Pathogenesis of leishmania infection in human macrophages:

role of leishmania chaperonin 10

and modulation of host miRNA

by

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Abstract

Protozoan parasites of the genus *Leishmania* infect macrophages in their mammalian hosts causing a spectrum of diseases known as the leishmaniases. The search for parasite and host factors that support macrophage infection is a focus of significant interest. Here, I studied two aspects of the host-pathogen interaction: the role of the *leishmania* protein chaperonin 10 (CPN10) and the impact of *leishmania* infection on host cell micro RNA (miRNA) expression.

The heat shock protein CPN10 is secreted by *leishmania* in exosomes and its human homolog has immunosuppressive properties. Two genetically modified strains of *Leishmania donovani* (Ld) were generated: one overexpressing CPN10 (CPN10+++) and the second, a CPN10 single allele knockdown (CPN10+/−). When compared with the wild-type (WT) parental strain, CPN10+/− Ld showed higher infection rates and parasite loads in human macrophages after 24 h of infection. Conversely, CPN10+++ Ld was associated with lower initial infection rates. We found that this unexpected apparent gain-of-function for the knockdown was a result of CPN10+/− Ld being more readily internalized than WT Ld. However, the CPN10+/− strain also displayed significantly impaired intracellular survival. Taken together, these findings identify *leishmania* CPN10 as a novel effector that negatively regulates the rate of parasite uptake by macrophages while being required for intracellular survival.

miRNAs have recently emerged as ubiquitous regulators of gene expression. Here, we used miRNA expression profiling to ask whether *leishmania* target their host cell miRNA machinery as a novel virulence strategy. The data show that *leishmania* infection of human macrophages induces a genome-wide downregulation of host miRNAs. This repression takes
place at the level of miRNA gene transcription as the synthesis rates of primary miRNAs are markedly decreased in infected cells. Expression of the host transcription factor c-Myc is markedly upregulated by infection and silencing of c-Myc both reverses miRNA suppression and brings about a significant reduction in leishmania survival. These findings identify c-Myc as a novel and essential leishmania virulence factor by proxy that targets the host miRNA machinery and promotes pathogen survival.
**Lay Summary**

Leishmaniases affect approximately 12 million people worldwide with ~1.3 million new cases each year. These protozoan parasites cause different syndromes, from cutaneous forms with severe skin ulcers to visceral forms affecting internal organs. There are no vaccines against human leishmaniasis, and current treatments have limited efficacy and significant toxicities. Leishmania are transmitted by the bite of a sandfly. Once under the skin, they encounter immune cells called phagocytes, which ingest the intruders to kill them. But leishmania have developed mechanisms that trick the phagocytes by preventing the production of microbicidal compounds: these allow the parasite to live and replicate within phagocytes instead of being killed. Here, I found two mechanisms promoting leishmania intracellular survival: (1) the leishmania protein chaperonin 10 slows the parasite internalization while increasing their survival and (2) leishmania increase the host protein c-Myc that enhances their survival and represses host cells' microRNAs, essential regulators of gene expression.

**Résumé**

Les leishmanioses affectent environ 12 millions de personnes à travers le monde, avec ~1.3 million de nouveaux cas annuels. Ces parasites protozoaires provoquent différents syndromes, des formes cutanées avec de sévères ulcères, aux formes viscérales touchant les organes internes. Il n'existe pas de vaccin et les traitements actuels sont toxiques et peu efficaces. Les leishmanias sont transmises par la piqûre d'insectes phlébotomes. Une fois sous la peau, elles
rencontrent des cellules immunitaires appelées phagocytes, qui les ingèrent pour les tuer. Mais les leishmanias manipulent les phagocytes en bloquant la production de composés microbicides, ce qui permet aux parasites de survivre et se répliquer à l'intérieur des cellules. Ici, j'ai révélé deux mécanismes favorisant la survie intracellulaire des leishmanias. Premièrement, la protéine chaperonin 10 de leishmania ralentit l'internalisation des parasites tout en améliorant leur survie. Deuxièmement, les leishmanias augmentent la protéine c-Myc de leurs cellules-hôtes, ce qui améliore la survie des parasites, et réprime les microARN de la cellule-hôte, qui sont des régulateurs critiques de l'expression génique.
Preface

Chapter 1. Figures are used with permission from the respective copyright owners.

Chapter 2. This chapter is based on collaborative work performed in the laboratories of Dr. Reiner (Department of Medicine, UBC), Dr. Clos (Bernhard-Nocht-Institut für Tropenmedizin, Hamburg, Germany) and Dr. Foster (Department of Biochemistry, UBC). A version of this chapter has been published as:

Colineau L, Clos J, Moon KM, Foster LJ, Reiner NE. Leishmania donovani chaperonin 10 regulates parasite internalization and intracellular survival in human macrophages. Medical Microbiology and Immunology (2017)

I principally designed the project and the experiments, performed the experiments, interpreted the data and wrote the manuscript for the article. Joachim Clos helped with experimental design to create the genetically modified leishmania strains and provided reagents and other materials. Leonard Foster helped with experimental design of the proteomics analyses, and Jenny Moon conducted the mass spectrometry experiments and data generation. Neil Reiner was the supervisory author on this project and participated in conceptual design and manuscript revision and editing. Permission to reproduce the figures and parts of the text has been granted by the Copyright holder Springer-Verlag Berlin Heidelberg 2017.

Chapter 3. This chapter is based on work performed in the laboratory of Dr. Reiner. A version of this chapter has been submitted for publication as:

Colineau L, Lambertz U, Reiner NE. Leishmania hijacks c-Myc to act as a virulence factor by proxy targeting host miRNAs.

This project was initiated by Dr. Ulrike Lambertz, she designed and performed the first
experiments, namely the analysis of miRNA expression in infected cells by nanostring and RT-qPCR (Figure 3.1). I then took over the project and designed the following experiments, conducted the experiments, interpreted the data and wrote the manuscript. Neil Reiner was the supervisory author on this project and participated in conceptual design and manuscript revision and editing.

**Ethics approvals.** Human blood was obtained through netCAD (reference nc0010) with approval from Canadian Blood Services Research Ethics (reference 2015.035 "Activities of white blood cells in response to infection"). All work with animal in this study was reviewed and approved by The University of British Columbia Animal Care Committee (protocol license number: A14-0218 "Mononuclear Phagocyte Cell Signaling"). The animal care and use protocol adhered to the standards and regulations provided by the Canadian Council on Animal Care in Science.
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<tbody>
<tr>
<td>Ago2</td>
<td>argonaute 2</td>
</tr>
<tr>
<td>Akti</td>
<td>Akt1/2 kinase inhibitor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>activator protein</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid assay</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone-marrow derived macrophage</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<td>BSA</td>
<td>bovine serum albumine</td>
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<td>cluster of differentiation 14</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
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<td>CHX</td>
<td>cycloheximide</td>
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<tr>
<td>CL</td>
<td>cutaneous leishmaniasis</td>
</tr>
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<td>CMI</td>
<td>cell-mediated immunity</td>
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<td>chaperonin</td>
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<td>complement receptor</td>
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<td>DAPI</td>
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<td>diffuse cutaneous leishmaniasis</td>
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<td>dimethyl sulfoxide</td>
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<td>DNA</td>
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<td>DTH</td>
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<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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F phenylalanine
FBS fetal bovine serum
FC fold change
FITC fluorescein isothiocyanate
FOXO1 forkhead box protein O1
GM-CSF granulocyte-macrophage colony-stimulating factor
GO gene ontology
GSK-3β glycogen synthase kinase 3β
HBSS Hanks' balanced salt solution
HIV human immunodeficiency virus
HMDM human monocyte-derived macrophage
HSP heat shock protein
IC50 half maximal inhibitory concentration
IFN-γ interferon-γ
IL interleukin
iNOS inducible nitric oxide synthase
IPTG isopropyl β-D-1-thiogalactopyranoside
JAK Janus kinase 2
JNK c-Jun N-terminal kinase
KMP-11 kinetoplastid membrane protein 11
L leucine
LAIR leukocyte-associated immunoglobulin-like receptor
LAL limulus amebocyte lysate
LB Luria-Bertani (LB) broth
Ld Leishmania donovani
LPG lipophosphoglycan
LPS lipopolysaccharide
LR leishmaniasis recidivans
MAPK mitogen-activated protein kinase
MARCKS myristoylated alanine-rich C-kinase substrate
MHC major histocompatibility complex
<table>
<thead>
<tr>
<th>Term</th>
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<tr>
<td>miRNA</td>
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</tr>
<tr>
<td>ML</td>
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</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<tr>
<td>MP</td>
<td>macrophage</td>
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<tr>
<td>mRNA</td>
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<td>MRP</td>
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<tr>
<td>SD</td>
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<td>siRNA</td>
<td>small (or short) interfering RNA</td>
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Acknowledgements

First, I want to thank my supervisor Dr. Neil Reiner, for giving me the opportunity to work in his lab and explore the world of host-pathogen interactions. Thanks for his guidance over the years.

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"Je suis de ceux qui pensent que la science est d’une grande beauté. Un scientifique dans son laboratoire est non seulement un technicien : il est aussi un enfant placé devant des phénomènes naturels qui l’impressionnent comme des contes de fées."

"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."

- Marie Curie
Chapter 1: Introduction

1.1 Leishmaniasis

Leishmaniasis refers to the group of diseases caused by the protozoan parasites of the genus *Leishmania* (order *Kinetoplastida* and family *Trypanosomatidae*). Leishmaniasis comprise a spectrum of clinical symptoms, and each of the leishmania species has its own geographic distribution, biology, ecology, mammalian reservoir and sand fly vector (Table 1.1) (Bennett et al., 2014).

The oldest descriptions of Old World cutaneous leishmaniasis (CL) date from 1500 to 2500 BC (Cox, 2002). In the New World, depictions of leishmaniasis can be dated as far as the first century AD: pre-Incan pottery from Ecuador and Peru show skin lesions and facial deformities that are typical of cutaneous and mucocutaneous leishmaniasis (Tuon et al., 2008). The disease became known as leishmaniasis after William Leishman and Charles Donovan independently characterized the disease in 1903: Leishman discovered ovoid bodies in the spleen of a British soldier who was experiencing bouts of fever, anemia, muscular atrophy and swelling of the spleen, in Dum Dum (India) (Leishman, 1903); Donovan found the parasites in spleen tissue and in the blood of an infected young boy in Madras (Ross, 1903). The "Leishman-Donovan bodies" (amastigotes) were described as causative agents of the "Dum Dum fever" or "kala-azar" (black fever in Hindi), as visceral leishmaniasis was called then (Steverding, 2017). It was not until 1921 that the sand fly vector was discovered, while the actual mode of transmission through the bite of sand flies was formally demonstrated in 1941 (Sergent et al., 1921; Adler and Ber, 1941).
### Table 1.1 Leishmania species, clinical manifestations and geographical locations.
Adapted from (Bennett et al., 2014). In the species name: 'L. (V.)' indicates members the Leishmania Viannia sub-genus. 'L. (L.)' species belong to the Leishmania Leishmania sub-genus.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Species</th>
<th>Geographical area</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL</td>
<td>L. (L.) donovani</td>
<td>Indian subcontinent [can relapse as PKDL]</td>
</tr>
<tr>
<td></td>
<td>L. (L.) infantum</td>
<td>Middle East, Mediterranean littoral, Central and southwestern Asia, North Africa</td>
</tr>
<tr>
<td></td>
<td>L. (L.) infantum</td>
<td>Latin America</td>
</tr>
<tr>
<td></td>
<td>L. (L.) donovani/infantum</td>
<td>East Africa [can relapse as PKDL]</td>
</tr>
<tr>
<td>Old</td>
<td>L. (L.) major</td>
<td>Middle East, India, Pakistan, Africa, Central and western Asia, northern and western China</td>
</tr>
<tr>
<td>World</td>
<td>L. (L.) tropica</td>
<td>Mediterranean littoral, Middle East, India, Central and western Asia [can relapse as LR]</td>
</tr>
<tr>
<td>CL</td>
<td>L. (L.) aethiopica</td>
<td>Ethiopia, Kenya, Yemen</td>
</tr>
<tr>
<td></td>
<td>L. (L.) donovani/infantum</td>
<td>East Africa</td>
</tr>
<tr>
<td>New</td>
<td>L. (L.) mexicana</td>
<td>Central and South America, Texas</td>
</tr>
<tr>
<td>World</td>
<td>L. (L.) amazonensis</td>
<td>Amazon Basin, neighbouring areas, Bahia and other states of Brazil</td>
</tr>
<tr>
<td>CL</td>
<td>L. (L.) pifanoi, garnhami, venezuelensis</td>
<td>Venezuela</td>
</tr>
<tr>
<td></td>
<td>L. (V.) braziliensis</td>
<td>Central and South America</td>
</tr>
<tr>
<td></td>
<td>L. (V.) guyanensis</td>
<td>Guyana, Surinam, northern Amazon Basin</td>
</tr>
<tr>
<td></td>
<td>L. (V.) peruviana</td>
<td>Peru</td>
</tr>
<tr>
<td></td>
<td>L. (V.) panemensis</td>
<td>Panama, Costa Rica, Colombia</td>
</tr>
<tr>
<td></td>
<td>L. (V.). colombiensis</td>
<td>Colombia and Panama</td>
</tr>
<tr>
<td></td>
<td>L. (L.) infantum</td>
<td>Central and South America</td>
</tr>
<tr>
<td>Diffuse</td>
<td>L. (L.) amazonensis</td>
<td>Amazon Basin, neighbouring areas, Bahia and other states of Brazil</td>
</tr>
<tr>
<td>CL</td>
<td>L. (L.) pifanoi</td>
<td>Venezuela</td>
</tr>
<tr>
<td></td>
<td>L. (L.) mexicana</td>
<td>Central and South America, Texas</td>
</tr>
<tr>
<td></td>
<td>L. (L.) aethiopica</td>
<td>Ethiopia, Kenya, Yemen</td>
</tr>
<tr>
<td>ML</td>
<td>L. (V.) braziliensis</td>
<td>Central and South America; most cases from Bolivia, Brazil, and Peru</td>
</tr>
</tbody>
</table>

### 1.1.1 Global importance, epidemiology

Leishmaniases affect a huge number of people, even though only estimates can be made. Approximately twelve million people are thought to suffer from leishmaniasis, with one to two million new cases annually, and an estimated 530 million people are at risk (Alvar et al., 2012). The leishmaniases can be found in tropical, subtropical and temperate regions in 89 countries (Torres-Guerrero et al., 2017). A large majority of these countries are in developing areas of the world. In the Eastern Hemisphere (The Old World), leishmaniasis is found in some regions of Asia, the Middle East, Africa, and southern Europe. In the Western Hemisphere (the New World), it is found in some regions of Mexico, Central America, and South America. Among the three forms of the disease, both cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) are endemic to countries in both Old and New World, while mucocutaneous (ML) is almost
exclusively found in the New World (Table 1.1) (Pigott et al., 2014). 90% of all VL cases are found in three regions: (1) the drainage basin of the Ganges in eastern India, the neighbouring Nepal, and Bangladesh; (2) the Sudan, and (3) Brazil (Alvar et al., 2012). 90% of all CL cases are found in Iran, Saudi Arabia in the Middle East, in Afghanistan in Central Asia, and in Brazil and Peru in Latin America.

In addition to causing severe morbidity and mortality, leishmaniases present additional socio-economic burdens to already impoverished people. These diseases are responsible every year for 2.4 million disability-adjusted life-years, mostly due to the stigmatizing effects of leishmania associated ulcers and scars - the respective disability-adjusted life-year is 95 years for CL and 4 years for VL (Desjeux, 2004). Poverty is indeed one of the main risk factors associated with a contracting leishmaniasis (Oryan and Akbari, 2016). The sand fly vectors breed in cracks in the walls of dwellings, in rubbish or rubble, or in rodent burrows, more prevalent in areas with poor living conditions. Leishmaniases also thrive in wars and conflicts, where populations movements introduce leishmania in areas where the disease is not endemic but the vector in present, or where non-immune populations move to endemic regions (Oryan and Akbari, 2016). The rubble from destruction of buildings constitute new breeding places for the sandflies. Food insecurity and lack of shelter also exposes people to increased risks of developing the disease. The living conditions in refugee camps increase human exposure to leishmania. Finally, the disruption of health care facilities also contributes to the development of epidemics. Recently, leishmaniasis outbreaks have been reported among the refugees of the Syrian civil war in Lebanon, Syria, Jordan, Turkey (Alawieh et al., 2014; Sharara and Kanj, 2014; Özkeklikçi et al., 2017).

VL is a common opportunistic infection associated with human immunodeficiency virus (HIV) infection (Alvar et al., 2008). VL and HIV are mutually reinforcing, therefore co-infection
has serious clinical and epidemiological implications. In southern Europe, up to 70% of VL cases in adults are HIV co-infections (WHO | Leishmaniasis and HIV coinfection).

1.1.2 Transmission and life cycle

Out of approximately 1000 known species of sandflies, 70 are proven vectors of leishmania; they belong to the genus Lutzomyia in the Americas, and Phlebotomus elsewhere (Bennett et al., 2014). The life cycle of leishmania is reviewed here (Cox, 1993). Humans get infected through the bite of a female sand fly. When the sand fly takes a blood meal on an infected animal, it ingests macrophages carrying leishmania amastigotes (Figure 1.1). Amastigotes are the ovoid-shaped, small, non-motile form of the parasite that live inside macrophages. Once in the digestive tract of the sand fly, amastigotes differentiate into procyclic promastigotes, which are highly replicative and weakly motile. These undergo further transformation while migrating to the anterior midgut of the sand fly, into metacyclic promastigotes, which are needle-like, non-dividing and flagellated. After a total of about 10 days differentiation, metacyclic promastigotes are the highly infective form of leishmania that will be transmitted to human and other vertebrate hosts during the next feeding. Promastigotes that reach the puncture wound are ingested by phagocytes, mostly macrophages and neutrophils. Once inside phagolysosomes, leishmania transform into amastigotes, and multiply by division until infected macrophages rupture and parasites are released in the extracellular environment. Released amastigotes are taken up by uninfected macrophages and multiply further. The life cycle is completed when infected macrophages are ingested by sand flies during blood meals.

In addition to humans, leishmania can infect many other vertebrates (dogs, mice, hamsters, foxes, racoons, sloths, opossums, forest rodents); they constitute a reservoir for leishmaniasis - one or a complex of species responsible for maintaining the parasite in nature.
Figure 1.1 Leishmania donovani life cycle.
The sandfly (top) and mammalian stage (bottom) of the leishmania life cycle. Starting in the top: Leishmania live and proliferate in the midgut of sandflies as procyclic promastigotes. The leishmania differentiate into highly infective metacyclic promastigotes. Upon a sandfly bite, leishmania promastigotes are injected into the dermis of the mammalian host. They attach to phagocytes and are taken up by phagocytosis. Within phagolysosomes of macrophages, they transform into amastigotes. Increasing proliferation and parasite burden leads to lysis of infected macrophages and release of amastigotes which then reinvade other macrophages to multiply further. Infected cells are taken up by sandflies, completing the cycle. Reprinted with permission from (Chappuis et al., 2007).

(Ashford, 1996). Leishmaniasis can therefore be considered a zoonosis: in many geographic areas, infected people are not needed to maintain the transmission cycle of the parasite in nature (they are called incidental hosts); infected animals constitute the reservoir (Pigott et al., 2014). However, in other parts of the World, humans can act as reservoirs, this type of transmission
(human – sand fly – human) is called anthroponotic (Oryan and Akbari, 2016). Studying reservoirs is necessary to develop effective control strategies.

1.1.3 Clinical manifestations

The spectrum of disease caused by leishmania infection is traditionally divided into three major syndromes: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) and mucosal leishmaniasis (ML) (McGwire and Satoskar, 2014). Post kala-azar dermal leishmaniasis (PKDL) is a complication of VL, while diffuse cutaneous leishmaniasis (DCL) and leishmaniasis recidivans (LR) constitute special clinical forms of CL. Table 1.1 represents the leishmania species responsible for each syndrome in different geographical areas.

The clinical spectrum of leishmaniasis is caused by the balance between parasite burden and immune response (Figure 1.2): at one end, VL and later-stage DCL are characterized by very high parasite burdens (polyparasitic), no cell-mediated immunity (anergy), and a detectable antibody response (Murray et al., 2005). At the other end, syndromes such as ML and LR (leishmaniasis recidivans) are characterized by very low parasite numbers (oligoparasitic), an exaggerated cell-mediated immune responses and minimal antibody responses.

VL, also called kala-azar, is the most severe of the three syndromes, and is usually fatal if not treated (Chappuis et al., 2007). It is mostly caused by L. donovani, and L. infantum. Many people living in endemic areas have positive leishman skin tests without having been diagnosed or treated for leishmaniasis, which suggests that a large number of cases are subclinical and self-resolving (up to 9 out of 10 infections are thought to remain asymptomatic) (Singh et al., 2014; Farrar et al., 2013). After inoculation of promastigotes by sand flies, the appearance of clinical signs might take anywhere between 10 days to 34 months (World Health Organization, 2010). The disease is slowly progressive and characterized by a subacute onset of fever,
hepatosplenomegaly, anaemia, leukopenia and thrombocytopenia. These are both signs of a chronic systemic infection (fever, fatigue, weakness, loss of appetite and weight) and of parasite invasion of the mononuclear phagocyte system (enlarged lymph nodes, spleen and liver). If not treated, the disease evolves into cachexia, and is fatal in over 90% of cases, death being usually caused by superimposed infections, severe anaemia or haemorrhages (Ready, 2014). Post Kala-azar Dermal Leishmaniasis (PKDL) is a complication of VL, characterized by a chronic rash, caused by *L. donovani* in endemic areas (parts of Asia and Africa) (Ready, 2014). Skin lesions appear within days (in East Africa) or months to years (Asia) after treatment in patients who are otherwise well.

Figure 1.2 Dynamic relation in the clinical spectrum of human leishmaniasis
Balance between effective cell-mediated immunity (CMI), delayed-type hypersensitivity (DTH), and parasite burden: CMI/DTH and parasite burden are inversely related. (1) Asymptomatic infection involves vigorous but not pathologic CMI/DTH and a low parasite burden. (2, 3) Self-healing disease, is shown separately in CL and VL since clinical disease is different and (3) presents lower CMI/DTH and higher parasite burden than (2). (4) Non-healing chronic disease is found at either side of the spectrum, produced by either (a) uncontrolled infection because of absent (DCL) or ineffective CMI/DTH (VL, PKDL), or (b) unrestrained CMI/DTH and pathologic inflammation despite low parasite burden (ML, chronic CL). Reprinted with permission from (Murray et al., 2005).
Simple or localized CL is characterized by skin lesions developing at the site of inoculation of promastigotes by sand flies (Reithinger et al., 2007). The incubation period varies from 2 weeks to several months. The appearance and type of the lesions vary greatly, even among people infected with the same leishmania species, but are characterized by chronic, painless ulcers or nodules. In most cases, it evolves toward spontaneous cure after months to years, depending on the species. Healing very often leads to marked scars that can be disfiguring. Diffuse CL is characterized by multiple, slowly developing nodules or plaques without ulcerations, large numbers of parasites in the lesions, and a negative leishman skin test (Torres-Guerrero et al., 2017). *L. aethiopica* in East Africa and *L. amazonensis* in Latin America are responsible of most cases of this rare variant of CL (Pigott et al., 2014). The disease persists over years and responds poorly to drugs. Leishmaniasis recidivans (LR) is a chronic form of leishmaniasis, caused mainly by *L. tropica* in the Old World and occasionally by *L. braziliensis* in the New World (Reithinger et al., 2007). Lesions enlarge slowly and often persist for years, healing at the center, while the margins expand. The lesions contain a small number of parasites and are due to an exaggerated cell-mediated immune response.

