). This confirms the importance of A2 in the virulence of these clinical isolates and

highlights the importance of this virulence factor in clinical isolates as well as laboratory strains.

4.6 Discussion

Leishmaniasis is the second most fatal tropical disease, with an estimated 20,000 to 40,000 deaths per year [395]. Given that visceral leishmaniasis is associated with most of the morbidity and mortality caused by *Leishmania* parasites, identifying the factors that mediate the development of visceral disease is of primordial importance. Comparison of the *L. major* and *L. donovani* genomes has highlighted a number of candidate genes that are involved in visceral virulence [124,125]. However, transfection of these genes into *L. major* was not able to lead to infection levels comparable to those caused by *L. donovani*. Therefore the identification of novel determinants of visceral disease is required. Although both from the Old World, *L. major* and *L. donovani* are evolutionarily quite distant [3]. Studying closely related parasite strains that cause different disease manifestations could help identify key genes that mediate differential parasite virulence.

L. donovani is the only known causative agent of cutaneous leishmaniasis in Sri Lanka and more typical visceral disease cases caused by *L. donovani* are rare there [156]. Comparison of cutaneous and visceral strains from Sri Lanka therefore represent an excellent model to identify the key changes that allowed a normally visceral parasite to cause cutaneous disease.

Parasites isolated from cutaneous and visceral leishmaniasis patients (SL-CL and SL-VL) caused the same disease phenotypes in BALB/c mice as they do in humans: only SL-CL causes cutaneous swelling, manifested by increased footpad swelling following subcutaneous infection, and SL-VL was significantly more virulent in the visceral organs than SL-CL. Maintenance of disease phenotype in our inbred animal model indicates that the atypical cutaneous leishmaniasis cases in Sri Lanka are due to parasite rather than host genetic background. Western blotting showed that the A2 expression pattern also differed between these two isolates, indicating that cutaneous and visceral cases are caused by different parasite strains. This highlights the advantage and clinical relevance of comparing protein expression patterns of virulence factors such as A2 to differentiate between clinical isolates.

A2 is a multigene family of proteins found in *L. donovani* and *L. infantum* but absent in *L. major* [83,88] and is a major virulence factor required for *L. donovani* infection in visceral organs [54,99]. Ectopic expression of A2 in *L. major* increased its ability to survive in visceral organs [54]. In addition, decreased A2 levels in laboratory strains of *L. donovani* have been tied to decreased virulence [54,99]. Western blot comparison of A2 levels in SL-CL and SL-VL showed that SL-CL expressed lower A2 levels and fewer A2 isoforms. We therefore investigated whether this lowered A2 expression could be a factor mediating the decreased visceral virulence of the SL-CL isolate. Ectopic expression of A2 in SL-CL was associated with increased visceral parasite burden in BALB/c mice. In parallel, we confirmed that A2 is a key mediator of

SL-VL virulence by downregulating A2 expression in these parasites with an antisense construct. Decreased A2 expression in SL-VL was associated with attenuated visceral virulence. Lowered A2 expression is therefore one of the factors that cause the decreased virulence of SL-CL parasites. A2 protects *L. donovani* from host defenses such as heat shock and oxidative stress [57,342] and therefore lowered A2 levels may render SL-CL more susceptible to parasite killing *in vivo*, although no differences in *in vitro* infectivity were observed between SL-CL and SL-VL. However, ectopic A2 expression could not restore SL-CL virulence to levels comparable to those of SL-VL. Other factors are therefore involved and we are currently investigating them by whole genome sequencing of these two isolates.

Overall, this work provides insight into the basis of the atypical cutaneous leishmaniasis observed in Sri Lanka. This will form the basis for improved understanding of the disease and could help guide case management and treatment in this region.

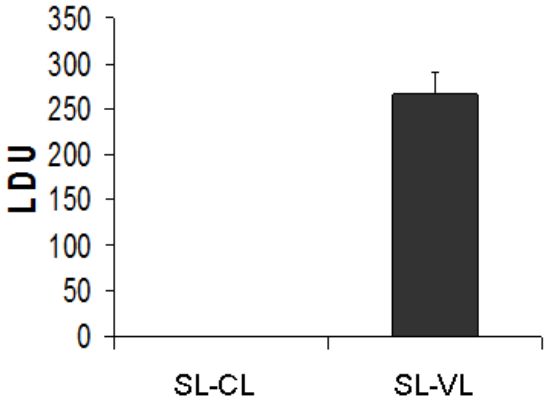
4.7 Acknowledgements

This work was supported by a research grant from the Canadian Institute of Health Research (CIHR), and scholarships from the Fonds de la Recherche en Santé du Québec (FRSQ) and from CIHR to LIM.

4.8 Figures

Figure 4.1 SL-CL is avirulent in the liver and spleen of BALB/c mice compared to the SL-VL that is highly virulent in these organs.

BALB/c (five mice per group) were injected in the tail vein and four weeks after infection, liver parasite burden (LDU, **A**) and spleen parasite burden (**B**) were determined from liver prints and spleen homogenates.



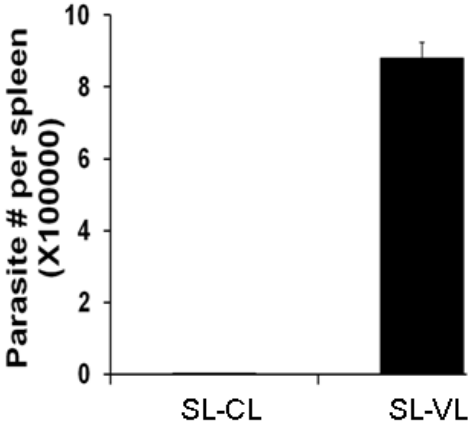


Figure 4.2 Only SL-CL causes footpad swelling in BALB/c mice.

BALB/c mice (five mice per group) were injected subcutaneously in the rear footpad and footpad swelling measured over 11 weeks.

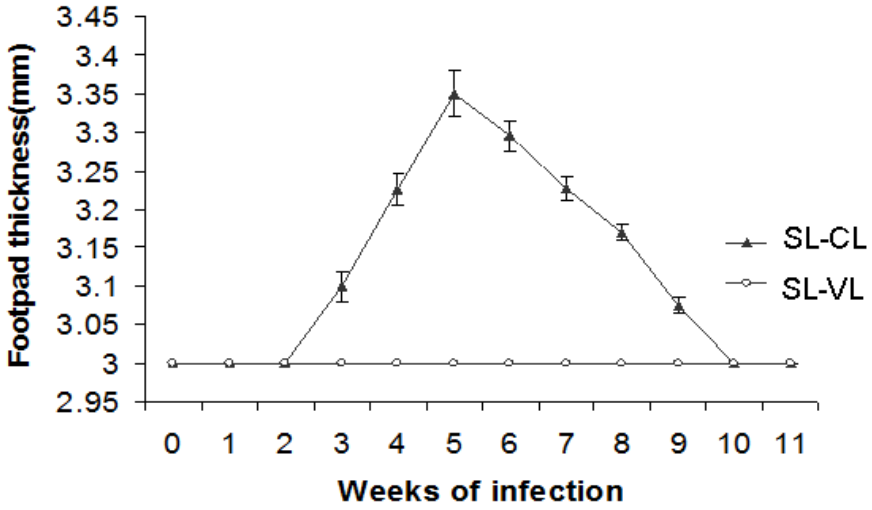
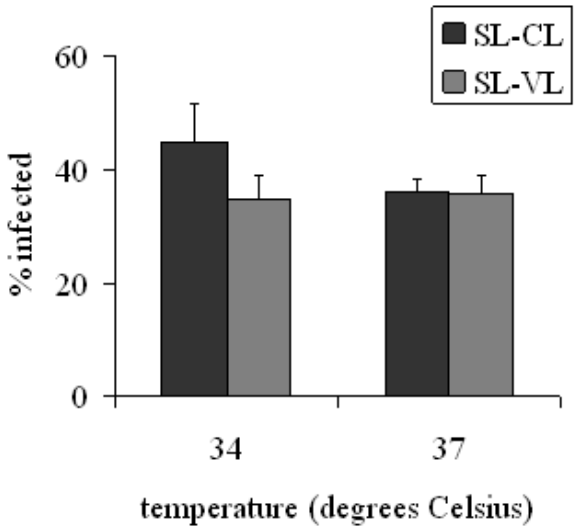


Figure 4.3 SL-CL and SL-VL parasites are equally infective to macrophages *in vitro.*

B10R macrophages were infected with stationary phase SL-CL or SL-VL promastigotes at a 2:1 parasite to macrophage ratio. 24 h post-infection, cells were stained with DiffQuick. The percentage of infected macrophages (**A**) and parasites per macrophage (**B**) were determined by direct counting on a light microscope. Average values + standard error are shown and represent the average of three independent experiments.

A


SL-CL
SL-VL
60
40
20
0
% infected
34
37
temperature (degrees Celsius)

B

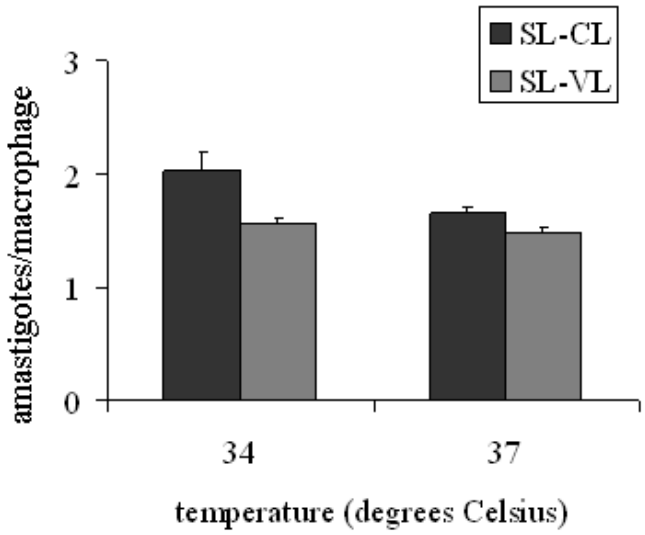

SL-CL
SL-VL
3
2
1
0
amastigotes/macrophage
34
37
temperature (degrees Celsius)

Figure 4.4 A2 expression in SL-CL and SL-VL.

SL-CL and SL-VL were heat shocked at 40°C for 8 h. The A2 proteins and tubulin were detected by Western blot. Top, A2 expression. A2 was detected with a 1:8,000 dilution of mouse monoclonal anti-A2 antibody (ascites). Note the different patterns of A2 bands between cutaneous (CL) and visceral isolates (VL) and the lower overall levels of A2 in cutaneous parasites. Bottom, Tubulin loading control.

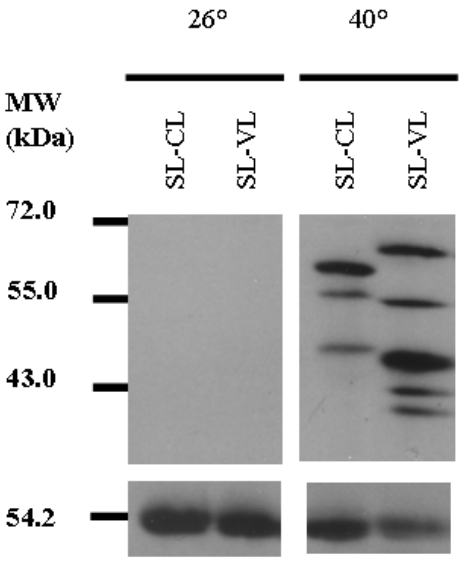
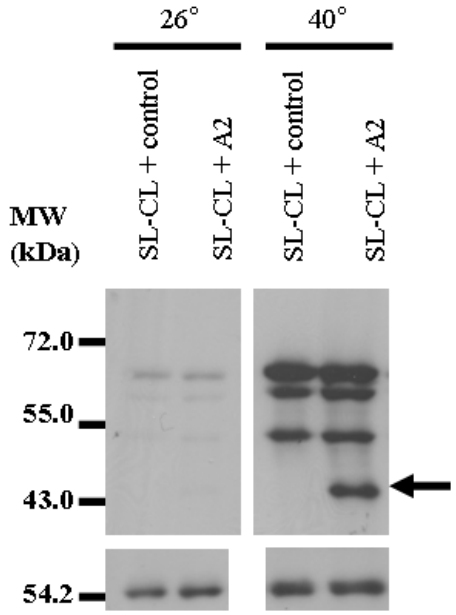


Figure 4.5 Ectopic expression of an additional A2 copy in SL-CL increases parasite virulence in visceral organs.

A, SL-CL was transfected with the KSneo vector encoding an extra copy of the A2 gene (SL-CL + A2, arrow) or the empty KSneo vector (SL-CL + control). Top, A2 expression was detected by Western blot following a 4h heat shock at 40°C. Bottom, tubulin loading control. **B,** Expression of an extra copy of the A2 gene increases SL-CL virulence in the visceral organs. BALB/c mice were injected in the tail vein and four weeks post-infection, spleen parasite burden was determined by limiting dilution of spleen homogenates. Values +/- standard error are shown and are representative of two independent experiments with five mice per group. $p<0.05$.

A


26°
40°
SL-CL + control
SL-CL + A2
SL-CL + control
SL-CL + A2
MW
(kDa)
72.0
55.0
43.0
54.2

B

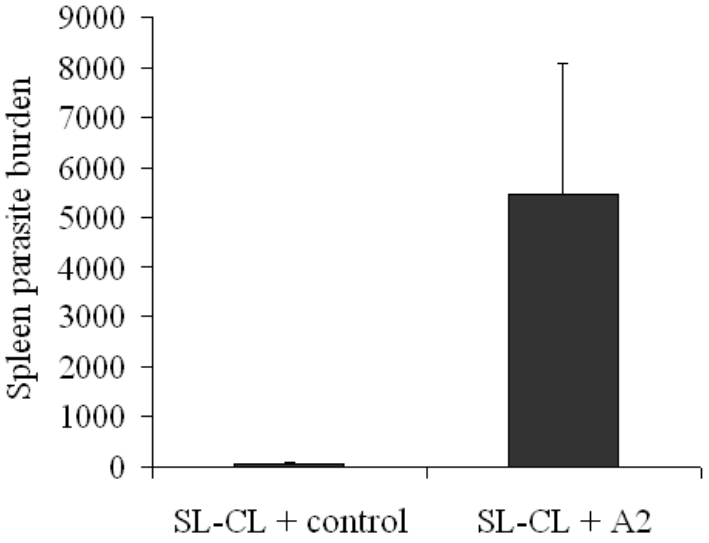
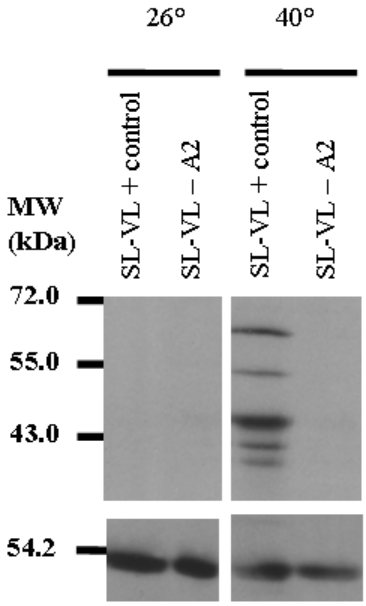

Spleen parasite burden
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
SL-CL + control
SL-CL + A2

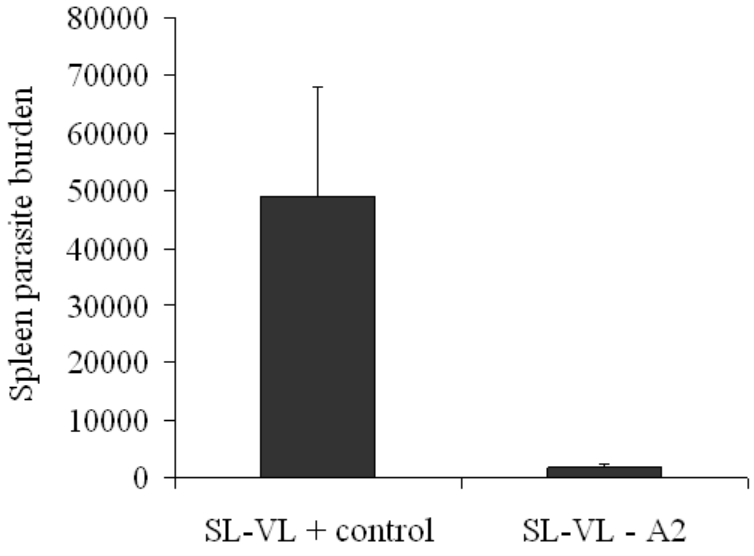
Figure 4.6 Downregulation of A2 protein expression decreases the virulence of SL-VL in visceral organs.

A, SL-VL was transfected with the KSneo vector encoding an antisense construct that downregulates A2 expression (SL-VL - A2) or the empty KSneo vector (SL-VL + control). Top, A2 expression was detected by Western blot following a 4h heat shock at 40°C. Bottom, tubulin loading control. **B,** Downregulation of A2 expression decreases SL-VL virulence in the visceral organs. BALB/c mice were injected in the tail vein and four weeks post-infection, spleen and liver parasite burden were determined by limiting dilution of organ homogenates. Values +/- standard error are shown and are representative of two independent experiments. $p<0.05$.

A


26°
40°
SL-VL + control
SL-VL – A2
SL-VL + control
SL-VL – A2
MW
(kDa)
72.0
55.0
43.0
54.2

B


Spleen parasite burden
80000
70000
60000
50000
40000
30000
20000
10000
0
SL-VL + control
SL-VL - A2

Chapter 5

Leishmanization revisited: immunization with a naturally attenuated cutaneous *Leishmania donovani* isolate from Sri Lanka protects against visceral leishmaniasis

Laura-Isobel McCall, Wen-Wei Zhang, Shanlindra Ranasinghe, and Greg Matlashewski

Adapted from *Vaccine* with permission from Elsevier.

McCall LI, Zhang WW, Ranasinghe S, Matlashewski G (2013), Leishmanization revisited: immunization with a naturally attenuated cutaneous *Leishmania donovani* isolate from Sri Lanka protects against visceral leishmaniasis. Vaccine, 31(10):1420-5.

5.1 Preface

Cutaneous leishmaniasis caused by *L. donovani* is common in Sri Lanka, while visceral leishmaniasis is present but rare [156]. No host genetic factors have been identified that could explain this increase in cutaneous disease and the scarcity of visceral cases. One possibility is that prior infection with the cutaneous *L. donovani* strain can protect against visceral leishmaniasis. We investigate this here by immunizing BALB/c mice subcutaneously with the cutaneous *L. donovani* clinical isolate to mimic the natural infection route, and challenging them intravenously with the visceral isolate.

5.2 Abstract

Leishmaniasis is a neglected tropical disease caused by *Leishmania* protozoa and associated with three main clinical presentations: cutaneous, mucocutaneous and visceral leishmaniasis. Visceral leishmaniasis is the second most lethal parasitic disease after malaria and there is so far no human vaccine. *L. donovani* is a causative agent of visceral leishmaniasis in South East Asia and Eastern Africa. However, in Sri Lanka, *L. donovani* causes mainly cutaneous leishmaniasis, while visceral leishmaniasis is rare. We investigate here the possibility that the cutaneous form of *L. donovani* can provide immunological protection against the visceral form of the disease, as a potential explanation for why visceral leishmaniasis is rare in Sri Lanka. Subcutaneous immunization with a cutaneous clinical isolate from Sri Lanka was significantly protective against visceral leishmaniasis in BALB/c mice. Protection was associated with a mixed Th1/Th2 response. These results provide a possible rationale for the scarcity of visceral leishmaniasis in Sri Lanka and could guide leishmaniasis vaccine development efforts.

5.3 Introduction

Leishmaniasis is a tropical disease caused by *Leishmania* protozoa. Parasites are transmitted to the mammalian host by a sand fly vector and replicate intracellularly, mainly in macrophage phagolysosomes. Infection is associated with three main forms of disease: cutaneous leishmaniasis, where parasites remain contained at the site of the sand fly bite, mucocutaneous leishmaniasis in which

there is metastasis to the mucosal tissues of the nose, mouth and throat, and visceral leishmaniasis where parasites disseminate to visceral organs, including the bone marrow, liver and spleen [192]. Visceral leishmaniasis is one of the most lethal tropical parasitic diseases, with a mortality second only to malaria [23]. Leishmaniasis is also the cause of over two million DALYs lost (disability-adjusted life years) [23]. Although visceral leishmaniasis is such a deadly disease, there are as yet no human vaccines licensed for use, and most vaccines studies have focused on the less severe cutaneous form of the disease [396].

Studying self-resolving infections or naturally immune patients can provide valuable insight into vaccine development. The practise of leishmanization arose from the observation that healing of a cutaneous leishmaniasis lesion was associated with protection against re-infection. Leishmanization involves inoculation with *Leishmania* parasites in a hidden area to protect against lesion development in visible areas, and was performed on a large scale in Israel and Iran in the 1970s and 1980s, but was abandoned due to concerns about decreased immunity to other vaccines and non-healing lesions. Uzbekistan is currently the only country where it is still performed [325].

Evidence that leishmanization could also protect against visceral leishmaniasis is more limited. A study in Sudan observed that only individuals unresponsive to the leishmanin skin test (not previously exposed to *Leishmania*) developed visceral disease. The authors suggested that prior exposure to a cutaneous parasite could lead to a positive leishmanin skin reaction and protection

against visceral disease [327]. The positive skin test could however have been due to asymptomatic *L. donovani* infections.

Immunization with *L. donovani* antigens is protective against cutaneous *Leishmania* species [299,397,398,399]. In contrast, although immunization with *L. major* antigen fractions such as soluble promastigote exogenous antigens was protective against visceral leishmaniasis [400], immunization with live *L. major* promastigotes did not protect against *L. chagasi* or *L. infantum* [309,310]. Indeed, prior infection with *L. major* was even associated with increased spleen parasite burden following *L. infantum* challenge [309]. Live immunization against visceral leishmaniasis may therefore require the use of parasites closely related to the challenge strain.