Mucosal or mucocutaneous leishmaniasis (ML) is due to a reactivation of *L. braziliensis*, and occasionally other leishmania species, months to years after the cutaneous lesions have healed (Strazzulla et al., 2013). Destructive mucosal lesions appear then on the nose and septum at first, followed by oral mucosa: palate, lips, down to the oropharynx or larynx. The sociopsychological consequences of this disfigurement are dramatic, even after healing.

### 1.1.4 Diagnosis, treatment and vaccine status

The diagnosis of leishmaniasis is based on three approaches: clinical, parasitological, and immunologic (Elmahallawy et al., 2014). Amastigotes can be detected by microscopic analysis.
of lesion smears from CL and MCL or splenic/bone marrow aspirates in the case of VL, stained with Giemsa or Leishman's stain. Promastigotes can be grown and isolated in culture from samples, leading to more sensitive results (Sundar and Rai, 2002). Polymerase chain-reaction (PCR)-based methods can be used to amplify Leishmania-specific DNA or RNA, they are highly sensitive and specific but require expensive equipment and material making them impossible to use in endemic regions (Pereira et al., 2014). This molecular parasitological method can also be used to monitor the progression of the disease and the efficacy of treatment. Concerning immunological tests: a number of serological tests that detect anti-leishmania antibodies are available (ELISA, indirect fluorescent antibody test, Western blot, direct agglutination test, rK39 antigen-based dipstick), but their use in CL or ML is limited, as antibodies tend to be present in very low quantities due to very poor humoral response (Elmahallawy et al., 2014). The leishmanin skin test is based on the delayed hypersensitivity response to intradermal inoculation of killed leishmania parasite, with the response read after 48 hours, it is frequently used to detect patients who have been exposed to leishmania (Kar, 1995). Diagnosis of leishmaniasis is limited by specificity and sensitivity, but also availability, feasibility, and field applicability.

A variety of drugs are available to treat leishmaniasis (McGwire and Satoskar, 2014), and finding the optimal drug depends on the demonstrated regional efficacy (based on the leishmania species and strains), available resources and risk-benefit assessment. Pentavalent antimony (Sb) has been considered the gold standard for treating leishmaniasis for decades (Frézard et al., 2009). Despite the increasing resistance (especially in India) and multiple side effects of the treatment, Sb is still in use (Croft et al., 2006). Other drugs used to treat VL include miltefosine, amphotericin B deoxycholate, pentamidine isethionate, and paromomycin; however, they all present strong adverse effects (Singh and Sundar, 2014a). Liposomal amphotericin B has much
less side effects and is less toxic, but its use in developing areas is limited by its high price and limited availability (Murray et al., 2005). Concerning CL, because most cases are self-resolving, and treatments are not without risks, the decision whether to recommend specific treatment depends on the gravity of the lesions, their location, and the immune status of the patient (Reithinger et al., 2007). Current chemotherapies often result in clinical cure (resolution of clinical symptoms) but seldom in parasitological cure (parasites are undetectable in smears, culture or by PCR) (Singh and Sundar, 2014b). Persistence of leishmania in the host tissues after treatment is the rule, not the exception, and allows for reactivation and opportunistic infections in the immunocompromised (Aebischer, 1994). This, along with the poor patient compliance to the current treatments due to the route and frequency of administration, and the high toxicity argues for the development of new therapies against leishmaniases.

"Leishmanization" is an ancient practice similar to cowpox immunization, that pre-dates modern vaccinations, and is widely used in the Middle East and in Central Asia (Saljoughian et al., 2014). People were immunized using live parasites to induce a cutaneous infection, in an unexposed site on the body in order to prevent the risk of facial lesions through natural infection. However, there are risks associated with this practice: the occurrence of persisting lesions, the difficulty to standardize and control the parasites and the risk of disseminated leishmaniasis in immunocompromised individuals, limit the actual deployment of this approach (Khamesipour et al., 2005).

There is currently no form of active (vaccine) or passive (immunoglobulin) immunoprophylaxis that is safe and efficient against human leishmaniasis - there are four canine vaccines used commercially (Foroughi-Parvar and Hatam, 2014) - but advances in the development of a human vaccine have been made (Gillespie et al., 2016). The first candidates to
be used consisted of killed or inactivated leishmania parasites, but they yielded inconsistent clinical results (Chakravarty et al., 2011; Coler et al., 2015). The second-generation vaccines are based on leishmania antigens (delivered as DNA or recombinant proteins) and are at various stages of pre-clinical and clinical development (Duthie et al., 2016). Without any prophylactic drug or vaccine, only personal protection measures against sand fly bites (DEET-based insect repellent and insecticides-impregnated clothing and mesh nets) and community-based efforts aimed at reducing sand fly habitat and multiplication can prevent the transmission of leishmania (Stockdale and Newton, 2013).

1.2 Immunopathogenesis of leishmania infection

Leishmania promastigotes are injected into the host dermis by the sand fly, which initiates a complex network of host-pathogen interactions (Torres-Guerrero et al., 2017). Leishmania can infect multiple cell types, including macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) and T-cells. However, it is established that the mononuclear phagocytes, macrophages and DCs are the most central to leishmania replication and long-term survival (Liu and Uzonna, 2012). Some observations suggest that neutrophils could act as "Trojan horses" to help promastigotes to enter macrophages without triggering their innate antimicrobial defenses (van Zandbergen et al., 2004). In the mean time, macrophages are recruited to the injection site where they take up free promastigotes or infected neutrophils.

Leishmania's host cells of choice, macrophages, are central to the immune system, acting with the first line of defense by engulfing and destroying pathogens, as well as playing a role in activating and informing both the innate and the adaptive immune responses. In order to establish a successful long-term infection, leishmania parasites need both to be taken up by macrophages, without activating them, and to block any attempt from the host cell to destroy the
parasites once they are inside phagosomes (Bhardwaj et al., 2010).

1.2.1 Leishmania uptake by macrophages

Leishmania enter phagocytes through receptor-mediated phagocytosis; several mechanisms of phagocytic uptake have been reported for different types of macrophages in vitro (Rittig et al., 1998), but the mechanisms used by leishmania in vivo are not known. The early interaction of leishmania with macrophages, such as the mode of entry used, influence the outcome of the infection (Liu and Uzonna, 2012).

The complement receptors (CR) 1, CR3 (Mac-1), fibronectin receptor, and the mannose-fucose receptor on the surface of macrophages play important roles in promastigote binding (Liu and Uzonna, 2012). A number of markers expressed on the surface of promastigotes such as lipophosphoglycan (LPG), GP63 and proteophosphoglycans (PPG) play important roles in initiating phagocytosis and subsequent intracellular survival of leishmania (Naderer and McConville, 2011). They are the target of various opsonins such as the complement components C3b and iC3b (Brittingham and Mosser, 1996), mannose-binding protein (Green et al., 1994), and galectins (Pelletier et al., 2003).

It is believed that leishmania use mechanisms of entry that do not activate macrophages, to maximise the chances of parasite survival. The "Trojan horse" hypothesis (as described above) may be one of these mechanisms: apoptotic neutrophils are taken up routinely by macrophages for which apoptotic cell clearance is a physiological event (Ribeiro-Gomes and Sacks, 2012). In contrast to phagocytosis of pathogens, removal of apoptotic cells is immunologically quiescent under physiological circumstances, and even promotes an anti-inflammatory response at the tissue level (Poon et al., 2014). Leishmania could use neutrophils to enter macrophages 'silently' and unrecognized.
Apoptotic death and apoptotic mimicry have been described in leishmania as participating in "silent" entry into macrophages (Wanderley and Barcinski, 2010). One characteristic of apoptotic cells is the expression of phosphatidylserine (PS) at the cell surface. *L. amazonensis* amastigotes can expose PS at their surface without dying, a process called apoptotic mimicry, which allows them to be recognized and engulfed maintaining the anti-inflammatory properties of an apoptotic cell (Wanderley et al., 2006). In promastigotes, it was described that a fraction of the metacyclic population undergoes programmed cell death during the differentiation. These apoptotic promastigotes get injected along with infective live leishmania, and can inhibit production of nitric oxide by activated macrophages (Wanderley et al., 2009).

**1.2.2 Intracellular fate of leishmania**

Macrophages are the major effector cells responsible for destruction of the parasites. Ideally, leishmania are internalized inside phagosomes, which fuse with lysosomes and mature into phagolysosomes, where the parasites are digested (Naderer and McConville, 2011). When activated - in response to interferon-\(\gamma\) (IFN-\(\gamma\)) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) - the macrophage produces reactive oxygen and nitrogen species, neutral proteases and lysosomal hydrolases, and anti-microbial proteins and peptides, which all participate in the killing of pathogens. Nitric oxide, secreted by inducible Nitric Oxide Synthase (iNOS), plays a central microbicidal role in macrophages infected with leishmania (Carneiro et al., 2016; Panaro et al., 2001), as exemplified by the finding that iNOS deficient mice on a normally resistant background are highly susceptible to *L. major* infection despite having a strong Th1 response (Wei et al., 1995). Moreover, activated macrophages secrete pro-inflammatory cytokines to induce a local anti-microbial response. Clearance of leishmania infection requires the production of interleukin-12 (IL-12) by antigen presenting cells and IFN-\(\gamma\) by T cells both of which activate
the T helper 1 (Th1) immune response. Together with TNF-α, these cytokines induce the microbicidal activities in infected macrophages to kill intracellular parasites. The cytokines IFN-γ, IL-12 and TNF-α are therefore critical for parasite killing, while IL-4, IL-13, transforming growth factor β (TGF-β) and IL-10 are associated with failure to cure and progressing disease (Cummings et al., 2010). Finally, antigens derived from digested pathogens are presented to T-cells, initiating the adaptive immune response.

In summary, an appropriate activation of macrophages is central to clearance of the parasite and resolution of the infection. Macrophage polarization exists in a spectrum, but the concept of classical versus alternative activation (M1 versus M2) can clearly be correlated with the intracellular fate of leishmania (Liu and Uzonna, 2012), and constitutes a good model to understand the role of macrophage polarization in leishmania infection. Classical activation (M1) is induced by intracellular pathogens, bacterial cell wall components, lipoproteins, and Th1 cytokines such as IFN-γ and TNF-α (Murray et al., 2014). M1 macrophages express iNOS to produce nitric oxide, secrete reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as pro-inflammatory cytokines such as IL-1, TNF-α, and IL-6. In contrast, alternative activation (M2) of macrophages is induced by fungal cells, parasites, immune complexes, complement components, apoptotic cells, IL-4, IL-13, IL-10, TGF-β (Murray et al., 2014). M2 macrophages secrete high amounts of IL-10 and low levels of IL-12. Classical activation is correlated with clearance of leishmania while alternative activation leads to survival of the parasite (Liu and Uzonna, 2012).

1.2.3 Leishmania interfere with macrophage signaling and cellular functions

Leishmania modulates many of the macrophages' cellular functions to its benefit. Following internalization, promastigotes delay the maturation of phagosomes into
phagolysosomes, allowing the transformation of promastigotes into amastigotes which can better sustain the exposure to lysosomal contents and acidic pH (Desjardins and Descoteaux, 1997; Scianimanico et al., 1999). From within the phagolysosomes, leishmania amastigotes prevent the production of ROS, by inducing heme degradation (Pham et al., 2005) and by blocking the assembly of NADPH oxidase at the phagosome membrane (Lodge et al., 2006). Leishmania also prevent the apoptosis of their host cell (Moore and Matlashewski, 1994), through several mechanisms, including the downregulation of programmed cell death-1 (PD-1) (Roy et al., 2017), the induction of suppressors of cytokine signalling (SOCS) proteins (Srivastav et al., 2014) and the regulation of glycogen synthase kinase 3β (GSK-3β) / β-catenin / Forkhead box protein O1 (FOXO-1) axis (Gupta et al., 2016).

To evade killing by macrophages, leishmania must manipulate macrophage activation pathways in a way that favours their survival and proliferation. These parasites have evolved strategies to efficiently evade or subvert macrophage microbicidal mechanisms, through interfering with several signalling pathways.

Leishmania infection induces an imbalance of protein kinase levels (inhibited) relative to protein phosphatases activities (activated) in macrophages, resulting in a dramatic hypophosphorylation of many signalling effectors (Stafford et al., 2002). The host cell protein tyrosine phosphatase (PTP) SHP-1 displays an increased activity in L. donovani infected macrophages, that correlates with reduced response to pro-inflammatory cytokines, decreased nitric oxide production, and prolonged survival of intracellular parasites (Nandan et al., 1999). Moreover, this increase in SHP-1 activity is associated with impaired IFN-γ -triggered Janus kinase 2 (JAK2) activation (Blanchette et al., 1999). A defect in JAK/ signal transducers and activators of transcription (STAT) signalling explains the unresponsiveness of infected cells to...
IFN-γ. A more recent article suggests that the activation of SHP-1 prevents IFN-γ dependent nitric oxide production also through inactivation of extracellular signal-regulated kinase 1/2 (ERK1/2) and prevention of nuclear translocation of nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) (Forget et al., 2006). Other PTPs have been described as playing a role in leishmania pathogenesis, such as PTP1B and TCPTP (Gomez et al., 2009) and the mitogen-activated protein kinase (MAPK)-directed phosphatases MKP1 and MPK3 (Kar et al., 2010).

MAPK signalling is also modulated by leishmania. MAPKs regulate various cellular functions to a variety of extracellular stimuli, including the production of pro-inflammatory cytokines. In murine bone marrow derived macrophages, *L. donovani* prevents the activation of the MAPKs p38, c-Jun N-terminal kinase (JNK), and ERK1/2 (Privé and Descoteaux, 2000). However, in human macrophages, *L. donovani* is reported to modulate the TLR2-stimulated MAPK pathway by repressing p38 and activating ERK1/2 phosphorylation (Chandra and Naik, 2008a). On the other hand, activation of p38 is reported to reduce leishmania survival (Junghae and Raynes, 2002). The modulation of MAPK pathways is linked to the suppression of IL-12 and activation of IL-10, required for parasite survival (Chandra and Naik, 2008b).

Protein kinase C (PKC) activity is particularly important for a number of macrophage-mediated processes, including phagocytosis and microbe killing, *L. donovani* LPG inhibits the activity of PKC isolated from rat brain (McNeely and Turco, 1987). This inhibition of PKC by *L. donovani* and LPG attenuates the oxidative burst and protein phosphorylation (Olivier et al., 1992; Descoteaux et al., 1992), as well as PKC-dependent depolymerization of periphagosomal F-actin, necessary for phagosome maturation (Holm A et al., 2001; Holm et al., 2003).

The PI3K/Akt pathway has also been shown to play a role in leishmania pathogenesis. RAW264.7 macrophages infected with *L. major*, *L. pifanoi* and *L. amazonensis* display a rapid
and pronounced phosphorylation of Akt, that prevents host cell apoptosis (Ruhland et al., 2007). Moreover, activation of PI3K by *L. amazonensis* leads to the inhibition of IL-12 production (Ruhland and Kima, 2009), while the induction of IL-10 production by *L. donovani* is mediated through the PI3K-dependent inactivation of GSK-3β (Nandan et al., 2012).

Altogether, the modulation of these signalling pathways by leishmania in the macrophage results in the inhibition of the induction of inflammatory mediators (TNF-α, IL-12), the induction of the production of IL-10, the prevention of host cell apoptosis, the impairment of oxidative burst, the suppression of iNOS expression and nitric oxide production, the downregulation of various genes, such as major histocompatibility complex (MHC)-I and II, required for mounting the adaptive immunity (Stafford et al., 2002).

### 1.3 Leishmania heat shock proteins and adaptation to stress

During their digenetic lifestyle, leishmania parasites are exposed to and adapt to very different and adverse environments. Harsh environmental conditions they encounter include nutrient deficiency, hypoxia, oxidative stress, changing pH, and shifts in temperature; all potential triggers of cellular stress. These stressful factors may damage cellular structures and interfere with essential functions of the parasite.

The intracellular stage of leishmania, when promastigotes are taken up by macrophages, transform into amastigotes, and survive and proliferate within phagolysosomes, requires from the parasite remarkable adaptation capacities (Naderer and McConville, 2011). Survival and proliferation of leishmania in host macrophages requires the activation of heat-shock responses (Requena et al., 2015), of oxidative and nitrosative defence mechanisms (Van Assche et al., 2011), and uptake and catabolism of essential nutrients (McConville et al., 2015). Heat shock
proteins (HSPs) play a central role in leishmania adaptation, but also differentiation, stage development, and virulence.

1.3.1 Heat shock response and protein quality control

The heat shock response is a highly conserved and organized response to cells stress. A small increase in temperature can cause protein unfolding, entanglement, and non-specific aggregation (Richter et al., 2010). The aggregation of proteins and the resulting imbalance in protein homeostasis can alone explain many of the morphological and phenotypic effects of heat stress. It is believed that the heat shock response is triggered by the detection of the accumulation of unfolded proteins in the cells. Misfolded proteins can accumulate and aggregate together, which is toxic to the cell, as seen in many neurodegenerative diseases such as Alzheimer’s disease, Parkinson's disease, Huntington's disease and Creutzfeldt–Jakob disease (Chaudhuri and Paul, 2006). The most important feature of heat shock response is the production of a group of proteins known as heat-shock proteins (HSPs). These proteins can help protect the cell to survive conditions that would otherwise be lethal. It is therefore crucial for the cell to detect the accumulation of misfolded proteins and activate the heat shock response.

One specific form of cellular stress is endoplasmic reticulum (ER) stress (Galluzzi et al., 2017; Dolai and Adak, 2014). Potential triggers of ER stress include not only shifts in temperature, but also nutrient deficiency, hypoxia, oxidative stress, and changing pH. ER stress is characterized by accumulations of unfolded proteins within the ER lumen caused by perturbations of ER homeostasis. A series of adaptive intracellular signalling pathways are then activated to reduce this stress, termed the unfolded protein response, and involving ER chaperones. Unresolved ER stress leads to the initiation of apoptosis and eventually cell death.

Even without external stress, cells need to ensure that their proteins are properly folded
and functional at the right time and place. The concept called 'protein quality control' includes all the cellular processes that regulate protein synthesis, folding, unfolding and turnover; it is mediated by chaperones and proteases (Chen et al., 2011).

HSPs are defined as a class of highly conserved chaperone proteins that are rapidly induced in response to temperature increases and other environmental stresses, through dedicated heat shock transcription factors. 'Molecular chaperones' refers to any protein that shares the ability to interact with non-native conformations of other proteins: chaperones assist proteins to fold properly during or after synthesis, to refold after partial denaturation, and to translocate to the cellular compartments where they reside and function (Hartl et al., 2011). In case the damaged protein is irreparable, chaperones may direct them to the protein degradation machinery where they are degraded and recycled (Young et al., 2004). Molecular chaperones are grouped in 7 main classes, according to their apparent molecular weight: small HSPs, HSP40, CCT/TriC, type I chaperonins (CPN), HSP70, HSP90 and HSP100.

1.3.2 Heat shock proteins in leishmania

Leishmania are able to sense environmental changes, and trigger stage differentiation that adapts parasite biology to extracellular or intracellular growth. Leishmania HSPs have been linked by pharmacological and genetic analyses to stage differentiation, viability and intracellular survival (Clos and Hombach, 2015). Interestingly, kinetoplastids lack classical mechanisms of transcriptional regulation and, unlike fungal, plant and animal cells, show largely constitutive expression of HSPs in the absence of stress or heat shock (Brandau et al., 1995; Späth et al., 2015). However, a strong induction of HSPs can still be observed in response to the shift in temperature that leishmania experience upon injection into their vertebrate host. Other stresses such as a low pH or nitric oxide also induce a number of HSPs in leishmania, in the
absence of temperature change (Salotra et al., 1994; Adhuna et al., 2000). Importantly, several leishmania HSPs have been shown to participate in leishmania pathogenesis.

A number of leishmania HSPs have been studied specifically, and they all play interesting roles in parasite biology and for some of them in pathogenesis as listed in Table 1.2. These observations were made by knocking-down the gene or overexpressing it in leishmania strains and studying the phenotype of the resulting parasite. Interestingly, knocking down some of the heat shock proteins and molecular chaperones has no effect on resistance to cellular stresses, suggesting a redundancy in function between the various heat shock proteins. *Leishmania donovani* HSP100 shows a strong induction during axenic amastigote differentiation (Krobitsch and Clos, 1999); it is dispensable for axenic growth of promastigotes and amastigotes but essential for normal development of the parasite inside mammalian host cells, thus suggesting an important role for HSP100 in parasite virulence (Hübel et al., 1997). Moreover, HSP100 is thought to play a very relevant function in packaging of exosomes: exosomes from wild type (WT) and HSP100 knockout *L. donovani* displayed very distinct protein cargos (Silverman et al., 2010a). *L. donovani* HSP90 (also called HSP83) plays a role in the control of stage differentiation into amastigotes and in cellular stress response (Wiesgigl and Clos, 2001). HSP70 abundance was shown to correlate with the heat shock response and with resistance to macrophage-induced oxidative stress in *Leishmania infantum* (Miller et al., 2000). The small heat shock protein HSP23 was found to be essential for thermotolerance and intracellular survival of *L. donovani* (Hombach et al., 2014). Finally, CPN60 (or HSP60) showed increased expression during in vitro differentiation into amastigotes (Schlüter et al., 2000, 60). In *Leishmania donovani*, CPN10 has been described as localized in the parasite mitochondrion/kinetoplast complex, like its partner CPN60, and significantly increased during
axenic amastigote differentiation (Zamora-Veyl et al., 2005). Cyclophilin 40, similar to HSP100 is not required for axenic growth, or axenic promastigote-to-amastigote differentiation, but is essential to establish intracellular infection in macrophages (Yau et al., 2014, 2016).

Table 1.2 Leishmania heat shock proteins and their roles.
Adapted from (Requena et al., 2015).

<table>
<thead>
<tr>
<th>HSP</th>
<th>Function</th>
<th>Role in leishmania biology &amp; infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP100</td>
<td>unfolding, stabilisation of aggregates, proteolysis</td>
<td>- dispensable for axenic growth but essential for infection (Hübel et al., 1997, 100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- role in exosomes packaging (Silverman et al., 2010a)</td>
</tr>
<tr>
<td>HSP90</td>
<td>protein maturation of components of cellular signalling pathways</td>
<td>- sensing and response to stress, triggering factor for stage differentiation (Wiesgigl and Clos, 2001)</td>
</tr>
<tr>
<td>(HSP83)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP70</td>
<td>folding of nascent and denatured proteins, translocation across membranes</td>
<td>- induced during heat shock, role in resistance to oxidative stress (Miller et al., 2000)</td>
</tr>
<tr>
<td>HSP60/</td>
<td>protein folding and prevention of aggregation (mitochondrial)</td>
<td>- induced by heat shock &amp; axenic amastigote differentiation (Schlüter et al., 2000; Zamora-Veyl et al., 2005)</td>
</tr>
<tr>
<td>HSP10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCT</td>
<td>folding of cytoskeleton components</td>
<td></td>
</tr>
<tr>
<td>HSP40</td>
<td>HSP70 cochaperone</td>
<td>- increased during amastigote differentiation (Tsiganov et al., 2014)</td>
</tr>
<tr>
<td>cyclophilins</td>
<td>protein folding, protein complex stabilization, protein trafficking</td>
<td>- cyclophilin 40: dispensable for axenic growth but essential for intracellular multiplication (Yau et al., 2014, 2016)</td>
</tr>
<tr>
<td>small HSPs</td>
<td>prevention of aggregation, probable role in membrane homeostasis</td>
<td>- HSP23 induced by stress, essential for stress tolerance (Hombach et al., 2014)</td>
</tr>
</tbody>
</table>

As mentioned earlier, control of leishmania infection relies mostly on chemotherapy, but treatment failure is increasing, due to the emergence of resistance to drugs. Not surprisingly considering their role in resistance to stress, some HSPs have been observed to play a role in resistance to antileishmanial drugs. Antimony resistant *L. tarentolae* and *L. donovani* express much higher levels of HSP70 than WT cells (Brochu et al., 2004; Vacchina et al., 2016). Moreover, when HSP70 was overexpressed in *L. tarentolae*, increased resistance to antimony was observed (Brochu et al., 2004). Increased levels of HSP90 were observed in isolates from patients with antimonial-resistant *L. donovani* infection, as compared to those infected with antimonial-sensitive *L. donovani* (Vergnes et al., 2007). Overexpressing HSP90 in a sensitive strain led to resistance to antimony, as well as to another antileishmanial drug, miltefosine.
(Vergnes et al., 2007). It is thought that high expression of HSP70 and/or HSP90 negatively regulate the mitochondria-dependent apoptotic pathway that antimonials induce in leishmania (Brochu et al., 2004).

1.3.3 Chaperonin 10

Type I chaperonins are one subgroup of molecular chaperones that assist in the folding of polypeptide chains to an active conformation upon synthesis, unfolding or following translocation. They consist of CPN10 and CPN60 which are essential for protein folding under both normal and stressful conditions. They are induced by stress, especially heat shock, where they prevent aggregation by binding non-native proteins, and facilitate the refolding of damaged or misfolded proteins (Boshoff, 2015). They are also sometimes called HSP60 and HSP10, and GroEL and in bacteria. The best characterized chaperonins are the GroEL and GroES of Escherichia coli, and they were used to characterize the structure and mode of action of the GroES/GroEL complex (Xu et al., 1997). The GroE complex consists of 14 GroEL subunits, arranged into two heptametrical rings that each form a cylinder, and a 'lid' formed by the cochaperone GroES, also arranged into a heptameric ring (Figure 1.3a). Unfolded proteins are captured by the hydrophobic amino acids that line the inside of each ring of GroEL (Clare et al., 2012) (Figure 1.3b). Upon ATP binding, the conformation of the GroEL ring changes and allows for the binding of the cochaperonin GroES. This assembly induces a reorganization of GroEL, and the lining of the inside of the rings changes from hydrophobic to hydrophilic. This forces the folding of nonnative proteins with exposed hydrophobic surfaces. The slow hydrolysis of ATP allows time for the correct folding of the encapsulated protein. Another ATP can then bind to the other GroEL ring, which releases GroES from the complex, and the substrate protein into the cell. An estimated 10-15% of all cytoplasmic proteins rely on GroEL to fold correctly under
normal cellular growth conditions, and this increases to 30% under stressful conditions (Ewalt et al., 1997).

Human CPN10 was first described as 'early pregnancy factor', an extracellular protein that appears in pregnant women's sera 48 hours after fertilization and is necessary for embryonic survival (Morton et al., 1977; Fan and Zheng, 1997). It was also described as having immunosuppressive and growth factor properties (Morton et al., 1992). The rosette inhibition test is based on the detection of early pregnancy factor to diagnose pregnancies (Morton et al., 1982). In eukaryotes, CPN10 is classically considered a mitochondrial protein, which is where it plays its role of assisting protein folding with CPN60 (Corrao et al., 2010). However, CPN10 has been found in extra-mitochondrial sites, such as the secretory system and in circulation (Velez-Granell et al., 1994; Sadacharan et al., 2001).

Importantly, CPN10 was shown to have immunosuppressive activity in several models of inflammation and autoimmunity. Treatment with recombinant human CPN10 was shown to
prolong allogeneic skin allograft survival (Morton et al., 2000) and to protect against experimental autoimmune encephalomyelitis (Zhang et al., 2000) and delayed-type hypersensitivity (Zhang et al., 2003). CPN10 from *Mycobacterium tuberculosis* has been shown to attenuate adjuvant arthritis in rats (Ragno et al., 1996; Agnello et al., 2002). Treatment with CPN10 attenuated lipopolysaccharide-induced NF-κB activation and secretion of inflammatory cytokines (Johnson et al., 2005). A recombinant form of CPN10, known as XToll (CBio Ltd) is currently being tested in phase IIa clinical trials of rheumatoid arthritis, psoriasis and multiple sclerosis (van Eden, 2008). However, the immunosuppressive activity of CPN10 may not be conserved across species: HSP10 of *Chlamydia pneumoniae* was reported to induce pro-inflammatory cytokines in the monocytic THP-1 cells (Zhou et al., 2011).

1.4 Micro-RNAs

Micro-RNAs (miRNAs), are small, highly conserved non-coding RNA molecules that play essential roles in the regulation of gene expression. Through a process called RNA interference, miRNA molecules silence gene expression, by neutralizing targeted messenger RNA (mRNA). It is thought that at least 60% of all genes are regulated by miRNAs (Lewis et al., 2005). miRNAs are involved in the regulation of many physiological processes, and a deregulation of their expression can have serious consequences, as was described in cancer, disorders of the cardiovascular and nervous system, and during infection (Li and Kowdley, 2012).

1.4.1 Biogenesis and mode of action

miRNA genes can be found in various genomic contexts. The majority of miRNAs is located in the non-coding regions between genes and transcribed on their own: they are called intergenic miRNAs (Cullen, 2004). Intronic miRNAs are derived from the intron regions of gene
transcripts: they share the same promoter with their host gene, are spliced out of the transcript before being processed into mature miRNAs (Ramalingam et al., 2014). Finally, a small subset of miRNAs are exonic; they are located in noncoding RNA genes of which the only known function is being the miRNA host gene (Slezak-Prochazka et al., 2013). Often, miRNAs are encoded in clusters, with several miRNAs transcribed as polycistrons, and displaying similar expression patterns (Cullen, 2004).

miRNAs are generated through a complex processing as shown in Figure 1.4. The majority of miRNAs is transcribed by RNA Polymerase II (Lee et al., 2004), but a few miRNAs linked to regulating cell cycle and growth are transcribed by RNA Polymerase III (Borchert et al., 2006). The resulting primary miRNA (pri-miRNA) transcript can be more than 1000 nucleotides in length, and contains at least a hairpin loop. It is recognized by the DGCR8 protein, which associates with the ribonuclease III enzyme Drosha, forming the microprocessor complex (Denli et al., 2004). The hairpin is cleaved from the pri-miRNA by the microprocessor complex, forming the precursor miRNA (pre-miRNA), a shorter structure of approximately 70 nucleotides. The pre-miRNA is transported into the cytoplasm by the karyopherin exportin 5 and Ran-GTP complex (Lund et al., 2004). The pre-miRNA is recognized by another ribonuclease III, Dicer, along with TBRP that cuts the loop end, creating a 22 nucleotide duplex (Bernstein et al., 2001; Chendrimada et al., 2005). The duplex is comprised of the paired mature miRNA and its passenger strand. TRBP and Dicer direct the duplex to be incorporated into the RNA-induced silencing complex (RISC), the final effector complex in which catalytic activity is performed by Argonaute 2 (Ago2) (Gregory et al., 2005). Once the miRNA complex is loaded, the passenger strand is degraded.
Mechanisms of biogenesis and function of miRNAs. RNA polymerase II and III transcribe miRNA genes, generating long primary transcripts (pri-miRNAs). Pri-miRNAs are then cleaved by Drosha and DGCR8, yielding a hairpin precursors (pre-miRNA). Pre-miRNAs are exported into the cytosol by Exportin-5 and its partners. Pre-miRNAs are cleaved by Dicer and TRBP into a miRNA duplex. One of the two strands in the duplex is degraded while the other (mature miRNA) is incorporated into the RNA-induced silencing complex (RISC), which regulates protein expression. Reprinted with permission from (Winter and Diederichs, 2011).

Once in the mature RISC complex, the miRNA is guided to its target mRNA, usually recognizing complementary sequences in the 3' untranslated region (3' UTR) of the mRNA (Gregory et al., 2005). The miRNA-mRNA pairing is imperfect in most cases, resulting in translational repression, although the mechanism of how miRNAs interfere with translation is
not completely understood (Valinezhad Orang et al., 2014). In the few cases where miRNA-mRNA pairing is perfect, cleavage of the mRNA occurs (Hutvágner and Zamore, 2002). Due to miRNAs' ability to bind mRNAs that are not perfectly complementary to their sequence, one miRNA can target multiple mRNAs, and in turn one mRNA can be targeted by multiple miRNAs (MacFarlane and Murphy, 2010).

1.4.2 Regulation of miRNA expression

The biogenesis and function of miRNAs themselves are tightly regulated, and their deregulation is often associated with disease, as will be described in the next subsection. miRNA regulation can take place at multiple steps, including transcription, processing into mature miRNAs, and their stability (Ha and Kim, 2014).

Pri-miRNA transcription is controlled by RNA Polymerase II-associated transcription factors and epigenetic regulators. Among the transcription factors that positively or negatively regulate miRNA expression are p53 (Rihani et al., 2015), c-Myc (Chang et al., 2008), Zeb1 and Zeb2 (Burk et al., 2008) and MyoD (Chen et al., 2006). Interestingly, many miRNAs target these transcription factors in turn, creating positive or negative feedback loops (Martinez and Walhout, 2009). For example, members of the miR-200 family target Zeb1 and Zeb2 mRNAs and destabilize them, while Zeb1 and Zeb2 inhibit the transcription of miR-200 miRNAs (Diepenbruck et al., 2017). c-Myc is especially interesting, because unlike other transcription factors, it both induces some miRNAs, and represses others (Psathas and Thomas-Tikhonenko, 2014). Epigenetic control, including DNA methylation and histone modification also contribute to regulating miRNA transcription (Sato et al., 2011).

The proteins involved in miRNA processing, such as Drosha, DGCR8, Dicer and TRBP, can be targeted for miRNA regulation, both in term of expression and activity. For example, a
correct ratio between DGCR8 and Drosha is required, each one of the proteins having a stabilising and controlling action on the other (Han et al., 2009), moreover, a strong upregulation of DGCR8 dramatically inhibits Drosha activity in vitro (Gregory et al., 2004). Similarly, the expression of Dicer depends on its partner TRBP: a defect in TRBP leads to destabilization of Dicer and impaired miRNA processing (Chendrimada et al., 2005). Several cancer types display global decreases in miRNAs that are due to mutations or depletions in the proteins responsible for miRNA processing Drosha, Dicer, or Exportin-5 (Wu et al., 2013; Muralidhar et al., 2007; Melo et al., 2010).

Finally, miRNA expression can be regulated by modulation of miRNA decay. Mature miRNAs persist for many hours or even days after their production is stopped by chemical inhibitors or by depletion of miRNA processing enzymes (Baccarini et al., 2011; Gantier et al., 2011). Individual miRNAs or groups of miRNAs can be subject to accelerated decay, for example as part of cell cycle changes (Rissland et al., 2011), or in response to external factors, such as growth factors (Avraham et al., 2010), or light in retinal cells (Krol et al., 2010). Several miRNA-degrading exoribonucleases have been identified, such as exoribonuclease 1 (XRN-1), ribosomal RNA processing protein 41 (RRP41) and polynucleotide phosphorylase (PNPase) in human cells (Rüegger and Großhans, 2012). Moreover, some viruses use their own RNAs to destabilize host miRNAs through specific base pairing: for example, murine cytomegalovirus produces the m169 RNA that contains miR-27-binding sites in its 3’ UTR and induces the degradation of miR-27 (Libri et al., 2012). Similarly, human cytomegalovirus codes for a short RNA molecule that targets host miRNAs of the miR-17-92 cluster and induces their degradation, which is beneficial for the viral infection (Lee et al., 2013).
1.4.3 miRNAs in infection & disease

Many miRNAs play key roles in physiological cellular processes, such as cell cycle and death (Ng et al., 2012), cell differentiation (Ivey and Srivastava, 2010), intracellular signalling (Zhang et al., 2012), and immunity (Taganov et al., 2006). Therefore, deregulated miRNA expression should have consequences on these critical processes, and lead to various pathological outcomes.

Since the beginning of miRNA research, cancer has proven to be the most prominent of human diseases with a clear role for miRNA deregulation. A striking effect that is reported in many types of cancer is a widespread downregulation of miRNAs that promotes cellular transformation and tumorigenesis (Lu et al., 2005; Chang et al., 2008; Kumar et al., 2007). Several mechanisms have been described, including amplification or deletion of miRNA genes (Calin et al., 2002; Tagawa and Seto, 2005), abnormal transcriptional regulation of miRNAs (Chang et al., 2008), dysregulated epigenetic changes (Weber et al., 2007) and defects in the miRNA biogenesis machinery (Dedes et al., 2011; Melo et al., 2010). miRNA-based anticancer therapies are being developed and tested, either alone or in combination with current targeted therapies (Iorio and Croce, 2012). For example, overexpression of miR-15a and miR-16-1 inhibit the proliferation of leukemic cells (Gao et al., 2011). More striking, the systemic delivery of a single miRNA (miR-26 or miR-375) in a mouse hepatocellular carcinoma model successfully halted disease progression (Kota et al., 2009; He et al., 2012).

Immune-related diseases have allowed for the identification of miRNAs that mediate inflammatory responses, such as miR-155 (Vigorito et al., 2013) and miR-146 (Xu et al., 2012). MiR-155 is induced in macrophages and DCs after Toll-like receptor (TLR) stimulation with lipopolysaccharide (LPS), via both the NF-κB and JNK pathways (O’Connell et al., 2007; Tili et
Targets of miR-155 include genes that play roles in inflammatory activation of DCs such as SOCS1, which negatively regulates the antigen-presenting activity of DCs (Yang et al., 2015b), and TAB2, which is an important signal transduction molecule of the TLR/IL-1 pathway (Ceppi et al., 2009). MiR-146 is also upregulated after LPS stimulation and it represses the expression of IRAK1 and TRAF6, both key elements of the TLR signalling cascades (Taganov et al., 2006). Therefore, miR-146 provides a negative feedback mechanism to attenuate TLR responses and prevent excessive inflammation.

The expression of these miRNAs and others can be modulated during infection with viruses, bacteria, parasites and fungi, as part of the host inflammatory response. On the other hand, the expression of miRNAs can be modulated by pathogens, to prevent anti-microbial responses and facilitate pathogen replication and persistence. For example, miR-155 and miR-146 are also increased in response to infection with several bacteria, such as Salmonella enterica, Helicobacter pylori, Francisella tularensis, Listeria monocytogenes and mycobacteria (Maudet et al., 2014a). Many more miRNAs have been shown to be induced by infection, and some play a role in the anti-bacterial response (Maudet et al., 2014a).

Several viruses are also able to regulate the expression of host miRNAs for their own benefit. For example, Epstein-Barr virus induces miR-155 which promotes viral persistence (Gatto et al., 2008; Lu et al., 2008), while liver-specific miR-122 favours hepatitis C virus replication (Jopling et al., 2005). Other viruses interfere with the host RNA interference pathway: Nodamura virus produces a protein, NoV B2, that functions as an inhibitor of RNA interference by binding to pre-Dicer substrate RNA, leading to inhibition of host miRNA expression (Sullivan and Ganem, 2005).
Table 1.3 Literature review of the effect of leishmania infection on host miRNA in phagocytes

<table>
<thead>
<tr>
<th>Article</th>
<th>Cell type</th>
<th>Leish. species</th>
<th>Method</th>
<th>Deregulated miRNAs</th>
<th>Other observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lemaire et al. Plos NTD 2013</td>
<td>Human monocyte derived MPs</td>
<td>L. major</td>
<td>TaqMan Human MicroRNA Array</td>
<td>64 consistently deregulated (both up- and down-), varies at each timepoint</td>
<td>GO enrichment analysis revealed that several pathways and molecular functions were disturbed</td>
</tr>
<tr>
<td>Geraci et al. Parasite Immunol. 2015</td>
<td>Human monocyte derived DCs, MPs</td>
<td>L. major, L. donovani</td>
<td>Next gen sequencing, qPCR</td>
<td>DC/Lm: 8 up-, 87 down-, DC/Ld: 21 up-, 12 down-, MP/Lm: 9 up-, 19 down-, MP/Ld: 10 up-, 17 down-</td>
<td>Pathway enrichments using in silico predicted gene targets</td>
</tr>
<tr>
<td>Frank et al. Parasites and vectors 2015</td>
<td>BMDMs from BALB/c mice</td>
<td>L. major</td>
<td>Affymetrix chip</td>
<td>26 deregulated miRNAs (both up- and down-)</td>
<td>miRNAs linked to autophagy: miRNAs miR-101c, miR-129-5p, miR-155-5p, and miR-210-5p.</td>
</tr>
<tr>
<td>Singh et al. Autophagy 2016</td>
<td>THP-1 cells</td>
<td>L. donovani</td>
<td>Human miRNA 8´15 k arrays (Agilent Tech)</td>
<td>38 deregulated miRNAs (both up- and down-)</td>
<td>miR-30a-3p inhibitor followed by infection promoted autophagy and decreased the intracellular parasite burden</td>
</tr>
<tr>
<td>Tiwari et al. Front Microbiol. 2017</td>
<td>RAW 264.7</td>
<td>L. donovani</td>
<td>Next gen Sequencing</td>
<td>59 down-, 26 up-</td>
<td>10 miRNAs: regulate MP effector functions (apoptosis inhibition, phagocytosis, drug response, and T cell phenotypic transitions)</td>
</tr>
<tr>
<td>Muxel et al. Scientific Reports 2017</td>
<td>BMDMs from BALB/c mice</td>
<td>L. amazonensis WT, arginase KO</td>
<td>miScript miRNA PCR Array</td>
<td>23 deregulated miRNAs with WT (18 up-, 5 down-)</td>
<td>argKO La: distinct regulation of miRNA profile of infected macrophages (miRNAs mostly down-)</td>
</tr>
</tbody>
</table>

DC; dendritic cells, MP: macrophages, BMDMs: bone marrow-derived macrophages
1.4.4 Host miRNAs in leishmania infection

Several studies have aimed at understanding the effects of leishmania infection on host miRNAs. Table 1.3 lists all the articles looking at miRNA expression in macrophages and DCs infected with leishmania. Various cell types - murine and human, primary and cell lines - were used, as well as various leishmania species. A deregulation in miRNA expression is reported in all the articles, but no specific trend was observed. Moreover, the underlying mechanisms for targeting host cell miRNA expression have yet to be identified.

Lemaire et al. described first the deregulation of host miRNAs in response to *L. major* infection in human monocyte-derived macrophages, showing both up- and down-regulation of miRNAs in infected cells (Lemaire et al., 2013). Another group looked at miRNA expression in *L. major* and *L. donovani* infected monocyte-derived macrophages and DCs: they observed a strong downregulation of miRNAs in DCs infected with *L. major*, while in the other combinations of cells and parasites, the miRNAs were both up- and down-regulated, including in *L. donovani* infected macrophages, which is the system we use in this work (Geraci et al., 2015).

A link between deregulated miRNAs and autophagy in infected cells was studied both in murine and human macrophages. These studies show that several miRNAs linked to autophagy are deregulated in infected cells, and that modulating these miRNAs (miR-30a-3p inhibitor, miR-210-5p inhibitor, miR-101c mimic and miR-129-5p mimic) led to increased autophagy and clearance of the parasites (Frank et al., 2015; Singh et al., 2016). Another study linked deregulated miRNAs to macrophage effector function: miR-3620, miR-6385, miR-6973a, miR-6996, miR-328, miR-8113, miR-3473f, miR-763, miR-6540, and miR-1264 were shown by target gene prediction and biological interaction analysis to be involved in apoptosis inhibition, phagocytosis, drug response, and T cell phenotypic transitions (Tiwari et al., 2017). Muxel et al.
observed that infection with arginase knockout *L. amazonensis* leads to different modulation of host miRNAs, as compared to WT parasites, with more downregulated miRNAs, suggesting that parasite arginase activity alters the regulation of host miRNAs by the parasite (Muxel et al., 2017).

Finally, in a study that did not focus on phagocytes only (therefore not listed in Table 1.3) miR-122 was found to be downregulated in the livers of infected mice, resulting in a reduction of serum cholesterol and increased parasite persistence (Ghosh et al., 2013). This downregulation is due to the reduction of Dicer by GP63. This last work shows that the alteration of a miRNA can lead to a modification of metabolism at the tissue level, and benefit the parasite.

### 1.5 Research questions and objectives

A central aspect of leishmania pathogenesis is the ability of these parasites to manipulate macrophage functions to establish the infection, replicate intracellularly and ultimately cause clinical manifestations. Understanding how leishmania interacts with its host macrophage is necessary to develop new therapies and vaccines. Two aspects of the host-pathogen interaction are studied in this thesis, one on each side of the interaction: the first describing the role of the leishmania protein CPN10 in pathogenesis, and the second focusing on at the alteration of a critical host cell regulatory mechanism, RNA interference, by leishmania.

Heat shock proteins play essential roles in leishmania resistance to stress, amastigote differentiation, and intracellular survival. CPN10 is increased in infection, but the role that it plays in pathogenesis had not been studied. I hypothesized that CPN10 plays a role during leishmania infection, promoting leishmania intracellular survival and fitness. Chapter 2 aimed at understanding whether leishmania CPN10 contributes to leishmania pathogenesis, with a focus
on early stages of infection.

MiRNAs are central to regulating gene expression, and leishmania are known for altering the phenotype of their host cells through multiple mechanisms. I therefore hypothesized that leishmania might alter host miRNA expression, leading to the deregulation of hundreds of target genes. Chapter 3 shows how leishmania strongly modulates host miRNA expression. The mechanism through which leishmania regulates the expression of host miRNAs was also investigated.
Chapter 2: *Leishmania donovani* chaperonin 10 regulates parasite internalization and intracellular survival in human macrophages

2.1 Background

The mechanisms by which leishmania survive within macrophages are beginning to be unraveled. It is believed that leishmania pathogenesis is related to the ability of the parasite to prevent macrophage activation - a necessary step to induce digestion and killing of macrophage prey - by hijacking critical intracellular signaling pathways (Nandan et al., 1999; Junghae and Raynes, 2002; Nandan et al., 2002). Another requirement for leishmania is to withstand the extreme conditions within acidified phagolysosomes (McConville et al., 2007).

Upon transmission from poikilothermic sandfly vector to mammalian hosts, leishmania experience heat shock and must respond accordingly. Heat shock is especially important in parasites such as leishmania because their digenetic life cycle requires adaptation and protection against deleterious effects of higher temperature, as well as other stressors such as hypoxia, nutrient deficiency, low pH and oxidative attack (Pérez-Morales and Espinoza, 2015). Heat shock proteins (HSPs) are central in the cell stress response, in addition to being required for normal homeostasis. The change in temperature that leishmania experience upon introduction into their mammalian hosts is accompanied by strong induction of HSPs (Requena et al., 2015), and several of them have been shown to support leishmania pathogenesis (Requena et al., 2015; Folgueira and Requena, 2007; Krobitsch and Clos, 1999; Wiesgigl and Clos, 2001; Hombach et al., 2014; Miller et al., 2000; Schlüter et al., 2000). Type I chaperonins - CPN60 and CPN10 - are one subgroup of molecular chaperones that are essential for folding of newly synthesized
proteins and in the refolding of denatured proteins, under both normal and stressful conditions. They are induced by stress, especially heat shock, where they contribute to the refolding of damaged or misfolded proteins (Boshoff, 2015).

In humans, CPN10 was first described as early pregnancy factor, an extracellular protein with immunosuppressive and growth factor properties, necessary for embryonic survival and detected in serum of pregnant women by the rosette inhibition test (Morton et al., 1977; Morton, 1998). Thus, it appears that despite the fact that the CPN10/CPN60 complex is found in relative abundance in the mitochondrial matrix of eukaryotic cells, CPN10 is neither exclusively mitochondrial nor even intracellular, and any differences between secreted and intracellular CPN10, and the secretion system involved are not known.