In Sri Lanka, there have been over 2,000 cases of cutaneous leishmaniasis caused by an atypical *L. donovani* strain, while visceral leishmaniasis is rare [156]. No host genetic polymorphisms associated with altered susceptibility to leishmaniasis have been observed in the population [177,178]. Therefore, either parasite factors or acquired host differences may play a role. One possibility is that prior exposure to a more prevalent cutaneous parasite protects against visceral leishmaniasis. We have recently observed virtually no visceral leishmaniasis in districts reporting high levels of cutaneous leishmaniasis in Sri Lanka [401].

We investigate this here and show that immunization of BALB/c mice with a cutaneous clinical isolate from Sri Lanka protects against visceral disease caused by a visceral clinical isolate from Sri Lanka. Protection is associated with

a mixed Th1/Th2 response prior to challenge. The protection resulting from this immunization may be due to the fact that both cutaneous and visceral *L. donovani* isolates are closely related, although the virulence of the cutaneous isolate is significantly attenuated since Sri Lanka *L. donovani* is almost exclusively associated with cutaneous rather than visceral leishmaniasis [156]. Overall, this work provides valuable insight into leishmaniasis disease dynamics in Sri Lanka but also has a broader impact on vaccination perspectives for visceral leishmaniasis.

5.4 Materials and methods

5.4.1 Parasite strains and culture

Both *L. donovani* isolates were obtained in Sri Lanka. The cutaneous SL-CL isolate was obtained from a cutaneous lesion on the nose from a 28 year old male. The SL-VL isolate was derived from the third autochthonous visceral leishmanaisis patient in Sri Lanka, a 57 year old visceral leishmaniasis patient with chronic fever, hepatosplenomegaly, lymphadenopathy, low haemoglobin and seropositivity for rK39. SL-VL was identified as *L. donovani* MON-37 based on multilocus isoenzyme electrophoresis and sequencing of the 6-phosphogluconate dehydrogenase (6PGDH) gene [401]. It has previously been established that the causative agent of cutaneous leishmaniasis in Sri Lanka is likewise *L. donovani* MON-37 [161,164] and we have confirmed by sequencing the 6PGDH gene that the SL-CL isolate used in this study is the same. Sequencing of the 6PGDH gene

in the SL-CL and SL-VL isolates used in this study was performed at the McGill University Genome Quebec Innovation Centre.

Leishmania promastigotes were cultured in M199 medium (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum, 40 mM HEPES (pH 7.4), 0.1 mM adenine, 5 mg/L hemin, 1 mg/L biotin, 1 mg/L biopterin, 100 U/mL penicillin and 100 µg/mL streptomycin. Parasites were maintained in this medium at 26°C.

5.4.2 Immunizations

Female BALB/c mice (17-20 g) were purchased from Charles River Laboratories and maintained in the animal care facility under pathogen-free conditions. All experiments were performed in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

Mice were immunized subcutaneously in the left hind footpad with $1x10^3$ to $1x10^6$ SL-CL stationary phase promastigotes in 50 µL PBS. Control mice were immunized with 50 µL PBS alone. Footpad parasite burden prior to challenge was determined by limiting dilution of footpad homogenates.

5.4.3 Challenge infections

Seven weeks post-immunization, mice were challenged intravenously with $5x10^7$ SL-VL stationary phase promastigotes. Mice were sacrificed 28 days following challenge. Liver parasite burden was determined by direct counting of

amastigotes from Diff-Quick stained liver impressions and expressed as Leishman-Donovan Units (LDU): number of amastigotes per 1000 cell nuclei × liver weight (g) [99].

5.4.4 *In vitro* splenocyte restimulation

Splenocytes were separated into single cell suspension by passing through a fine wire mesh and red blood cells were lysed with 0.17 M NH_4Cl pH 7.2. Splenocytes were then resuspended at $2x10^6$ cells/mL in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mM HEPES, 10% heat-inactivated FBS, 20 U/mL penicillin, 20 µg/mL streptomycin and 50 µM β-mercaptoethanol (Sigma). Cells were restimulated at 37°C in 5% CO_2 with 50 µg/ml of soluble *Leishmania* antigen (SLA) prepared from SL-VL promastigotes or left unstimulated (null) for 72 h. IFNγ, IL4, and IL10 cytokine concentration in the culture supernatant were determined by sandwich ELISA according to the manufacturer's instructions (eBioscience).

Soluble *Leishmania* antigen (SLA) was obtained by sequential freeze-thaw cycles of stationary phase SL-VL promastigotes. Solutions were cleared by centrifugation and the protein concentration in the supernatant was determined by Bradford reaction (BioRad).

5.4.5 Serum antibody analysis

To determine total IgG serum antibody levels, ELISA plates were coated with 2.5 μg/mL SLA overnight. Wells were blocked in 2.5% FBS in PBS. Two-fold serial dilutions of serum samples in 2.5% FBS in PBS were added to the wells for 2 hours, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:20,000; Rockland). Color development was performed with 3,3',5,5'-tetramethylbenzidine substrate (eBioscience) and the reaction was stopped with 2 N H_2SO_4. Absorbance measurements were performed at 450 and 570 nm. The antibody titer was identified as the highest dilution where absorbance values still exceeded 3×(mean blank OD+S.D. of blanks) as described in [105].

To determine antibody isotypes, ELISAs were performed as above, using 1 in 50 dilutions of serum samples. Incubation with serum samples was followed by 1 h incubation with anti-mouse IgG1 or IgG2a antibodies (1:1,000) and 1 h incubation with horseradish peroxidase conjugated anti-goat IgG (1:5,000).

5.4.6 Statistical analysis

The significance of the data was evaluated by two-tailed Student's *t* test.

5.5 Results

5.5.1 Subcutaneous immunization with low doses of SL-CL is protective against visceral leishmaniasis

In Sri Lanka, cutaneous leishmaniasis caused by *L. donovani* is common, while visceral leishmaniasis is rare [156]. We were therefore interested to investigate whether cutaneous infection with *L. donovani* derived from a human cutaneous leishmaniasis case can provide immunological protection against visceral leishmaniasis and this was tested in BALB/c mice. Clinical isolates were obtained from a cutaneous leishmaniasis patient (SL-CL) and a visceral leishmaniasis patient (SL-VL) from Sri Lanka. Both isolates are *L. donovani* since this species is reported to be the causative agent of cutaneous leishmaniasis [161,163,164]) and visceral leishmaniasis [401] in Sri Lanka as detailed in methods. Parasites used in this study were confirmed as *L. donovani* MON-37 by sequencing of the 6-phosphogluconate dehydrogenase (6PGDH) gene [394].

Since the Sri Lanka *L. donovani* causes cutaneous rather than visceral leishmaniasis, and since only four cases of visceral leishmaniasis have been reported in Sri Lanka compared to over 2,000 cutaneous cases in the past ten years [156], the cutaneous *L. donovani* SL-CL isolate can be considered a naturally attenuated parasite. Furthermore, whereas the SL-CL isolate was unable to cause significant liver and spleen parasitemia, the SL-VL isolate was able to survive in visceral organs in BALB/c mice (**chapter 4, Figure 4.1**). The SL-CL isolate was therefore used in this study as a live cutaneous vaccine.

Mice were immunized subcutaneously with the cutaneous SL-CL clinical isolate to mimic the natural course of infection, and seven weeks post-immunization mice were challenged intravenously with a clinical isolate from a visceral leishmaniasis patient (SL-VL). Immunization with 10^4 to 10^6 SL-CL promastigotes was associated with significantly decreased liver parasite burden ($p<0.01$), indicating that these SL-CL doses are protective against visceral leishmaniasis. Lower SL-CL doses were not protective (**Figure 5.1**).

5.5.2 Immune response post-challenge

The cellular and humoral immune response was investigated post-challenge. Splenocytes were restimulated *in vitro* with soluble *Leishmania* antigen (SLA) prepared from the visceral SL-VL promastigotes, and IFNγ, IL4 and IL10 levels measured after 72 h. IFNγ production post-challenge was comparable in all groups. In contrast, IL4 and IL10 levels were significantly higher in protected mice (SL-CL 10^4 to SL-CL 10^6, $p<0.05$) (**Figure 5.2 A-C**). In accordance with this increased Th2 cytokine production, total IgG titers and IgG1 antibody levels were elevated in protected mice (**Figure 5.2D, Table 5.1**). Similarly, the lack of significant differences in IFNγ and Th1 cytokine production was associated with no significant differences in IgG2a antibody levels post-challenge (**Table 5.1**).

5.5.3 Determinants of protection pre-challenge

To identify further determinants of protection, the cellular and humoral immune responses were compared at 7 weeks post-immunization, before challenge. Protection by SL-CL cutaneous immunization was associated with significantly higher IFNγ and IL4 production before challenge (**Figure 5.3A and B**). With regards to IL10, no significant difference was observed between SLA-stimulated and unstimulated (null) splenocytes, indicating that there was no antigen-specific IL10 response before challenge in any of the groups (**Figure 5.3C**). Significant antibody responses were only detected in mice immunized with 10^6 SL-CL promastigotes and both IgG1 and IgG2a were detected (**Figure 5.3D, Table 5.1**). Therefore protection was associated with a mixed Th1/Th2 response, as evidenced by the IFNγ and IL4 production and an IgG1 to IgG2a ratio of 1.82.

Finally, to assess whether protection was associated with parasite persistence at the site of immunization, we measured the footpad parasite burden at the time of challenge, 7 weeks post immunization. Low but detectable footpad parasitemia was observed in protected mice (**Figure 5.4**), with the highest footpad parasite burden as expected in mice immunized with 10^6 SL-CL. No remaining parasites were detected in mice immunized with 10^3 SL-CL. Parasite persistence does not however seem to be required for durable vaccine protection in the liver since $5x10^6$ SL-CL was protective against challenge with SL-VL 3 months post-immunization, even though footpad parasite burden becomes low to undetectable at time points greater than 2 months post-infection (**Figure S1**).

Even though persistent parasites were detected in the footpad of mice immunized with 10^4 to 10^6 SL-CL, no footpad lesions or footpad swelling were observed in any of the mice immunized with these SL-CL doses. We did however observe minor but transient footpad swelling when mice were immunized with $5x10^6$ SL-CL (see **chapter 4**, **Figure 4.2**). Dissemination of the SL-CL parasite to the spleen following subcutaneous immunization in the footpad was also assessed. No growth was observed by limiting dilution of spleen homogenates in any of the groups, indicating that parasite levels in the spleen prior to challenge are below the detection limit for spleen limiting dilutions (fewer than 40 parasites per spleen).

5.6 Discussion

The major observation from this study was that subcutaneous immunization with the live *L. donovani* SL-CL isolate was associated with significantly decreased liver parasite burden following challenge with the virulent SL-VL isolate (**Figure 5.1**). A number of previous studies have assessed whether immunization with live *Leishmania* parasites protects against visceral leishmaniasis. However, in these studies, immunization involved intravenous infection of mice with live *L. chagasi* or *L. infantum* parasites, resulting in the presence of parasite in visceral organs [290,321]. In contrast, subcutaneous immunization with the *L. donovani* SL-CL isolate performed in our study was not associated with dissemination to the spleen.

Since SL-CL does not visceralize, induction of protective responses in the spleen may involve the movement of dendritic cells that have taken up parasite antigens from peripheral tissues into the bloodstream, possibly via lymphatic vessels and lymph nodes. These cells can then migrate to the liver, bone marrow and spleen and activate T cells there [402,403]. Alternatively, some T cells may become activated in lymph nodes draining the site of subcutaneous immunization and then migrate to other organs, including the liver and spleen [404]. Effector memory T cells can also exit the lymph nodes to localize to peripheral tissues [405]. Both of these mechanisms could be involved in the induction of the protective cytokine response that we observed. Several live vaccines delivered subcutaneously are protective against visceral leishmaniasis even though the parasites used for immunization do not visceralize (see for instance [292,310,318]). In addition, many second and third generation vaccines are delivered subcutaneously [396]. It is therefore not surprising that our vaccine was protective even though SL-CL did not visceralize.

SL-CL mediated protection against challenge infection with SL-VL was associated with a mixed Th1/Th2 response prior to challenge (**Figure 5.3A and B, Figure 5.3D, Table 5.1**). This is typical of other successful experimental vaccines against visceral leishmaniasis (see for instance [105,290,291,292]). Notably, there was no antigen-specific IL10 production observed prior to challenge (**Figure 5.3C**). Low antigen-specific IL10 production has previously been identified as a key predictor of vaccine success [291]. Significantly higher IL4 production was observed in all protected groups prior to challenge (10^4 to

10^6) and this may be involved in vaccine-mediated protection. IL4 plays an essential role in vaccine-mediated protection against visceral leishmaniasis by priming CD8+ T cells for long-term protection and promoting granuloma resolution [243]. Finally, only low antibody levels were observed in most protected groups prior to challenge, except in mice immunized with 10^6 SL-CL (**Figure 5.3D**). The role of antibodies in protection against visceral leishmaniasis is still controversial but antibody levels are elevated during active visceral leishmaniasis [406] and IgG exacerbates disease during experimental murine leishmaniasis [407].

Higher IL10 levels were also observed post-challenge in protected groups (**Figure 5.2C**). It is possible that this is why there was not a complete protection against challenge infection. Moreover, elevated IL10 has been observed for other protective vaccine formulations. For instance, higher IL10 than IFNγ levels were detected post-challenge in mice immunized with 10^4 live *L. infantum* intravenously [290]. Similarly, higher IL10 production compared to IFNγ levels was also observed post-immunization (prior to challenge) in mice injected with either live *L. chagasi* or killed but metabolically active *L. chagasi* [292]. The observed production of IL10 may be involved in limiting organ damage, as previously suggested [246]. It is possible that pre-challenge immune responses rather than post-challenge cytokine production are the main determinants of vaccine-mediated protection. This observation also highlights the fact that correlates of vaccine-mediated protection are still not full understood for visceral leishmaniasis [323,408].

Protection against re-infection in cutaneous leishmaniasis has been associated with parasite persistence [312,313]. Protected mice in these experiments had low but detectable footpad parasitemia at the time of challenge (**Figure 5.4**). The highest footpad parasite burden at the time of challenge was observed in mice immunized with 10^6 SL-CL promastigotes, the group in which the highest degree of protection was observed, suggesting that low level parasite persistence may have contributed to the observed protection. However, parasite persistence may not be essential for protection since protection was still observed when mice were challenged at 3 months post-immunization rather than at seven weeks post-immunization, a time point at which parasite burden is undetectable (**Figure S1**).

Immunization with cutaneous *Leishmania* species has had limited success against visceral leishmaniasis. No cross-protection between *L. major* and *L. chagasi* or *L. infantum* was observed in a BALB/c mouse model of visceral leishmaniasis [309,310]. Similarly, autoclaved *L. major* in combination with BCG did not provide any vaccine-mediated protection against visceral leishmaniasis compared to BCG alone in human clinical trials [311]. The lack of success of these immunization strategies may be due to the approximately 14-24 million year divergence between Old World cutaneous and visceral species [3]. In contrast, both the cutaneous immunization strain and the challenge visceral strain used in this study belong to the *L. donovani* species, as confirmed by DNA sequencing of the 6PGDH gene, and are therefore closely related. Using such a naturally attenuated *L. donovani* strain that has evolved to persist in the skin may

therefore prove to be a more successful vaccination strategy against visceral disease caused by *L. donovani.* It would however be necessary to further attenuate the SL-CL *L. donovani* by genetic engineering so that it is unable to cause cutaneous lesions, before it can be considered as a potential live vaccine for visceral leishmaniasis [289].

Overall, this work shows that immunization with a naturally attenuated cutaneous *L. donovani* strain from Sri Lanka provides significant protection against visceral leishmaniasis in the liver of BALB/c mice. The level of protection against visceral leishmaniasis provided by SL-CL may however be more substantial in humans since resolution of cutaneous lesions is common in Sri Lanka and this could indicate protective immunity. These results also provide a potential rationale for the low levels of visceral disease observed in Sri Lanka and work is currently under way to investigate this in the population there. In addition, this successful immunization with a naturally attenuated parasite could also have a broader impact on vaccine development efforts for visceral leishmaniasis worldwide.

5.7 Acknowledgements

This work was supported by a research grant from the Canadian Institute of Health Research (CIHR), and scholarships from the Fonds de la Recherche en Santé du Québec (FRSQ) and from CIHR to LIM.

5.8 Tables

Table 5.1 Antibody isotype responses.

	IgG1	IgG2a	IgG1/IgG2a
Pre-challenge			
PBS	0.060 +/- 0.002	0.057 +/- 0.001	1.07
SL-CL 10^3	0.055 +/- 0.001	0.055 +/- 0.001	0.984
SL-CL 10^4	0.056 +/- 0.000	0.054 +/- 0.001	1.04
SL-CL 10^5	0.065 +/- 0.002	0.057 +/- 0.001	1.14
SL-CL 10^6	0.088 +/- 0.004	0.071 +/- 0.003	1.24
Post-challenge			
PBS	0.084 +/- 0.003	0.087 +/- 0.003	0.965
SL-CL 10^3	0.081 +/- 0.002	0.085 +/- 0.004	0.958
SL-CL 10^4	0.119 +/- 0.003	0.096 +/- 0.005	1.23
SL-CL 10^5	0.116 +/- 0.005	0.073 +/- 0.002	1.60
SL-CL 10^6	0.164 +/- 0.008	0.090 +/- 0.005	1.82

Average absorbance at 450 nm for two independent experiments plus or minus standard error are shown.

5.9 Figures

Figure 5.1 Liver parasite burden post-challenge.

Mice were immunized subcutaneously with 10^3 to 10^6 SL-CL in PBS or with PBS alone as a negative control. Seven weeks post-immunization, mice were challenged intravenously with SL-VL in PBS. Liver parasite burden was determined 28 days post-challenge by direct counting of amastigotes from Diff-Quick stained liver impressions. Average Leishman-Donovan values (LDU) plus standard error are shown and are representative of two independent experiments. ** $p<0.01$ compared to PBS.

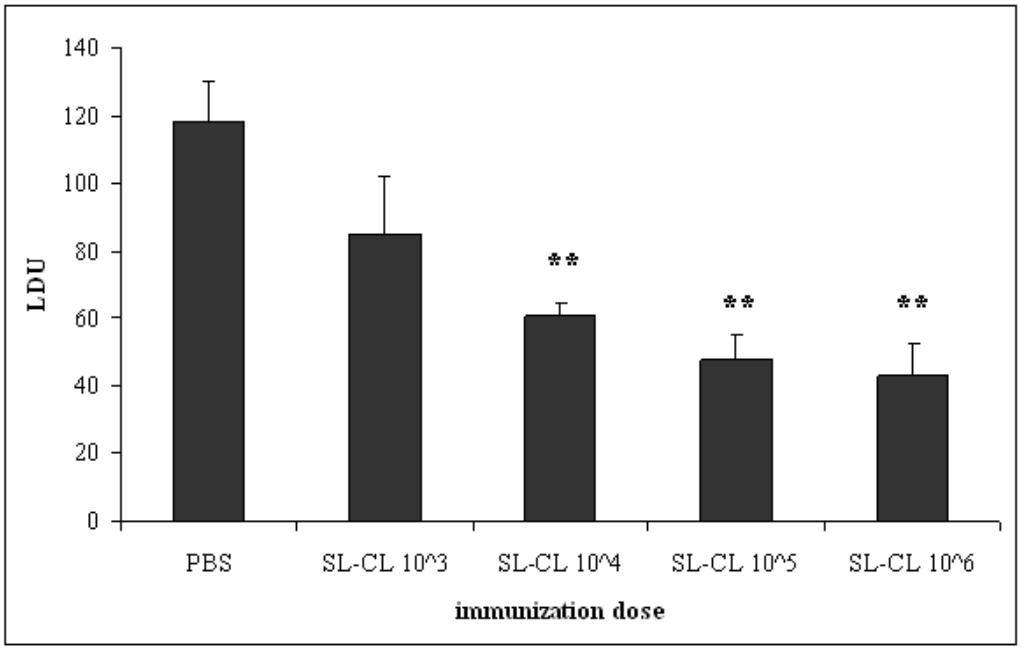
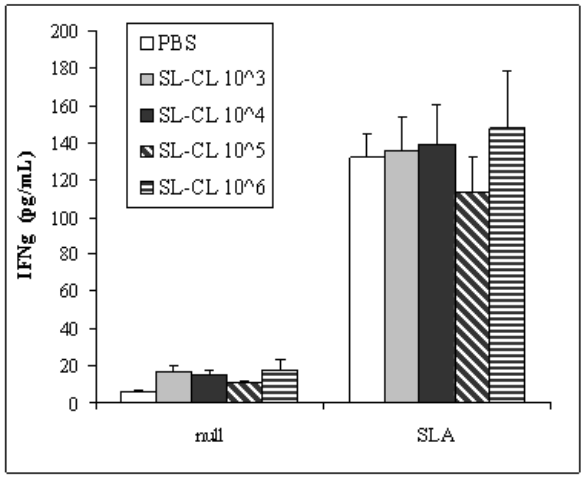


Figure 5.2 Immune response post-challenge.

Mice were immunized subcutaneously with 10^3 to 10^6 SL-CL in PBS or with PBS alone and challenged intravenously seven weeks post-immunization with SL-VL. Splenocytes were isolated 28 days post-challenge and restimulated *in vitro* with SLA prepared from SL-VL promastigotes (SLA) or left unstimulated (null) (**A-C**). Splenocyte culture supernatants were collected after 72 h and IFNγ (**A**), IL4 (**B**) and IL10 (**C**) concentrations determined by ELISA. Average values plus standard error are shown for two independent experiments. * $p<0.05$ compared to PBS. Serum was also collected 28 days post-challenge and IgG titers determined by ELISA (**D**). Average values of two independent experiments plus standard error are shown. ** $p<0.01$ compared to PBS.