Notably, CPN10 was shown to have immunosuppressive activity in several models of inflammatory responses. In murine macrophages and human peripheral blood mononuclear cells, it attenuated lipopolysaccharide-induced NF-κB activation and secretion of inflammatory cytokines (Johnson et al., 2005). Recombinant CPN10 inhibited inflammatory responses in rats and mice with experimental autoimmune encephalomyelitis as well delayed-type hypersensitivity (Zhang et al., 2000, 2003). CPN10 from Mycobacterium tuberculosis has also been shown to downregulate several animal models of autoimmunity (Ragno et al., 1996; Agnello et al., 2002). A recombinant form of CPN10, known as XToll (CBio Ltd) is currently being tested in phase IIa clinical trials of rheumatoid arthritis, psoriasis and multiple sclerosis (van Eden, 2008). However, these immunosuppressive activities do not appear to be conserved across all species of CPN10. Notably, HSP10 of Chlamydia pneumoniae was found to induce proinflammatory cytokines in THP-1 cells (Zhou et al., 2011).

In leishmania, CPN10 is found primarily in the mitochondrion (Zamora-Veyl et al.,
Its expression is significantly increased upon heat stress and in axenic amastigotes as compared to promastigotes in *L. donovani* (Zamora-Veyl et al., 2005). This suggests a role for CPN10 in the differentiation to the amastigote stage. Moreover, CPN10 was among the proteins identified in the secretome of *leishmania* (Silverman et al., 2008) as well as in *leishmania* exosomes, the latter having been shown to induce a suppressive macrophage phenotype, permissive for infection (Silverman et al., 2010b; a).

Based upon this array of findings supporting immune-modulatory roles for CPN10 in a variety of settings, in the current study, we asked whether *leishmania* CPN10 plays a role in pathogenesis. To study the role of *leishmania* CPN10 in infection, we generated two genetically modified *L. donovani* (Ld) strains expressing more or less of CPN10: a single allele knock-out (CPN10+/− Ld) and a CPN10 overexpressing strain (CPN10+++). Unexpectedly, CPN10+/− Ld showed higher infection rates than wild-type (WT) Ld, while CPN10+++ displayed lower infection rates than the control strain. However, when macrophages were lysed after infection, CPN10+/− Ld were significantly impaired in their survival and recovery, as compared to WT. Quantitative proteomic analyses identified a diverse array of *leishmania* proteins whose expression was positively regulated by CPN10. Conversely, many proteins involved in innate immunity, intracellular trafficking and transport were negatively regulated in macrophages infected with WT *leishmania* as compared with CPN10+/− Ld. These findings which are discussed in detail below validate important roles for CPN10 in *leishmania* pathogenesis and identify it as an attractive target for further study.
2.2 Results

2.2.1 Study of Leishmania chaperonin 10 gene and protein characteristics

*Leishmania donovani* chaperonin 10 is coded by two genes of 303 nucleotides, LdBPK_260590 and LdBPK_266010, that are located on chromosome 26 (Figure 2.1). They are 99.7% identical, with only one of the 303 nucleotides being different: either a C or a T in position four. They are 5000 base pairs apart, located on either side of a hypothetical gene (LDBPK_26600). We used the Basic Local Alignment Search Tool (BLAST) to look for genes in other species that had a similarity with LDBPK_26600, but it did not show any resemblance to a known gene that would allow us to infer the function that it may play in the parasite.

Figure 2.1 CPN10 loci, gene and protein sequences, alignment with other species' CPN10 proteins
Top: the two CPN10 genes (red) are located on chromosome 26 of *Leishmania donovani*. In blue is the unknown gene. Middle: sequence of the two genes and proteins (red highlights the differences between the two). Bottom: Alignment of CPN10 proteins from several *Leishmania* species (LbrM, LmjF, LmxM, LinJ), *Trypanosoma* species (TeCLB, Tb, Tbg, TvY), and human (Hs).
All other leishmania species whose genome has been sequenced have two CPN10 genes, as well as other Trypanosomatids such as *Trypanosoma cruzi* (TcCLB), *Trypanomosa brucei* (Tb) *Trypanosoma brucei gambiense* (Tbg) and *Trypanosoma vivax* (TvY). Two genes from the same species are highly similar together (Figure 2.1).

This extreme similarity of gene sequences translates, at the protein level, into 99% identity between the two proteins, with only one amino acid being different, leucine versus phenylalanine in position two (Figure 2.1). Leucine and phenylalanine are both hydrophobic amino acids, and both L and F are present at this position in chaperonin 10 proteins from other organisms of the Trypanosomatid order. This suggests that the substitution of L for F does not have any consequence for the function of the final protein. CPN10 proteins from leishmania, as well as other Trypanosomatids, and humans were aligned to show the similarities between all CPN10 proteins (Figure 2.1).

![Figure 2.2 Three dimensional model of leishmania and human CPN10](image)

3D models of the CPN10 protein from *Leishmania donovani* and human was made using The Phyre2 web portal for protein modeling, prediction and analysis (Kelley et al., 2015).

It is interesting to note that despite being only 44% identical in sequence, the predicted three-dimensional structure of human and leishmania CPN10 proteins is highly similar (Figure 2.2), showing that the structure of CPN10 is extremely important for its function, and that its function is so essential for all cells that it is under enormous evolutionary pressure to maintain its
2.2.2 Localization of CPN10 in leishmania cells and in infected cells

Leishmania CPN10 was described to be localized in the mitochondrion, where it plays its role in protein folding (Zamora-Veyl et al., 2005). However, these observations were done only in axenic promastigotes and amastigotes. We therefore wondered where leishmania CPN10 was localized within the parasite during infection of human cells. Moreover, CPN10 was identified in the secretome of L. donovani (Silverman et al., 2008) and in the exosomes secreted by L. donovani (Silverman et al., 2010a). We also aimed at determining whether CPN10 could be found in the cytosol of infected macrophages.

Differentiated human THP-1 cells were infected with *L. donovani* for 18 hours, fixed and processed for transmission electron microscopy. The samples were labeled using anti-leishmania CPN10 antibodies and gold-labeled secondary antibodies. Leishmania CPN10 was found in the mitochondrion of the parasite, as was previously described (Figure 2.3 A,B), but also in the cytosol of the parasite, with a certain accumulation at the cell membrane (Figure 2.3 C, black arrow). Finally, in infected cells, leishmania CPN10 was delivered to the cytosol of infected cells (Figure 2.3 C, white arrows). The mechanism of delivery is still not clear, nor is whether the delivery of CPN10 is directed towards any specific component of the host cell.

Immunofluorescence labeling and confocal microscopy were used to confirm these findings: infected THP-1 cells were stained with anti-leishmania CPN10 followed by fluorescent secondary antibody, and DAPI used to stain the nuclei. A very strong CPN10 staining marked the leishmania that has been taken up by the macrophage (Figure 2.3 D), and a more faint diffuse staining was present in the cytosol of the host cell, confirming that leishmania CPN10 is present in the cytosol of the host cell during infection.
Figure 2.3 Leishmania CPN10 is present in the cytosol of the parasite host cell
A-C: PMA-differentiated THP-1 cells were grown on grids, infected for 18 hours with Leishmania donovani. The samples were then fixed, processed by high pressure freezing and embedded to be cut into sections. The sections were labeled using anti-leishmania CPN10 antibodies and secondary antibodies labeled with gold 15 nm particles. The labeled grids were then analyzed by transmission electron microscopy. A: Internalized leishmania within the macrophage cytosol. The squares are respectively close-ups B (right) and C (left). B: "N" is the leishmania nucleus; "m" indicates parts of the mitochondrion that stretches on either side of the nucleus. C: the black arrow indicates the membrane between the leishmania and the host cell cytosol. White arrows point at gold particles labeling leishmania CPN10 in the macrophage's cytosol. D: PMA-differentiated THP-1 cells were grown on coverslips, infected for 18 hours, and fixed. The coverslips were stained with DAPI (top image) and with anti-leishmania CPN10 followed by secondary anti-rabbit Alexa Fluor 594 (bottom image). The white line shows the macrophages' outline, and was drown based on brightfield.
2.2.3 Engineering leishmania for reduced expression and overexpression of CPN10

In order to study the role of leishmania CPN10 in leishmania biology and especially during infection, a gene replacement strategy was designed to knock CPN10 genes out, based on the model to replace both alleles simultaneously as described in (Ommen et al., 2009). As described in 2.2.1, CPN10 in *L. donovani* is encoded by two genes separated by a stretch of 5000 base pairs, in which is found a hypothetical "unknown" gene. Our strategy consisted of replacing the whole section - containing the two CPN10 genes and the unknown gene - with a construct containing the unknown gene and an antibiotic resistance gene (Figure 2.4). By putting back the unknown gene, we made sure that the phenotype we would observe would be strictly due to CPN10, and not this gene. Leishmania are diploid cells, therefore we had to construct two different plasmids for gene replacement- one containing a puromycin resistance gene and one containing a bleomycin resistance gene - to allow for simultaneous replacement of both alleles. This technique is thought to be more time efficient and less prone to induce spontaneous amplification of the targeted gene, which would be highly counter-productive (Ommen et al., 2009). An episomal vector was also constructed, to be used as an addback vector in knockout leishmania.

Leishmania promastigotes were transfected with the constructs using a nucleofector. Three attempts were made to transf ect promastigotes with both constructs in order to obtain a complete removal of CPN10 genes, on both alleles. Unfortunately, no viable cell could be obtained. Several possible explanations exist on why a double knockout could not be obtained: (1) one or the other of the antibiotic resistance gene is not expressed properly, either not expressed at all, or not quickly enough to overcome antibiotic in the environment; (2) CPN10 is essential to leishmania, and therefore a complete knockout cell is not viable. There are several
arguments that support the idea that CPN10 may be essential: no CPN10 knockout in any species was described in the literature, it has a very central role in protein folding, and the protein is highly conserved between species (Zeilstra-Rylls et al., 1991).

It was decided to attempt a partial knockdown of CPN10, meaning using only one construct to remove half of the CPN10 genes. This strategy was successful, and led to the creation of CPN10 knockdown leishmania, called CPN10+/− Ld. The control strain for this CPN10 knockdown, called 'WT Ld', was generated in parallel with CPN10+/− Ld-. They underwent the same transfection procedure, but without DNA. These "mock transfectants" were then always processed in parallel with CPN10+/− Ld, going through the same cloning and equivalent numbers of passages and freezing steps.

It was important to confirm that this partial deletion led to a reduced amount of CPN10 at
all stages of the complex leishmania life cycle, especially since CPN10 is a heat shock protein which expression varies greatly depending on the temperature (Zamora-Veyl et al., 2005). In Wild Type (WT) leishmania, CPN10 was induced up to five fold in promastigotes incubated at 37°C for 3 days, and six fold in axenic amastigotes (incubated at 37°C, in pH 5.5 medium for 3 days), as compared to promastigotes grown at 26°C (Figure 2.5 A, C). CPN10+- Ld expressed approximately 50% of CPN10, as compared to WT Ld, at all cell cycle stage (Figure 2.5 A, C). This makes it a good tool to study the role of CPN10.

**Figure 2.5 Leishmania CPN10 expression in CPN10+- and CPN10++ leishmania**

Expression in promastigotes (P), axenic amastigotes (Ax.A) and heat shock promastigotes (HS.P) of WT Ld (WT), CPN10+- Ld (+/-), empty vector control Ld (Ø) and CPN10+++ Ld (+++). The culture condition determine cell cycle stage of leishmania: promastigotes (P) in pH=7 medium at 26°C for 3 days; axenic amastigotes in pH=5.5 medium at 37°C for 3 days; heat shock promastigotes in pH=7 medium at 37°C for 3 days. A,B: The cells were then lysed and western blotting was performed using anti-CPN10 and anti-tubulin antibodies. C: The relative amount of CPN10 to tubulin was determined by densitometry. Mean and SD of six independent experiments. Unpaired t test, compared to WT at each respective stage (p-values: * < 0.05, ** < 0.01, *** < 0.001).
In parallel, we produced a CPN10 overexpressing Ld using the episomal vector pCL2N, similarly to described in (Schäfer et al., 2014). This vector does not integrate in the genome like the gene replacement constructs but replicates independently, and it usually is present in the cell in many copies. The vector contains an antibiotic resistance gene and a CPN10 gene under the control of a very strong leishmania promoter (LmxCBP2). Its presence in the cell is maintained by keeping the cells under antibiotic pressure (G418 here). The expression of the CPN10 gene was therefore increased in these cells called CPN10+++ Ld (Figure 2.5 B,C). To create a proper control, some leishmania were transfected using a pCL2N vector but without CPN10 gene, these cells were called 'empty vector control'.

2.2.4 Phenotype of CPN10+/− and CPN10++ leishmania, and response to stress

Once the knockdown and overexpressing leishmania strains were created, we aimed at studying their phenotype, such as growth curves, cell morphology, and response to different kinds of stress. This is especially important, as these characteristics will influence the leishmania ability to infect macrophages and survive and grow within its host cell.

Figure 2.6 Growth curve of leishmania promastigotes and axenic amastigotes
A: Promastigotes were set at 5 x 10⁵ cells/ml in pH=7 medium at 26°C. B: Axenic amastigotes were set at 1x10⁶ cells/ml in pH=7 medium at 37°C for 24 hours and then transferred to pH=5.5 medium at 37°C, 5% CO₂. Cells were counted every 24 hours for 6 days. Mean and standard deviation of three independent experiments.
First we looked at their growth curves in culture, as promastigotes and axenic amastigotes. Axenic amastigotes were made by first transferring promastigotes at 1x10⁶ cells/ml to 37°C, still in pH=7 medium for 24 hours, at which point the cells were spun down and resuspended in pH=5.5 medium, at 37°C and 5% CO2. The growth curve of CPN10+/-, empty vector control and CPN10+++ was the same as WT cells, both as promastigotes and axenic amastigotes (Figure 2.6). This suggests that reducing or increasing the amount of CPN10 does not interfere with leishmania growth or ability to transform into amastigote-like form and survive and grow in pH=5.5 medium at 37°C.

![Figure 2.7 Morphology of leishmania cells](image)

**Figure 2.7 Morphology of leishmania cells**

Leishmania were cultured in pH=7 medium at 26° (stationary phase promastigotes), in pH=5.5 medium at 37°C (axenic amastigotes), or in pH=7 medium at 37°C (heat shocked promastigotes). Results are representative of three experiments. Cells were fixed and stained for tubulin (green), leishmania CPN10 (red) and DAPI (blue), and analyzed by confocal microscopy.
Next, we looked at whether the cell morphology might be altered in CPN10+/- and CPN10+++ cells. Cells were grown, differentiated, and fixed on coverslips. They were then stained for tubulin (green), leishmania CPN10 (red) and DAPI (blue). Stationary phase promastigotes were as expected needle-like with long flagella for all strains, cell body length of 5-10 µm (Figure 2.7); however CPN10+/- promastigotes were slightly shorter in length. All axenic amastigotes took on the characteristic morphology of amastigotes: round shaped, without flagella, approximately 5 µm in diameter. Heat shocked promastigotes displayed an intermediary morphology: short, heart-shaped, but still with a flagellum, similar in all strains.

We aimed at studying these leishmania strains in the context of cell stress, which is central to the ability of leishmania to infect and survive within host cells. Once in the macrophage, leishmania need to adapt to higher temperature as well as other stressors such as hypoxia, nutrient deficiency, low pH and oxidative stress (McConville et al., 2007). CPN10's main role is to refold denatured proteins, in particular during heat shock and other cells stress, where it is strongly induced, therefore its lower expression in CPN10+/- might lead to lower resistance to different stressors.

We first tested the ability of the leishmania strains to survive and grow under heat shock: promastigotes were grown at 25°C and 37°C (heat shock). Cells were counted after 3 days. An MTS assay was run in parallel to assess cell viability. The MTS assay is based on the conversion of MTS into soluble formazan by mitochondrial dehydrogenase of viable cells (Berg et al., 1994). Is it interesting to note that the absorbance measured in the cells grown at 37°C is much lower than in the 25°C cells: leishmania metabolism can change greatly, depending on the culture conditions (Saunders et al., 2014). Heat shock led to a lower growth and mitochondrial dehydrogenase activity in all leishmania strains, but we did not see any difference between
CPN10+/−, empty vector control, CPN10+++ and WT Ld (Figure 2.8). This suggests that cutting in half the amount of CPN10 or increasing it did not change the growth rate and viability of leishmania promastigotes.

Figure 2.8 Response of leishmania to heat shock, tunicamycin and H₂O₂-induced stress
A, B: Promastigotes growth in response to heat shock. Cells were seeded at 2x10⁶ cells/ml in pH=7 medium, at 25°C and 37°C. After 3 days, the cells were counted (A) and cell viability was assessed by MTS (B). ANOVA. C, D: Resistance to oxidative stress induced by H₂O₂. Promastigotes were incubated with H₂O₂ for 2 days. Cell viability was assessed using an MTS assay. Data is represented as % of untreated for each leishmania strain. A negative control was obtained by treating cells with Triton X-100. E, F: Resistance to tunicamycin-induced ER stress. Promastigotes were treated with tunicamycin (1, 2, 5 and 10 µg/ml) and counted after 2 days. Tukey's multiple comparisons test. (p-values: * < 0.05, ** < 0.01, *** < 0.001).
We then tested tunicamycin: it induces ER stress and the ER-unfolded protein response. The unfolded protein response triggers a cellular adaptation including an increase in mitochondrial metabolism, and past a threshold can trigger apoptotic pathways (Bravo et al., 2011; Chaudhari et al., 2014). During their digenetic lifestyle, ER stress can be induced in leishmania by many factors: nutrient deficiency, hypoxia, oxidative stress, changing pH and temperature changes (Dolai and Adak, 2014). 10 µg/ml of tunicamycin inhibited WT Ld growth by 50%. The growth of CPN10+/- Ld was approximately half of WT growth at all concentrations used, down to only 20% growth when using 10 µg/ml of tunicamycin. The empty vector control and CPN10+++ strains had a similar response to WT Ld (Figure 2.8 E,F). In conclusion, CPN10+/- leishmania were significantly more sensitive to tunicamycin-induced stress than their WT counterpart. However, it is interesting to note that this higher sensitivity to ER stress we see in CPN10+/- Ld does not alter the resistance of these parasites to heat shock or their ability to transform into axenic amastigotes (Figure 2.6, Figure 2.7).

Figure 2.9 Phosphatidylserine positive cells in CPN10+/- and WT Ld
Log phase promastigotes (Day 2) and stationary phase promastigotes (Day 5) were stained with Annexin-V-FITC and analyzed by flow cytometry to compare their surface exposure of phosphatidylserine. Some day 5 leishmania were heat killed to serve at positive control for staining (65°C for 45 min).
Leishmania are thought to use apoptotic mimicry to get phagocytozed by macrophages without activating their host immune cell. In *L. amazonensis* metacyclic promastigotes, a fraction of the population dies by apoptosis, allowing the viable promastigotes to be internalized in parallel and develop a productive infection in host macrophages (Wanderley et al., 2009). Apoptotic cells expose phosphatidylserine (PS) on their surface, which can be labeled by annexin-V-FITC and analyzed by flow cytometry. Day 2 (log phase) and day 5 (stationary phase) leishmania promastigotes were stained with annexin-V-FITC. As expected, a larger percentage of day 5 leishmania were positive for annexin-V, as compared to day 2: stationary phase leishmania are composed mostly of metacyclic parasites that are the infective stage (Figure 2.9). However, no difference in staining between WT and CPN10+/- Ld was observed.

### 2.2.5 Proteomic profiling of CPN10+/- leishmania

In order to characterize CPN10+/- leishmania further and understand their phenotype, we performed a comparison of proteomes between WT and CPN10+/- Ld, using stable isotope labeling by amino acids in cell culture (SILAC), followed by mass spectrometry. SILAC is a technique of quantitative proteomics based on mass spectrometry that detects differences in protein abundance among samples using metabolic non-radioactive isotopic labeling (Chen et al., 2015). Two cell populations are grown in culture media that are identical except that one of them contains a 'heavy' form of a particular amino acid (\(^{13}\)C\(_6\)-arginine and \(^2\)H\(_4\)-lysine), where the other one has normal isotopic abundance amino acids. The labeled analog and the natural amino acids are incorporated normally into all newly synthesized proteins. After enough cell divisions, all proteins in a cell will be labeled. Metabolic incorporation of the 'heavy' amino acids into the proteins results in a mass shift of the corresponding peptides. This mass shift can be detected by a mass spectrometer. When lysates from both cell populations are combined, the ratio of peak
Figure 2.10 Comparative proteomics of CPN10+/- and WT Ld

CPN10+/- and WT promastigotes were grown for two weeks in light or heavy SILAC medium, respectively. Cells from both cultures were lysed and the lysates were mixed together before being analyzed by mass spectrometry, allowing for quantitative comparison between the two strains.

A: log2 ratio of proteins which were significantly changed between CPN10+/- and WT: a negative value means that the protein was less abundant ("down-regulated") in CPN10+/- Ld, whereas a positive value means that the protein was more abundant ("up-regulated") in CPN10+/- Ld, as compared to WT Ld. B, C: Gene Ontology analysis of significantly changed proteins (Gene Ontology from PantherDB: biological process and protein class). The x axis is the number of proteins more or less abundant in CPN10+/- for each Gene Ontology term.
intensities in the mass spectrum reflects the relative protein abundance.

Three independent experiments were performed and a total of 2886 proteins were identified. The *Leishmania major* Friedlin genome was used as a reference, because it is the best annotated amongst all leishmania genomes thus far. This analysis returned a quantitative ratio for 2307 proteins, comparing its expression in WT and in CPN10+/− leishmania. A Z-test with Bonferroni multiple testing correction led us to identify 123 proteins whose abundance was significantly different in WT and CPN10+/− Ld across all three independent experiments. A majority of these proteins (99 proteins, 80.5%) were less abundant in CPN10+/− Ld as compared to WT Ld (Figure 2.10 A). Among the 123 proteins, 38 were hypothetical unknown proteins, with no known ortholog in any organism, while 84 were known proteins in the database (Table 2.1).

Table 2.1 lists the detail of all the proteins that were significantly changed. CPN10 was one of the significantly less abundant proteins, which confirmed this as a *bona fide* knockdown strain. Importantly, no protein known to play a role in leishmania pathogenesis (gp63, KMP-11 or enolase among others) were found up- or down-regulated in CPN10+/− Ld. Moreover, no other heat shock proteins or proteins involved in unfolded protein response or in heat shock response were found to be increased to compensate for reduced abundance of CPN10. Finally, the abundance of CPN60, the chaperonin that works in a complex with CPN10, was not modified when CPN10 was knocked-down.

**Table 2.1 Comparative proteomics of CPN10+/− Ld and WT Ld.**
The most highly changed proteins (log2 ratio < -0.3 and > 0.3) are listed here. CPN10+/− and WT promastigotes were cultured in light and heavy SILAC medium, and mass spectrometry was performed on a mix of both lysates. Proteins with a negative ratio were less abundant in CPN10+/− Ld, whereas proteins with a positive ratio were more abundant in CPN10+/− Ld, as compared to WT Ld.
<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Protein name – ortholog (Tritrypdb)</th>
<th>log2 ratio</th>
</tr>
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<tr>
<td>LmjF.06.046</td>
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<td>-0.628</td>
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<tr>
<td>Lipid metabolic process</td>
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<td>LmjF.05.018</td>
<td>Dihyrdroloamide branched chain transacylase, putative</td>
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2.2.6 CPN10 modulates the infection rates and parasite load in human macrophages

We then examined the effect of CPN10 knockdown or overexpression on macrophage infection in vitro. Infection experiments were carried on human primary monocyte-derived macrophages (HMDMs) and differentiated THP-1 - a human monocytic cell line. The macrophages were differentiated on coverslips and infected with stationary phase promastigotes for 24 hours. Two multiplicities of infection (MOI) were used, 20:1 and 10:1. The MOI 20:1 led to only slightly higher infection rates than 10:1, which suggests that a MOI of 10:1 is already close to saturating the in vitro model (Figure 2.11).

Figure 2.11 Infection rate and parasite load in human macrophages
A,C: Human monocytes were differentiated on coverslips using GM-CSF for 7 days. B,D: THP-1 cells were differentiated using PMA. The macrophages were infected for 24 hours with WT, CPN10+/-, empty vector control and CPN10+++, stationary phase promastigotes, at a multiplicity of infection (MOI) of 10:1 and 20:1. The coverslips were washed, fixed and mounted on microscopy slides using a DAPI-containing mounting media. Images were taken with a fluorescence microscope, and the percentage of infected cells (infection rate, A & B) and the number of parasites per infected cell (parasite load, C & D) were calculated. Mean and SD (n=3). Paired t-test. (p-values: * < 0.05, ** < 0.01, *** < 0.001).
A distinctly higher infection rate could be seen with CPN10+/- Ld, as compared to WT Ld: at MOI 20:1, 75% of HMDMs were infected with CPN10+/- versus 47% with WT Ld, at MOI 10:1, 64% versus 37% (Figure 2.11 A). A similar effect was observed in THP-1 cells: with MOI 20:1, 56% of macrophages were infected with CPN10+/- versus 38% with WT, and at MOI 10:1, 53% versus 36% (Figure 2.11 B). The number of leishmania per infected cells, called parasite load, was also higher in cells infected with CPN10+/- Ld, as compared to WT Ld, both in HMDMs and THP-1 cells (Figure 2.11C, D).

Concerning the overexpressing strain CPN10+++, it led to lower infection rates than both WT and the empty vector control - this latter did not differ in infection rate or parasite load from WT Ld (Figure 2.11). The infection rate was lower by 10-15% in HMDMs and 10-20% in THP-1, at both MOIs. The parasite load was very slightly decreased in cells infected with CPN10+++, but the difference was so small that it did not appear significant.

These increased infection rate and parasite load in cells infected with CPN10+/- were highly unexpected, similar to a lower infection rate when cells were infected with CPN10++. This suggests a gain-of-function phenotype when CPN10 is knocked-down in leishmania. Looking further, the infection rate at a relatively late timepoint such as this one depends on both the uptake of parasites by macrophages and the intracellular survival of leishmania. We then attempted to look whether the increased infection rate displayed by CPN10+/- Ld was due to increased internalization of leishmania, as compared to WT.

2.2.7 CPN10 modulates the internalization of leishmania

To assess the internalization rate of leishmania by macrophages, differentiated THP-1 cells were infected with stationary phase leishmania, and infection rate and parasite load were monitored at early time of infection: at 2h, 4h, and 6h post-infection.
Figure 2.12 Time-course analysis of leishmania internalization into macrophages
THP-1 cells were differentiated on coverslips using PMA and infected with stationary phase promastigotes (WT, CPN10+/−, emptyVC and CPN10++/+). At 2h, 4h and 6h post-infection, the coverslips were washed, fixed and mounted on microscopy slides with a DAPI-containing mounting media. Images were taken with a fluorescence microscope, and the infection rate (A) and parasite load (B) were calculated. Mean and SD (n=3). Paired t-test. The graphic inserts represent the nonlinear regression (variable slope, four parameters) of the same data (0h to 24h post-infection). The p-values of the comparison of fits are represented (semilog fits). (p-values: * < 0.05, ** < 0.01, *** < 0.001, **** <0.0001).

The infection rate and parasite load were both significantly higher (1.5 fold) at every time point in cells infected with CPN10+/− Ld as compared to WT Ld (Figure 2.12 A,B). A non-linear regression analysis and comparison of fits showed that the rate of internalization was significantly higher in cells infected with CPN10+/− than in cells infected with WT Ld (Figure 2.12 A, B: graphic inserts). CPN10+++ showed a lower progression of infection rate than WT and empty vector control, but not parasite load (Figure 2.12 A,B). Altogether, this suggests that CPN10 restricts the uptake of leishmania by macrophages, explaining the differences in infection rate and parasite load noted previously at 24 h post-infection.

2.2.8 CPN10 is required for intracellular survival

We then looked at whether CPN10 might play a role in leishmania intracellular survival.
PMA-differentiated THP-1 cells were infected with WT, CPN10+/-, empty vector control and CPN10+++ leishmania for 4 hours. At that point, the cells were thoroughly washed to remove any uninternalized parasite, and the cells were incubated for another 20 hours and 44 hours. Coverslips were fixed at 4 hours, 24 hours, and 48 hours, and the number of parasites per 100 macrophages was calculated. The values are represented as part of the initial number of parasites, at 4 hours post-infection, to allow a monitoring of the intracellular survival and/or growth of leishmania (Figure 2.13 A). For WT Ld, the number of intracellular parasites was constant between 4 hours and 24 hours, then increased to 1.5 the initial number at 48 hours post-infection. The same pattern can be observed for empty vector control. In contrast, the number of intracellular CPN10+/- Ld shows a notable and steady decrease down to half its initial numbers at 48 hours post-infection: this shows that CPN10+/- intracellular survival is strongly impaired. On the other hand, the number of intracellular CPN10+++ leishmania increased strongly between 4 and 24 hours post-infection, after which the numbers stayed stable: this suggests that CPN10 overexpression allows the leishmania to begin multiplying intracellularly faster than the empty vector control.

Another method was used to assess intracellular survival of leishmania, paired with recovery of parasites after infection: the parasite rescue and transformation assay (Jain et al., 2012). Briefly, macrophages were infected with leishmania and at different timepoints they were thoroughly washed to remove any uninternalized leishmania. Some samples were incubated further. The macrophages were then lysed with a gentle detergent that does not damage the leishmania. The released leishmania are then allowed to transform back into promastigotes and grow at 26°C for 2 days, at which point they are counted. The cell number reflects then the ability of the leishmania to transform and grow again as promastigotes. Since CPN10+/- Ld
**Figure 2.13 Intracellular survival and recovery of leishmania after infection**

**A:** THP-1 cells were differentiated on coverslips with PMA, and infected with stationary phase promastigotes for 4 hours. The cells were then thoroughly washed to remove unbound leishmania. The coverslips were washed and fixed at 4, 24 and 48 h post-infection, then mounted on slides using DAPI-mounting media. Images were taken with a fluorescence microscope, and leishmania and macrophages were counted to obtain the number of parasites per 100 macrophages under each condition. These numbers are represented as part of initial number for each leishmania strain. Mean and SD (n=3). Two-way ANOVA (black stars are p-value of WT vs CPN10+/-, grey stars are p-value of emptyVC vs CPN10+++). **B:** Parasite rescue and transformation assay: THP-1 cells were infected and lysed at different timepoints to release intracellular leishmania, which were then grown in M199 at 26°C for 48 hours. Recovered leishmania were counted. In parallel, the total number of internalized leishmania was assessed by microscopy as described previously. Represented here is the rescue rate (recovered/internalized ratio) '4h+20h': the cells were infected with leishmania, and at 4h post-infection, uninternalized leishmania were washed away, and the cells were incubated another 20h before cell lysis. '24h': the cells were infected for 24h, washed and lysed. '24h+24h': the cells were infected for 24 hours, then washed away and incubated another 24 hours before lysis. Mean and SD (n=4). Paired t test. (p-values: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001).
started with more internalized parasites inside macrophages before lysis, the counts of recovered leishmania were normalized by dividing them by the number of internalized leishmania at each time point, and this ratio was called parasite rescue rate (Figure 2.13 B). The parasite rescue rate was decreased for CPN10+/- Ld as compared to WT Ld, for all timepoints: when the cells were infected 4 hours, washed thoroughly and incubated another 20 hours ('4h+20h'), the parasite rescue rate of WT was 105, versus only 49 for CPN10+/-, meaning that for every parasite internalized, 105 WT promastigotes were counted 2 days after release, but only 49 when the same procedure was done with CPN10+/- Ld. After 24 hours infection, the parasite rescue rate of WT was 100 versus 55 for CPN10+/-, and after 24 + 24 hours, it was 59 versus 33. In conclusion, CPN10+/- showed significantly impaired intracellular survival compared to WT Ld.

No significant difference was observed in the parasite rescue rate of CPN10+++ and empty vector control, using this method: both were similar to the WT rate at '4h+20h' post-infection (Figure 2.13 B), largely because the very low number of internalized leishmania at this short time of infection led to high variation in parasite rescue rates. With 24 hours of infection, the rescue rate of CPN10+++ was not significantly different from the empty vector control, while at '24h+24h', the rescue rate of CPN10+++ was increased compared to WT but not to empty vector control. Although this method was not as clear-cut as the first one used to assess intracellular survival, this suggests that, CPN10+++ are more successful at intracellular survival than WT Ld, after a long infection time.

Altogether, these results suggest that leishmania CPN10 is required for optimal intracellular survival.

### 2.2.9 Proteomic profiling of infected macrophages

Our results showed so far that CPN10 knockdown increased parasite internalization by
macrophages while also reducing intracellular survival of the leishmania. Together, this suggests that CPN10+/- Ld may interact with macrophages in a different manner than WT, resulting in increased phagocytosis and intracellular killing of parasites.

In an attempt to grasp the molecular basis of this phenotype, the proteome of macrophages infected with CPN10+/- Ld was analyzed and compared to the proteome of macrophages infected with WT Ld. THP-1 cells were grown in heavy and light SILAC medium for at least five doublings, then differentiated using PMA, and infected with leishmania for 24 hours. The 'heavy' THP-1 cells were infected with WT Ld, while the 'heavy' THP-1 cells were infected with CPN10+/- Ld. In order to be able to analyze the proteome only of infected cells, the leishmania were labeled with CFSE before infection. The infected CFSE+ macrophages were sorted out using a cell sorter, combined, and analyzed by mass spectrometry.

Mass spectrometry allowed us to identify 1109 proteins, 999 human proteins, 62 leishmania proteins, and 50 bovine proteins and other contaminants. A z-test was used to identify the 45 proteins that were significantly changed between WT and CPN10+/- infection. The majority of the proteins (37 proteins, 82.2%) was more abundant in CPN10+/- infected cells as compared to WT infected cells (Figure 2.14 A). Table 2.2 lists all the proteins which abundance was significantly different in WT and CPN10+/- infection.

The lists of significantly changed proteins were analyzed for Gene Ontology using InnateDB (Figure 2.14 B,C). The gene ontology terms referring to the most proteins upregulated in CPN10+/- infected cells were 'innate immune response', 'gene expression', 'membrane organization', and 'apoptotic process'. Concerning molecular function, the most common term was 'protein binding' (25 upregulated proteins, 5 downregulated), followed by 'poly(A) RNA binding' (8 upregulated proteins, 6 downregulated).
Figure 2.14 Comparative proteomics of infected macrophages with CPN10+-/- and WT Ld
THP-1 monocyte-like cells were grown in either light or heavy SILAC media for 2 weeks, then differentiated with PMA and infected with CFSE-labeled promastiogtes. 'Light' cells were infected with CPN10+-/- Ld, while 'heavy' cells were infected with WT Ld. 24 h post-infection, the cells were collected and infected CFSE+ macrophages were sorted out with a cell sorter. Lysate from 'Heavy' and 'Light' was combined in equal parts and mass spectrometry was performed on this mixture to allow for quantitative analysis. A z-test was performed to select the proteins significantly changed in all three independent experiments. A: all significantly changed proteins and their log2 ratio; a ratio<0 means the protein is less abundant ("down-regulated") in cells infected with CPN10+-/- Ld, while a ratio>0 means the protein is more abundant ("up-regulated") in cells infected with CPN10+-/-, as compared to cells infected with WT Ld. B: All significantly changed proteins were analyzed for Gene Ontology. The x axis represents the number of proteins more or less abundant in infection with CPN10+-/- Ld as compared to infection with WT Ld, for each term.
Table 2.2 Comparative proteomics of macrophages infected with CPN10+/− Ld or WT Ld.
The log2 ratio of proteins significantly changed across all 3 replicates (adjusted p-value < 0.05) is represented here. A positive log2 ratio means the protein is more abundant in CPN10+/− infected macrophages, if negative the protein is less abundant in CPN10+/− infected macrophages as compared to macrophages infected with WT Ld.

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<td>Heterogeneous nuclear ribonucleoprotein A/B (HNRNPAB)</td>
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<tr>
<td>P49750</td>
<td>YLP motif-containing protein 1 (YLPM1)</td>
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<tr>
<td>O14979</td>
<td>Heterogeneous nuclear ribonucleoprotein D-like (HNRPD1)</td>
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<tr>
<td></td>
<td><strong>METABOLISM</strong></td>
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<tr>
<td>P19440, P36268</td>
<td>Gamma-glutamyltransferase 1 (GGT1), 3P (GGT3P) and 2 (GGT2)</td>
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<tr>
<td>O94903</td>
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<td><strong>Lipid transport and metabolism</strong></td>
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<td>Perilipin 2 (PLIN2) and Canopy 2 homolog (CNPY2)</td>
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<td><strong>Inorganic ion transport and metabolism</strong></td>
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<tr>
<td>P02792</td>
<td>Ferritin, light polypeptide (FTL)</td>
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</table>

### 2.2.10 Effect of recombinant CPN10 (leishmania and human) on infection rate

In order to check whether CPN10 by itself could modulate the infection rate similarly to what is seen with the genetically modified leishmania expressing more or less of CPN10, differentiated THP-1 cells were treated with recombinant leishmania CPN10, and subsequently infected with WT leishmania. After 24 hours, the infection rates were assessed. Human CPN10 was used as a comparison.

Recombinant leishmania CPN10 (rLCPN10) and human CPN10 (rHCPN10) were produced in *E. coli*, purified by immobilized metal affinity chromatography followed by polymyxin columns for endotoxin removal. Figure 2.15 A shows Western blotting of leishmania and human whole cell lysates (WCL) and of the purified recombinant proteins rHCPN10 and rLCPN10. As expected, the recombinant proteins appear slightly bigger than their native counterparts, mostly because of the His tag: leishmania CPN10 has a mass of 10.708 KDa, rLCPN10 is 12.6 KDa, human CPN10 is 10.932 KDa, rHCPN10 is 12.9 KDa.
Figure 2.15 Effect of recombinant leishmania CPN10 and human CPN10 on infection rates.

A: Western blotting for human and leishmania CPN10 on whole cell lysate (WCL) from leishmania cells and THP-1 cells, and recombinant proteins rHCPN10 and rLCPN10.

B: PMA-differentiated THP-1 cells were treated either with free or Profect-delivered rHCPN10 or rLCPN10 (1 and 10 µg/ml) and infected with WT Ld for 24h. Cells were treated with Profect delivery reagent for 2h before infection, following the manufacturer’s indications. Paired t-test. (p-values: * < 0.05).

In absence of the delivery reagent, the infection rate was unchanged when cells were treated with either 1 µg/ml or 10 µg/ml of free protein added to the culture medium (Figure 2.15 B). However, cells treated with 10 µg/ml of rHCPN10 plus Profect showed an increase in infection rate (150% of untreated). Cells treated with 1 µg/ml of rHCPN10 + Profect tend to show the same increase. On the other hand, when treated with either 1 or 10 µg/ml of rLCPN10
plus Profect, cells did not show any change in infection rate. Therefore, recombinant human CPN10 induced an increase in infection rate but recombinant leishmania CPN10 did not alter the infection rate.

### 2.3 Discussion

In this work, we aimed at understanding the role of leishmania CPN10 in the parasite biology, and especially during the infection of human macrophages. Several tools were used, including the generation of CPN10 knock-down and overexpressing leishmania strains, and the expression of recombinant leishmania and human CPN10.

#### 2.3.1 CPN10 knock-down and overexpressing leishmania strains as a tool to study the role of CPN10

To examine the role of CPN10 in pathogenesis, we repeatedly attempted to generate a null mutant of CPN10 in *L. donovani*. This involved a one-step double-allele replacement strategy (Ommen et al., 2009), but no viable strains could be obtained. Based on the fact that CPN10 plays a critical role in protein folding, and that to date, no viable complete knockout of CPN10 has been described in any organism, this suggested that CPN10 is essential in leishmania.

As an alternative strategy, we generated a partial CPN10 knockdown in *L. donovani* (CPN10+/- Ld), via single allele replacement (Figure 2.4, Figure 2.5), and used this to study the role of CPN10 in pathogenesis. Reduced expression of CPN10 did not impair axenic growth, either as promastigotes or amastigotes (Figure 2.6). CPN10+/-Ld promastigotes displayed a normal ability to differentiate into amastigotes under axenic conditions, having similar growth and morphology when compared with WT Ld (Figure 2.6, Figure 2.7). Moreover, CPN10+/-Ld were able to resist heat shock as well as WT organisms (Figure 2.8 A,B). These findings indicate
that reduced amounts of CPN10 are sufficient for axenic growth or for resistance to heat shock.

Resistance to heat stress is known to depend on at least two other chaperones, HSP23 and HSP100 (Hübel et al., 1997; Hombach et al., 2014).

In contrast to their resistance to heat shock, CPN10+-Ld were much more sensitive to tunicamycin-induced stress than WT Ld (Figure 2.8 E). Tunicamycin is an antibiotic that inhibits N-linked glycosylation during glycoprotein synthesis thereby inducing ER stress, the unfolded protein response and apoptosis, as well as increased mitochondrial metabolism. ER stress occurs in response to adverse environmental changes such as shifts in temperature, oxidative stress, changing pH, hypoxia, and nutrient deficiency, all of which leishmania have to confront in order to establish a productive infection. This suggests that even though they can tolerate heat shock, CPN10+-/- Ld are more sensitive to ER stress than WT Ld, underlining the need for leishmania to express WT amounts of CPN10 for optimal fitness.

2.3.2 CPN10+-/- Ld shows a gain-of-function for macrophage uptake and a loss-of-function for intracellular survival.

CPN10+-/- Ld displayed an interesting, novel phenotype of increased uptake by macrophages, but decreased intracellular survival when compared to WT Ld (Figure 2.11, Figure 2.12, Figure 2.13). Leishmania's preferred host cells are macrophages, and leishmania exploit macrophage phagocytosis to enter the cells and replicate within phagolysosomes. Using CPN10+-/- Ld, we found that reducing the amount of CPN10 in leishmania surprisingly led to increased infection rates due to increased parasite internalization (Figure 2.11, Figure 2.12). The mechanism accounting for this is unknown at this point and may very well be multifactorial, but it is interesting to note that macrophages infected with CPN10+-/- Ld demonstrated higher abundances of multiple proteins involved in membrane organization (Figure 2.14 B), perhaps
accounting for the increased uptake of CPN10+/- Ld as compared to WT Ld.

The finding of increased macrophage internalization of CPN10+/- Ld implies that CPN10 normally acts to restrict the rate of uptake of leishmania promastigotes. This seems somewhat paradoxical since leishmania that fail to invade macrophages are quickly eliminated by cytotoxic NK cells, neutrophils and eosinophils in the host (Pimenta et al., 1987). This raises the question of why high levels of CPN10 were maintained evolutionarily in Ld if they act to reduce pathogen internalization.

In considering this apparent paradox, it must be kept in mind that invading macrophages is not all leishmania need to do to establish chronic infection, as they must subsequently survive in phagolysosomes. We examined the ability of CPN10+/- Ld to survive intracellularly and be recovered after lysis of their host cells, and indeed their intracellular survival and recovery were strongly impaired compared to WT Ld (Figure 2.13). Consistent with this, over-expressing CPN10 in Ld, even modestly, led to decreased internalization of leishmania by macrophages, but improved intracellular survival and recovery after 48 hours of infection, as compared to WT Ld (Figure 2.12, Figure 2.13). These findings show clearly that CPN10 is required for intracellular survival, explaining its evolutionary conservation.

An important question that arises here is what advantage if any would there be for leishmania CPN10 restricting parasite internalization. This may relate to the fact that in order to establish a long-term infection, leishmania prefer – as much as possible - to enter macrophages silently without activating them as the latter would promote parasite killing. It is interesting to hypothesize, therefore, that higher amounts of leishmania CPN10 in the cell - by restricting the rate of internalization - would favor "silent" entry, so as not to promote macrophage activation and death of leishmania. This hypothesis is supported by the lower intracellular survival shown
by CPN10+/- Ld in host macrophages (Figure 2.13). Thus, CPN10+/- Ld are internalized more efficiently by macrophages, but also are more readily killed, perhaps related to their propensity to promote greater degrees of macrophage activation. This latter argument is supported further by the upregulation of multiple proteins involved in the innate immune response – which may contribute to parasite killing - in macrophages infected with CPN10+/- Ld as compared to macrophages infected with WT Ld (Table 2.2.).

2.3.3 Comparative, quantitative proteomics provides insight into the novel phenotype of CPN10+/- Ld.

Comparative proteomics of WT and CPN10+/- Ld

Comparative proteomics of WT and CPN10+/- Ld showed clearly that lowering the amount of leishmania CPN10 available in the cell resulted in changed abundance of many leishmania proteins. The majority (80.5%) of leishmania proteins that showed altered abundance were less abundant in CPN10+/- Ld as compared to the parental WT strain (Figure 2.10A). This suggests that a lower pool of CPN10 in leishmania leads to a less efficient protein folding or refolding, resulting in increased degradation of these unfolded proteins and their reduced expression. The importance of CPN10 in protein folding is, therefore, underlined here, revealing that despite normal axenic growth and resistance to heat shock, many proteins are affected by reduced levels of CPN10. Interestingly, although CPN10 was confirmed to be less abundant in CPN10+/- Ld, the same could not be said of CPN60, which is likely to work as a complex with CPN10 in leishmania, similarly to all other species studied. The abundance of CPN60 was indeed unchanged by infection, raising the question on what happens to CPN60 when CPN10 is knock-downed.

Gene ontology analysis revealed that a large number of the proteins which abundance
was lower in CPN10+/− Ld than in WT Ld are involved in metabolic processes and cellular processes (Figure 2.10 B). In particular, multiple proteins involved in oxidation-reduction processes were less abundant in CPN10+/− Ld, with 6 oxidoreductases showing reduced expression compared to WT Ld (Figure 2.10B). A robust system to regulate the cellular redox state is essential for optimal fitness in leishmania, especially in intracellular amastigotes when they are targeted by the macrophage effectors within phagolysosomes. For example, proline oxidase - also called proline dehydrogenase - which was reduced in CPN10+/− Ld (Table 2.1) contributes to the regulation of redox state and oxidative metabolism in another trypanosomatid, Trypanosoma cruzi, and its overexpression led to resistance to hydrogen peroxide (Paes et al., 2013). Also less abundant in CPN10+/− Ld was cytochrome-b5-reductase. Lack of this protein resulted in decreased linoleate synthesis, increased oxidative stress and apoptosis in Leishmania major (Mukherjee et al., 2012). Taken together, these findings suggest that CPN10+/− Ld would not be able to deal with oxidative stress as well as WT Ld, and this is consistent with their lowered resistance to tunicamycin-induced stress (Figure 2.8 E).

We also found that many proteins involved in fatty acid metabolism (Table 2.1) were less abundant in CPN10+/− Ld and reduction of any one or more of these may contribute to its reduced fitness. For example, dihydriolipamide branched chain transacylase is involved in fatty-acyl-coA biosynthesis. This is an important function as acyl-coA dehydrogenase catalyses the initial step of fatty acid beta-oxidation, playing roles both breaking down fatty acids and in energy production. Also reduced in abundance were Acyl carrier protein and C-5 sterol desaturase, two proteins involved in fatty acid biosynthesis and their products are used to form cell and organellar membranes. Notably, the lipid composition of the leishmania plasma membrane plays a role in pathogenesis as well as resistance to drugs (Rakotomanga et al., 2005;
Altered lipid composition of the leishmania plasma membrane might also modify its interactions with the macrophage plasma membrane resulting in the phenotype of higher internalization of CPN10+/− Ld as compared to WT Ld.

Several proteins involved in nucleoside, nucleotide and nucleic acid metabolic processes were also less abundant in CPN10+/− Ld than in WT Ld (Figure 2.10 C). For example, methylthioadenosine phosphorylase was reduced in CPN10+/− Ld (Table 2.1). This is an important drug target, through its key function in purine and polyamine metabolism and the methionine salvage pathway (Bacchi et al., 1991). Together with downregulation of cytidine deaminase which is involved in pyrimidine salvage, this suggests that both purine and pyrimidine metabolism might be impaired in CPN10+/− Ld (Koszalka and Krenitsky, 1986).