A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SL-CL 10^4
SL-CL 10^5
SL-CL 10^6
IFNg (pg/mL)
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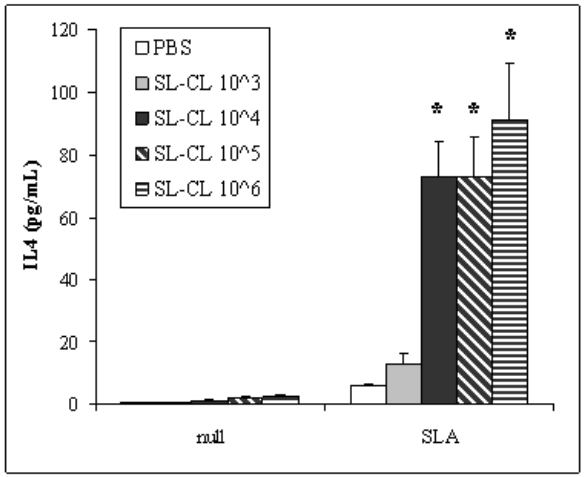
20
40
60
80
100
120
140
160
180
200
null
SLA

B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SL-CL 10^4
SL-CL 10^5
SL-CL 10^6
IL4 (pg/mL)
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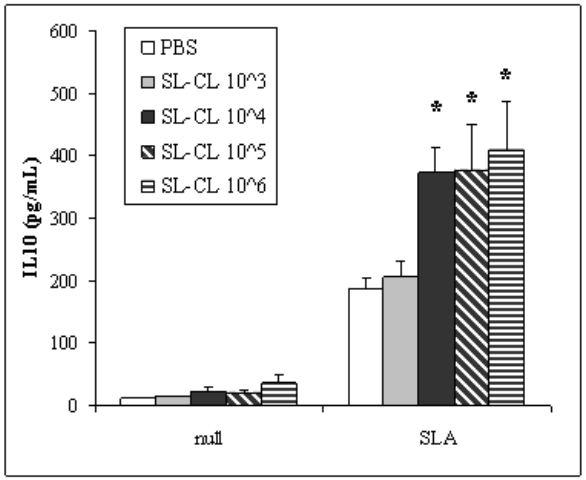
20
40
60
80
100
120
*
*
*
null
SLA

C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SL-CL 10^4
SL-CL 10^5
SL-CL 10^6
IL10 (pg/mL)
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200
300
400
500
600
*
*
*
null
SLA

D

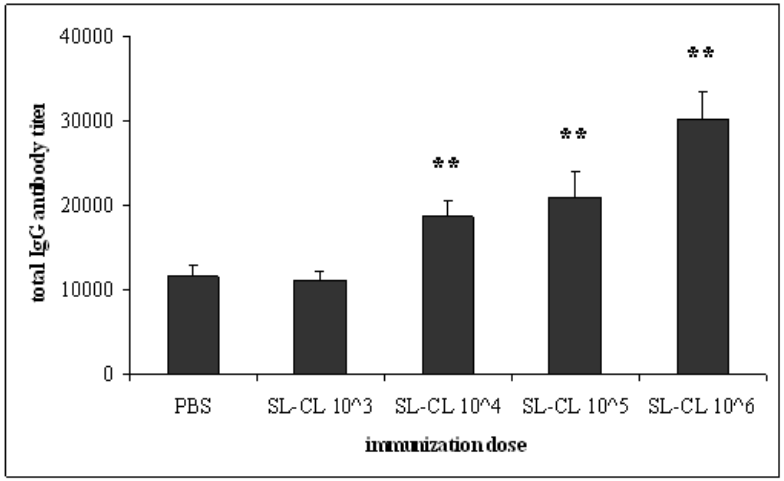
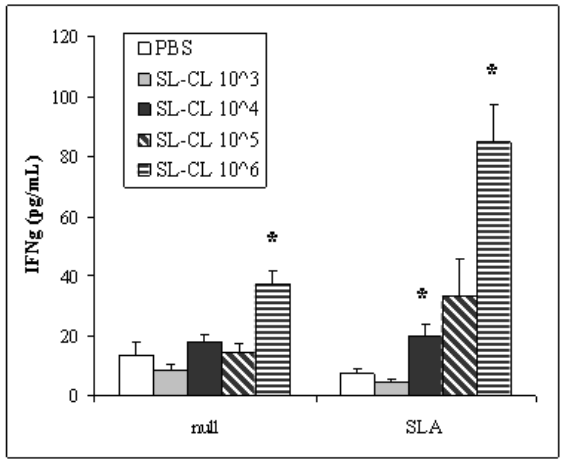

total IgG antibody titer
0
10000
20000
30000
40000
**
**
**
PBS
SL-CL 10^3
SL-CL 10^4
SL-CL 10^5
SL-CL 10^6
immunization dose

Figure 5.3 Immune response pre-challenge.

Mice were immunized subcutaneously with 10^3 to 10^6 SL-CL in PBS or with PBS alone. Splenocytes were isolated seven weeks post-immunization and restimulated *in vitro* with SL-VL SLA (SLA) or left unstimulated (null) (**A-C**). Splenocyte culture supernatants were collected after 72 h and IFNγ (**A**), IL4 (**B**) and IL10 (**C**) concentrations determined by ELISA. Average values plus standard error are shown for two independent experiments. * $p<0.05$ compared to PBS. Serum was also collected seven weeks post-immunization and IgG titers pre-challenge were determined by ELISA (**D**). Average values of two independent experiments plus standard error are shown. ** $p<0.01$ compared to PBS.

A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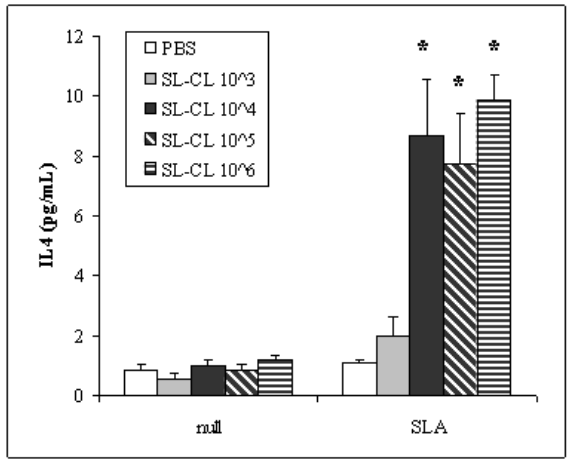
SL-CL 10^3
SL-CL 10^4
SL-CL 10^5
SL-CL 10^6
IFNg (pg/mL)
0
20
40
60
80
100
120
null
SLA
*
*
*

B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SL-CL 10^5
SL-CL 10^6
IL4 (pg/mL)
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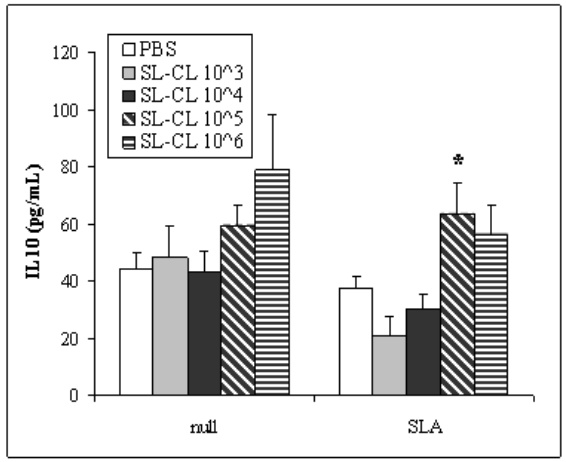
2
4
6
8
10
12
null
SLA
*
*
*

C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SL-CL 10^4
SL-CL 10^5
SL-CL 10^6
IL10 (pg/mL)
0
20
40
60
80
100
120
null
SLA
*

D

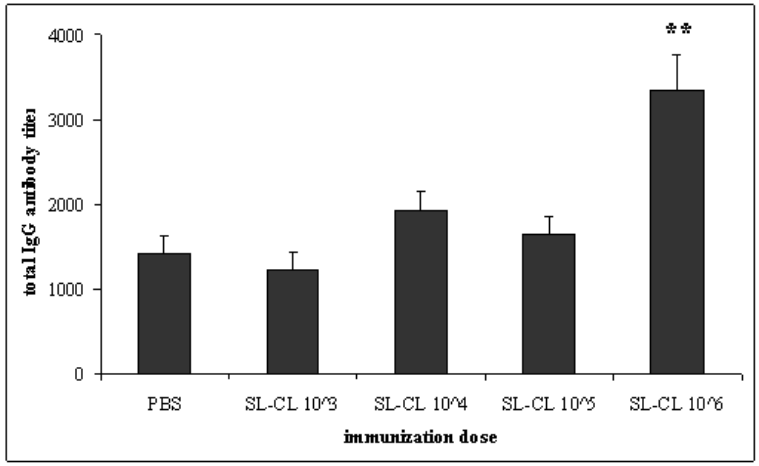

total IgG antibody titer
0
1000
2000
3000
4000
PBS
SL-CL 10^3
SL-CL 10^4
SL-CL 10^5
SL-CL 10^6
immunization dose
**

Figure 5.4 Footpad parasite burden at challenge.

Mice were immunized subcutaneously with 10^3 to 10^6 SL-CL in PBS. Seven weeks post-immunization, prior to parasite challenge, footpad parasite burden was determined by limiting dilution of footpad homogenates.

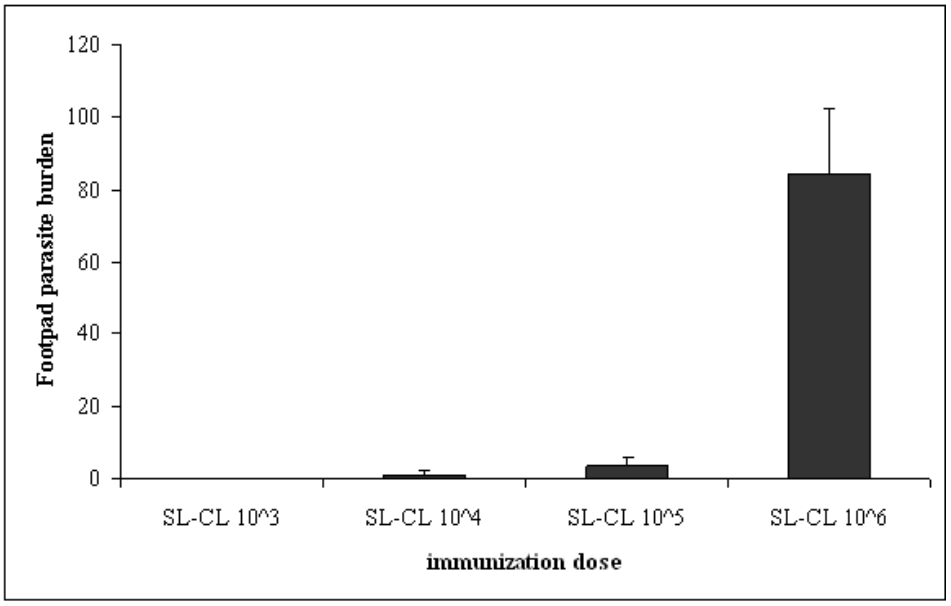
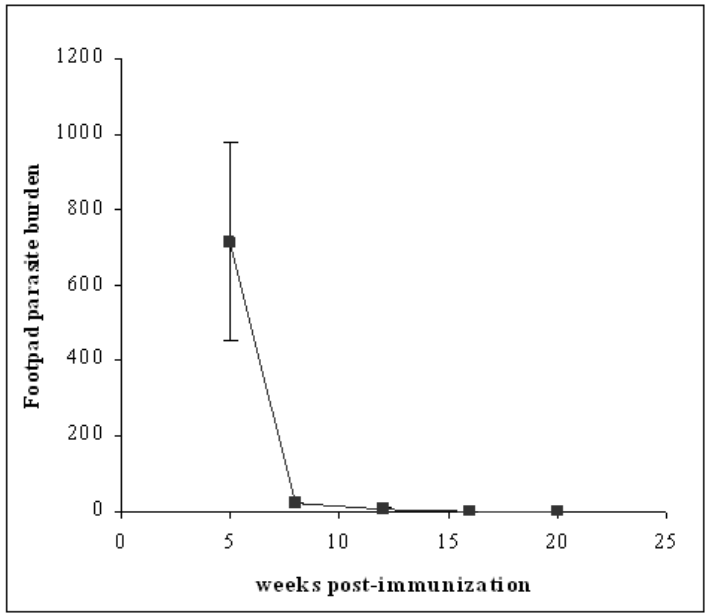


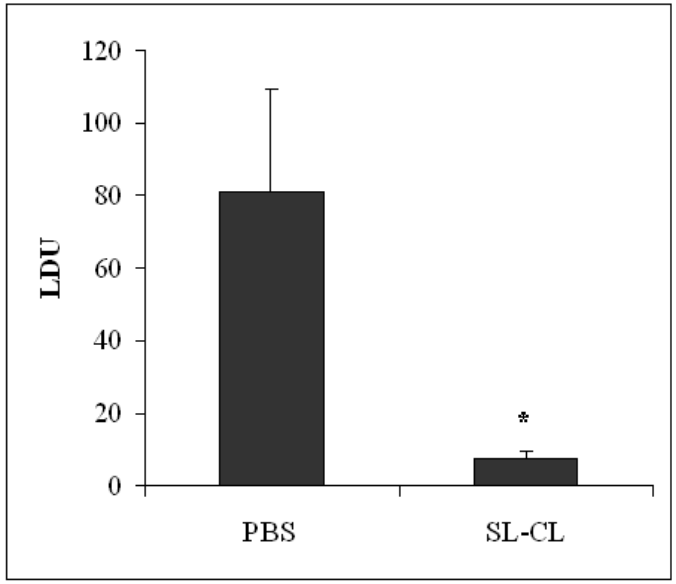
Figure S1 Long-term protection in the absence of persistent footpad parasitemia.

Mice were immunized subcutaneously with $5x10^6$ SL-CL in PBS. Footpad parasite burden was assessed by limiting dilution at five weeks post-infection and at 2, 3, 4 and 5 months post-infection (**A**). Values +/- standard error are shown. Three months post-immunization, mice were challenged intravenously with $5x10^7$ SL-VL in PBS. Liver parasite burden was determined 28 days post-challenge by direct counting of amastigotes from Diff-Quick stained liver impressions (**B**). Average Leishman-Donovan values (LDU) plus standard error are shown. * $p<0.05$ compared to PBS.

A


Footpad parasite burden
1200
1000
800
600
400
200
0
0
5
10
15
20
25
weeks post-immunization

B


LDU
120
100
80
60
40
20
0
*
PBS
SL-CL

Chapter 6

Conclusions and perspectives

A key question in the field of leishmaniasis is why some *Leishmania* species cause self-resolving lesions limited to the skin, while other species are associated with parasite dissemination to the visceral organs, leading to fatal visceral leishmaniasis. Host, vector and parasite factors have all been implicated. This thesis highlights the importance of host immunity and of parasite factors, particularly the A2 virulence factor family, in the development of visceral disease.

6.1 The A2 virulence factor

A number of parasite factors play a key role in visceral disease [125]. Of these, A2 proteins have the most significant impact on visceralization [54,99]. However, the mechanism by which they promote visceral disease is unknown and was the focus of chapters 2 and 3 of this thesis.

6.1.1 Mechanism of action of A2

A2 promotes increased parasite survival in the visceral organs when expressed ectopically in *L. major*, and conversely, decreasing A2 levels by knocking out some of the A2 genes or by antisense mRNA expression was associated with decreased parasite virulence [54,99]. In chapters 2 and 3, we investigate the mechanism by which this occurs. A key difference between

cutaneous and visceral species is the temperature to which parasites are exposed. We show that ectopic expression of A2 in *L. major* promastigotes is associated with increased resistance to heat shock at temperatures comparable to those found in visceral leishmaniasis (**Figure 2.7**). Downregulating A2 levels in *L. donovani* promastigotes and amastigotes was similarly associated with increased sensitivity to heat shock (**Figure 3.1**).

In addition to fever, host defenses include the production of reactive oxygen and nitrogen species, and recently cutaneous *Leishmania* species have been shown to be more sensitive to oxidants than visceral species [61]. We therefore examined the role of A2 in protection against oxidative stress and showed that A2-deficient amastigotes are more sensitive to hydrogen peroxide and nitric oxide than A2-expressing cells (**Figure 3.5**).

To investigate the mechanism by which A2 promotes increased resistance against host defenses, we examined the subcellular localization of A2 and showed that it is localized in the endoplasmic reticulum of the parasites. In addition, a common link between heat shock and oxidative stress is the production of intracellular oxidants. During both heat shock and oxidative stress, internal ROS levels were lower in A2-expressing cells compared to A2-deficient parasites (**Figures 3.2** and **3.4**). However, it is unclear whether A2 directly decreases internal ROS or whether A2 limits the toxic effects of the stress treatment, leading to increased parasite viability and decreased internal ROS.

A2 is a highly repetitive protein. Protein structure modelling using the CPH3.0 server suggests that A2 has a structure similar to type I collagen (**Figure**

6.1). Interestingly, glycine-proline repeats, which are key to the helical structure of collagen [409], are also found in A2. Overall, this suggests that A2 may play a structural rather than enzymatic role. However, confirmation of this model, for instance via X-ray crystallography, would be desirable.

Heat shock is associated with significant cellular destabilization and in particular with endoplasmic reticulum fragmentation [137]. A2 may therefore promote endoplasmic reticulum structural integrity. Alternatively, membrane destabilization is a major characteristic of heat shock [136]. A2-expressing cells had increased membrane stability during heat shock, compared to A2-deficient cells (**Figure 6.2**). A2 could alter membrane fluidity either by direct interaction via its hydrophobic N-terminal region or by interacting with other membrane-associated proteins. The interactions of A2 with membranes are currently being investigated.

A2 function may also involve protein-protein interactions. A2 interacts with the endoplasmic reticulum chaperone BiP. Protein misfolding is observed during heat shock and A2 may counter these effects by altering BiP activity either directly or indirectly. Alternatively, structural proteins implicated in protection from heat shock have been proposed to serve as scaffolds for protein binding [393] and this may also be the case for A2. We are currently investigating possible additional A2 binding partners by co-immunoprecipitation and mass spectrometry.

6.1.2 A2 and the *L. mexicana* species complex

A2 is also found in the *L. amazonensis* and *L. mexicana* genomes [88]. A2 can be detected by Western blot in these species, confirming that both express A2. *L. amazonensis* expresses a single 53 kDa isoform of A2 [121] while three large molecular weight (>170 kDa) isoforms are detected in *L. mexicana* (our observations). The larger A2 sizes in *L. mexicana* could be due to longer A2 sequences in the parasite genome. Alternatively, post-translational modifications could have occurred. Interestingly, the sizes of the two A2 genes in the published *L. mexicana* genome are considerably smaller (LmxM.22.0691 and LmxM.22.0692, 74.5 and 84.8 kDa respectively [410]) than the A2 proteins detected by Western blot in this parasite. However, given the difficulty in sequencing repetitive regions of genomes [411], this cannot be interpreted as automatic support of post-translational modifications. In addition, the molecular weight of A2 proteins in *L. donovani* corresponds to the expected weight based on gene length [46], suggesting that post-translational modifications of A2 are minor or do not occur in *L. donovani*. Any post-translational modifications would therefore be *L. mexicana*-specific.

A2 has been shown to be an important visceralization-promoting factor [54,99]. However, members of the *L. mexicana* species complex, which are considered essentially cutaneous, also express A2. The presence of A2 in this species complex may help account for the cases of visceral leishmaniasis caused by *L. amazonensis* (see **section 1.4.4.3**), especially given the fact that the size of

A2 detected in *L. amazonensis* is similar to the main A2 isoform in *L. chagasi* [121].

However, while there are multiple reports of *L. amazonensis* causing visceral disease [207,208,209], reports of visceral *L. mexicana* are scarcer [212]. This would suggest that although *L. mexicana* species express A2, these proteins do not promote visceralization, nor do they inhibit cutaneous growth of theses parasites. While this may seem contradictory, there are a number of differences between *L. donovani* and *L. mexicana* A2 proteins. First, as described above, *L. mexicana* and *L. donovani* A2 have significantly different sizes. Longer A2 isoforms may be inactive. Alternatively, if the larger protein size in *L. mexicana* is due to post-translational modifications, these may alter protein function.

In addition, the published *L. mexicana* genome sequence has highlighted a number of differences between *L. donovani* and *L. mexicana* A2 sequences. In particular, several prolines are replaced by serines in *L. mexicana,* a change from a hydrophobic to a hydrophilic amino acid. Similarly, many uncharged glycines are replaced with negatively-charged aspartic acids [410]. A2 has been predicted to have a structure similar to type I collagen. Glycine-proline repeats are essential for the structural integrity of collagen [409] and a single amino acid mutation affecting glycine can lead to significant disruption of collagen function, as observed for example in osteogenesis imperfecta. In particular, a single glycine to aspartic acid mutation is lethal and is associated with decreased collagen thermal stability [412]. Many such glycine to aspartic acid mutations are found in the *L. mexicana* A2 sequence. Therefore, the changes in A2 sequence in *L. mexicana*

may render the A2 proteins of *L. mexicana* inactive or less stable. Finally, the last common ancestor of *L. donovani* and *L. mexicana* was at least 40-80 million years ago [4]. Many genetic differences therefore exist between *L. mexicana* and *L. donovani* and these could affect A2 function.

Cloning the *L. mexicana* A2 gene and transfecting it into *L. major* would allow us to determine whether *L. mexicana* A2 is inactive or whether it is other *L. mexicana* genes that account for the cutaneous localization of these parasites. Experiments to perform would be similar to those in which an *L. donovani* A2 gene was transfected into *L. major* and could involve testing parasite virulence *in vivo*, as in [54], and testing sensitivity to heat shock, as in chapter 2. In addition, mutations (glycine to aspartic acid, for instance) could be introduced in the A2-encoding KSneo plasmid. This plasmid could then be transfected into *L. major* to determine whether the mutations in the *L. mexicana* A2 sequence inhibit A2 function. The impact and function of A2 in the *L. mexicana* species complex therefore still requires investigation.

6.1.3 Applications for drug development

Most anti-*Leishmania* drugs have significant disadvantages, including major side effects, counter-indications, and the development of resistance (see **Section 1.1.4.2**). Virulence factors are increasingly being investigated as drug targets. Advantages include decreased risk of resistance because of lower selective pressure, and increased specificity [413].

Suitable drug targets must play an essential role in pathogen growth (particularly in the case of classical antibiotics) or in disease progression (so-called "disease-modifying" genes), and they must be "druggable" [414,415]. Previous work [54,99] indicated that A2 plays a key role in disease progression. The results presented here detail mechanisms by which A2 promotes visceral disease and provide avenues that could be explored to develop *in vitro* methods to test inhibitors. A2 therefore fulfills the first requirement concerning pathogen growth or disease progression. However, to be druggable, a protein target must have a binding pocket for the drug [414]. If the predicted structural model is correct, no such pockets are found in A2, suggesting that it may not be druggable.

In contrast, a number of species-specific genes that have been implicated in visceral disease play an enzymatic role in the cell. These include LinJ.15.0900, a nucleotide sugar transporter [126], and LinJ.36.2480, a cytosolic glyceraldehyde 3-phosphate dehydrogenase GAPDH [127]. The latter may be a better target since LinJ.36.2480 knockouts had significantly decreased liver and spleen parasite burden [127], while knocking out LinJ.15.0900 had no significant effect on *L. donovani* visceral virulence [126]. Even more promising, glyceraldehyde 3-phosphate dehydrogenases are able to bind viable drug leads [415]. Studying *L. donovani* virulence factors can therefore help identify potential drug targets.

6.2 Virulence of the Sri Lanka clinical isolates

6.2.1 Involvement of A2

Given the importance of A2 in *L. donovani* virulence, we investigated whether A2 expression was altered in an atypical cutaneous *L. donovani* isolate

from Sri Lanka (SL-CL). Lower overall A2 levels and fewer A2 isoforms were detected in SL-CL parasites (**Figure 4.4**). This was shown to be a major determinant of the attenuation of this isolate (**Figure 4.5**). These results highlight the importance of A2 in a clinical setting. A2 can be considered an "on/off switch" for virulence in *Leishmania*: introducing A2 into SL-CL changes parasite phenotype from complete avirulence in the spleen to the ability to cause significant parasitemia.

Given that *L. donovani* is normally a visceral parasite and that the visceral isolate from Sri Lanka (SL-VL) expresses more sizes of A2 than the cutaneous isolate, the most likely hypothesis is loss of A2 isoforms in SL-CL. An alternative hypothesis is A2 gene amplification from an original cutaneous parasite, leading to the generation of visceral strains with additional A2 copies. Direct repeats have been associated with gene amplification via homologous recombination in *Leishmania* [416] and A2 genes are organized in tandem arrays interspersed with A2rel genes (**Figure 1.6**) [46]. Therefore, both A2 and A2rel genes could be sites for homologous recombination. Repetitive sequences are unstable [417] and replication splippage could explain the differences in A2 protein sizes. Comparison of A2 protein patterns between the Sri Lanka isolates and Indian *L. donovani* strains has been performed, but no significant similarities were observed in A2 patterns between three clinical isolates from India and the Sri Lanka isolates.

6.2.2 Identification of other factors involved in the attenuation of the SL-CL isolate

Although decreased A2 levels in the SL-CL isolate clearly plays a key role in its attenuation, reconstituting A2 expression to levels comparable to those found in the SL-VL isolate did not fully restore parasite virulence (**Figure 4.5**). Other factors are therefore involved. Whole genome sequencing of both isolates is ongoing with our collaborators at the Seattle Biomedical Research Institute to look for any missense or nonsense mutations. Nonsense mutations could lead to the generation of pseudogenes and may have a major effect on parasite virulence: all four *L. donovani* species-specific genes that increased *L. major* virulence (A2, LinJ.15.0900, LinJ.28.0340 and LinJ.36.2480) are pseudogenes in *L. major* [46,125,126,127]. Missense mutations may significantly alter protein activity and stability. Any genes that show mutations will be investigated by cloning the corresponding gene from SL-VL and transfecting it into SL-CL. The virulence of the SL-CL transfectants will then be examined in our BALB/c mouse infection model, to determine whether these genes play a role in *L. donovani* virulence.

Whole genome sequencing should therefore help us identify a number of novel determinants of *L. donovani* virulence. To identify other factors involved in visceral disease, serial intravenous infections with SL-CL are ongoing to select for increased survival in the visceral organs. The original clinical isolate was used to infect mice intravenously. Twenty-eight days later, mice were sacrificed and parasites were recovered from the liver and spleen. Recovered parasites are then used to reinfect mice intravenously. This procedure will be repeated until

significantly increased parasite virulence is observed. At each infection endpoint, liver and spleen weight and visceral organ parasitemia are assessed. Genomic DNA from the isolated SL-CL with increased virulence will then be used for whole genome sequencing to identify differences compared to the original clinical isolate.

So far six serial infections have been performed. Visceral organ parasitemia is increasing, while no significant difference in organ weight have been observed so far (**Figure 6.3**). Serial infections will continue to be performed to determine whether this trend is maintained.

In addition, we have also transfected the SL-CL cutaneous isolate with a cosmid library from a virulent *L. donovani* strain to try and complement the genetic defects found in the SL-CL isolate. Transfection is followed by selection *in vivo* and recovery of parasites from the visceral organs 28 days post-infection. Cosmids are then isolated from recovered parasites and sequenced to identify which genes they contain. This allows us to determine *in vivo* which genes are most important for increased survival in the visceral organs.

Finally, SL-CL and SL-VL were equally infective to macrophages. Recent work has shown that a high-virulence *L. major* strain was more infective to dendritic cells than a strain with low virulence, although unlike SL-CL and SL-VL, differences were also observed during monocyte infection [418]. Differences in dendritic cell migration have also been observed following infection with *L. donovani* compared to infection with *L. major*, suggesting a role for dendritic cells in parasite dissemination to the visceral organs [46]. Dendritic cells also

migrate from the dermis to present *L. major* antigens in the lymph nodes [419] and are an important source of IL12 [243]. It would therefore be interesting to compare the infectivity of SL-CL and SL-VL to dendritic cells.

6.2.3 Public health considerations in Sri Lanka

Visceral leishmaniasis is currently rare in Sri Lanka. There are two possible explanations for this observation. First, the SL-CL parasite may be as virulent as the SL-VL parasite, and host characteristics specific to the Sri Lanka population or to certain ethnic groups in Sri Lanka prevent parasites from establishing a visceral infection. Second, SL-CL and SL-VL may represent distinct parasite strains. The SL-CL parasite would be attenuated and unable to establish a visceral infection.

The results presented in chapter 4 indicate that differences in disease phenotype between these two isolates are maintained when infecting inbred mice, demonstrating that the atypical disease manifestations in Sri Lanka are due to parasite rather than host characteristics. In addition, the differences in A2 protein expression suggest that the SL-CL and SL-VL parasites represent different *L. donovani* strains.

A major public health concern in Sri Lanka is the risk of an epidemic of visceral leishmaniasis. The fact that the different disease manifestations are due to parasite rather than host characteristics suggests that the SL-VL strain may spread, leading to many visceral leishmaniasis cases. A second possibility is the reversion of the SL-CL parasite to a viscerotropic phenotype. The serial

infections described above (**Section 6.2.2**) will help us investigate this possibility. Preliminary results do suggest that virulence can increase following passage through multiple hosts. These results indicate that the situation in Sri Lanka should be actively monitored since reversion of SL-CL to a more virulent phenotype could occur in the field.

6.3 Immunizations with SL-CL

The low rates of visceral leishmaniasis in Sri Lanka could also be due to protection against visceral disease by a prior cutaneous infection. We investigated this and showed that immunization with SL-CL did provide some protection against visceral disease in BALB/c mice (**Figure 5.1**). These results support further work to investigate whether this occurs in the field in Sri Lanka. A longitudinal study could be performed by taking advantage of the fact that infection with SL-CL does not lead to rK39 seropositivity [166]. Patients that have been exposed to SL-CL alone can be differentiated from patients exposed to SL-VL: the former are Leishmanin skin test positive and rK39 negative, while the latter are positive for both tests. Individuals that have not been exposed to either strain would be negative for both tests. The incidence of visceral leishmaniasis could be compared over time between individuals that have not been exposed to either parasite and individuals that were originally exposed only to SL-CL. However, this study would require very large sample sizes since visceral leishmaniasis is so rare in Sri Lanka. The study would be further hampered by the

long incubation time for visceral leishmaniasis: while the average incubation time is 3-8 months, incubation may last many years [8].

Preliminary whole genome sequencing results suggest that there are very few differences between the SL-CL and the SL-VL isolates. Therefore, subcutaneous immunization with the SL-VL isolate would most likely also be protective against intravenous challenge with SL-VL. Indeed, results at five weeks post-infection by subcutaneous route indicated no significant difference in splenocyte immune responses between SL-CL and SL-VL infections. However, immunization with SL-CL is far more interesting from a vaccine safety perspective since SL-CL is already partially attenuated. Further modifications would still be required to make SL-CL suitable as a vaccine. Knocking out virulence factors or other genes could be performed to further attenuate these parasites. For instance, knockouts of the biopterin transporter BT1 or centrin, and silent information regulatory 2 (SIR2)-deficient parasites were protective against visceral disease [323] and these genes could also be knocked out in the SL-CL isolate. The addition of suicide genes could also be considered.

While knockout parasites have previously been found undesirable for clinical applications because of the introduction of drug resistance genes during the knockout process, novel techniques are being developed to knockout genes without introducing permanent drug resistance genes. For instance, a double transfection strategy using a first construct with both a negative selectable marker and a positive selectable marker, followed by transfection with a null cassette, was used to knock out one of the cysteine peptidase arrays in *L. infantum* [420].

Similarly, the CRE/*LoxP* system was successfully used to knock out genes without introducing permanent drug resistance in *T. brucei* [421]. This system could also be applied in *Leishmania.*

Finally, the addition of CpG oligonucleotides has also been suggested to increase the safety of immunization with *L. major.* Immunization with SL-CL in combination with CpG oligonucleotides should be investigated to determine whether protection is still observed against visceral leishmaniasis.

6.4 Overall conclusions

Leishmaniasis is a major issue in tropical and subtropical regions of the world, with over 350 million people at risk in 88 countries [24]. Visceral leishmaniasis is especially deadly [22,23,26]. Global warming, immunodeficiencies and population movement may further expand the range of this disease [348]. However, current treatment options have many limitations and there is no human vaccine.

Programs are underway in India, Nepal and Bangladesh with the goal of eliminating visceral leishmaniasis by 2015 [29]. However, a cornerstone of these programs is the anthroponotic transmission cycle of *L. donovani* in this region. Such elimination programs may not be suitable in the case of *L. infantum* which has a zoonotic transmission cycle. In addition, visceral cases are on the rise in Brazil and especially in Sudan and South Sudan [22]. Therefore, drug and vaccine development are still required.

Studying virulence factors such as A2 provide valuable insight into disease pathogenesis and could guide drug development. Novel virulence factors could also represent new drug targets. Finally, studying naturally attenuated parasites could help us identify desirable vaccine characteristics and formulate better vaccines. This work therefore has potential applications in anti-*Leishmania* drug and vaccine development.

Neglected tropical diseases affect at least a billion people worldwide [422]. Hopefully, further work on the causative organisms of these diseases will lead to new treatment and immunization regimens so that one day these diseases may be eradicated.

6.5 Figures

Figure 6.1 A2 is predicted to have a helical structure similar to the structure of type I collagen.

The sequence of A2 deposited in TritrypDB (LinJ.22.0670, http://tritrypdb.org/tritrypdb/) was input into the CPHmodels 3.0 server (http://www.cbs.dtu.dk/services/CPHmodels/). A2 is predicted to have a structure similar to type I collagen. Surface representation highlighting the repetitive glycine-proline units. Glycines are labelled in green and prolines in red.

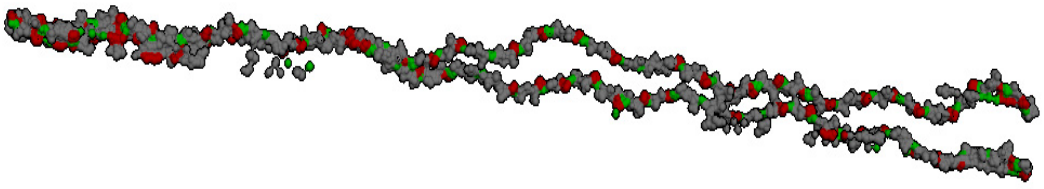


Figure 6.2 A2 promotes decreased membrane fluidity during heat shock. Promastigotes of *L. donovani* transfected with the empty KSneo plasmid (*L. d.* + control) or the antisense A2 plasmid (*L. d.* – A2) were stained with 1,6-diphenylhexa-1,3,5-triene (DPH) to assess membrane fluidity. Fluorescence anisotropy (r) of DPH was measured in a temperature-controlled fluorometer, with temperatures increasing from room temperature at t = 0 min to 40°C at t = 14 min. Temperatures were then maintained at 40°C for the remainder of the experiment. Note the decrease in anisotropy as the temperature increases, indicating increased membrane fluidity caused by heat shock. Higher anisotropy values in *L. d.* + control indicate increased membrane stability (lower fluidity). $p<0.05$ for t = 10-12 min and $p<0.01$ for t = 14-120 min. Values represent the average of four independent experiments.

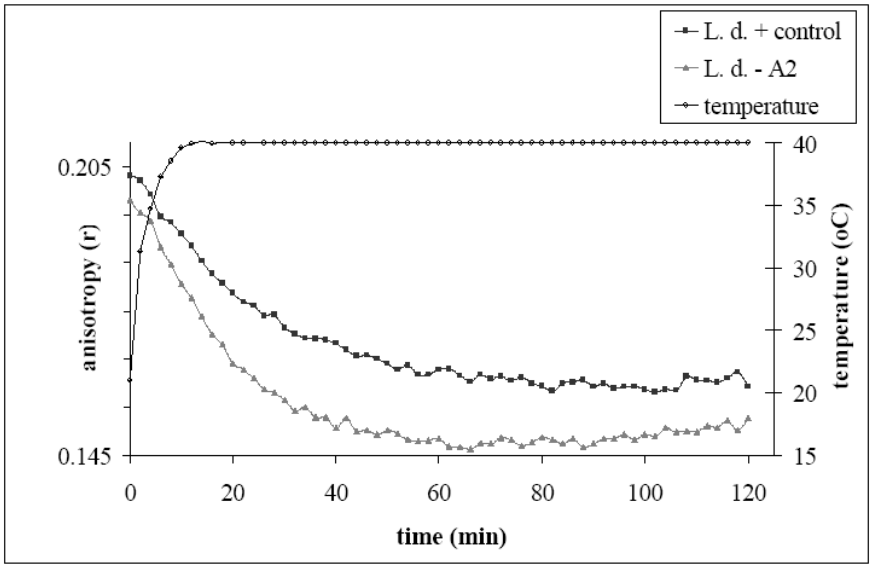
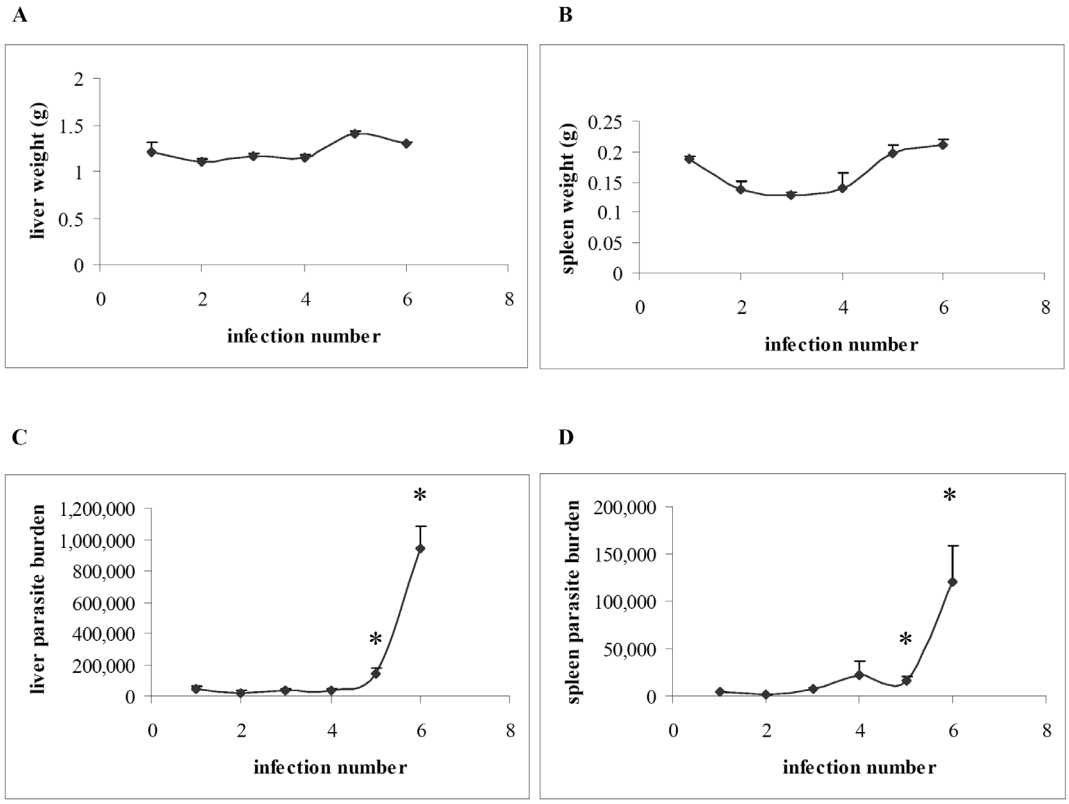


Figure 6.3 Selecting for increased SL-CL virulence.

BALB/c mice were infected intravenously with $1x10^8$ SL-CL stationary phase promastigotes. Twenty-eight days post-infection, amastigotes were isolated from the liver and spleen and used to reinfect mice. Liver (**A**) and spleen (**B**) weights were determined, and liver (**C**) and spleen (**D**) parasitemia were assessed by limiting dilution. *, $p<0.05$ compared to infection number 1.



References

1. Banuls AL, Hide M, Prugnolle F (2007) *Leishmania* and the leishmaniases: A parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. Advances in Parasitology, Vol 64 64: 1-+.
2. Tuon FF, Amato V, Amato VS (2008) *Leishmania*: origin, evolution and future since the Precambrian. Fems Immunology and Medical Microbiology 54: 158-166.
3. Lukes J, Mauricio IL, Schonian G, Dujardin JC, Soteriadou K, et al. (2007) Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. Proceedings National Academy of Sciences U S A 104: 9375-9380.
4. 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? Immunological Reviews 201: 206-224.
5. Leblois R, Kuhls K, Francois O, Schonian G, Wirth T (2011) Guns, germs and dogs: On the origin of *Leishmania chagasi*. Infection Genetics and Evolution 11: 1091-1095.
6. Ribeiro-Gomes FL, Peters NC, Debrabant A, Sacks DL (2012) Efficient Capture of Infected Neutrophils by Dendritic Cells in the Skin Inhibits the Early Anti-*Leishmania* Response. PLoS Pathogens 8: e1002536.
7. Sacks D, Kamhawi S (2001) Molecular aspects of parasite-vector and vector-host interactions in Leishmaniasis. Annual Review of Microbiology 55: 453-483.
8. Pearson RD, Sousa AQ (1996) Clinical spectrum of Leishmaniasis. Clinical Infectious Diseases 22: 1-13.
9. Tsigankov P, Gherardini PF, Helmer-Citterich M, Zilberstein D (2012) What has proteomics taught us about *Leishmania* development? Parasitology 139: 1146-1157.
10. Wenzel UA, Bank E, Florian C, Forster S, Zimara N, et al. (2012) *Leishmania major* parasite stage-dependent host cell invasion and immune evasion. Faseb Journal 26: 29-39.
11. Pescher P, Blisnick T, Bastin P, Spath GF (2011) Quantitative proteome profiling informs on phenotypic traits that adapt *Leishmania donovani* for axenic and intracellular proliferation. Cellular Microbiology 13: 978-991.
12. Gupta N, Goyal N, Rastogi AK (2001) In vitro cultivation and characterization of axenic amastigotes of *Leishmania*. Trends in Parasitology 17: 150-153.
13. Rochette A, Raymond F, Corbeil J, Ouellette M, Papadopoulou B (2009) Whole-genome comparative RNA expression profiling of axenic and intracellular amastigote forms of *Leishmania infantum*. Molecular and Biochemical Parasitology 165: 32-47.
14. Banuls AL, Bastien P, Pomares C, Arevalo J, Fisa R, et al. (2011) Clinical pleiomorphism in human leishmaniases, with special mention of

asymptomatic infection. Clinical Microbiology and Infection 17: 1451-1461.
15. Bailey MS, Lockwood DN (2007) Cutaneous leishmaniasis. Clinics in Dermatology 25: 203-211.
16. WHO (2010) Control of the leishmaniases Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010 Introduction. WHO Technical Report Series 949: Xii-+.
17. David CV, Craft N (2009) Cutaneous and mucocutaneous leishmaniasis. Dermatologic Therapy 22: 491-502.
18. Ives A, Ronet C, Prevel F, Ruzzante G, Fuertes-Marraco S, et al. (2011) *Leishmania* RNA Virus Controls the Severity of Mucocutaneous Leishmaniasis. Science 331: 775-778.
19. Duthie MS, Raman VS, Piazza FM, Reed SG (2012) The development and clinical evaluation of second-generation leishmaniasis vaccines. Vaccine 30: 134-141.
20. Duprey ZH, Steurer FJ, Rooney JA, Kirchhoff LV, Jackson JE, et al. (2006) Canine visceral leishmaniasis, United States and Canada, 2000-2003. Emerging Infectious Diseases 12: 440-446.
21. Rose K, Curtis J, Baldwin T, Mathis A, Kumar B, et al. (2004) Cutaneous leishmaniasis in red kangaroos: isolation and characterisation of the causative organisms. International Journal for Parasitology 34: 655-664.
22. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, et al. (2012) Leishmaniasis Worldwide and Global Estimates of Its Incidence. PLoS One 7: e35671.
23. Mathers CD, Ezzati M, Lopez AD (2007) Measuring the Burden of Neglected Tropical Diseases: The Global Burden of Disease Framework. PLoS Neglected Tropical Diseases 1: e114.
24. Desjeux P (2004) Leishmaniasis: current situation and new perspectives. Comparative immunology, microbiology and infectious diseases 27: 305-318.
25. Machado-Coelho GL, Caiaffa WT, Genaro O, Magalhaes PA, Mayrink W (2005) Risk factors for mucosal manifestation of American cutaneous leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 99: 55-61.
26. Hotez PJ, Remme JHF, Buss P, Alleyne G, Morel C, et al. (2004) Combating tropical infectious diseases: Report of the disease control priorities in developing countries project. Clinical Infectious Diseases 38: 871-878.
27. Sundar S, Chakravarty J, Agarwal D, Rai M, Murray HW (2010) Single-Dose Liposomal Amphotericin B for Visceral Leishmaniasis in India. New England Journal of Medicine 362: 504-512.
28. Pandey K, Sinha PK, Das VR, Bimal S, Singh SK, et al. (2009) Pharmacotherapeutic options for visceral leishmaniasis-current scenario. Clinical Medicine Pathology 2: 1-4.
29. Matlashewski G, Arana B, Kroeger A, Battacharya S, Sundar S, et al. (2011) Visceral leishmaniasis: elimination with existing interventions. Lancet Infectious Diseases 11: 322-325.