Concerning RNA metabolic processes, DNA-directed RNA Pol I, transcription elongation regulator-like protein SPT5, and an ATP-dependent RNA helicase were all reduced in abundance suggesting that transcription may be affected in CPN10+/− Ld (Table 2.1).

The abundance of several protein phosphatases was also reduced in CPN10+/− Ld (Figure 2.10 C). Protein phosphatases allow for the reversible protein phosphorylation that is essential for the regulation of signaling pathways controlling a variety of processes including metabolic pathways, cell-cell communication, cell growth and proliferation and gene transcription. Other proteins involved in protein modifications and transport were also reduced in CPN10+/−. Two peptidases were also strongly downregulated. All these proteins act on other proteins to change their activity, their stability, their localization, thereby playing important roles in many metabolism processes, and essential to cellular homeostasis.

Proteins involved in localization were also less abundant, especially transfer/carrier proteins, membrane traffic proteins and transporters (Figure 2.10 C). The only protein class
which was overall more abundant in CPN10+/-Ld was cytoskeletal proteins, this could be linked to the fact that CPN10+/- promastigotes are slightly shorter than their WT counterpart (Figure 2.7).

Taken together, this proteomic analysis establishes leishmania CPN10 as a pleiotropic regulator of the leishmania proteome. This likely promotes the expression of leishmania effectors and contributes to pathogenesis and warrants further study.

**Comparative proteomics of macrophages infected with WT versus CPN10+/- Ld.**

In order to allow for their survival and propagation within their hosts, leishmania target signaling pathways to inhibit macrophage activation and block the induction of innate and adaptive immunity (Shio et al., 2012). How this is orchestrated is a focus of significant interest. Here, using quantitative proteomics we found that macrophages infected with CPN10+/- Ld – in contrast to WT leishmania - were strongly biased towards the increased abundance of proteins associated with innate immune responses (Figure 2.14 B, Table 2.2). These findings suggest that during infection with WT leishmania expressing WT levels of CPN10, this heat shock protein acts in some fashion to inhibit the expression of critical host defense proteins.

One of the proteins which abundance is most highly increased in CPN10+/-Ld infected cells compared to WT infected cells was CD14, an innate immunity pattern recognition receptor expressed specifically by monocytes and macrophages (Table 2.2). CD14 works in concert with other proteins to recognize a variety of ligands, including potentially leishmania-derived ligands such as Leishmania pifanoi P8 glycolipid complex, which it delivers to TLR4. This in turn promotes MD2 and MyD88-dependent signaling and the production of proinflammatory cytokines (Dogra et al., 2007). Consistent with our finding of enhanced expression of CD14 in CPN10+/- Ld-infected cells, cell surface expression of CD14 was previously found to be
downregulated in monocyte-derived macrophages when infected with *Leishmania major* (Mock et al., 2012). Taken together, our findings suggest that WT levels of CPN10 act to tonically limit CD14 expression thereby restricting the innate immune response.

Like CD14, leukocyte-associated immunoglobulin-like receptors 1 & 2 (LAIR1, LAIR2) were also more abundant in CPN10+/- Ld infected macrophages than in WT infection (Table 2.2). LAIR1 and 2 belong to a family of immune inhibitory receptors that regulate immune system balance. LAIR-1 is a collagen-receptor that inhibits immune cell function, while LAIR-2 is expressed as a soluble receptor able to antagonize the collagen/LAIR-1 inhibitory immune interaction (Lebbink et al., 2008). LAIR-1 and LAIR-2 have 74 to 100% identity, depending on isoforms, which likely explains why mass spectrometry did not allow us to differentiate between them. Nevertheless, it is interesting to speculate that downregulation of the expression of either LAIR1 or LAIR2 or both by leishmania CPN10 may contribute to defective induction of innate or adaptive anti-leishmania immunity.

Endoglin (CD105), also more abundant in macrophages infected with CPN10+/- Ld (Table 2.2), is a transmembrane protein component of the TGF-β receptor complex. It seems to have variable modulatory roles in TGF-β signaling in macrophages: in some cases inhibitory (Lastres et al., 1996) and in others enhancing it (Aristorena et al., 2014). A direct link between endoglin and leishmania infection has not been described, but TGF-β is known to promote parasite survival.

Promyelocytic leukemia protein (PML) is a multifunctional protein that plays critical roles in growth suppression, induction of apoptosis and cellular senescence, but also in innate immunity, potentially through the TLR/NF-kB prosurvival pathway (Lunardi et al., 2011). Here we show that the abundance of PML is higher in macrophages infected with CPN10+/- Ld than
in cells infected with WT Ld (Table 2.2). This may lead to activation of the TLR/NF-kB pathway, which contributes to host defense against leishmania (Cameron et al., 2004), but it could also play a role in inducing apoptosis.

Proteins of the ubiquitin family were also more abundant in CPN10+/− Ld-infected macrophages (Table 2.2). Ubiquitin proteins are central to a variety of cellular processes in which they attach covalently to other proteins thereby changing the stability, activity or localization of their target proteins. Amongst others, protein ubiquitination regulates the activities of toll-like receptors (TLRs), which are targeted by leishmania as a mean to escape the host immune response (Gupta et al., 2014). In this context, it was shown that *L. donovani* upregulates the host deubiquitinating enzyme A20, leading to a drastic reduction of TRAF6 ubiquitination, explaining modulation of the TLR2-mediated signaling pathway in infected macrophages (Srivastav et al., 2012). Ubiquitination is involved in many different pathways, and the increased abundance of ubiquitin in the cell may indicate that protein ubiquitination is a more frequent event in cells infected with CPN10+/− leishmania compared with WT. How this may influence intracellular survival of leishmania is not clear at this stage, but the identification of proteins that undergo changes in ubiquitination seems to be a logical next step.

Other miscellaneous proteins previously linked to the fate of leishmania infection were found to be more abundant in cells infected with CPN10+/−Ld than in WT infected cells. One example is ferritin (Table 2.2), a protein responsible for cytosolic storage of iron in a redox-inert form, decreasing access of the parasite to free iron, which is essential for the intracellular replication of leishmania (Andrews, 2012).

MARCKS, a protein kinase C substrate, was one of the most strongly up-regulated proteins in macrophages infected with CPN10+/− Ld (Table 2.2). MARCKS has been described
as being strongly downregulated in leishmania-infected cells, together with its partner MARCKS-related protein (MRP) (Corradin et al., 1999). Leishmania has been shown to interfere with many host cell signaling pathways, including protein kinase C (PKC)-dependent signaling (Olivier et al., 1992; Moore et al., 1993), a pathway required for optimal macrophage activation (Castrillo et al., 2001). These findings suggest that CPN10+/- Ld may not interfere with host PKC-dependent signaling to the same extent as WT leishmania. This would allow for more effective macrophage activation resulting in decreased intracellular survival of CPN10+/-Ld as we observed (Figure 2.13).

Another protein of interest found to be more abundant in cells infected with CPN10+/-Ld was cystatin, a cysteine protease inhibitor (Table 2.2). Cystatin targets parasite cysteine proteases thought to play a role in infection by directing the immune response towards a Th2 response, favorable to parasite proliferation (Descoteaux, 1998). The ability of leishmania CPN10 to repress macrophage cystatin expression may confer a survival advantage and this is consistent with reduced intracellular survival of CPN10+/- Ld as compared to WT (Figure 2.13). This notion is supported further by the finding that chicken cystatin has been used successfully as a treatment against murine visceral leishmaniasis by activating an NF-kB-mediated proinflammatory responses (Das et al., 2001; Kar et al., 2011).

2.3.4 Recombinant leishmania CPN10 did not decrease the infection rate

During infection, leishmania CPN10 can be found in the cytosol of the host cell (Figure 2.3), suggesting that it might directly interact with some components of the human macrophage, resulting in decreased uptake, as seen with CPN10+++ Ld or WT Ld in comparison to CPN10+/-Ld (Figure 2.12). In an attempt to mimic this delivery of CPN10 protein to the host cell's cytosol, we treated human macrophages with recombinant human (HCPN10) and leishmania CPN10
(LCPN10) before infection with WT Ld. Treatment with HCPN10 increased the infection rate by 50%, while LCPN10 did not change the infection rate at all (Figure 2.15). The increase due to HCPN10 is consistent with its anti-inflammatory properties, suggesting that HCPN10 inhibited the activation of macrophages, thereby allowing leishmania to infect macrophages more readily. The fact that treatment with LCPN10 did not change the infection rate suggests that LCPN10 does not act directly but rather would modify some other factors in the parasite itself to lower the uptake of leishmania (Figure 2.11). However, more experiments would be needed to confirm this observation.

2.3.5 Conclusion

In summary, the results of the present study define novels roles for leishmania CPN10 and identify it as is a novel bona fide virulence factor. CPN10 is shown to both attenuate the rate of leishmania internalization -which may limit the degree of macrophage activation- while at the same time promoting their intracellular survival. Using a systems biology based approach, we show that leishmania CPN10 is a pleiotropic regulator of the leishmania proteome. The abundance of numerous leishmania proteins belonging to diverse classes were found to be positively regulated by CPN10 and any one or more of these candidate effectors may contribute to pathogenesis. Not only did leishmania CPN10 regulate expression of the leishmania proteome, but it also influenced the abundance of a large and diverse group of macrophage proteins in infected cells. In particular, leishmania CPN10 was strongly biased towards downregulation of an interesting array of proteins linked to innate immunity and host defense. This notable and unanticipated property of leishmania CPN10 to reciprocally up regulate candidate leishmania effectors while down regulating various host innate immune effectors places it at a critical nidus at the host-pathogen interface. These findings identify leishmania CPN10 as an interesting
candidate virulence factor. Combined with its probable essentiality, CPN10 also appears to be an attractive drug target and such a notion is supported by the precedent that GroES and GroEL inhibitors have already been identified as potential antibiotics, and tested against a panel of Gram-positive and Gram-negative bacteria (Johnson et al., 2014; Abdeen et al., 2016).

2.4 Material and Methods

2.4.1 Leishmania strains and culture

The *Leishmania donovani* Sudan strain 2S was obtained from Dr. Kwang Poo Chang (Rockefeller University, NY, USA). Promastigotes were routinely cultured in M199 medium (Sigma) with 10% heat inactivated fetal bovine serum (FBS, Gibco), 20 mM HEPES (StemCell), 6 µg/ml hemin (Sigma), 10 µg/ml folic acid (Sigma), 2 mM L-Glutamine (StemCell), 100 U/ml penicillin/streptomycin (StemCell) and 100 mM adenosine (Sigma). This medium will be called later pH=7 medium, as opposed to axenic amastigote medium which pH is 5.5. Promastigotes were incubated at 26°C in closed culture flasks. The leishmania were subcultured every 3 days by diluting them 1:15 in fresh medium for a maximum of 20-25 passages.

The virulence of the leishmania was maintained by regular passage through Syrian Golden hamsters: they were infected using promastigotes, and once the infection was established (manifested by weight loss), the animal was sacrificed, and fresh amastigotes were purified from the spleen. The leishmania were then transformed back into promastigotes by culturing for 7 days at 26°C in promastigotes medium as described above.

Axenic amastigotes were obtained by first incubating stationary phase promastigotes at 1x10^6 cells/ml in complete M199 (pH=7, as described above) at 37°C, 5% CO₂ for 16-18 h. The leishmania were then spun down at 2000 RPM for 10 minutes and resuspended in pH=5.5
medium. This acidic medium contains RPMI-1640 (HyClone) with 25% heat inactivated FBS, 2 mM L-Glutamine, 100 mM adenosine, 100 U/ml penicillin/streptomycin and 20 mM MES buffer (Sigma). The pH of the medium was adjusted to 5.5 using 2N HCl. Axenic amastigotes were cultured in pH=5.5 medium at 37°C, 5% CO₂.

2.4.2 Creation of gene replacement plasmids and episomal vector

The gene replacement plasmids were designed to contain the unknown gene LdBPK_260600 and an antibiotic resistance gene (puromycin or bleomycin) flanked by approximately 1000 base pairs (bp) of (1) the 5' UTR of LdBPK_260610 and (2) the 3' UTR of LdBPK_260590 (Figure 2.4). This allowed the replacement of the whole region and reintroduction of the LdBPK_260600 gene simultaneously.

Genomic DNA was extracted from L. donovani using the Genetra Systems Puregene Tissue Core Kit A (Qiagen). The primers CPN10-P1fwd and CPN10-P2rev were used to amplify a large fragment containing LdBPK_260600, as well as add BamHI and XbaI sites on either side of the fragment. These restriction enzymes were used to digest the fragment which was ligated into a pUC19 vector previously digested with the same enzymes. A fragment of approximately 1000 bp was deleted from this vector using the primers CPN10-P3rev and CPN10-P4fwd, and the pUC-260600 was recircularized by ligation. The UTRs of CPN10 were amplified by PCR: the primers CPN10-P5fwd and CPN10-P6rev amplified CPN10-5'UTR and added EcoRI and KpnI sites to the fragment, while the primers CPN10-P7fwd and CPN10-P8rev amplified CPN10-3'UTR and added Sall and HindIII sites. The fragments were digested with the restriction enzymes listed above and ligated in pUC19 previously digested with the same enzyme pairs. The obtained pUC19-CPN10-5'UTR plasmid was digested with EcoRI and KpnI and the CPN10-5'UTR fragment was ligated into pUC-260600 to obtain pUC19-CPN10-5'UTR-260600.
In a similar way, pUC19-CPN10-3'UTR was digested with SalI and EcoRI and the CPN10-3'UTR was ligated in pUC19-CPN10-5'UTR-260600 to obtain pUC19-CPN10-5'UTR-260600-CPN10-3'UTR. A puromycin resistance gene was digested with KpnI and BamHI out of a pUC-puroAC vector and ligated into the construct to yield pUC19-CPN10-5'UTR-puroAC-260600-CPN10-3'UTR. Similarly, a bleomycin was introduced into the same construct to yield pUC19-CPN10-5'UTR-belO-260600-CPN10-3'UTR.

Primers and sequences:

- CPN10-P1fwd: GGGAAGGATCCGAAACGGGTGGAGAAGAGAC
- CPN10-P2rev: GGGAGTCTAGAGCGACTGGTGCGTCCCCCTC
- CPN10-P3rev: ACATCAATTTCGCCACCAACC
- CPN10-P4fwd: GGCAGGTTGTCGTCGTATG
- CPN10-P5fwd: GGGGAATTCATTCCGGTGGAGAAGAGAC
- CPN10-P6rev: GGGGTACCGAGAAAAAGTTTGAAAGGTAGTCC
- CPN10-P7fwd: GGGGTACGGT GGAGAAAAAGTTTGAAAGGTAGTCC
- CPN10-P8rev: GGGGTACCGAGAAAAAGTTTGAAAGGTAGTCC
- CPN10-P9fwd: GGGGTACCGAGAAAAAGTTTGAAAGGTAGTCC
- CPN10-P10rev: GGGGTACCGAGAAAAAGTTTGAAAGGTAGTCC

PCR amplifications were performed using iProof High Fidelity Master Mix (Bio-Rad).

The isolation and purification of PCR fragments and digestion products were done by running the DNA on a 1% agarose gel and cutting out the band of interest using a gel cutting tip. The DNA was then purified from the gel using Nucleospin Gel and PCR Clean-Up kit, following the manufacturer's instructions (Macherey-Nagel). Restriction digest was performed with the 10X CutSmart Buffer (New England Biolabs) and restriction enzymes (New England Biolabs). Ligation was done using 10X ligation buffer and ligase (New England Biolabs). After overnight incubation, the ligation reaction was used to transform competent DH5α E. coli: the ligation reaction was incubated with the thawing E. coli on ice for 30 minutes, followed by 20 seconds at 42°C (heat shock), and 2 minutes on ice. Warm LB-medium with ampicillin was added to the tubes, which were incubated at 37°C for 1 hour with shaking at 700 RPM. The bacterial
suspension was plated on LB agar with Ampicillin. The plates were incubated overnight at 37°C. In the morning, the number of colonies was counted and a plasmid DNA Mini Prep was performed, followed by restriction digest to confirm the presence of the ligated plasmid.

The plasmids were extracted from transformed E. coli DH5a, and purified using CsCl continuous gradient centrifugation (Sambrook and Russell, 2001). The gene replacement plasmids were linearized by digestion with the restriction enzyme SwaI and digestion fragments were separated by agarose electrophoresis and purified. The targeting fragments were used for transfection of leishmania.

For the episomal vector, the CPN10 open reading frame (LdBPK_260610) was amplified by PCR using primers CPN10-P9fwd and CPN10-P10rev that added KpnI and BglIII sites to either side of the fragment. The PCR fragment was digested with KpnI and BglIII and ligated into pCL2N (Hombach et al., 2014; Schäfer et al., 2014), digested with the same enzymes to yield pCL2N-CPN10.

2.4.3 Leishmania transfection and selection

Log phase promastigotes were transfected with the gene replacement fragments and episomal vector by electroporation with a Nucleofector. Leishmania were spun down at 2500 RPM at 4°C, washed twice in ice-cold PBS, and resuspended in ice-cold PBS at 10x10^6 cells/ml. 400 µl of cell suspension were distributed in eppendorf tubes (1 per transfection sample), and they were spun down in a microcentrifuge for 5 minutes at 4500 RPM, 4°C. All supernatant was removed, and the cell pellets were resuspended in 100 µl Nucleofection solution (Lonza) and placed on ice. 5 µl of linearized DNA or 10 µg of episomal vector DNA was added to the right tubes. No DNA was added to the 'mock' control. The cell suspension was transferred to Electroporation cuvettes. The electroporation was done using the Amaxa Nuclefector (Lonza),
program U-033. The cuvettes were incubated on ice for 10 minutes. 500 µl of complete M199 was added to the cuvettes. The cells were transferred to 10 ml culture flasks containing 10 ml of warm M199 medium. The leishmania were incubated at 26°C for 24 hours. After 24 hours, antibiotics were added to the flasks to apply the selection pressure, depending on the DNA they were given: bleomycin (1 µg/ml), puromycin (10 µg/ml), and G418 (35 µg/ml). After 10 days of antibiotic treatment, the effect of the selection can be seen, starting with the 'mock' transfected cells that were not able to grow.

The expression of CPN10 was tested by Western blotting to confirm the knockdown or overexpression of CPN10. Aliquots of the newly created leishmania were stored in liquid nitrogen, and fresh batches of leishmania were thawed every two months, so the leishmania used in experiments did not exceed 20-25 passages.

The WT leishmania used in all the experiments are the strain used to create CPN10+/-, empty vector control, and CPN10++. They were subjected to the exact same procedure as the other strains: mock transfection, freeze/thaw cycles and in vitro passages.

2.4.4 Purification, culture and differentiation of human cells

The THP-1 monocytic cell line was purchased from ATCC, and cultured in RMPI-1640 supplemented with 10% heat inactivated FBS and 2 mM L-Glutamine (called 'complete RPMI'). THP-1 cells were maintained between 0.2x10^6 and 1x10^6 cells/ml by subculture every 2-3 days. Differentiation into macrophage-like cells was induced by incubation with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) for 16-18 hours. The adherent cells were washed three times with warm Hank's balanced cell solution (HBSS, Sigma). The cells were then rested in supplemented RMPI without PMA for 24 hours.

Buffy coats from healthy donors were obtained from Canadian Blood Services Network
Centre for Applied Development (netCAD). Monocytes were enriched by centrifugation over a Ficoll-Paque gradient (GE Healthcare) followed by adherence on plastic. Buffy coats were distributed in 50 ml tubes and diluted 1:2.5 in sterile PBS containing 2 mM EDTA, and 10 ml of Ficoll was underlayed. The tubes were centrifuged for 20 minutes at 500g at room temperature, without brakes. The lymphocyte layer was collected and washed repeatedly with PBS (3-4 times), decreasing the centrifuge speed at every step, to remove platelets. The cell pellets were then resuspended in warm complete RPMI and transferred to 150 cm² culture flasks (Corning). The cells were incubated for an hour at 37°C, 5% CO₂ to allow monocytes to adhere to the surface of the flasks. The adherent cell layer was washed three times in warm HBSS to remove non-adherent cells. The adherent cells were scraped off using a cell scraper into complete RPMI, and counted using Türk's solution. This cell suspension enriched in monocytes was diluted to 0.75x10⁶ cells/ml in complete RPMI containing 10 ng/ml GM-CSF (StemCell), and distributed in culture plates (2 ml/well in 6-well plate, 1 ml/well in 12-well plates, 0.5 ml/well in 24-well plates). After 3 days incubation at 37°C, 5% CO₂, the cells were washed 3 times with warm HBSS and given fresh complete RPMI containing 10 ng/ml GM-CSF. Three days later, the cells were washed 3 times with warm HBSS and given fresh complete RPMI. After 24 hours rest without GM-CSF, the cells were used for infection.

2.4.5 In vitro infection experiments

Day 5 (stationary phase) promastigotes were counted, washed two times with HBSS, resuspended in HBSS and used to infect human cells at MOIs 10:1 and 20:1. The cells were incubated at 37°C with leishmania for 4 hours or 24 hours, depending on the experiment. They were then either harvested or washed to remove uninternalized leishmania, and incubated for an additional 24 or 48 hours.
Infection rates were assessed using cells grown on coverslips and subsequently infected. At different timepoints, the coverslips were washed three times with PBC and fixed with 2% paraformaldehyde in PBS for 15 minutes at room temperature. The coverslips were washed in PBS and mounted on microscopy slides using ProLong Diamond Antifade Mountant with DAPI (ThermoFischer). Fluorescence microscopy images were taken using an Axioplan II epifluorescence microscope (Zeiss), and AxioCam MRm Camera (Zeiss) and the AxioVision software Version 4.8.2 (Zeiss). At least 10 images were collected for each coverslip, at 40X magnification. DAPI stained the macrophage nuclei and the leishmania nuclei and kinetoplast, which allowed us to count the total number of human cells, the number of infected cells, and the number of leishmania in each image. The infection rate (number of infected cells/total number of cells x 100) and the parasite load (number of parasites / number of infected cells) were calculated on each image, as well as the average of the 10 images.

Human cells were also subjected to parasite rescue and transformation assays (Jain et al., 2012): differentiated macrophages were infected with stationary phase leishmania for 4 or 24 hours, after which the wells were thoroughly washed to remove uninternalized parasites. The plates were then incubated further at 37°C. After 24 or 48 hours, the wells were washed three times with HBSS. The macrophages were lysed by incubating them in 0.01% SDS in HBSS for 30 minutes at 37°C, 5% CO2. The lysis of macrophages was confirmed by visually checking under the microscope. 300 µl of complete M199 was added to each well. The plates were then incubated at 26°C for 2 days to allow the released leishmania to transform and grow as promastigotes. After 2 days, the leishmania were counted.

2.4.6 SDS-PAGE and Western blotting

Anti-leishmania CPN10 antibodies were raised in rabbits by Genemed Synthesis, Inc
(San Antonio, Texas, USA). The CPN10 peptide used as an antigen was H-Cys-Val-Glu-Gly-Glu-Glu-Leu-Phe-Leu-Tyr-Asp-Glu-Ser-Val-Leu-Leu-Gly-Val-Leu-Ser-Ser-OH. IgGs were purified from the serum using Protein A Sepharose. Western blotting was used to confirm that this antibody was specific for leishmania CPN10 and did not recognize human CPN10.

Leishmania were washed two times with cold PBS, and lysed in protein lysis buffer, containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X-100, 1 mM EDTA, 2.5 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin. The samples were incubated on ice for 5 minutes, and 4X Laemmlli loading buffer was added (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% LDS, 0.005% Bromophenol Blue) and the samples were boiled for 7 minutes. The lysates were loaded on 15% Tris-Tricine gel, run at 100V, followed by semi-dry transfer to a PVDF membrane (37 mA for 90 minutes). For the CPN10 Western blotting, the membrane was blocked in 5% milk in TBST0.05% (TBS + 0.05% Tween-20) for one hour at room temperature. The membrane was then incubated with the primary anti-leishmania CPN10 antibody diluted in blocking buffer for 3 hours at room temperature. After three washes in TBST, the membrane was incubated for 1 hour with secondary anti-rabbit Alexa Fluor 700 antibodies (Life Technologies). Concerning the tubulin Western blotting, the membrane was blocked in 5% milk in TBST0.1% (TBS + 0.1% Tween-20) for one hour at room temperature. The membrane was then incubated with the primary anti-alpha tubulin (Sigma, T6074) antibody diluted in blocking buffer for 2 hours at room temperature. After three washes in TBST, the membrane was incubated for 1 hour with secondary anti-mouse Alexa Fluor 700 antibodies (Life Technologies). For both proteins, the membrane was washed again and read with the Odyssey CLx Imaging Systems (LiCor). Densitometry analysis was done using ImageJ.
2.4.7 Immunofluorescence microscopy on infected macrophages

THP-1 cells were differentiated on coverslips using PMA and infected with leishmania promastigotes for 18 hours. The coverslips were washed and fixed as described above (2.4.5). The cells were permeabilized with 0.3% Triton X-100 in PBS for 10 minutes at room temperature, and washed three times in PBS 0.1% Tween-20 (PBST). Blocking was done by incubating for 1 hour in 1% BSA in PBST. The coverslips were incubated overnight with the primary anti-leishmania CPN10 antibody. After three washes in PBST, the coverslips were incubated for 1 hour with anti-rabbit Alexa Fluor 594 (Life Technologies). After another three washes, the coverslips were mounted on microscopy glass slides using ProLong Diamond Antifade Mountant with DAPI (ThermoFisher). The slides were analyzed with a Zeiss LSM 780 confocal microscope (Zeiss). Negative controls of staining were performed using only the secondary antibody on infected cells, and using both primary and secondary antibodies on uninfected control cells. No fluorescence was detected in either control.

2.4.8 Immunofluorescence microscopy on leishmania

Leishmania were counted, washed twice in PBS and resuspended in PBS at 10 x 10^6 cells/ml. 100 µl of leishmania suspension was added on top of sterile coverslips, followed by 400 µl of ice-cold methanol. The coverslips were incubated at room temperature for 15 minutes, washed in PBS + 0.1% Triton X-100, and stored in PBS. The cells were stained for tubulin: blocking in 2% BSA 0.1% Triton X-100 in PBS for 1 hour at room temperature. The primary anti-tubulin antibody was used at 1:1000 in blocking buffer, for 1 hour at room temperature. After three washed in PBS + 0.1% Triton X-100, the coverslips were incubated with the secondary anti-mouse FITC antibody (1:100 in blocking buffer) for 1 hour at room temperature. The coverslips were washed three times in 0.2% Triton X-100 in PBS, followed by once in PBS.
They were then mounted on microscopy slides using DAPI-containing mounting media. Fluorescence microscopy images were taken using an Axioplan II epifluorescence microscope (Zeiss), and AxioCam MRm Camera (Zeiss) and the AxioVision software Version 4.8.2 (Zeiss).

2.4.9 Electron microscopy

Carbon coated 3-mm sapphire discs (Leica) were coated with poly-L-lysine for 30 min, rinsed five times with sterile water, and dried for 1 h at 37 °C. The cells were seeded directly onto the discs: 5 x 10^4 THP-1 cells in complete RPMI with 10 ng/ml PMA were added on each disc, and incubated at 37°C, 5% CO_2 for 16–18 h. The discs were washed three times with HBSS and rested in complete RPMI for 24 h. *Leishmania donovani* stationary phase promastigotes were added at a MOI of 10:1. 18 h post-infection, the cells were fixed with 4% paraformaldehyde in PBS for 30 minutes, and washed three times with PBS.

Samples were high-pressure frozen using a Leica HPM 100 high pressure freezer and hexadecane as a space filler between the sapphire disk and the 100um side of a Leica type A HPF specimen carrier. The samples were freeze-substituted in acetone using a Leica AFS freeze-substitution machine at -90 °C for 3 days, then infiltrated with and low-temperature embedded in UV polymerized HM20 resin at −50 °C. The samples were sectioned with a Leica UC7 ultramicrotome at 70 nm section thickness with sections deposited on 200 mesh formvar coated nickel grids.

The grids were immunolabeled using anti-leishmania CPN10 antibodies. Grids were first incubated in 50 mM NH_4Cl for 30 min, then blocked in PBS with 1% BSA and 0.05% Tween 20 (PBS-BSAT) for 1 h at room temperature. Grids were incubated with anti-leishmania CPN10 antibodies in PBSBSAT for 1 h then washed three times for 5 min each in PBS-BSAT and incubated in secondary anti-rabbit antibody coupled to 15 nm gold particles for 1 h. The grids
were washed for 10 min in PBS with 3% BSA, then 10 min in PBS with 1% BSA, then in PBS for 10 min and finally in distilled water for 10 min. A control using only secondary antibodies was prepared in parallel, as well as staining on uninfected samples, which did not show any labeling after incubation with anti-leishmania CPN10 and secondary antibodies. Grids were then dried and analyzed using a Hitachi H-7600 TEM operating at 80 kV and equipped with an AMT XR50 side mount CCD camera.

2.4.10 Leishmania stable isotope labeling by amino acids in cell culture (SILAC)

Leishmania promastigotes were grown in special SILAC medium, prepared using powdered M199 minus L-arginine and L-lysine with Earle’s salts (Caisson labs), supplemented with 10% heat inactivated dialyzed fetal bovine serum (Gibco), 10 mM HEPES (Stemcell), 6 µg/ml hemin (Sigma), 10 µg/ml folic acid (Sigma–Aldrich), 2 mM L-Glutamine (Stemcell), 100 U/ml penicillin/streptomycin (Stemcell) and 100 mM adenosine (Sigma). This medium was then split into two, and the “light” medium was supplemented with normal isotopic abundance arginine (42 mg/l) and lysine (73 mg/l), while the “heavy” medium was supplemented with $^{13}$C$_6$-arginine (43.5 mg/l) and $^2$H$_4$-lysine (75 mg/l).

Fresh WT and CPN10+/− promastigotes (less than 2 passages) were centrifuged at 2000 RPM for 10 minutes and resuspended in light and heavy SILAC medium, respectively. Selection pressure was maintained on CPN10+/− Ld with 25 µg/ml of puromycin in the media. The leishmania were cultured at 26 °C, diluted 1:10 every 3–4 days, and their growth was monitored. After four doublings, which took about 2 weeks, stationary phase promastigotes were collected, centrifuged, washed three times in PBS, and pellets were frozen at −80 °C.

2.4.11 Macrophage SILAC

The macrophage SILAC media was prepared using RPMI-1640 minus L-Glutamine and
L-Lysine (Caisson labs), supplemented with 10% heat inactivated dialyzed fetal bovine serum (Gibco) and 2mM L-glutamine. This medium was split in two, and the "light" SILAC medium was supplemented with normal isotopic abundance arginine (42 mg/l) and lysine (73 mg/l), while the "heavy" SILAC medium was supplemented with $^{13}$C$_6$-arginine (43.5 mg/l) and $^2$H$_4$-lysine (75 mg/l). Fresh THP-1 cells (less than 2 passages) were centrifuged, split in two, and half was resuspended in "light" medium, while the other half was resuspended in "heavy" medium. The SILAC THP-1 were cultured at 37°C, passaged every 3 or 4 days. Cells were counted every day to monitor growth.

After 2 weeks, during which the cells had gone through at least five doublings, the cells were differentiated using 10 ng/ml of PMA for 16–18 h, before being washed three times with HBSS and rested for 24 h in fresh supplemented SILAC media. For the infection, WT and CPN10+/− Ld were labeled in CFSE. Stationary phase promastigotes were centrifuged, washed two times in PBS, resuspended at $50 \times 10^6$ cells/ml in PBS. CFSE (Life Technologies) was added at 5 µM, and the leishmania were incubated at 37°C for 10 min in the dark. The leishmania were washed two times in large volumes of PBS with 10% FBS, and used to infect the differentiated THP-1 cells (WT Ld for heavy THP-1, and CPN10+/− for light THP-1). After 24 h of infection, the plates were washed two times in PBS, and THP-1 cells were collected using a cell scraper, centrifuged, and resuspended in PBS-2% FBS. Samples were then run on a BD FACSAria cell sorter, and CFSE+ macrophages were collected for each of the two samples. Cells were centrifuged and pellets were frozen at −80 °C.

2.4.12 Mass spectrometry

Cell pellets were lysed in RIPA buffer (1% NP40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris pH8) and proteins were quantified using BCA assay (Pierce). Fifteen
microgram of protein from each label were mixed and run on a short 10% SDS–PAGE gel. Proteins were visualized by colloidal coomassie and digested out of the gel as described. Peptide samples were purified by solid phase extraction on C-18 Stop And Go Extraction (STAGE) Tips, and run on LC-MS/MS (Bruker IMpact II Qtof).

Purified peptides were analyzed using a quadrupole time of flight mass spectrometer (Impact II; Bruker Daltonics) online coupled to an Easy nano LC 1000 HPLC (ThermoFisher Scientific) using a Captive spray nanospray ionization source (Bruker Daltonics) including a 2-cm- long, 100-µm-inner diameter fused silica fritted trap column, 75-µm-inner diameter fused silica analytical column with an integrated spray tip (6–8 µm-diameter opening, pulled on a P-2000 laser puller from Sutter Instruments), fixed on an in-house constructed column heater set at 50°C. The trap column was packed with 5 µm Aqua C-18 beads (Phenomenex) while the analytical column was packed with 1.9 µm-diameter Reprosil-Pur C-18-AQ beads (Dr. Maisch). Buffer A consisted of 0.1% aqueous formic acid, and buffer B consisted of 0.1% formic acid and 80% acetonitrile in water. Samples were resuspended in buffer A and loaded with the same buffer. A standard 120 min run gradient was from 10% B to 35% B over 105 min, then held at 100% B for 15 min. Before each run the trap column was conditioned with 20 µL buffer A, the analytical with 4 µL of the same buffer and the sample loading was set at 5 µL. The LC termostat temperature was set at 7 °C. The analysis was performed at a flow rate of 0.25 µL/min. The Impact II was set to acquire in a data-dependent auto-MS/MS mode with inactive focus fragmenting the 20 most abundant ions (one at the time at 18 Hz rate) after each full-range scan from m/z 200 Th to m/z 2000 Th (at 5 Hz rate). The isolation window for MS/MS was 2–3 Th depending on parent ion mass to charge ratio and the collision energy ranged from 23 to 65 eV depending on ion mass and charge. Parent ions were then excluded from MS/MS for the next 0.4
min and reconsidered if their intensity increased more than five times. Singly charged ions were excluded since in ESI mode peptides usually carry multiple charges. Strict active exclusion was applied. Mass accuracy: error of mass measurement was typically within 5 ppm and was not allowed to exceed 10 ppm. The nano ESI source was operated at 1700 V capillary voltage, 0.20 Bar nano buster pressure, 3 L/min drying gas and 150 °C drying temperature.

2.4.13 Proteomics analysis

Peak lists were created using Compass 1.9 for OTOF version 4.0.15.3248 (Bruker Daltonik GmbH). MaxQuant 1.5.1.0 was used for database searching. Searches were performed against a database comprised the protein sequences from the source organism plus common contaminants using the following parameters: peptide mass accuracy 10 parts per million; fragment mass accuracy 0.05 Da; trypsin enzyme specificity, fixed modifications—carbamidomethyl, variable modifications—methionine oxidation and N-acetyl proteins; allowing for up to two miscleavages. Only those peptides exceeding the individually calculated 99% confidence limit (as opposed to the average limit for the whole experiment) were considered as accurately identified.

The peptides were searched in the following databases: the human database from MaxQuant group, and the Leishmania major Friedlin database (custom—8324 proteins). False positive determination was done by “revert” decoy search; in infected THP-1 cells, 12 decoy proteins were found out of 1121.

The expression of individual proteins between heavy and light was statistically analyzed using z-test on Excel, with p-value threshold of <0.05.

2.4.14 Production of recombinant proteins

The bacterial expression vector for human CPN10 pMCSG7-HSPE1 with His-tag was
obtained from the DNASU plasmid repository (Clone: HsCD00343194).

The leishmania CPN10 gene was cloned into the bacterial expression plasmid pET28a, along with a His tag (Addgene). E. coli were transformed with the plasmids and used to express the recombinant proteins that were then purified on Nickel columns. Endotoxins were removed using endotoxin removal columns, and their levels were tested using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript). The size of the recombinant proteins, and their concentrations were tested before using them to treat human cells.

Total DNA was extracted from L. donovani cells. The CPN10 genes were cloned by PCR using Pfu polymerase (ThermoScientific) and the following primers:

Chap-LeishF 5’-GACCATATGATGTTCCGCTTCACCATCCCCG-3’
Chap-LeishR 5’-GATGCGGCCGCTCAGCTTGACAGCACGCAAG-3’

The PCR fragments were purified using the PCR Clean-up kit (Qiagen). Both PCR fragments and pET-28a vector were digested with the restriction enzymes NdeI and XhoI and NEBuffer 3.1 (New England Biolabs). The digested fragments were run on a 2% agarose gel and extracted using the PCR Clean-Up kit (Qiagen). The fragments were ligated together using 10X ligation buffer and ligase (New England Biolabs). Competent DH5α E. coli were transformed with the ligation reactions by adding the DNA on top of frozen bacteria. The reactions were then incubated for 30 minutes on ice, followed by 20 seconds at 42°C (heat shock), and 2 minutes on ice. 950 µl of warm LB-medium with Kanamycin was added, and the bacteria were incubated for 1 hour at 37°C, with shaking at 700 RPM. 100 µl of the cell suspension was plated on a Kanamycin-LB agar plates. These plates were incubated overnight at 37°C and colonies were counted and a colony PCR was done on several colonies: a PCR master mix with specific primers for CPN10 was prepared. Each single colony was numbered, picked from the agar plate with a pipet tip, used for a streak on a new Kanamycin-LB agar plate and dipped into a tube
containing the PCR mix. The PCR was run on a thermo cycler, after which the samples were run on a 2% agarose gel to identify the colonies that had internalized the vector. One or two of these colonies were collected from the streak plate (after overnight incubation), and used for DNA MaxiPrep (Qiagen). The vectors were sequenced to confirm the proper introduction of the gene into the plasmid and the absence of mismatch.

BL21 *E. coli* were used for protein expression. They were transformed with pMCSG7-HCPN10 and pET28-LCPN10, with the same protocol as described previously for DH5α cells. After overnight growth, colonies of BL21/plasmid were used to inoculate 4 tubes of 20 ml of LB with ampicillin (HCPN10) or kanamycin (LCPN10) which were incubated overnight at 37°C, shaking at 700 RPM. The next day, the 20 ml of overnight culture were diluted in 750 ml of fresh LB with antibiotics. The bacteria were grown at 30°C until OD 600 was 0.5-0.8. 0.5 mM of IPTG was added, and the bacteria were incubated at 18°C overnight. The cells were centrifuged at 5000 RPM for 5 minutes and washed with PBS. The pellets were resuspended with 5 ml of lysis buffer per gram of cells (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, 0.0005% Tween 20, 0.01% PMSF, pH adjusted to 8). Lysozyme was added to the lysis buffer, to a final concentration of 1 mg/ml. The cell suspensions were incubated on ice for 20 minutes, sonicated on ice (power 6, 6 times, 20 seconds, rest for 20 seconds), and centrifuged at 10000 RPM for 20 minutes. The supernatant containing the solubilized protein was collected.

His tag purification was performed using Nickel-NTA slurry on columns (ThermoScientific). The slurry was washed with 30 column volumes (CV) of lysis buffer. The bacterial supernatant was loaded slowly onto the columns. The columns were washed with 10 CV of lysis buffer, 1 CV at a time. The columns were washed with 4 CV of Wash buffer II (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 150 mM Imidazole, 0.01% PMSF). 6 CV of elution buffer I were
added (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM Imidazole, 0.01% PMSF). 6 fractions were collected, each 1 CV. 6 CV of elution buffer II were added (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 500 mM Imidazole, 0.01% PMSF). 6 fractions were collected, each 1 CV. A sample from each fraction was mixed with Western blotting loading buffer, boiled for 7 minutes, and run on a 15% acrylamide gel, followed by Coomassie staining. This allowed us to identify the fractions in which the protein was contained. The fractions were stored at 4°C.

The fractions containing CPN10 were combined and subjected to dialysis and desalting to concentrate the protein samples. The fractions were loaded in Spectra/Por Dialysis Membranes (Spectrum Labs), and incubated overnight with slow stirring at 4°C in protein buffer (PBS with 5% glycerol). In the morning, the buffer was replaced with fresh protein buffer, and incubated another 2-3h. The sample was collected.

Detoxigel columns (ThermoScientific) were used to remove endotoxins, following the manufacturer’s recommendations. The level of endotoxin was measured using a LAL Endotoxin Detection Assay (Lonza). The samples contained 0.14 and 0.16 EU/mg of protein for rLCPN10 and rHCPN10, respectively. Of note, the acceptable endotoxin level for commercially available recombinant protein is 0.1 EU/µg of protein (R&D and Biolegend for example), which is at least three orders of magnitude higher than in our recombinant proteins. The amount of protein in the samples was measured with the absorbance at 280 nm using a Nanodrop and the following formula:

\[
\text{[protein]} \text{in } M = \frac{\text{Abs 280 nm}}{\text{extinction coeff} \times \text{path length in cm}}
\]

\[
\text{[protein]} \text{ in } M \times MW = \frac{\text{[protein]} \text{ in mg/ml}}{\text{LCPN10 Extinction coefficient} = 8480 \text{ liter/mol.cm}}
\]

LCPN10 MW = 11530.3
Profect delivery reagent (Targeting Systems) was used to deliver the recombinant proteins to THP-1 cells grown on coverslips in 24-well plates. A transfection mix containing the recombinant protein, the Profect reagent (2 µl), a serum-free DMEM (50 µl) was prepared, vortexed, and incubated at room temperature for 20 minutes. 200 µl of DMEM was added to the transfection mix. The serum-containing culture medium was removed for the wells of the culture plate, the cells were washed with serum-free medium. 200 µl of transfection mix was added to the wells. The plates were incubated at 37°C for 2 hours, and 200 µl of complete RPMI was added to each well.

2.4.15 Statistical analysis

Most statistical analyses were performed using Prism 6.0 (GraphPad): t-tests, Tukey’s, and nonlinear regression followed by comparison of fits. Excel 2011 (Microsoft) was used for the z-tests of proteomics data. Data are presented as mean +/- standard deviation. The p-values are represented as stars: (* < 0.05, ** < 0.01, *** < 0.001, **** <0.0001).
Chapter 3: Leishmania hijacks c-Myc to act as a virulence factor by proxy, repressing host miRNAs

3.1 Background

How leishmania infection modifies macrophage gene expression and phenotype is a focus of considerable interest and one possibility to consider is through targeting of host miRNAs. MiRNAs are small non-coding RNAs that play key roles in the regulation of gene expression at the post-transcriptional level (Kim, 2005). Given the ubiquitous importance of miRNAs in regulating gene expression, targeting of the host miRNA machinery by leishmania could constitute a powerful mechanism for modifying the abundance of critical proteins and host cell phenotype. By disrupting host cell miRNA expression, leishmania could act on many target genes simultaneously.

Indeed, a number of pathogens have been found to modify host cell miRNA expression including: *Salmonella enterica* (Maudet et al., 2014b). *Listeria monocytogenes* (Schnitger et al., 2011; Izar et al., 2012), *Helicobacter pylori* and *Mycobacterium* (Fehri et al., 2010; Koch et al., 2012; Xiao et al., 2009; Ghorpade et al., 2012). A number of reports have aimed at studying alterations of host miRNAs during leishmania infection (Geraci et al., 2015; Lemaire et al., 2013; Frank et al., 2015; Tiwari et al., 2017; Singh et al., 2016). All show that Leishmania infection alters host miRNA expression, using different species and cell types, no specific trend has been identified. These reports are of considerable interest, but the underlying mechanisms for targeting host cell miRNA expression have yet to be identified.

The expression of miRNAs can be regulated at multiple levels. At the transcriptional level, transcription factors and DNA methylation can induce or repress pri-miRNAs synthesis
(Suzuki et al., 2012; Wang et al., 2011). At the post-transcriptional level, regulation can take place through targeting the proteins involved in miRNA biogenesis (Wang et al., 2013; Poursadegh Zonouzi et al., 2015; Sellier et al., 2013). Finally, the stability of mature miRNAs can be altered (Winter and Diederichs, 2011).

c-Myc is a transcription factor that regulates many essential cellular processes, including cell cycle, cell growth and differentiation, metabolism, protein synthesis, cell adhesion, migration and apoptosis (Meyer and Penn, 2008). It is a proto-oncogene that participates in the genesis of many human cancers (Dang, 2012). c-Myc can act both as a transcriptional activator, as a heterodimer with its partner MAX, and as a transcriptional repressor, although the mechanisms of transcriptional repression is still not entirely understood and may be indirect (Wanzel et al., 2003). c-Myc regulates the expression of thousands of genes including a large number of miRNAs, which can be either induced or repressed by c-Myc (Tao et al., 2014). Finally, c-Myc was shown to be upregulated in cells infected with *Toxoplasma gondii*, mycobacteria, *Salmonella* typhimurium and *Theileria* parasites (Yim et al., 2011; Franco et al., 2014; Seong et al., 2009; Dessauge et al., 2005)

To understand whether leishmania interferes with its host miRNAs, miRNA expression profiling was used to define the effects of *Leishmania donovani* infection on the expression of human macrophage miRNAs and to identify the underlying mechanisms involved. Infection of HMDMs with *L. donovani* brought about the downregulation of a group of 19 host miRNAs that are widely distributed throughout the genome. Eleven of these had been shown previously to be negatively regulated by the transcription factor and oncogene c-Myc. Indeed, we found that repression of the group of 19 miRNAs occurred at the level of miRNA gene transcription and was reversed by c-Myc silencing. The expression of c-Myc itself was strongly upregulated in
infected cells and silencing of c-Myc also brought about a dramatic reduction in the intracellular survival of leishmania. Taken together, these findings identify c-Myc as novel, essential virulence factor by proxy that acts to bring about a global, genome-wide repression of host miRNAs, thereby promoting pathogen survival.

3.1.1 Host miRNAs are largely downregulated in leishmania-infected macrophages

Ulrike Lambertz initiated this project, and generated the first results, presented in this following subsection and in Figure 3.1. The experiments in my project are based on the observations she made.