30. Lee BY, Bacon KM, Shah M, Kitchen SB, Connor DL, et al. (2012) The Economic Value of a Visceral Leishmaniasis Vaccine in Bihar State, India. American Journal of Tropical Medicine and Hygiene 86: 417-425.
31. Jha T (2011) Epidemiology of Drug-Resistant Kala-Azar in India and Neighboring Countries; Jha T, Noiri E, editors. New York: Springer. 290 p.
32. Alvar J, Yactayo S, Bern C (2006) Leishmaniasis and poverty. Trends in Parasitology 22: 552-557.
33. Yardley V, Croft SL, De Doncker S, Dujardin JC, Koirala S, et al. (2005) The sensitivity of clinical isolates of *Leishmania* from Peru and Nepal to miltefosine. American Journal of Tropical Medicine and Hygiene 73: 272-275.
34. Croft SL, Sundar S, Fairlamb AH (2006) Drug resistance in leishmaniasis. Clinical Microbiology Reviews 19: 111-+.
35. Maltezou HC (2010) Drug Resistance in Visceral Leishmaniasis. Journal of Biomedicine and Biotechnology 2010: 8.
36. Sundar S (2001) Drug resistance in Indian visceral leishmaniasis. Tropical Medicine & International Health 6: 849-854.
37. Sundar S, Singh A, Rai M, Prajapati VK, Singh AK, et al. (2012) Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. Clinical Infectious Diseases 55: 543-550.
38. Srivastava P, Prajapati VK, Rai M, Sundar S (2011) Unusual case of resistance to amphotericin B in visceral leishmaniasis in a region in India where leishmaniasis is not endemic. Journal of Clinical Microbiology 49: 3088-3091.
39. Moreira W, Leprohon P, Ouellette M (2011) Tolerance to drug-induced cell death favours the acquisition of multidrug resistance in *Leishmania*. Cell Death & Disease 2.
40. Kumar D, Kulshrestha A, Singh R, Salotra P (2009) In Vitro Susceptibility of Field Isolates of *Leishmania donovani* to Miltefosine and Amphotericin B: Correlation with Sodium Antimony Gluconate Susceptibility and Implications for Treatment in Areas of Endemicity. Antimicrobial Agents and Chemotherapy 53: 835-838.
41. WHO (2000) The leishmaniases and *Leishmania*/HIV co-infections. WHO Fact Sheet No 116 WHO Fact Sheet No 116.
42. Bates PA (2007) Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. International Journal for Parasitology 37: 1097-1106.
43. de Souza EP, Pereira APE, Machado FCS, Melo MF, Souto-Padron T, et al. (2001) Occurrence of *Leishmania donovani* parasitemia in plasma of infected hamsters. Acta Tropica 80: 69-75.
44. Thalhofer CJ, Chen YN, Sudan B, Love-Homan L, Wilson ME (2011) Leukocytes Infiltrate the Skin and Draining Lymph Nodes in Response to the Protozoan *Leishmania infantum chagasi*. Infection and Immunity 79: 108-117.

45. Peters NC, Kimblin N, Secundino N, Kamhawi S, Lawyer P, et al. (2009) Vector Transmission of *Leishmania* Abrogates Vaccine-Induced Protective Immunity. PLoS Pathogens 5: e1000484.
46. Zhang WW, Mendez S, Ghosh A, Myler P, Ivens A, et al. (2003) Comparison of the A2 gene locus in *Leishmania donovani* and *Leishmania major* and its control over cutaneous infection. Journal of Biological Chemistry 278: 35508-35515.
47. Laskay T, Diefenbach A, Rollinghoff M, Solbach W (1995) Early parasite containment is decisive for resistance to *Leishmania major* infection. European Journal of Immunology 25: 2220-2227.
48. Melby PC, Yang YZ, Cheng J, Zhao WG (1998) Regional differences in the cellular immune response to experimental cutaneous or visceral infection with *Leishmania donovani*. Infection and Immunity 66: 18-27.
49. Ahmed S, Colmenares M, Soong L, Goldsmith-Pestana K, Munstermann L, et al. (2003) Intradermal infection model for pathogenesis and vaccine studies of murine visceral leishmaniasis. Infection and Immunity 71: 401-410.
50. Gomes R, Teixeira C, Teixeira MJ, Oliveira F, Menezes MJ, 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. Proceedings of the National Academy of Sciences U S A 105: 7845-7850.
51. Maia C, Seblova V, Sadlova J, Votypka J, Volf P (2011) Experimental transmission of *Leishmania infantum* by two major vectors: A comparison between a viscerotropic and a dermotropic strain. PLoS Neglected Tropical Diseases 5: 6 e1181.
52. Engwerda CR, Ato M, Kaye PM (2004) Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis. Trends in Parasitology 20: 524-530.
53. Nieto A, Dominguez-Bernal G, Orden JA, De La Fuente R, Madrid-Elena N, et al. (2011) Mechanisms of resistance and susceptibility to experimental visceral leishmaniosis: BALB/c mouse versus syrian hamster model. Veterinary Research 42.
54. Zhang WW, Matlashewski G (2001) Characterization of the A2-A2rel gene cluster in *Leishmania donovani*: involvement of A2 in visceralization during infection. Molecular Microbiology 39: 935-948.
55. Scott P (1985) Impaired macrophage leishmanicidal activity at cutaneous temperature. Parasite Immunology 7: 277-288.
56. Wittner M, Tanowitz HB (2000) Leishmaniasis in infants and children. Seminars in Pediatric Infectious Diseases 11: 196-201.
57. McCall LI, Matlashewski G (2010) Localization and induction of the A2 virulence factor in *Leishmania*: evidence that A2 is a stress response protein. Molecular Microbiology 77: 518-530.
58. Callahan HL, Portal IF, Bensinger SJ, Grogl M (1996) *Leishmania* spp: Temperature sensitivity of promastigotes in vitro as a model for tropism in vivo. Experimental Parasitology 84: 400-409.

59. Hoyer C, Mellenthin K, Schilhabel M, Platzer M, Clos J (2001) Use of genetic complementation to identify gene(s) which specify species-specific organ tropism of *Leishmania*. Medical Microbiology and Immunology 190: 43-46.
60. Beachy SH, Repasky EA (2011) Toward establishment of temperature thresholds for immunological impact of heat exposure in humans. International Journal of Hyperthermia 27: 344-352.
61. Sarkar A, Ghosh S, Pakrashi S, Roy D, Sen S, et al. (2012) *Leishmania* strains causing self-healing cutaneous leishmaniasis have greater susceptibility towards oxidative stress. Free Radical Research 46: 665-673.
62. De Trez C, Magez S, Akira S, Ryffel B, Carlier Y, et al. (2009) iNOS-producing inflammatory dendritic cells constitute the major infected cell type during the chronic *Leishmania major* infection phase of C57BL/6 resistant mice. PLoS Pathogens 5: e1000494.
63. Kaye P, Scott P (2011) Leishmaniasis: complexity at the host-pathogen interface. Nature Reviews Microbiology 9: 604-615.
64. Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, et al. (2005) Macrophage receptors and immune recognition. Annual Review of Immunology 23: 901-944.
65. Olivier M, Tanner CE (1987) Susceptibilities of macrophage populations to infection in vitro by *Leishmania donovani*. Infection and Immunity 55: 467-471.
66. Redmond HP, Shou J, Gallagher HJ, Kelly CJ, Daly JM (1993) Macrophage-dependent candidacidal mechanisms in the murine system - Comparison of murine kupffer cell and peritoneal macrophage candidacidal mechanisms. Journal of Immunology 150: 3427-3433.
67. Liu GW, Xia XP, Gong SL, Zhao Y (2006) The macrophage heterogeneity: Difference between mouse peritoneal exudate and splenic F4/80(+) macrophages. Journal of Cellular Physiology 209: 341-352.
68. Rutherford MS, Witsell A, Schook LB (1993) Mechanisms generating functionally heterogeneous macrophages - Chaos revisited. Journal of Leukocyte Biology 53: 602-618.
69. Warburg A, Saraiva E, Lanzaro GC, Titus RG, Neva F (1994) Saliva of *Lutzomyia longipalpis* sibling species differs in its composition and capacity to enhance leishmaniasis. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 345: 223-230.
70. Rohousova I, Subrahmanyam S, Volfova V, Mu JB, Volf P, et al. (2012) salivary gland transcriptomes and proteomes of *Phlebotomus tobbi* and *Phlebotomus sergenti*, vectors of leishmaniasis. PLoS Neglected Tropical Diseases 6: e1660.
71. Gradoni L, Gramiccia M (1994) *Leishmania infantum* tropism - Strain genotype or host immune status. Parasitology Today 10: 264-267.
72. Sulahian A, Garin YJF, Pratlong F, Dedet JP, Derouin F (1997) Experimental pathogenicity of viscerotropic and dermotropic isolates of *Leishmania infantum* from immunocompromised and immunocompetent patients in a murine model. Fems Immunology and Medical Microbiology 17: 131-138.

73. Campos-Ponce M, Ponce C, Ponce E, Maingon RDC (2005) *Leishmania chagasi/infantum*: further investigations on *Leishmania* tropisms in atypical cutaneous and visceral leishmaniasis foci in Central America. Experimental Parasitology 109: 209-219.
74. Louassini M, Adroher FJ, Foulquie MR, Benitez R (1998) Investigations on the in vitro metacyclogenesis of a visceral and a cutaneous human strain of *Leishmania infantum*. Acta Tropica 70: 355-368.
75. Mendez S, Fernandez-Perez FJ, Santin N, De la Fuente C, Cuquerella M, et al. (2001) Correlation between in vitro and in vivo infectivity of *Leishmania infantum* clones. Journal of Eukaryotic Microbiology 48: 616-621.
76. Lira R, Mendez S, Carrera L, Jaffe C, Neva F, et al. (1998) *Leishmania tropica*: The identification and purification of metacyclic promastigotes and use in establishing mouse and hamster models of cutaneous and visceral disease. Experimental Parasitology 89: 331-342.
77. Gregory DJ, Sladek R, Olivier M, Matlashewski G (2008) Comparison of the effects of *Leishmania major* or *Leishmania donovani* infection on macrophage gene expression. Infection and Immunity 76: 1186-1192.
78. Anstead GM, Chandrasekar B, Zhao WG, Yang J, Perez LE, et al. (2001) Malnutrition alters the innate immune response and increases early visceralization following *Leishmania donovani* infection. Infection and Immunity 69: 4709-4718.
79. Kropf P, Fuentes JM, Fahnrich E, Arpa L, Herath S, et al. (2005) Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. Faseb Journal 19: 1000-+.
80. Matte C, Olivier M (2002) *Leishmania*-induced cellular recruitment during the early inflammatory response: Modulation of proinflammatory mediators. Journal of Infectious Diseases 185: 673-681.
81. McDowell MA, Marovich M, Lira R, Braun M, Sacks D (2002) *Leishmania* priming of human dendritic cells for CD40 ligand-induced interleukin-12p70 secretion is strain and species dependent. Infection and Immunity 70: 3994-4001.
82. Meddeb-Garnaoui A, Zrelli H, Dellagi K (2009) Effects of tropism and virulence of *Leishmania* parasites on cytokine production by infected human monocytes. Clinical and Experimental Immunology 155: 199-206.
83. Charest H, Matlashewski G (1994) Developmental gene expression in *Leishmania donovani* - Differential cloning and analysis of an amastigote-stage-specific gene. Molecular and Cellular Biology 14: 2975-2984.
84. Charest H, Zhang WW, Matlashewski G (1996) The developmental expression of *Leishmania donovani* A2 amastigote-specific genes is post-transcriptionally mediated and involves elements located in the 3'-untranslated region. Journal of Biological Chemistry 271: 17081-17090.
85. Zhang WW, Charest H, Ghedin E, Matlashewski G (1996) Identification and overexpression of the A2 amastigote-specific protein in *Leishmania donovani*. Molecular and Biochemical Parasitology 78: 79-90.
86. Downing T, Imamura H, Decuypere S, Clark TG, Coombs GH, et al. (2011) Whole genome sequencing of multiple *Leishmania donovani* clinical

isolates provides insights into population structure and mechanisms of drug resistance. Genome Research 21: 2143-2156.
87. Charest H, Matlashewski G (1994) Developmental gene expression in *Leishmania donovani*: differential cloning and analysis of an amastigote-stage-specific gene. Molecular and Cellular Biology 14: 2975-2984.
88. Ghedin E, Zhang WW, Charest H, Sundar S, Kenney RT, et al. (1997) Antibody response against a *Leishmania donovani* amastigote-stage-specific protein in patients with visceral leishmaniasis. Clinical and Diagnostic Laboratory Immunology 4: 530-535.
89. Ghedin E, Charest H, Matlashewski G (1998) A2rel: a constitutively expressed *Leishmania* gene linked to an amastigote-stage-specific gene. Molecular and Biochemical Parasitology 93: 23-29.
90. Alcolea PJ, Alonso A, Gomez MJ, Sanchez-Gorostiaga A, Moreno-Paz M, et al. (2010) Temperature increase prevails over acidification in gene expression modulation of amastigote differentiation in *Leishmania infantum*. Bmc Genomics 11: 31.
91. Alcolea PJ, Alonso A, Gomez MJ, Moreno I, Dominguez M, et al. (2010) Transcriptomics throughout the life cycle of *Leishmania infantum*: High down-regulation rate in the amastigote stage. International Journal for Parasitology 40: 1497-1516.
92. Gosline SJC, Nascimento M, McCall LI, Zilberstein D, Thomas DY, et al. (2011) Intracellular eukaryotic parasites have a distinct unfolded protein response. PLoS One 6: e19118.
93. Barak E, Amin-Spector S, Gerliak E, Goyard S, Holland N, et al. (2005) Differentiation of *Leishmania donovani* in host-free system: analysis of signal perception and response. Molecular and Biochemical Parasitology 141: 99-108.
94. Harder S, Thiel M, Clos J, Bruchhaus I (2010) Characterization of a subunit of the outer dynein arm docking complex necessary for correct flagellar assembly in *Leishmania donovani*. PLoS Neglected Tropical Diseases 4: e586.
95. Cloutier S, Laverdiere M, Chou MN, Boilard N, Chow C, et al. (2012) Translational control through eIF2alpha phosphorylation during the *Leishmania* differentiation process. PLoS One 7: e35085.
96. Boucher N, Wu Y, Dumas C, Dube M, Sereno D, et al. (2002) A common mechanism of stage-regulated gene expression in *Leishmania* mediated by a conserved 3 '-untranslated region element. Journal of Biological Chemistry 277: 19511-19520.
97. Rochette A, Raymond F, Ubeda JM, Smith M, Messier N, et al. (2008) Genome-wide gene expression profiling analysis of *Leishmania major* and *Leishmania infantum* developmental stages reveals substantial differences between the two species. Bmc Genomics 9: 255.
98. McNicoll F, Muller M, Cloutier S, Boilard N, Rochette A, et al. (2005) Distinct 3'-untranslated region elements regulate stage-specific mRNA accumulation and translation in *Leishmania*. Journal of Biological Chemistry 280: 35238-35246.

99. Zhang WW, Matlashewski G (1997) Loss of virulence in *Leishmania donovani* deficient in an amastigote-specific protein, A2. Proceedings of the National Academy of Sciences U S A 94: 8807-8811.
100. Azizi H, Hassani K, Taslimi Y, Najafabadi HS, Papadopoulou B, et al. (2009) Searching for virulence factors in the non-pathogenic parasite to humans *Leishmania tarentolae*. Parasitology 136: 723-735.
101. Mizbani A, Taslimi Y, Zahedifard F, Taheri T, Rafati S (2011) Effect of A2 gene on infectivity of the nonpathogenic parasite *Leishmania tarentolae*. Parasitology Research 109: 793-799.
102. Sharma P, Gurumurthy S, Duncan R, Nakhasi HL, Salotra P (2009) Comparative in vivo expression of amastigote up regulated *Leishmania* genes in three different forms of Leishmaniasis. Parasitol International 59: 262-264.
103. Anderson TJC, Day KP (2000) Geographical structure and sequence evolution as inferred from the *Plasmodium falciparum* S-antigen locus. Molecular and Biochemical Parasitology 106: 321-326.
104. Resende DM, Caetano BC, Dutra MS, Penido MLO, Abrantes CF, et al. (2008) Epitope mapping and protective immunity elicited by adenovirus expressing the *Leishmania* amastigote specific A2 antigen: Correlation with IFN-gamma and cytolytic activity by CD8+ T cells. Vaccine 26: 4585-4593.
105. 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: 59-66.
106. Ghosh A, Labrecque S, Matlashewski G (2001) Protection against *Leishmania donovani* infection by DNA vaccination: increased DNA vaccination efficiency through inhibiting the cellular p53 response. Vaccine 19: 3169-3178.
107. Zanin FHC, Coelho EAF, Tavares CAP, Marques-da-Silva EA, Costa MMS, et al. (2007) Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against *Leishmania chagasi* and *Leishmania amazonensis* experimental infections. Microbes and Infection 9: 1070-1077.
108. Mizbani A, Taheri T, Zahedifard F, Taslimi Y, Azizi H, et al. (2009) Recombinant *Leishmania tarentolae* expressing the A2 virulence gene as a novel candidate vaccine against visceral leishmaniasis. Vaccine 28: 53-62.
109. Fernandes AP, Costa MM, Coelho EA, Michalick MS, de Freitas E, et al. (2008) Protective immunity against challenge with *Leishmania (Leishmania) chagasi* in beagle dogs vaccinated with recombinant A2 protein. Vaccine 26: 5888-5895.
110. Fernandes AP, Coelho EA, Machado-Coelho GL, Grimaldi GJ, Gazzinelli RT (2012) Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives. Current Opinion in Microbiology 15:476-85.

111. Goto Y, Bhatia A, Raman VS, Liang H, Mohamath R, et al. (2011) KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. Clinical and Vaccine Immunology 18: 1118-1124.
112. Gomes R, Teixeira C, Oliveira F, Lawyer PG, Elnaiem DE, 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 Neglected Tropical Diseases 6: e1610.
113. Coelho EA, Tavares CA, Carvalho FA, Chaves KF, Teixeira KN, et al. (2003) Immune responses induced by the *Leishmania (Leishmania) donovani* A2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania (Leishmania) amazonensis* infection. Infection and Immunity 71: 3988-3994.
114. Saar Y, Ransford A, Waldman E, Mazareb S, Amin-Spector S, et al. (1998) Characterization of developmentally-regulated activities in axenic amastigotes of *Leishmania donovani*. Molecular and Biochemical Parasitology 95: 9-20.
115. Somanna A, Mundodi V, Gedamu L (2002) In vitro cultivation and characterization of *Leishmania chagasi* amastigote-like forms. Acta Tropica 83: 37-42.
116. Goyard S, Segawa H, Gordon J, Showalter M, Duncan R, et al. (2003) An in vitro system for developmental and genetic studies of *Leishmania donovani* phosphoglycans. Molecular and Biochemical Parasitology 130: 31-42.
117. Debrabant A, Joshi MB, Pimenta PF, Dwyer DM (2004) Generation of *Leishmania donovani* axenic amastigotes: their growth and biological characteristics. International Journal for Parasitology 34: 205-217.
118. Ghedin E, Charest H, Zhang WW, Debrabant A, Dwyer D, et al. (1998) Inducible expression of suicide genes in *Leishmania donovani* amastigotes. Journal of Biological Chemistry 273: 22997-23003.
119. Costa MM, Penido M, dos Santos MS, Doro D, de Freitas E, et al. (2012) Improved canine and human visceral leishmaniasis immunodiagnosis using combinations of synthetic peptides in enzyme-linked immunosorbent assay. PLoS Neglected Tropical Diseases 6: e1622.
120. Porrozzi R, Santos da Costa MV, Teva A, Falqueto A, Ferreira AL, et al. (2007) Comparative evaluation of enzyme-linked immunosorbent assays based on crude and recombinant leishmanial antigens for serodiagnosis of symptomatic and asymptomatic *Leishmania infantum* visceral infections in dogs. Clinical Vaccine Immunology 14: 544-548.
121. Carvalho FAA, Charest H, Tavares CAP, Matlashewski G, Valente EP, et al. (2002) Diagnosis of American visceral leishmaniasis in humans and dogs using the recombinant *Leishmania donovani* A2 antigen. Diagnostic Microbiology and Infectious Disease 43: 289-295.
122. Rogers MB, Hilley JD, Dickens NJ, Wilkes J, Bates PA, et al. (2011) Chromosome and gene copy number variation allow major structural change between species and strains of *Leishmania*. Genome Research 21: 2129-2142.

123. Peacock CS, Seeger K, Harris D, Murphy L, Ruiz JC, et al. (2007) Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. Nature Genetics 39: 839-847.
124. Zhang WW, Peacock CS, Matlashewski G (2008) A genomic-based approach combining in vivo selection in mice to identify a novel virulence gene in leishmania. PLoS Neglected Tropical Diseases 2: e248.
125. Zhang WW, Matlashewski G (2010) Screening *Leishmania donovani*-specific genes required for visceral infection. Molecular Microbiology 77: 505-517.
126. Zhang WW, Chan KF, Song ZW, Matlashewski G (2011) Expression of a *Leishmania donovani* nucleotide sugar transporter in *Leishmania major* enhances survival in visceral organs. Experimental Parasitology 129: 337-345.
127. Zhang WW, McCall LI, Matlashewski G The role of cytosolic glyceraldehyde-3-phosphate dehydrogenase in visceral organ infection by *Leishmania donovani*. Eukaryotic Cell, in press.
128. Wilson ME, Jeronimo SMB, Pearson RD (2005) Immunopathogenesis of infection with the visceralizing *Leishmania* species. Microbial Pathogenesis 38: 147-160.
129. Murray HW, Nathan CF (1999) Macrophage microbicidal mechanisms in vivo: Reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. Journal of Experimental Medicine 189: 741-746.
130. Avery SV (2011) Molecular targets of oxidative stress. Biochemical Journal 434: 201-210.
131. Mehlotra RK (1996) Antioxidant defense mechanisms in parasitic protozoa. Critical Reviews in Microbiology 22: 295-314.
132. Chan J, Fujiwara T, Brennan P, Mcneil M, Turco SJ, et al. (1989) Microbial glycolipids - Possible virulence factors that scavenge oxygen radicals. Proceedings of the National Academy of Sciences U S A 86: 2453-2457.
133. Van Assche T, Deschacht M, da Luz RAI, Maes L, Cos P (2011) *Leishmania*-macrophage interactions: Insights into the redox biology. Free Radical Biology and Medicine 51: 337-351.
134. Castro H, Tomas AM (2008) Peroxidases of trypanosomatids. Antioxidants & Redox Signaling 10: 1593-1606.
135. Olivier M, Gregory DJ, Forget G (2005) Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. Clinical Microbiology Reviews 18: 293-+.
136. Park HG, Han SI, Oh SY, Kang HS (2005) Cellular responses to mild heat stress. Cellular and Molecular Life Sciences 62: 10-23.
137. Richter K, Haslbeck M, Buchner J (2010) The heat shock response: Life on the verge of death. Molecular Cell 40: 253-266.
138. Salotra P, Ralhan R, Bhatnagar R (1994) Differential expression of stress proteins in virulent and attenuated promastigotes of *Leishmania-donovani*. Biochemistry and Molecular Biology International 33: 691-697.

139. Adhuna A, Saltora P, Bhatnagar R (2000) Nitric oxide induced expression of stress proteins in virulent and avirulent promastigotes of *Leishmania donovani*. Immunology Letters 71: 171-176.
140. Brochu C, Haimeur A, Ouellette M (2004) The heat shock protein HSP70 and heat shock cognate protein HSC70 contribute to antimony tolerance in the protozoan parasite *Leishmania*. Cell Stress & Chaperones 9: 294-303.
141. Vergnes B, Gourbal B, Girard I, Sundar S, Drummelsmith J, et al. (2007) A proteomics screen implicates HSP83 and a small kinetoplastid calpain-related protein in drug resistance in *Leishmania donovani* clinical field isolates by modulating drug-induced programmed cell death. Molecular & Cellular Proteomics 6: 88-101.
142. Hubel A, Krobitsch S, Horauf A, Clos J (1997) *Leishmania major* Hsp100 is required chiefly in the mammalian stage of the parasite. Molecular and Cellular Biology 17: 5987-5995.
143. Isnard A, Shio MT, Olivier M (2012) Impact of *Leishmania* metalloprotease GP63 on macrophage signaling. Frontiers in Cellular and Infection Microbiology 2: 72.
144. Matlashewski G (2001) *Leishmania* infection and virulence. Medical Microbiology and Immunology 190: 37-42.
145. Lincoln LM, Ozaki M, Donelson JE, Beetham JK (2004) Genetic complementation of *Leishmania* deficient in PSA (GP46) restores their resistance to lysis by complement. Molecular and Biochemical Parasitology 137: 185-189.
146. Kedzierski L, Montgomery J, Bullen D, Curtis J, Gardiner E, et al. (2004) A leucine-rich repeat motif of *Leishmania* parasite surface antigen 2 binds to macrophages through the complement receptor 3. Journal of Immunology 172: 4902-4906.
147. Lodge R, Descoteaux A (2005) Modulation of phagolysosome biogenesis by the lipophosphoglycan of *Leishmania*. Clinical Immunology 114: 256-265.
148. Ilg T (2000) Lipophosphoglycan is not required for infection of macrophages or mice by *Leishmania mexicana*. Embo Journal 19: 1953-1962.
149. Spath GF, Epstein L, Leader B, Singer SM, Avila HA, et al. (2000) Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite leishmania major. Proceedings of the National Academy of Sciences U S A 97: 9258-9263.
150. Gabriel C, McMaster WR, Girard D, Descoteaux A (2010) *Leishmania donovani* promastigotes evade the antimicrobial activity of neutrophil extracellular traps. Journal of Immunology 185: 4319-4327.
151. Vinet AF, Fukuda M, Turco SJ, Descoteaux A (2009) The *Leishmania donovani* lipophosphoglycan excludes the vesicular proton-ATPase from phagosomes by impairing the recruitment of synaptotagmin V. PLoS Pathogens 5: e1000628.
152. Lodge R, Diallo TO, Descoteaux A (2006) *Leishmania donovani* lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane. Cellular Microbiology 8: 1922-1931.

153. Denise H, McNeil K, Brooks DR, Alexander J, Coombs GH, et al. (2003) Expression of multiple CPB genes encoding cysteine proteases is required for *Leishmania mexicana* virulence in vivo. Infection and Immunity 71: 3190-3195.
154. Denise H, Poot J, Jimenez M, Ambit A, Herrmann DC, et al. (2006) Studies on the CPA cysteine peptidase in the *Leishmania infantum* genome strain JPCM5. Bmc Molecular Biology 7.
155. Mottram JC, Coombs GH, Alexander J (2004) Cysteine peptidases as virulence factors of *Leishmania*. Current Opinion in Microbiology 7: 375-381.
156. Karunaweera ND (2009) *Leishmania donovani* causing cutaneous leishmaniasis in Sri Lanka: a wolf in sheep's clothing? Trends in Parasitology 25: 458-463.
157. Naotunne TD, Rajakulendran S, Abeywickreme W, Kulasiri CD, Perera J, et al. (1990) Cutaneous leishmaniasis in Sri-Lanka - an imported disease linked to the Middle-East and African employment boom. Tropical and Geographical Medicine 42: 72-74.
158. Athukorale DN, Seneviratne JKK, Ihalamulla RL, Premaratne UN (1992) Locally acquired cutaneous leishmaniasis in Sri-Lanka. Journal of Tropical Medicine and Hygiene 95: 432-433.
159. Abeygunasekara PH, Costa YJ, Seneviratne N, Ratnatunga N, Wijesundera Mde S (2007) Locally acquired visceral leishmaniasis in Sri Lanka. Ceylon Medical Journal 52: 30-31.
160. Rajapaksa US, Ihalamulla RL, Karunaweera ND (2005) First report of mucosal tissue localisation of leishmaniasis in Sri Lanka. Ceylon Medical Journal 50: 90-91.
161. Karunaweera ND, Pratlong F, Siriwardane HVYD, Ihalamulla RL, Dedet JP (2003) Lankan cutaneous leishmaniasis is caused by *Leishmania donovani* zymodeme MON-37. Transactions of the Royal Society of Tropical Medicine and Hygiene 97: 380-381.
162. Siriwardana H, Noyes HA, Beeching NJ, Chance ML, Karunaweera ND, et al. (2007) *Leishmania donovani* and cutaneous leishmaniasis, Sri Lanka. Emerging Infectious Diseases 13: 476-478.
163. Yatawara L, Wickramasinghe S, Rajapakse RPVJ, Siyambalagoda RRMLR, Le TH, et al. (2008) Morphological and molecular studies on Sri Lankan *Leishmania*. Tropical medicine and health: 811190040.
164. Siriwardana HVYD, Noyes HA, Beeching NJ, Chance ML, Karunaweera ND, et al. (2007) *Leishmania donovani* and cutaneous leishmaniasis, Sri Lanka. Emerging Infectious Diseases 13: 476-478.
165. Alam MZ, Haralambous C, Kuhls K, Gouzelou E, Sgouras D, et al. (2009) The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. Microbes and Infection 11: 707-715.
166. Siriwardana HVYD, Thalagala N, Karunaweera ND (2010) Clinical and epidemiological studies on the cutaneous leishmaniasis caused by

Leishmania (Leishmania) donovani in Sri Lanka. Annals of Tropical Medicine and Parasitology 104: 213-223.
167. Ozbel Y, Sanjoba C, Alten B, Asada M, Depaquit J, et al. (2011) Distribution and ecological aspects of sand fly (Diptera: Psychodidae) species in Sri Lanka. Journal of Vector Ecology 36: S77-S86.
168. Ranasinghe S, Maingon RDC, Bray DP, Ward RD, Udagedara C, et al. (2012) A morphologically distinct *Phlebotomus argentipes* population from active cutaneous leishmaniasis foci in central Sri Lanka. Memorias Do Instituto Oswaldo Cruz 107: 402-+.
169. Lane RP, Pile MM, Amerasinghe FP (1990) Anthropophagy and aggregation behaviour of the sandfly *Phlebotomus argentipes* in Sri Lanka. Medical and Veterinary Entomology 4: 79-88.
170. Gajapathy K, Surendran SN (2012) Report of the presence of *Phlebotomus (Phlebotomus) salehi* Mesghali in Sri Lanka: a potential cutaneous leishmaniasis vector. Journal of the National Science Foundation of Sri Lanka 40: 169-173.
171. Siriwardana HVYD, Noyes HA, Beeching NJ, Wickremasinghe AR, Chance ML, et al. (2008) Clinical fatures of ctaneous lishmaniasis in Sri Lanka and mlecular ientification of *L. donovani* as the cause. International Journal of Infectious Diseases 12: E387-E387.
172. Nawaratna SSK, Weilgama DJ, Rajapaksha K (2009) Cutaneous leishmaniasis in Sri Lanka: a study of possible animal reservoirs. International Journal of Infectious Diseases 13: 513-517.
173. Rosypal AC, Tripp S, Kinlaw C, Hailemariam S, Tidwell RR, et al. (2010) Surveillance for antibodies to *Leishmania* spp. in dogs from Sri Lanka. Journal of Parasitology 96: 230-231.
174. Rajapaksa US, Ihalamulla RL, Udagedera C, Karunaweera ND (2007) Cutaneous leishmaniasis in southern Sri Lanka. Transactions of the Royal Society of Tropical Medicine and Hygiene 101: 799-803.
175. Nawaratna SSK, Weilgama DJ, Wijekoon CJ, Dissanayake M, Rajapaksha K (2007) Cutaneous leishmaniasis, Sri Lanka. Emerging Infectious Diseases 13: 1068-1070.
176. Samaranayake TN, Dissanayake VHW, Fernando SD (2008) Clinical manifestations of cutaneous leishmaniasis in Sri Lanka - possible evidence for genetic susceptibility among the Sinhalese. Annnals of Tropical Medicine and Parasitology 102: 383-390.
177. Samaranayake TN, Fernando SD, Dissanayake VHW (2010) Candidate gene study of susceptibility to cutaneous leishmaniasis in Sri Lanka. Tropical Medicine & International Health 15: 632-638.
178. Samaranayake TN, Fernando SD, Jayasekara RW, Dissanayake VHW (2008) Inherited susceptibility to cutaneous leishmaniasis in Sri Lanka. International Journal of Infectious Diseases 12: E386-E386.
179. Sharma NL, Mahajan VK, Kanga A, Sood A, Katoch VM, et al. (2005) Localized cutaneous leishmaniasis due to *Leishmania donovani* and *Leishmania tropica*: Preliminary findings of the study of 161 new cases

from a new endemic focus in Himachal Pradesh, India. American Journal of Tropical Medicine and Hygiene 72: 819-824.
180. Pulimood SA, Rupali P, Ajjampur SSR, Thomas M, Mehrotra S, et al. (2012) Atypical mucocutaneous involvement with *Leishmania donovani*. National Medical Journal of India 25: 148-150.
181. Svobodova M, Alten B, Zidkova L, Dvorak V, Hlavackova J, et al. (2009) Cutaneous leishmaniasis caused by *Leishmania infantum* transmitted by *Phlebotomus tobbi*. International Journal for Parasitology 39: 251-256.
182. Knio K, Baydoun E, Tawk R, Nuwayri-Salti N (2000) Isoenzyme characterization of *Leishmania* isolates from Lebanon and Syria. American Journal of Tropical Medicine and Hygiene 63: 43-47.
183. Azmi K, Schonian G, Nasereddin A, Schnur LF, Sawalha S, et al. (2012) Epidemiological and clinical features of cutaneous leishmaniases in Jenin District, Palestine, including characterisation of the causative agents in clinical samples. Transactions of the Royal Society of Tropical Medicine and Hygiene 106: 554-562.
184. Antoniou M, Haralambous C, Mazeris A, Pratlong F, Dedet JP, et al. (2008) *Leishmania donovani* leishmaniasis in Cyprus. Lancet Infectious Diseases 8: 6-7.
185. Mebrahtu YB, Vaneys G, Guizani I, Lawyer PG, Pamba H, et al. (1993) Human cutaneous leishmaniasis caused by *Leishmania-donovani* Sl in Kenya. Transactions of the Royal Society of Tropical Medicine and Hygiene 87: 598-601.
186. Pratlong F, Bastien P, Perello R, Lami P, Dedet JP (1995) Human cutaneous leishmaniasis caused by *Leishmania-donovani* sensu-stricto in Yemen. Transactions of the Royal Society of Tropical Medicine and Hygiene 89: 398-399.
187. Elamin EM, Guizani I, Guerbouj S, Gramiccia M, El Hassan AM, et al. (2008) Identification of *Leishmania donovani* as a cause of cutaneous leishmaniasis in Sudan. Transactions of the Royal Society of Tropical Medicine and Hygiene 102: 54-57.
188. Ponce C, Ponce E, Morrison A, Cruz A, Kreutzer R, et al. (1991) *Leishmania-donovani-chagasi* - New clinical variant of cutaneous leishmaniasis in Honduras. Lancet 337: 67-70.
189. De Lima H, Rodriguez N, Feliciangeli MD, Barrios MA, Sosa A, et al. (2009) Cutaneous leishmaniasis due to *Leishmania chagasi/Le. infantum* in an endemic area of Guarico State, Venezuela. Transactions of the Royal Society of Tropical Medicine and Hygiene 103: 721-726.
190. Noyes H, Chance M, Ponce C, Ponce E, Maingon R (1997) *Leishmania chagasi*: Genotypically similar parasites from Honduras cause both visceral and cutaneous leishmaniasis in humans. Experimental Parasitology 85: 264-273.
191. Zeledon R, Hidalgo H, Viquez A, Urbina A (1989) Atypical cutaneous leishmaniasis in a semiarid region of North-West Costa-Rica. Transactions of the Royal Society of Tropical Medicine and Hygiene 83: 786-786.

192. Murray HW, Berman JD, Davies CR, Saravia NG (2005) Advances in leishmaniasis. Lancet 366: 1561-1577.
193. Gouzelou E, Haralambous C, Amro A, Mentis A, Pratlong F, et al. (2012) Multilocus microsatellite typing (MLMT) of strains from Turkey and Cyprus reveals a novel monophyletic *L. donovani* sensu lato group. PLoS Neglected Tropical Diseases 6: e1507.
194. Kuhls K, Chicharro C, Canavate C, Cortes S, Campino L, et al. (2008) Differentiation and gene flow among European populations of *Leishmania infantum* MON-1. PLoS Neglected Tropical Diseases 2: e261.
195. Pratlong F, Rioux JA, Marty P, Faraut-Gambarelli F, Dereure J, et al. (2004) Isoenzymatic analysis of 712 strains of *Leishmania infantum* in the south of France and relationship of enzymatic polymorphism to clinical and epidemiological features. Journal of Clinical Microbiology 42: 4077-4082.
196. Khanra S, Bandopadhyay SK, Chakraborty P, Datta S, Mondal D, et al. (2011) Characterization of the recent clinical isolates of Indian Kala-azar patients by RAPD-PCR method. Journal of Parasitic Diseases 35: 116-122.
197. Alborzi A, Rasouli M, Shamsizadeh A (2006) *Leishmania tropica* - Isolated patient with visceral leishmaniasis in Southern Iran. American Journal of Tropical Medicine and Hygiene 74: 306-307.
198. Alborzi A, Pouladfar GR, Fakhar M, Motazedian MH, Hatam GR, et al. (2008) Case report: Isolation of *Leishmania tropica* from a patient with visceral leishmaniasis and disseminated cutaneous leishmaniasis, southern Iran. American Journal of Tropical Medicine and Hygiene 79: 435-437.
199. Mebrahtu Y, Lawyer P, Githure J, Were JB, Muigai R, et al. (1989) Visceral leishmaniasis unresponsive to pentostam caused by *Leishmania tropica* in Kenya. American Journal of Tropical Medicine and Hygiene 41: 289-294.
200. GuessousIdrissi N, Berrag B, Riyad M, Sahibi H, Bichichi M, et al. (1997) Short report: *Leishmania tropica*: Etiologic agent of a case of canine visceral leishmaniasis in northern Morocco. American Journal of Tropical Medicine and Hygiene 57: 172-173.
201. Lemrani M, Nejjar R, Pratlong F (2002) A new *Leishmania tropica* zymodeme - causative agent of canine visceral leishmaniasis in northern Morocco. Annals of Tropical Medicine and Parasitology 96: 637-638.
202. Hajjaran H, Mohebali M, Zarei Z, Edrissian GH (2007) *Leishmania tropica*: Another etiological agent of canine visceral leishmaniasis in Iran. Iranian Journal of Public Health 36: 85-88.
203. CDC (1992) Viscerotropic leishmaniasis in persons returning from Operation Desert Storm--1990-1991. MMWR Morbidity and Mortality Weekly Report 41: 131-134.
204. Magill AJ, Grogl M, Johnson SC, Gasser RA (1994) Visceral infection due to *Leishmania tropica* in a veteran of operation desert storm who presented 2 years after leaving Saudi-Arabia. Clinical Infectious Diseases 19: 805-806.

205. Magill AJ, Grogl M, Gasser RA, Sun W, Oster CN (1993) Visceral infection caused by *Leishmania tropica* in veterans of operation desert storm. New England Journal of Medicine 328: 1383-1387.
206. Schwenkenbecher JM, Wirth T, Schnur LF, Jaffe CL, Schallig H, et al. (2006) Microsatellite analysis reveals genetic structure of *Leishmania tropica*. International Journal for Parasitology 36: 237-246.
207. Barral A, Badaro R, Barralnetto M, Grimaldi G, Momem H, et al. (1986) Isolation of *Leishmania-mexicana-amazonensis* from the bone-marrow in a case of American visceral leishmaniasis. American Journal of Tropical Medicine and Hygiene 35: 732-734.
208. Barral A, Pedralsampaio D, Grimaldi G, Momen H, Mcmahonpratt D, et al. (1991) Leishmaniasis in Bahia, Brazil - Evidence that *Leishmania-amazonensis* produces a wide spectrum of clinical-disease. American Journal of Tropical Medicine and Hygiene 44: 536-546.
209. Aleixo JA, Nascimento ET, Monteiro GR, Fernandes MZ, Ramos AMO, et al. (2006) Atypical American visceral leishmaniasis caused by disseminated *Leishmania amazonensis* infection presenting with hepatitis and adenopathy. Transactions of the Royal Society of Tropical Medicine and Hygiene 100: 79-82.
210. Rodriguez-Bonfante C, Bonfante-Garrido R, Grimaldi G, Jr., Momen H, Cupolillo E (2003) Genotypically distinct *Leishmania colombiensis* isolates from Venezuela cause both cutaneous and visceral leishmaniasis in humans. Infection, Genetics and Evolution 3: 119-124.
211. Schonian G, Mauricio I, Cupolillo E (2010) Is it time to revise the nomenclature of *Leishmania*? Trends in Parasitology 26: 466-469.
212. Mestra L, Lopez L, Robledo SM, Muskus CE, Nicholls RS, et al. (2011) Transfusion-transmitted visceral leishmaniasis caused by *Leishmania (Leishmania) mexicana* in an immunocompromised patient: a case report. Transfusion 51: 1919-1923.
213. Almeida RP, BarralNetto M, DeJesus AMR, DeFreitas LAR, Carvalho EM, et al. (1996) Biological behavior of *Leishmania amazonensis* isolated from humans with cutaneous, mucosal, or visceral leishmaniasis in BALB/c mice. American Journal of Tropical Medicine and Hygiene 54: 178-184.
214. Chusri S, Hortiwakul T, Silpapojakul K, Siriyasatien P (2012) Case report: Consecutive cutaneous and visceral leishmaniasis manifestations involving a novel *Leishmania* species in two HIV patients in Thailand. American Journal of Tropical Medicine and Hygiene 87: 76-80.
215. Suankratay C, Suwanpimolkul G, Wilde H, Siriyasatien P (2010) Autochthonous visceral leishmaniasis in a human immunodeficiency virus (HIV)-infected patient: the first in thailand and review of the literature. The American journal of tropical medicine and hygiene 82: 4-8.
216. Bualert L, Charungkiattikul W, Thongsuksai P, Mungthin M, Siripattanapipong S, et al. (2012) Autochthonous disseminated dermal and visceral leishmaniasis in an AIDS patient, southern Thailand, caused by *Leishmania siamensis*. The American journal of tropical medicine and hygiene 86: 821-824.

217. Sukmee T, Siripattanapipong S, Mungthin M, Worapong J, Rangsin R, et al. (2008) A suspected new species of *Leishmania*, the causative agent of visceral leishmaniasis in a Thai patient. International Journal for Parasitology 38: 617-622.
218. Reuss SM, Dunbar MD, Mays MB, Owen JL, Mallicote MF, et al. (2012) Autochthonous *Leishmania siamensis* in Horse, Florida, USA. Emerging Infectious Diseases 18: 1545-1547.
219. Muller N, Welle M, Lobsiger L, Stoffel MH, Boghenbor KK, et al. (2009) Occurrence of *Leishmania* sp. in cutaneous lesions of horses in Central Europe. Veterinary Parasitology 166: 346-351.
220. Chargui N, Amro A, Haouas N, Schonian G, Babba H, et al. (2009) Population structure of Tunisian *Leishmania infantum* and evidence for the existence of hybrids and gene flow between genetically different populations. International Journal for Parasitology 39: 801-811.
221. Gelanew T, Kuhls K, Hurissa Z, Weldegebreal T, Hailu W, et al. (2010) Inference of population structure of *Leishmania donovani* strains isolated from different Ethiopian visceral leishmaniasis endemic areas. PLoS Neglected Tropical Diseases 4: e889.
222. Kelly JM, Law JM, Chapman CJ, Van Eys GJ, Evans DA (1991) Evidence of genetic recombination in *Leishmania*. Molecular and Biochemical Parasitology 46: 253-263.
223. Dujardin JC, Banuls AL, Llanoscuentas A, Alvarez E, Dedoncker S, et al. (1995) Putative *Leishmania* hybrids in the Eastern Andean valley of Huanuco, Peru. Acta Tropica 59: 293-307.
224. Nolder D, Roncal N, Davies CR, Llanos-Cuentas A, Miles MA (2007) Multiple hybrid genotypes of *Leishmania (Viannia)* in a focus of mucocutaneous leishmaniasis. American Journal of Tropical Medicine and Hygiene 76: 573-578.
225. Belli AA, Miles MA, Kelly JM (1994) A putative *Leishmania-panamensis Leishmania-braziliensis* hybrid is a causative agent of human cutaneous leishmaniasis in Nicaragua. Parasitology 109: 435-442.
226. Banuls AL, Guerrini F, LePont F, Barrera C, Espinel I, et al. (1997) Evidence for hybridization by multilocus enzyme electrophoresis and random amplified polymorphic DNA between *Leishmania braziliensis* and *Leishmania panamensis/guyanensis* in Ecuador. Journal of Eukaryotic Microbiology 44: 408-411.
227. Delgado O, Cupolillo E, BonfanteGarrido R, Silva S, Belfort E, et al. (1997) Cutaneous leishmaniasis in Venezuela caused by infection with a new hybrid between *Leishmania (Viannia) braziliensis* and *L. (V.) guyanensis*. Memorias Do Instituto Oswaldo Cruz 92: 581-582.
228. Odiwuor S, De Doncker S, Maes I, Dujardin JC, Van der Auwera G (2011) Natural *Leishmania donovani/Leishmania aethiopica* hybrids identified from Ethiopia. Infection Genetics and Evolution 11: 2113-2118.
229. Ravel C, Cortes S, Pratlong F, Morio F, Dedet JP, et al. (2006) First report of genetic hybrids between two very divergent *Leishmania* species:

Leishmania infantum and *Leishmania major*. International Journal for Parasitology 36: 1383-1388.
230. Sadlova J, Yeo M, Seblova V, Lewis MD, Mauricio I, et al. (2011) Visualisation of *Leishmania donovani* fluorescent hybrids during early stage development in the sand fly vector. PLoS One 6: e19851.
231. Akopyants NS, Kimblin N, Secundino N, Patrick R, Peters N, et al. (2009) Demonstration of genetic exchange during cyclical development of *Leishmania* in the sand fly vector. Science 324: 265-268.
232. Cortes S, Esteves C, Mauricio I, Maia C, Cristovao JM, et al. (2012) In vitro and in vivo behaviour of sympatric *Leishmania (V.) braziliensis*, *L. (V.) peruviana* and their hybrids. Parasitology 139: 191-199.
233. Sreenivas G, Raju BVS, Singh R, Selvapandiyan A, Duncan R, et al. (2004) DNA polymorphism assay distinguishes isolates of *Leishmania donovani* that cause kala-azar from those that cause post-kala-azar dermal leishmaniasis in humans. Journal of Clinical Microbiology 42: 1739-1741.
234. Salotra P, Duncan RC, Singh R, Raju BVS, Sreenivas G, et al. (2006) Upregulation of surface proteins in *Leishmania donovani* isolated from patients of post kala-azar dermal leishmaniasis. Microbes and Infection 8: 637-644.
235. Singh S, Sharma U, Mishra J Post-kala-azar dermal leishmaniasis: recent developments. International Journal of Dermatology 50: 1099-1108.
236. Gasim S, Elhassan AM, Khalil EAG, Ismail A, Kadaru AMY, et al. (1998) High levels of plasma IL-10 and expression of IL-10 by keratinocytes during visceral leishmaniasis predict subsequent development of post-kala-azar dermal leishmaniasis. Clinical and Experimental Immunology 111: 64-69.
237. Katara GK, Ansari NA, Verma S, Ramesh V, Salotra P (2011) Foxp3 and IL-10 expression correlates with parasite burden in lesional tissues of post kala azar dermal leishmaniasis (PKDL) patients. PLoS Neglected Tropical Diseases 5: e1171.
238. Saha S, Mondal S, Ravindran R, Bhowmick S, Modak D, et al. (2007) IL-10- and TGF-beta-mediated susceptibility in kala-azar and post-kala-azar dermal leishmaniasis: the significance of amphotericin B in the control of *Leishmania donovani* infection in India. Journal of Immunology 179: 5592-5603.
239. Ganguly S, Das NK, Panja M, Pal S, Modak D, et al. (2008) Increased levels of interleukin-10 and IgG3 are hallmarks of Indian post-kala-azar dermal leishmaniasis. Journal of Infectious Diseases 197: 1762-1771.
240. Ansari NA, Ramesh V, Salotra P (2006) Interferon (IFN)-gamma , tumor necrosis factor-alpha , interleukin-6, and IFN-gamma receptor 1 are the major immunological determinants associated with post-kala azar dermal leishmaniasis. Journal of Infectious Diseases 194: 958-965.
241. Ansari NA, Katara GK, Ramesh V, Salotra P (2008) Evidence for involvement of TNFR1 and TIMPs in pathogenesis of post-kala-azar dermal leishmaniasis. Clinical and Experimental Immunology 154: 391-398.

242. Salih MA, Ibrahim ME, Blackwell JM, Miller EN, Khalil EAG, et al. (2007) IFNG and IFNGR1 gene polymorphisms and susceptibility to post-kala-azar dermal leishmaniasis in Sudan. Genes and Immunity 8: 75-78.
243. Stanley AC, Engwerda CR (2007) Balancing immunity and pathology in visceral leishmaniasis. Immunology and Cell Biology 85: 138-147.
244. Singh OP, Gidwani K, Kumar R, Nylen S, Jones SL, et al. (2012) Reassessment of immune correlates in human visceral leishmaniasis as defined by cytokine release in whole blood. Clinical and Vaccine Immunology 19: 961-966.
245. Verma S, Kumar R, Katara GK, Singh LC, Negi NS, et al. (2010) Quantification of parasite load in clinical samples of leishmaniasis patients: IL-10 level correlates with parasite load in visceral leishmaniasis. PLoS One 5: e10107.
246. Nylen S, Sacks D (2007) Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends in Immunology 28: 378-384.
247. Coterell SEJ, Engwerda CR, Kaye PM (2000) Enhanced hematopoietic activity accompanies parasite expansion in the spleen and bone marrow of mice infected with *Leishmania donovani*. Infection and Immunity 68: 1840-1848.
248. Engwerda CR, Kaye PM (2000) Organ-specific immune responses associated with infectious disease. Immunology Today 21: 73-78.
249. Badaro R, Jones TC, Lorenco R, Cerf BJ, Sampaio D, et al. (1986) A prospective-study of visceral leishmaniasis in an endemic area of Brazil. Journal of Infectious Diseases 154: 639-649.
250. Jeronimo SMB, Duggal P, Ettinger NA, Nascimento ET, Monteiro GR, et al. (2007) Genetic predisposition to self-curing infection with the protozoan *Leishmania chagasi*: A genomewide scan. Journal of Infectious Diseases 196: 1261-1269.
251. Jamieson SE, Miller EN, Peacock CS, Fakiola M, Wilson ME, et al. (2007) Genome-wide scan for visceral leishmaniasis susceptibility genes in Brazil. Genes and Immunity 8: 84-90.
252. Sakthianandeswaren A, Foote SJ, Handman E (2009) The role of host genetics in leishmaniasis. Trends in Parasitology 25: 383-391.
253. Blackwell JM, Fakiola M, Ibrahim ME, Jamieson SE, Jeronimo SB, et al. (2009) Genetics and visceral leishmaniasis: of mice and man. Parasite Immunology 31: 254-266.
254. Lipoldova M, Demant P (2006) Genetic susceptibility to infectious disease: lessons from mouse models of leishmaniasis. Nature Reviews Genetics 7: 294-305.
255. Bucheton B, Abel L, Kheir MM, Mirgani A, El-Safi SH, et al. (2003) Genetic control of visceral leishmaniasis in a Sudanese population: candidate gene testing indicates a linkage to the NRAMP1 region. Genes and Immunity 4: 104-109.
256. Karplus TM, Jeronimo SMB, Chang H, Helms BK, Burns TL, et al. (2002) Association between the tumor necrosis factor locus and the clinical

outcome of *Leishmania chagasi* infection. Infection and Immunity 70: 6919-6925.
257. Mohamed HS, Ibrahim ME, Miller EN, Peacock CS, Khalil EAG, et al. (2003) Genetic susceptibility to visceral leishmaniasis in the Sudan: linkage and association with IL4 and IFNGR1. Genes and Immunity 4: 351-355.
258. Frade AF, de Oliveira LC, Costa DL, Costa CHN, Aquino D, et al. (2011) TGFB1 and IL8 gene polymorphisms and susceptibility to visceral leishmaniasis. Infection Genetics and Evolution 11: 912-916.
259. Bucheton B, Argiro L, Chevillard C, Marquet S, Kheir MM, et al. (2007) Identification of a novel G245R polymorphism in the IL-2 receptor beta membrane proximal domain associated with human visceral leishmaniasis. Genes and Immunity 8: 79-83.
260. Mehrotra S, Fakiola M, Oommen J, Jamieson SE, Mishra A, et al. (2011) Genetic and functional evaluation of the role of CXCR1 and CXCR2 in susceptibility to visceral leishmaniasis in north-east India. Bmc Medical Genetics 12.
261. Alonso DP, Ferreira AFB, Ribolla PEM, Santos IKFDM, Cruz MDSPE, et al. (2007) Genotypes of the mannan-binding lectin gene and susceptibility to visceral leishmaniasis and clinical complications. Journal of Infectious Diseases 195: 1212-1217.
262. Mehrotra S, Fakiola M, Mishra A, Sudarshan M, Tiwary P, et al. (2012) Genetic and functional evaluation of the role of DLL1 in susceptibility to visceral leishmaniasis in India. Infection Genetics and Evolution 12: 1195-1201.
263. Dye C, Williams BG (1993) Malnutrition, age and the risk of parasitic disease: visceral leishmaniasis revisited. Proceedings Biological Sciences 254: 33-39.
264. Maciel BLL, Lacerda HG, Queiroz JW, Galvao J, Pontes NN, et al. (2008) Association of nutritional status with the response to infection with *Leishmania chagasi*. American Journal of Tropical Medicine and Hygiene 79: 591-598.
265. Malafaia G (2009) Protein-energy malnutrition as a risk factor for visceral leishmaniasis: a review. Parasite Immunology 31: 587-596.
266. Bern C, Courtenay O, Alvar J (2010) Of cattle, sand flies and men: A systematic review of risk factor analyses for South Asian visceral leishmaniasis and implications for elimination. PLoS Neglected Tropical Diseases 4: e599.
267. Desjeux P, Alvar J (2003) *Leishmania*/HIV co-infections: epidemiology in Europe. Annals of Tropical Medicine and Parasitology 97 Suppl 1: 3-15.
268. Santos-Oliveira JR, Da-Cruz AM, Pires LHS, Cupolillo E, Kuhls K, et al. (2011) Case report: Atypical lesions as a sign of cutaneous dissemination of visceral leishmaniasis in a human immunodeficiency virus-positive patient simultaneously infected by two viscerotropic *Leishmania* species. American Journal of Tropical Medicine and Hygiene 85: 55-59.

269. Rosenthal E, Marty P, del Giudice P, Pradier C, Ceppi C, et al. (2000) HIV and *Leishmania* coinfection: A review of 91 cases with focus on atypical locations of *Leishmania*. Clinical Infectious Diseases 31: 1093-1095.
270. Gramiccia M (2003) The identification and variability of the parasites causing leishmaniasis in HIV-positive patients in Italy. Annals of Tropical Medcine and Parasitology 97: 65-73.
271. Hassan MF, Zhang YB, Engwerda CR, Kaye PM, Sharp H, et al. (2006) The *Schistosoma mansoni* hepatic egg granuloma provides a favorable microenvironment for sustained growth of *Leishmania donovani*. American Journal of Pathology 169: 943-953.
272. Marques CS, Rolao N, Centeno-Lima S, Lousada H, Maia C, et al. (2005) Studies in a co-infection murine model of *Plasmodium chabaudi chabaudi* and *Leishmania infantum*: interferon-gamma and interleukin-4 mRNA expression. Memorias Do Instituto Oswaldo Cruz 100: 889-892.
273. Christodoulou V, Messaritakis I, Svirinaki E, Tsatsanis C, Antoniou M (2011) *Leishmania infantum* and *Toxoplasma gondii*: Mixed infection of macrophages in vitro and in vivo. Experimental Parasitology 128: 279-284.
274. Rousseau D, LeFichoux Y, Stien X, Suffia I, Ferrua B, et al. (1997) Progression of visceral leishmaniasis due to *Leishmania infantum* in BALB/c mice is markedly slowed by prior infection with *Trichinella spiralis.* Infection and Immunity 65: 4978-4983.
275. Murthy PK, Dixit S, Gaur RL, Kumar R, Sahoo MK, et al. (2008) Influence of *Brugia malayi* life stages and BmAFII fraction on experimental *Leishmania donovani* infection in hamsters. Acta Tropica 106: 81-89.
276. Kamhawi S (2000) The biological and immunomodulatory properties of sand fly saliva and its role in the establishment of *Leishmania* infections. Microbes and Infection 2: 1765-1773.
277. Menezes MJ, Costa DJ, Clarencio J, Miranda JC, Barral A, et al. (2008) Immunomodulation of human monocytes following exposure to *Lutzomyia intermedia* saliva. BMC Immunology 9: 12.
278. Rogers KA, Titus RG (2003) Immunomodulatory effects of Maxadilan and *Phlebotomus papatasi* sand fly salivary gland lysates on human primary in vitro immune responses. Parasite Immunology 25: 127-134.
279. Brodie TM, Smith MC, Morris RV, Titus RG (2007) Immunomodulatory effects of the *Lutzomyia longipalpis* salivary gland protein maxadilan on mouse macrophages. Infection and Immunity 75: 2359-2365.
280. Araujo-Santos T, Prates DB, Andrade BB, Nascimento DO, Clarencio J, et al. (2010) *Lutzomyia longipalpis* saliva triggers lipid body formation and prostaglandin E-2 production in murine macrophages. PLoS Neglected Tropical Diseases 4: e873.
281. Costa DJ, Favali C, Clarencio J, Afonso L, Conceicao V, et al. (2004) *Lutzomyia longipalpis* salivary gland homogenate impairs cytokine production and costimulatory molecule expression on human monocytes and dendritic cells. Infection and Immunity 72: 1298-1305.

282. Titus RG, Ribeiro JMC (1988) Salivary-gland lysates from the sand fly *Lutzomyia-longipalpis* enhance *Leishmania* infectivity. Science 239: 1306-1308.
283. Vinhas V, Andrade BB, Paes F, Bomura A, Clarencio J, et al. (2007) Human anti-saliva immune response following experimental exposure to the visceral leishmaniasis vector, *Lutzomyia longipalpis*. European Journal of Immunology 37: 3111-3121.
284. Vlkova M, Rohousova I, Drahota J, Stanneck D, Kruedewagen EM, et al. (2011) Canine antibody response to *Phlebotomus perniciosus* bites negatively correlates with the risk of *Leishmania infantum* transmission. PLoS Neglected Tropical Diseases 5: e1344.
285. Gomes RB, Brodskyn U, de Oliveira CI, Costa J, Miranda JC, et al. (2002) Seroconversion against *Lutzomyia longipalpis* saliva concurrent with the development of anti-*Leishmania chagasi* delayed-type hypersensitivity. Journal of Infectious Diseases 186: 1530-1534.
286. Aquino DMC, Caldas AJM, Miranda JC, Silva AAM, Barral-Netto M, et al. (2010) Short report: Epidemiological study of the association between anti-*Lutzomyia longipalpis* saliva antibodies and development of delayed-type hypersensitivity to *Leishmania* antigen. American Journal of Tropical Medicine and Hygiene 83: 825-827.
287. Rohousova I, Hostomska J, Vlkova M, Kobets T, Lipoldova M, et al. (2011) The protective effect against *Leishmania* infection conferred by sand fly bites is limited to short-term exposure. International Journal for Parasitology 41: 481-485.
288. Gomes R, Oliveira F (2012) The immune response to sand fly salivary proteins and its influence on *Leishmania* immunity. Frontiers in Immunology 3: 110.
289. Selvapandiyan A, Dey R, Gannavaram S, Lakhal-Naouar I, Duncan R, et al. (2012) Immunity to visceral leishmaniasis using genetically defined live-attenuated parasites. Journal of Tropical Medicine 2012: 631460.
290. Ferrua B, Luci C, Le Fichoux Y, Paul A, Marty P (2006) Imprinting of BALB/c mice with low *Leishmania infantum* parasite dose markedly protects spleen against high-dose challenge. Vaccine 24: 589-596.
291. Silvestre R, Cordeiro-Da-Silva A, Santarem N, Vergnes B, Sereno D, et al. (2007) SIR2-Deficient *Leishmania infantam* induces a defined IFN-gamma/IL-10 pattern that correlates with protection. Journal of Immunology 179: 3161-3170.
292. Bruhn KW, Birnbaum R, Haskell J, Vanchinathan V, Greger S, et al. (2012) Killed but metabolically active *Leishmania infantum* as a novel whole-cell vaccine for visceral leishmaniasis. Clinical and Vaccine Immunology 19: 490-498.
293. Bhowmick S, Ali N (2009) Identification of novel *Leishmania donovani* antigens that help define correlates of vaccine-mediated protection in visceral leishmaniasis. PLoS One 4: e5820.

294. Stager S, Alexander J, Kirby AC, Botto M, Rooijen NV, et al. (2003) Natural antibodies and complement are endogenous adjuvants for vaccine-induced CD8+ T-cell responses. Nature Medicine 9: 1287-1292.
295. Melby PC, Yang J, Zhao W, Perez LE, Cheng J (2001) *Leishmania donovani* p36(LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. Infection and Immunity 69: 4719-4725.
296. Tripathi P, Singh V, Naik S (2007) Immune response to *Leishmania*: paradox rather than paradigm. FEMS Immunology and Medical Microbiology 51: 229-242.
297. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, et al. (2007) Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. Nature Medicine 13: 843-850.
298. Coler RN, Goto Y, Bogatzki L, Raman V, Reed SG (2007) Leish-111f, a recombinant polyprotein vaccine that protects against visceral leishmaniasis by elicitation of CD4(+) T cells. Infection and Immunity 75: 4648-4654.
299. Selvapandiyan A, Dey R, Nylen S, Duncan R, Sacks D, et al. (2009) Intracellular replication-deficient *Leishmania donovani* induces long lasting protective immunity against visceral leishmaniasis. Journal of Immunology 183: 1813-1820.
300. Stager S, Rafati S (2012) CD8(+) T cells in *Leishmania* infections: friends or foes? Frontiers in Immunology 3: 5.
301. Mary C, Auriault V, Faugere B, Dessein AJ (1999) Control of *Leishmania infantum* infection is associated with CD8(+) and gamma interferon- and interleukin-5-producing CD4(+) antigen-specific T cells. Infection and Immunity 67: 5559-5566.
302. Gurunathan S, Klinman DM, Seder RA (2000) DNA vaccines: Immunology, application, and optimization. Annual Review of Immunology 18: 927-974.
303. Mohebali M, Khamesipour A, Mobedi I, Zarei Z, Hashemi-Fesharki R (2004) Double-blind randomized efficacy field trial of alum precipitated autoclaved *Leishmania major* vaccine mixed with BCG against canine visceral leishmaniasis in Meshkin-Shahr district, I.R. Iran. Vaccine 22: 4097-4100.
304. Dube A, Sharma P, Srivastava JK, Misra A, Naik S, et al. (1998) Vaccination of langur monkeys (Presbytis entellus) against *Leishmania donovani* with autoclaved *L. major* plus BCG. Parasitology 116: 219-221.
305. Misra A, Dube A, Srivastava B, Sharma P, Srivastava JK, et al. (2001) Successful vaccination against *Leishmania donovani* infection in Indian langur using alum-precipitated autoclaved *Leishmania major* with BCG. Vaccine 19: 3485-3492.
306. Gicheru MM, Olobo JO, Anjili CO (1997) Heterologous protection by *Leishmania donovani* for *Leishmania major* infections in the vervet monkey model of the disease. Experimental Parasitology 85: 109-116.

307. Kumari S, Samant M, Khare P, Misra P, Dutta S, et al. (2009) Photodynamic vaccination of hamsters with inducible suicidal mutants of *Leishmania amazonensis* elicits immunity against visceral leishmaniasis. European Journal of Immunology 39: 178-191.
308. Srivastava JK, Misra A, Sharma P, Srivastava B, Naik S, et al. (2003) Prophylactic potential of autoclaved *Leishmania donovani* with BCG against experimental visceral leishmaniasis. Parasitology 127: 107-114.
309. Nation CS, Dondji B, Stryker GA (2012) Previous exposure to a low infectious dose of *Leishmania major* exacerbates infection with *Leishmania infantum* in the susceptible BALB/c mouse. Parasitology Research.
310. Streit JA, Recker TJ, Gueiros F, Beverley SM, Wilson ME (2001) Protective immunity against the protozoan *Leishmania chagasi* is induced by subclinical cutaneous infection with virulent but not avirulent organisms. Journal of Immunology 166: 1921-1929.
311. Khalil EAG, El Hassan AM, Zijlstra EE, Mukhtar MM, Ghalib HW, et al. (2000) Autoclaved *Leishmania major* vaccine for prevention of visceral leishmaniasis: a randomised, double-blind, BCG-controlled trial in Sudan. Lancet 356: 1565-1569.
312. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL (2002) CD4(+)CD25(+) regulatory T cells control *Leishmania major* persistence and immunity. Nature 420: 502-507.
313. Uzonna JE, Wei GJ, Yurkowski D, Bretscher P (2001) Immune elimination of *Leishmania major* in mice: Implications for immune memory, vaccination, and reactivation disease. Journal of Immunology 167: 6967-6974.
314. Okwor I, Kuriakose S, Uzonna J (2010) Repeated inoculation of killed *Leishmania major* induces durable immune response that protects mice against virulent challenge. Vaccine 28: 5451-5457.
315. Joshi T, Rodriguez S, Perovic V, Cockburn IA, Stager S (2009) B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against *Leishmania donovani* infections. PLoS Pathogens 5: e1000431.
316. Alvar J, Aparicio P, Aseffa A, Den Boer M, Canavate C, et al. (2008) The relationship between leishmaniasis and AIDS: the second 10 years. Clinical Microbiology Reviews 21: 334-+.
317. Menon JN, Bretscher PA (1996) Characterization of the immunological memory state generated in mice susceptible to *Leishmania major* following exposure to low doses of *L. major* and resulting in resistance to a normally pathogenic challenge. European Journal of Immunology 26: 243-249.
318. Breton M, Tremblay MJ, Ouellette M, Papadopoulou B (2005) Live nonpathogenic parasitic vector as a candidate vaccine against visceral leishmaniasis. Infection and Immunity 73: 6372-6382.
319. Bhowmick S, Mazumdar T, Ali N (2009) Vaccination route that induces transforming growth factor beta production fails to elicit protective

immunity against *Leishmania donovani* infection. Infection and Immunity 77: 1514-1523.
320. Kaur S, Kaur T, Garg N, Mukherjee S, Raina P, 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. Parasitology Research 103: 1413-1419.
321. Rousseau D, Demartino S, Anjuere F, Ferrua B, Fragaki K, et al. (2001) Sustained parasite burden in the spleen of *Leishmania infantum*-infected BALB/c mice is accompanied by expression of MCP-1 transcripts and lack of protection against challenge. European Cytokine Network 12: 340-347.
322. Bhowmick S, Ali N (2008) Recent developments in leishmaniasis vaccine delivery systems. Expert Opinion on Drug Delivery 5: 789-803.
323. Evans KJ, Kedzierski L (2012) Development of Vaccines against Visceral Leishmaniasis. Journal of Tropical Medicine 2012: 892817.
324. Raman VS, Duthie MS, Fox CB, Matlashewski G, Reed SG (2012) Adjuvants for *Leishmania* vaccines: from models to clinical application. Frontiers in immunology 3: 144.
325. Noazin S, Modabber F, Khamesipour A, Smith PG, Moulton LH, et al. (2008) First generation leishmaniasis vaccines: A review of field efficacy trials. Vaccine 26: 6759-6767.
326. Noazin S, Khamesipour A, Moulton LH, Tanner M, Nasseri K, et al. (2009) Efficacy of killed whole-parasite vaccines in the prevention of leishmaniasis-A meta-analysis. Vaccine 27: 4747-4753.
327. Zijlstra EE, Elhassan AM, Ismael A, Ghalib HW (1994) Endemic kala-azar in Eastern Sudan - a longitudinal-study on the incidence of clinical and subclinical infection and post-kala-azar dermal leishmaniasis. American Journal of Tropical Medicine and Hygiene 51: 826-836.
328. Nagill R, Kaur S (2011) Vaccine candidates for leishmaniasis: A review. International Immunopharmacology 11: 1464-1488.
329. Nagill R, Mahajan R, Sharma M, Kaur S (2009) Induction of cellular and humoral responses by autoclaved and heat-killed antigen of *Leishmania donovani* in experimental visceral leishmaniasis. Parasitology International 58: 359-366.
330. FDA (2012) http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm093833.htm.
331. Mendez S, Tabbara K, Belkaid Y, Bertholet S, Verthelyi D, et al. (2003) Coinjection with CpG-containing immunostimulatory oligodeoxynucleotides reduces the pathogenicity of a live vaccine against cutaneous leishmaniasis but maintains its potency and durability. Infection and Immunity 71: 5121-5129.
332. Spath GF, Lye LF, Segawa H, Turco SJ, Beverley SA (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. Infection and Immunity 72: 3622-3627.
333. Reiling L, Jacobs T, Kroemer M, Gaworski I, Graefe S, et al. (2006) Spontaneous recovery of pathogenicity by Leishmania major hsp100(-/-)alters the immune response in mice. Infection and Immunity 74: 6027-6036.
334. Paul J, Karmakar S, De T (2012) TLR-mediated distinct IFN-gamma/IL-10 pattern induces protective immunity against murine visceral leishmaniasis. European Journal of Immunology 42: 2087-2099.
335. Modabber F (2010) Leishmaniasis vaccines: past, present and future. International Journal of Antimicrobial Agents 36: S58-S61.
336. Chakravarty J, Kumar S, Trivedi S, Rai VK, Singh A, 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: 3531-3537.
337. Velez ID, Gilchrist K, Martinez S, Ramirez-Pineda JR, Ashman JA, et al. (2009) Safety and immunogenicity of a defined vaccine for the prevention of cutaneous leishmaniasis. Vaccine 28: 329-337.
338. NIH (2012) http://clinicaltrials.gov/ct2/home.
339. Gradoni L, Manzillo VF, Pagano A, Piantedosi D, De Luna R, et al. (2005) Failure of a multi-subunit recombinant leishmanial vaccine (MML) to protect dogs from Leishmania infantum infection and to prevent disease progression in infected animals. Vaccine 23: 5245-5251.
340. Bhatia A, Reed SG (2012) Vaccines comprising non-specific nucleoside hydrolase and sterol 24-c-metyltransferase (SMT) polypeptides for the treatment and diagnosis of leishmaniasis. In: Application USP, editor. United States Patent Application. United States of America: Infectious Disease Research Institute, Seattle WA.
341. Moreno J, Vouldoukis I, Martin V, McGahie D, Cuisinier AM, et al. (2012) Use of a LiESP/QA-21 Vaccine (CaniLeish) Stimulates an Appropriate Th1-Dominated Cell-Mediated Immune Response in Dogs. PLoS Neglected Tropical Diseases 6: e1683.
342. McCall LI, Matlashewski G (2012) Involvement of the Leishmania donovani virulence factor A2 in protection against heat and oxidative stress. Experimental Parasitology 132: 109-115.
343. Bhaumik SK, Singh MK, Karmakar S, De T (2009) UDP-Gal: N-acetylglucosamine beta 1-4 galactosyltransferase expressing live attenuated parasites as vaccine for visceral leishmaniasis. Glycoconjugate Journal 26: 663-673.
344. Daneshvar H, Molaei MM, Kamiabi H, Burchmore R, Hagan P, et al. (2010) Gentamicin-attenuated Leishmania infantum: cellular immunity production and protection of dogs against experimental canine leishmaniasis. Parasite Immunology 32: 722-730.
345. Datta S, Manna M, Khanra S, Ghosh M, Bhar R, et al. (2012) Therapeutic immunization with radio-attenuated Leishmania parasites through i.m.

route revealed protection against the experimental murine visceral leishmaniasis. Parasitology Research 111: 361-369.
346. Sacks D, Noben-Trauth N (2002) The immunology of susceptibility and resistance to Leishmania major in mice. Nature Reviews Immunology 2: 845-858.
347. Bogdan C (2012) Leishmaniasis in rheumatology, haematology and oncology: epidemiological, immunological and clinical aspects and caveats. Annals of the Rheumatic Diseases 71: 60-66.
348. Desjeux P (2001) The increase in risk factors for leishmaniasis worldwide. Transactions of the Royal Society of Tropical Medicine and Hygiene 95: 239-243.
349. Gottlieb M, Dwyer DM (1982) Identification and Partial Characterization of an Extracellular Acid-Phosphatase-Activity of Leishmania-Donovani Promastigotes. Molecular and Cellular Biology 2: 76-81.
350. Shapira M, Pinelli E (1989) Heat-Shock Protein-83 of Leishmania-Mexicana-Amazonensis Is an Abundant Cytoplasmic Protein with a Tandemly Repeated Genomic Arrangement. European Journal of Biochemistry 185: 231-236.
351. Bangs JD, Uyetake L, Brickman MJ, Balber AE, Boothroyd JC (1993) Molecular cloning and cellular localization of a BiP homologue in *Trypanosoma brucei*. Divergent ER retention signals in a lower eukaryote. Journal of Cell Science 105 (Pt 4): 1101-1113.
352. Vasilescu J, Guo XC, Kast J (2004) Identification of protein-protein interactions using in vivo cross-linking and mass spectrometry. Proteomics 4: 3845-3854.
353. Shakarian AM, Dwyer DM (2000) Structurally conserved soluble acid phosphatases are synthesized and released by Leishmania major promastigotes. Experimental Parasitology 95: 79-84.
354. Dudek J, Benedix J, Cappel S, Greiner M, Jalal C, et al. (2009) Functions and pathologies of BiP and its interaction partners. Cellular and Molecular Life Sciences 66: 1556-1569.
355. Gruschus JM (2008) Do amyloid oligomers act as traps for misfolded proteins? A hypothesis. Amyloid-Journal of Protein Folding Disorders 15: 160-165.
356. Clos J, Klaholz L, Kroemer M, Krobitsch S, Lindquist S (2001) Heat shock protein 100 and the amastigote stage-specific A2 proteins of *Leishmania donovani*. Medical Microbiology and Immunology 190: 47-50.
357. Yao CQ, Donelson JE, Wilson ME (2003) The major surface protease (MSP or GP63) of Leishmania sp - Biosynthesis, regulation of expression, and function. Molecular and Biochemical Parasitology 132: 1-16.
358. Bates PA, Dwyer DM (1987) Biosynthesis and secretion of acid phosphatase by *Leishmania donovani* promastigotes. Molecular and Biochemical Parasitology 26: 289-296.
359. Bates PA, Hermes I, Dwyer DM (1990) Golgi-Mediated Posttranslational Processing of Secretory Acid-Phosphatase by Leishmania-Donovani Promastigotes. Molecular and Biochemical Parasitology 39: 247-256.

360. Ilg T, Stierhof YD, Etges R, Adrian M, Harbecke D, et al. (1991) Secreted acid-phosphatase of *Leishmania-mexicana* - a filamentous phosphoglycoprotein polymer. Proceedings of the National Academy of Sciences U S A 88: 8774-8778.
361. Wiesgigl M, Clos J (2001) Heat shock protein 90 homeostasis controls stage differentiation in Leishmania donovani. Molecular Biology of the Cell 12: 3307-3316.
362. Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. Journal of Molecular Biology 340: 783-795.
363. Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP and related tools. Nature Protocols 2: 953-971.
364. Bates PA, Hermes I, Dwyer DM (1989) Leishmania-Donovani - Immunochemical Localization and Secretory Mechanism of Soluble Acid-Phosphatase. Experimental Parasitology 68: 335-346.
365. Wei XQ, Charles IG, Smith A, Ure J, Feng CJ, et al. (1995) Altered Immune-Responses in Mice Lacking Inducible Nitric-Oxide Synthase. Nature 375: 408-411.
366. Kampinga HH (2006) Chaperones in preventing protein denaturation in living cells and protecting against cellular stress. Handbook of Experimental Pharmacology: 1-42.
367. Robinson KA, Beverley SM (2003) Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite Leishmania. Molecular and Biochemical Parasitology 128: 217-228.
368. Mikus J, Steverding D (2000) A simple colorimetric method to screen drug cytotoxicity against *Leishmania* using the dye Alamar Blue. Parasitology International 48: 265-269.
369. Mukherjee A, Roy G, Guimond C, Ouellette M (2009) The gamma-glutamylcysteine synthetase gene of *Leishmania* is essential and involved in response to oxidants. Molecular Microbiology 74: 914-927.
370. Moreira W, Leblanc E, Ouellette M (2009) The role of reduced pterins in resistance to reactive oxygen and nitrogen intermediates in the protozoan parasite Leishmania. Free Radical Biology and Medicine 46: 367-375.
371. Matsumoto R, Akama K, Rakwal R, Iwahashi H (2005) The stress response against denatured proteins in the deletion of cytosolic chaperones SSA1/2 is different from heat-shock response in Saccharomyces cerevisiae. Bmc Genomics 6.
372. Liu Y, Chang A (2008) Heat shock response relieves ER stress. Embo Journal 27: 1049-1059.
373. Schroder M, Kaufman RJ (2005) The mammalian unfolded protein response. Annual Review of Biochemistry 74: 739-789.
374. Zhang WW, Matlashewski G (2000) Analysis of antisense and double stranded RNA downregulation of A2 protein expression in *Leishmania donovani*. Molecular and Biochemical Parasitology 107: 315-319.

375. Militello KT, Refour P, Comeaux CA, Duraisingh MT (2008) Antisense RNA and RNAi in protozoan parasites: Working hard or hardly working? Molecular and Biochemical Parasitology 157: 117-126.
376. Naderer T, Dandash O, McConville MJ (2011) Calcineurin is required for *Leishmania major* stress response pathways and for virulence in the mammalian host. Molecular Microbiology 80: 471-480.
377. Gokool S (2003) sigma 1- and mu 1-adaptin homologues of Leishmania mexicana are required for parasite survival in the infected host. Journal of Biological Chemistry 278: 29400-29409.
378. Vince JE, Tull DL, Spurck T, Derby MC, McFadden GI, et al. (2008) Leishmania adaptor protein-1 subunits are required for normal lysosome traffic, flagellum biogenesis, lipid homeostasis, and adaptation to temperatures encountered in the mammalian host. Eukaryotic Cell 7: 1256-1267.
379. Malhotra JD, Miao H, Zhang K, Wolfson A, Pennathur S, et al. (2008) Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. Proceedings of the National Academy of Sciences U S A 105: 18525-18530.
380. Harding HP, Zhang YH, Zeng HQ, Novoa I, Lu PD, et al. (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Molecular Cell 11: 619-633.
381. Haynes CM, Titus EA, Cooper AA (2004) Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. Molecular Cell 15: 767-776.
382. Sanders SL, Whitfield KM, Vogel JP, Rose MD, Schekman RW (1992) Sec61p and Bip Directly Facilitate Polypeptide Translocation into the Er. Cell 69: 353-365.
383. Huang LE, Zhang H, Bae SW, Liu AY (1994) Thiol reducing reagents inhibit the heat shock response. Involvement of a redox mechanism in the heat shock signal transduction pathway. J Biol Chem 269: 30718-30725.
384. Zarley JH, Britigan BE, Wilson ME (1991) Hydrogen Peroxide-Mediated Toxicity for Leishmania-Donovani Chagasi Promastigotes - Role of Hydroxyl Radical and Protection by Heat-Shock. Journal of Clinical Investigation 88: 1511-1521.
385. Wilson ME, Andersen KA, Britigan BE (1994) Response of Leishmania-Chagasi Promastigotes to Oxidant Stress. Infection and Immunity 62: 5133-5141.
386. Channon JY, Blackwell JM (1985) A Study of the Sensitivity of Leishmania-Donovani Promastigotes and Amastigotes to Hydrogen-Peroxide .1. Differences in Sensitivity Correlate with Parasite-Mediated Removal of Hydrogen-Peroxide. Parasitology 91: 197-206.
387. Lemesre JL, Sereno D, Daulouede S, Veyret B, Brajon N, et al. (1997) Leishmania spp.: Nitric oxide-mediated metabolic inhibition of promastigote and axenically grown amastigote forms. Experimental Parasitology 86: 58-68.

388. Pimenta PF, Saraiva EM, Sacks DL (1991) The comparative fine structure and surface glycoconjugate expression of three life stages of *Leishmania major*. Experimental Parasitology 72: 191-204.
389. Thorne KJI, Blackwell JM (1983) Cell-mediated killing of protozoa; Baker JR, editor. London: Academic Press.
390. Nielsen M, Lundegaard C, Lund O, Petersen TN (2010) CPHmodels-3.0-remote homology modeling using structure-guided sequence profiles. Nucleic Acids Research 38: W576-W581.
391. Pal S, Dolai S, Yadav RK, Adak S (2010) Ascorbate Peroxidase from Leishmania major Controls the Virulence of Infective Stage of Promastigotes by Regulating Oxidative Stress. PLos One 5: e11271.
392. Iyer JP, Kaprakkaden A, Choudhary ML, Shaha C (2008) Crucial role of cytosolic tryparedoxin peroxidase in *Leishmania donovani* survival, drug response and virulence. Molecular Microbiology 68: 372-391.
393. Toivola DM, Strnad P, Habtezion A, Omary MB (2010) Intermediate filaments take the heat as stress proteins. Trends in Cell Biology 20: 79-91.
394. Ranasinghe S, Zhang WW, Wickremasinghe R, Abeygunasekera P, Chandrasekharan V, et al. (2012) Leishmania donovani zymodeme MON-37 isolated from an autochthonous visceral leishmaniasis patient in Sri Lanka. Pathogens and global health 106: 421-424.
395. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, et al. (2012) Leishmaniasis Worldwide and Global Estimates of Its Incidence. PLoS One 7: e35671.
396. Costa CHN, Peters NC, Maruyama SR, de Brito EC, Santos IKFD, et al. (2011) Vaccines for the Leishmaniases: Proposals for a Research Agenda. PLoS Neglected Tropical Diseases 5: e943.
397. Mitchell GF, Handman E (1987) Heterologous protection in murine cutaneous leishmaniasis. Immunology and Cell Biology 65 Pt 5: 387-392.
398. Campbell SA, Alawa J, Doro B, Henriquez FL, Roberts CW, et al. (2012) Comparative assessment of a DNA and protein Leishmania donovani gamma glutamyl cysteine synthetase vaccine to cross-protect against murine cutaneous leishmaniasis caused by L. major or L. mexicana infection. Vaccine 30: 1357-1363.
399. Bhaumik S, Basu R, Sen S, Naskar K, Roy S (2009) KMP-11 DNA immunization significantly protects against L. donovani infection but requires exogenous IL-12 as an adjuvant for comparable protection against L. major. Vaccine 27: 1306-1316.
400. Tonui WK, Titus RG (2007) Cross-protection against Leishmania donovani but not L-braziliensis caused by vaccination with L-major soluble promastigote exogenous antigens in BALB/c mice. American Journal of Tropical Medicine and Hygiene 76: 579-584.
401. Ranasinghe S, Zhang WW, Wickremasinghe R, Abeygunasekera P, Chandrasekharan V, et al. (2012) Leishmania donovani zymodeme MON-37 isolated from an autochthonous visceral leishmaniasis patient in Sri Lanka. Pathogens and Global Health 106: 421-424.

402. Cavanagh LL, Bonasio R, Mazo IB, Halin C, Cheng GY, et al. (2005) Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells. Nature Immunology 6: 1029-1037.
403. Bonasio R, von Andrian UH (2006) Generation, migration and function of circulating dendritic cells. Current Opinion in Immunology 18: 503-511.
404. Agace WW (2006) Tissue-tropic effector T cells: generation and targeting opportunities. Nature Reviews Immunology 6: 682-692.
405. Esser MT, Marchese RD, Kierstead LS, Tussey LG, Wang FB, et al. (2003) Memory T cells and vaccines. Vaccine 21: 419-430.
406. Caldas A, Favali C, Aquino D, Vinhas V, van Weyenbergh J, et al. (2005) Balance of IL-10 and interferon-gamma plasma levels in human visceral leishmaniasis: implications in the pathogenesis. BMC Infectious Diseases 5: 113.
407. Deak E, Jayakumar A, Cho KW, Goldsmith-Pestana K, Dondji B, et al. (2010) Murine visceral leishmaniasis: IgM and polyclonal B-cell activation lead to disease exacerbation. European Journal of Immunology 40: 1355-1368.
408. Sharma U, Singh S (2009) Immunobiology of leishmaniasis. Indian Journal of Experimental Biology 47: 412-423.
409. Prockop DJ, Kivirikko KI (1995) Collagens: molecular biology, diseases, and potentials for therapy. Annual Review of Biochemistry 64: 403-434.
410. Aslett M, Aurrecoechea C, Berriman M, Brestelli J, Brunk BP, et al. (2010) TriTrypDB: a functional genomic resource for the Trypanosomatidae. Nucleic Acids Research 38: D457-D462.
411. Treangen TJ, Salzberg SL (2012) Repetitive DNA and next-generation sequencing: computational challenges and solutions. Nature Reviews Genetics 13: 36-46.
412. Baldwin CT, Constantinou CD, Dumars KW, Prockop DJ (1989) A Single Base Mutation That Converts Glycine-907 of the Alpha-2(I) Chain of Type-I Procollagen to Aspartate in a Lethal Variant of Osteogenesis Imperfecta - the Single Amino-Acid Substitution near the Carboxyl Terminus Destabilizes the Whole Triple Helix. Journal of Biological Chemistry 264: 3002-3006.
413. Clatworthy AE, Pierson E, Hung DT (2007) Targeting virulence: a new paradigm for antimicrobial therapy. Nature Chemical Biology 3: 541-548.
414. Hajduk PJ, Huth JR, Tse C (2005) Predicting protein druggability. Drug Discovery Today 10: 1675-1682.
415. Hopkins AL, Groom CR (2002) The druggable genome. Nature Reviews Drug Discovery 1: 727-730.
416. Leprohon P, Legare D, Raymond F, Madore E, Hardiman G, et al. (2009) Gene expression modulation is associated with gene amplification, supernumerary chromosomes and chromosome loss in antimony-resistant Leishmania infantum. Nucleic Acids Research 37: 1387-1399.
417. Kajava AV (2012) Tandem repeats in proteins: From sequence to structure. Journal of Structural Biology 179: 279-288.

418. Markikou-Ouni W, Ben Achour-Chenik Y, Meddeb-Garnaoui A (2012) Effects of Leishmania major clones showing different levels of virulence on infectivity, differentiation and maturation of human dendritic cells. Clinical and Experimental Immunology 169: 273-280.
419. Moll H, Fuchs H, Blank C, Rollinghoff M (1993) Langerhans cells transport *Leishmania-major* from the infected skin to the draining lymph-node for presentation to antigen-specific T-cells. European Journal of Immunology 23: 1595-1601.
420. Denise H, Coombs GH, Mottram JC (2004) Generation of Leishmania mutants lacking antibiotic resistance genes using a versatile hit-and-run targeting strategy. Fems Microbiology Letters 235: 89-94.
421. Scahill MD, Pastar I, Cross GAM (2008) CRE recombinase-based positive-negative selection systems for genetic manipulation in Trypanosoma brucei. Molecular and Biochemical Parasitology 157: 73-82.
422. Hotez PJ, Fenwick A, Savioli L, Molyneux DH (2009) Rescuing the bottom billion through control of neglected tropical diseases. Lancet 373: 1570-1575.