The expression of a large number of miRNAs in the context of leishmania infection was assessed using the Nanostring nCounter Human v2 miRNA Expression Assay. Human monocytes-derived macrophages were infected with *L. donovani* at a multiplicity of infection (MOI) of 20:1 for 24 hours, 48 hours and 72 hours. Uninfected cells at each timepoint were used as controls. 800 miRNAs are available for detection in this assay. After background subtraction and normalization of the data, 46 miRNAs (the group of 46) were reproducibly detected (average > 50 counts) in the samples (Figure 3.1 A). A large majority of these miRNAs (35/46) appeared to be downregulated in infected cells, as compared to uninfected cells (Figure 3.1 A, B). The remaining 11 miRNAs did not display a consistent expression in the three donors. Statistical analysis revealed that the expression of 19 miRNAs (the group of 19) were significantly downregulated at one or more of the infection time frames (Figure 3.1 C). These results show that the expression of host miRNAs is broadly downregulated in the context of *L. donovani* infection.
Figure 3.1 miRNA expression profiles of uninfected and L. donovani infected human macrophages.
A, B, C: Human monocytes were purified from buffy coats of three healthy donors, differentiated into macrophages, and infected with L. donovani strain 1S stationary phase promastigotes (Ld) at MOI 20:1, or left uninfected (control). Time points were taken at 24, 48 and 72 h post infection; RNA was extracted and sent to Nanostring for miRNA expression analysis. A. Heat map (agglomerative cluster) showing z-scores of all 46 expressed miRNAs. B. Log2 ratios of all 46 expressed miRNAs in infected over uninfected cells at different time points, miRNA expression values were averaged for the three donors. C. Log2 ratios of significantly changed miRNAs in infected over uninfected cells at different time points (all 19 downregulated). Asterisk indicates p < 0.05. D, E: Infected or uninfected monocyte-derived macrophages were lysed, RNA was extracted, reverse transcribed into cDNA and amplified by qPCR using primers specific for mature miRNAs. Relative quantification was done using the small nuclear RNA U6 as a reference gene. Data are expressed as log2 of fold change, compared to control cells. One way ANOVA (p-values: * < 0.05, ** < 0.01, **** <0.0001). E: correlation between qRT-PCR and Nanostring values. Pearson correlation.
In order to validate the Nanostring results, six miRNAs from the group of 19 were selected from distinct regions of the genome and their expression was tested by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The 72 h timepoint was not included in this assay, since the Nanostring results showed that the expression of miRNAs was much less consistent across donors at this timepoint, qPCR analysis (Figure 3.1 D) confirmed the downregulation of miRNAs observed previously. The Nanostring results and RT-qPCR data were clearly tightly correlated (Figure 3.1 E).

Based on these observations, I aimed at understanding the mechanism that brings about this broad-based repression of host miRNAs.

3.2 Results

3.2.1 RNA profiles of infected and uninfected macrophages

The downregulation of many miRNAs that was observed in infected macrophages might have been due to a reduction or loss of all small RNAs in infected cells - or during the extraction. The RNA was extracted with a kit from Exiqon that preserves small RNA, unlike total RNA extraction kits (such as the one that was used for primary and precursor miRNA analysis). RNA purified from control and infected cells, using both RNA extraction kits, was analyzed using an Agilent Bioanalyzer. This instrument allows for sizing, quantification and quality control of RNA samples.

This analysis showed that despite the differences in the distributions of RNA sizes after infection, which was expected to some extent, there was no detectable downregulation of all small RNAs in infected cells (Figure 3.2). Moreover, these results confirm that the small RNA extraction kit does preserve small RNAs better than the total RNA extraction kit, making it ideal
for studying mature miRNAs.

**Figure 3.2 Profiles of total RNA/small RNA in infected cells versus uninfected cells.**
Human monocytes were differentiated with GM-CSF for 7 days, and infected with day 5 Leishmania donovani promastigotes. C is control cells, Ld is infected cells. Arrows indicate 28S and 18S ribosomal RNAs, as well as small RNAs. RNA was extracted with the Exiqon kit (small RNA enriched) and with the ThermoScientific kit (total RNA) and were run on an Agilent Bioanalyzer to compare the distribution of RNA sizes. The small RNA enriched samples were used in all the experiments looking at mature miRNAs, while total RNA was used when looking at pri-miRNAs and pre-miRNAs.

### 3.2.2 Phagocytosis of beads did not lead to downregulation of miRNAs

Leishmania enter host cells by phagocytosis, which in itself can trigger many changes in macrophage biology. In order to establish that the downregulation of miRNAs we observed was due to infection and not phagocytosis per se, cells were given inert latex beads and the levels of five miRNA were tested by RT-qPCR. Latex beads are routinely used as control of phagocytosis in articles studying leishmania infection (Fernandes et al., 2016; Forestier et al., 2011). In the following experiments, three or four of these five miRNAs were selected randomly to be tested. We did not test all five miRNAs and later omitted some of them in the figure: all the tested
miRNAs in a given experiment are presented in the figure.

Latex beads were readily taken up by macrophages (Figure 3.3 A, B). However, the set of miRNAs tested was unchanged, suggesting that miRNAs were not downregulated by the process of phagocytosis (Figure 3.3 C).

**Figure 3.3 Beads phagocytosis in HMDMs and miRNA expression**

HMDMs were given 2 µm inert latex beads as a control for phagocytosis, for 24 and 48h. **A:** Microscopy image showing the uptake of beads by macrophages **B:** phagocytosis rate (% of cells that took up beads) is the same as the infection rate with leishmania. **C:** RNA was extracted and reverse-transcribed. The expression of 5 miRNAs was analyzed by qPCR (log2 of the ratio between treated and control cells). No statistical differences between control and treated cells were observed.

### 3.2.3 Characteristics of downregulated miRNAs

The specificities of the 19 downregulated miRNAs identified with Nanostring are listed in Table 3.1. The Group of 19 miRNAs downregulated in leishmania-infected HMDMs. Further observation revealed that the downregulated miRNAs came from 11 different chromosomes, and
17 different loci (miR-23 and miR-93 are from the same cluster - cluster 9; let-7a-3 and let-7b are from cluster 3) (Table 3.1). Moreover, all types of miRNAs were represented: there were 36% intergenic miRNAs, 52% intronic and 10 exonic, this distribution being close to the general distribution of all miRNAs - approximately 40% intergenic, 40% introns, 10% exons, and 10% 'mixed' miRNAs that can be assigned to either intronic or exonic depending on the alternative splicing patterns. This reflects a genome-wide downregulation rather than being specific to a family of miRNAs, a cluster or a chromosome region.

**Table 3.1 The Group of 19 miRNAs downregulated in leishmania-infected HMDMs.**

In bold are the miRNAs selected for confirmation by qPCR and further study.

<table>
<thead>
<tr>
<th>ID</th>
<th>Accession</th>
<th>Genomic location</th>
<th>Type</th>
<th>Host gene</th>
<th>Cluster</th>
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<tr>
<td>hsa-mir-34a-5p</td>
<td>M10000268</td>
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<td>DALRD3,NDUFAF3</td>
<td># 35</td>
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</tr>
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<td>hsa-mir-378a-3p</td>
<td>M10000786</td>
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<td>M10000082</td>
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<td>MCM7</td>
<td># 9</td>
</tr>
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<td>Intron</td>
<td>MCM7</td>
<td># 9</td>
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</tr>
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<tr>
<td>hsa-let-7b-5p</td>
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<td>MIRLET7B</td>
<td># 3</td>
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<tr>
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</tbody>
</table>

3.2.4 The expression of proteins involved in miRNA biogenesis was not affected by leishmania infection, except for Drosha which was upregulated

Having confirmed that the downregulation of miRNA expression that we observed in
infected cells was not due to a reduction of all small RNAs (Figure 3.2) or induced in response to phagocytosis and not specific to leishmania infection (Figure 3.3), we aimed at understanding how this downregulation takes place.

**Figure 3.4 Expression of proteins involved in miRNA biogenesis.**

A: miRNA biogenesis scheme and its main components. B: Human monocyte-derived macrophages were infected with leishmania (Ld) or not (C), lysed and processed for western blot of Drosha, DGCR8, Dicer, TRBP and Ago2 (these western blots are representative of all 4 donors). C: densitometry allowed the measurement of fold change between infected and control cells at 24 and 48 hours post-infection (n=4 donors).

The findings that the downregulated miRNAs were very diverse in terms of chromosome localization of their genes and loci, and their type (Table 3.1), suggested to us that it might be worthwhile to look for a regulation mechanism that might be common to all of these miRNAs. One mechanism that could explain this global downregulation of miRNAs would be the that one or more of the proteins involved in miRNA processing was affected by infection, either
downregulated or sequestered in a compartment other than where it acts. The ribonuclease protein complex that processes the pri-miRNA after transcription is composed of Drosha, which targets and cleaves the flanking ends of the hairpin, and DCGR8 that stabilizes the complex. This step takes place in the nucleus. After being exported into the cytosol, the pre-miRNA is targeted by Dicer and its partner TRBP and cleaved into a mature miRNA duplex. The duplex is then loaded onto an Argonaute protein, and the passenger strand is discarded. Ago2 is the most common Argonaute protein and is responsible for cleaving the complementary mRNA, along with other RISC proteins (Figure 3.4 A).

**Figure 3.5 Subcellular localization of Drosha and DGCR8**

A: HMDMs were grown on coverslips and infected for 24 and 48h. Coverslips were fixed, and stained for Drosha or DGCR8 (red) and DAPI (blue) and analyzed by confocal microscopy. 3D images of the cells were reconstituted using z-stacks confocal images. B: The staining intensity of Drosha and DGCR8 in the nucleus versus the cytoplasm was measured using ImageJ on at least 50 cells (n=3 donors).
To examine the status of these proteins in the miRNA maturation pathway, human macrophages were infected with *L. donovani* for 24 and 48 hours, lysed and analyzed for protein expression by Western blot. DGCR8, Dicer, TRBP and Ago2 were all unchanged by infection, both at 24 and 48 hours (Figure 3.4 B, C). Surprisingly, Drosha was unchanged at 24 hours, but was strongly up-regulated at 48 hours post-infection, as compared to control cells.

Drosha and DGCR8 need to be present in the nucleus to process pri-miRNAs, therefore, we examined whether leishmania infection might block the nuclear localization of these proteins. Infected and non-infected cells were labeled using anti-Drosha and anti-DGCR8 antibodies, as well as DAPI to stain nuclei and analyzed by confocal microscopy (Figure 3.5 A). Nuclear versus cytosolic localization of the proteins was measured and the same distribution was observed in both control and infected cells (Figure 3.5 B).

Taken together, these findings suggest that, in this system, the observed downregulation of miRNAs by leishmania infection in HMDMs cannot be attributed to a decrease in miRNA processing proteins or a restricted cytosolic localization of Drosha and DGCR8, such that they are not available for nuclear processing of pri-miRNA.

### 3.2.5 The stabilities of mature miRNAs were not affected by infection

An alternative mechanism to explain downregulation of mature miRNAs in infected cells might have been faster rates of miRNA decay. To examine whether the stabilities of miRNAs were reduced in the context of infection, actinomycin D was used to inhibit transcription. RNA from both control and infected cells (24 hours of infection) was then collected at 0, 6 and 24 hours post addition of actinomycin D and RT-qPCR was done to measure the decay rates of three mature miRNAs.
Figure 3.6 Stability of mature miRNAs in control and infected cells

A: Human monocyte-derived macrophages were infected for 24 hours, while some uninfected cells were used as a control. Subsequently, the cells were given actinomycin D (act D) to inhibit transcription, allowing us to look at decay rates of mature miRNAs, while DMSO was used as a control. RNA was collected after 6 hours and 24 hours of treatment, and RT-qPCR was performed on three miRNAs. Represented here is the expression at each timepoint, normalized to DMSO treatment (4 donors). B: The slope of each individual curve was measured and is presented in the bottom graph. Statistical analysis was done using a 2-way ANOVA comparing each treatment. (p-values: ** < 0.01, *** < 0.001, **** < 0.0001).

Figure 3.6 A shows the amounts of each miRNA at each time point in control and infected cells +/- actinomycin D. The slope for each curve was then calculated and these are shown in Figure 3.6 B. Inspection of these results led to several conclusions. First of all, as expected, the slopes of actinomycin D-treated control cells were lower (more negative) than those in DMSO-treated control cells, showing the decay of miRNAs when their synthesis is blocked and validating this model. Second, when comparing actinomycin D treated samples,
thereby looking only at decay, the slopes were similar between control and infected cells, indicating that miRNAs did not have faster decay rates in infected cells. Finally, when cells were treated only with DMSO, therefore, taking into account both synthesis and decay, the slopes were lower in infected cells as compared to control cells. By default, this pointed towards a de facto defect in miRNA synthesis in infected cells, since as discussed above decay rates were not changed by infection. Taken together, these results suggest a defect in synthesis, but no differences in the stabilities of mature miRNAs in the context of infection.

3.2.6 Like mature miRNAs, levels of pri- and pre-miRNAs were also downregulated in infected cells

To examine further whether the synthesis of miRNAs was downregulated in infected cells, the levels of five pri-miRNAs and three pre-miRNAs were assessed by RT-qPCR.

![Graphs showing log2 FC compared to control for primary and precursor miRNAs](image)

Figure 3.7 Primary and precursor miRNA levels in infected macrophages. Monocytes from four donors were purified, differentiated with GM-CSF and infected with L. donovani (Ld) for 24 and 48 hours. Total RNA was extracted, reverse transcribed and a qPCR was performed using primers for primary and precursor miRNAs. Data is represented as fold change in infected cells compared to uninfected cells. A: primary miRNAs, B: precursor miRNAs. T-test. (p-values: * < 0.05, *** < 0.001).
The results indeed showed that the pri-miRNAs and pre-miRNAs tested were
downregulated in infected cells (respectively, Figure 3.7 A, B), as was earlier shown be the case
for the levels of their cognate mature miRNAs (Figure 3.1 D). This observation was found to be
the case for a big majority of the pri- and pre-miRNAs examined, especially at 24 hours post-
infection. These findings were suggestive of a defect in transcription of pri-miRNAs as
contributing to the global downregulation of mature miRNAs in infected cells.

3.2.7 The transcription rate of pri-miRNAs was lower in infected cells

To examine directly the transcription rates of pri-miRNAs, RNA in infected and control
cells was metabolically labeled using ethynyl-uridine (EU), and RNA was collected after 15, 30
and 60 minutes of EU treatment. The newly synthesized RNA (that incorporated EU) was
pulled-down using click-it technology and analyzed by RT-qPCR for several pri-miRNAs as
well as control genes. This allowed us to look in real time at the synthesis of four pri-miRNAs
and three control RNAs (Figure 3.8 A, B). To compare the synthesis rates of pri-miRNAs in
infected and control cells, the slopes of these curves were measured for each gene and each
donor, and are presented normalized to control cells (Figure 3.8 C, D). The miRNAs selected for
this experiment were of different transcriptional types: miR-15a and miR-15b are intronic
miRNAs (their transcription depends on their cognate host gene), let-7a is an exonic miRNA and
miR-34a is intergenic, in which case miRNAs are transcribed on their own.

The results of this experiment show that the transcription rates of the four pri-miRNAs
were significantly lower in infected cells as compared to control cells. Despite the variability
between donors, the transcription rates were reduced by half in infected cells as compared to
control cells, for all four miRNAs without regard to their transcriptional type (Figure 3.8 C).
Figure 3.8 Transcription rates of primary miRNAs. Ld-infected (Ld) (24 hours) and control (C) cells were treated with ethynyl-uridine for 15, 30 and 60 minutes. Total RNA was collected, and click-iT technology allowed for pull-down of nascent RNA. RT-qPCR was performed on total and nascent RNA for four pri-miRNAs and three control genes. A, B: newly synthesized RNAs (normalized to total RNA) in control and infected cells, mean values are shown for three experiments. C, D: the slope of the synthesis rate was assessed for each gene in control and infected cells. (n=4 donors). T-test. (p-values: * < 0.05, ** < 0.01, *** < 0.001).

3.2.8 DNA methylation is unchanged in CpG sites linked to downregulated miRNAs

The main factors that could explain decreased transcription rates such as these, are either epigenetic control or transcription factors being differentially expressed. In some cancer cells, miRNAs have been shown to be repressed by CpG island hypermethylation (Weber et al., 2007; Suzuki et al., 2012).

Another more recent article was published looking at DNA methylation changes in leishmania-infected macrophages (Marr et al., 2014). In analyzing the data from this latter study, we found 125 sites linked to the downregulated miRNAs identified in our study, but their
methylation status was unchanged, which was confirmed by A. Marr et al (personal communication, Figure 3.9). This suggests that the transcription of pri-miRNA in infected cells was not inhibited by any changes in DNA methylation.

![Graph showing methylation of CpG sites associated with the group of 19 miRNAs in infected and control cells.](image)

**Figure 3.9 Methylation of CpG sites associated with the group of 19 miRNAs in infected and control cells**
PMA-differentiated THP-1 cells were infected with leishmania for 48 hours, at which point total DNA was isolated and processed for analysis with Illumina Infinium HumanMethylation450 BeadChip arrays, derived from re-analysis of data in a study published by Marr et al., 2014. 125 CpG sites associated with the miRNAs downregulated in infected HMDMs were tested in this assay, and all were unchanged as compared to uninfected. The methylation values (M) are plotted here: x-axis is uninfected cells, y-axis is cells given either live Ld (red) or heat killed Ld (blue).

**3.2.9 c-Myc is upregulated and more stable in leishmania-infected cells**
Reduced transcription rates of pri-miRNAs in leishmania infected cells could have been due to aberrant activity of a transcription factor such as c-Myc. The latter has pleiotropic effects on cell growth, proliferation, differentiation and apoptosis, mediated by its ability to regulate – either positively or negatively- the expression of thousands of genes including many miRNAs (reviewed in (Tao et al., 2014)).

Figure 3.10 A shows the miRNAs that are known to be either induced or repressed by c-Myc (Gao et al., 2009; Chang et al., 2008). In regard to the miRNAs that are repressed by c-Myc, 11 of those belong to the group of 19 miRNAs that were downregulated by leishmania infection:
let-7g, let-7d, let-7i, let-7a, let-7b, miR-223, miR-23a, miR-15a, miR-181a, miR-34a, miR-98. Moreover, c-Myc is known to induce Drosha (Wang et al., 2013), the expression of which was markedly upregulated at 48 hours post-infection (Figure 3.4). Based upon these considerations, we decided to investigate whether c-Myc might be responsible for the downregulation of miRNAs observed in infected cells.

Figure 3.10 Upregulation of c-Myc in infected macrophages. 

A: schematic representation of either c-Myc repression or induction of miRNAs as reported in the literature. In bold, in the box on the right are shown the miRNA members of the group of 19 downregulated by leishmania infection. B: HMDMs were infected or not for 24 and 48 h and Ld-infected (Ld) and control (C) cells were analyzed by WB probed for c-Myc. Paired T-test. (p-values: * < 0.05). C: HMDMs were infected for 24 hours, and both control and infected cells were treated with cycloheximide to block translation for 20, 40 and 60 minutes. Cells were lysed and western blotting was performed to assess the decay rates of c-Myc.
Using Western blotting we found that c-Myc was strongly upregulated in infected cells, both at 24 and 48 hours post-infection (Figure 3.10 B). c-Myc is a highly unstable protein and is regulated mostly through alterations of its stability (Sears, 2004). Infected and control cells were treated with cycloheximide to block translation, for 20, 40 and 60 minutes. Cells were lysed at each time point and Western blotting was performed to look at the decay of c-Myc over time. We established that the stability of c-Myc is increased in infected cells, as compared to control cells, accounting for its increased abundance (Figure 3.10 C).

### 3.2.10 c-Myc plays a role in miRNA repression

To try and link c-Myc to miRNA repression, we used small interfering RNAs (siRNAs) to knockdown c-Myc before infection with leishmania. Macrophages were incubated with either control (scrambled) or c-Myc siRNAs and then infected with leishmania for 24 hours. c-Myc siRNAs did not alter the cell viability, in both control and infected cells (Figure 3.11 A). The cells were then lysed and Western blotting was used to confirm knockdown of c-Myc. Three c-Myc siRNAs were used: siRNA-A proved almost inefficient, while both siRNA-B and -C downregulated c-Myc to the same extent in control cells, but not in infected cells, where siRNA-C was more efficacious that siRNA-B (Figure 3.12 B). Drosha being induced by c-Myc (Figure 3.10 A), as well as increased in infected cells (Figure 3.4), we decided to test whether it would be decreased in cells treated with c-Myc siRNA. Drosha was unchanged in uninfected cells treated with siRNAs-B and -C, but it was decreased in infected cells treated with siRNA-C, as compared to infected cells treated with scrambled siRNA (Figure 3.11 C). This is consistent with the hypothesis that Drosha is induced by c-Myc in infected cells.

In parallel, RNA was collected and RT-qPCR was performed to assess the levels of three miRNAs from the group of 19 (miR-15a, miR-34a and miR-378). c-Myc siRNA-B led to a
Figure 3.11 Effect of siRNA-mediated knockdown of c-Myc on miRNA expression
HMDMs were treated for 48 hours with control (scrambled) and c-Myc siRNAs (A, B and C), followed by 24 hours rest or infection with leishmania (Ld). A: The viability of macrophages was evaluated by MTT assay. B: The cells were lysed and analyzed by western blotting for c-Myc protein levels. C: Western blotting for Drosha protein levels. T-test. D: RNA was collected from siRNA-treated cells (siRNAs B and C), both infected and control, and RT-qPCR was performed to assess the expression of U6, miR-15a, miR-34a and miR-378. Statistical analysis was done using a 2-way ANOVA comparing each condition. (p-values: * < 0.05, **** <0.0001).

Partial recovery of miRNA levels in infected cells, closer to control levels, while c-Myc siRNA-C induced a strong upregulation of the miRNAs of interest (Figure 3.12 D). These results show
that c-Myc levels and miRNA levels are inversely correlated: siRNA-B caused a modest downregulation of c-Myc and modest increases in miRNAs, while siRNA-C caused a strong downregulation of c-Myc along with robust recoveries of miRNA levels. Thus, knockdown of c-Myc protein reversed the downregulation of miRNA levels caused by infection.

Figure 3.12 Effects of c-Myc inhibition with 10058-F4 on miRNA levels
A: Leishmania growth after 24 hours incubation with 10058-F4 or DMSO. B: Macrophage viability was measured using MTT assay, after 48 hours incubation with 10058-F4 with or without 24 hours infection with leishmania (Ld). C: HMDMs were treated with c-Myc inhibitor 10058-F4 (25 µM or 50 µM) or DMSO for 24 hours then infected for 24 hours. c-Myc levels were measured by western blotting. D: Drosha expression was assessed in 10058-F4 treated cells by western blotting. E: RNA was extracted from 10058-F4 treated cells, both infected and control cells, reverse transcribed and analyzed by qPCR for the expression of U6, let-7a, miR-15a and miR-378. 2-way ANOVA. (p-values: * < 0.05, ** < 0.01, *** < 0.001, **** <0.0001).
To confirm this link between c-Myc and miRNA repression, we used an orthogonal approach consisting of a c-Myc specific inhibitor, compound 10058-F4. The latter blocks c-Myc/MAX interactions, and also downregulates the expression of c-Myc after prolonged exposure (Müller et al., 2014), which was confirmed with our cells (Figure 3.12 C). Cells were treated with either 25 µM or 50 µM 10058-F4 and then infected for 24 hours, after which RT-qPCR was performed to assess the levels of three miRNAs from the group of 19 (let-7a, miR-15a and miR-378). Treatment with 10058-F4 did not affect leishmania growth (Figure 3.12 A) or macrophage viability (Figure 3.12 B). Here, inhibiting c-Myc led to a strong downregulation of Drosha in both infected and uninfected cells (Figure 3.12 D), suggesting that the effect of 10058-F4 (downregulation plus inhibition) is more effective than the siRNA approach. Finally, uninfected cells treated with 50 µM of 10058-F4 displayed higher levels of miRNAs than untreated cells (Figure 3.12 E). Most importantly, infected cells, treated with either 25 µM or 50 µM of 10058-F4 showed similar levels of miRNAs as did uninfected cells (Figure 3.12 E). These findings show that the inhibition of c-Myc with 10058-F4 reversed the downregulation of miRNAs due to infection.

3.2.11 c-Myc is required for leishmania intracellular survival

We next asked whether c-Myc played a role in the establishment of the infection: siRNA-treated and 10058-F4 treated cells were infected with leishmania, and the infection rate was measured. Knockdown and inhibition of c-Myc did not alter the infection rate (Figure 3.13 A, C). This suggests that c-Myc does not play a role in leishmania uptake by macrophages.

Once inside the cells, the fate of intracellular leishmania can be either to be destroyed or to survive and grow inside the phagolysosomes. Therefore, in order to examine whether c-Myc is required for optimal intracellular survival of leishmania, a parasite rescue assay was performed.
under conditions of c-Myc inhibition. Briefly, macrophages were infected with leishmania and after 24 hours, they were thoroughly washed to remove any uninternalized leishmania. The macrophages were then lysed with a gentle detergent that does not damage the leishmania. The released leishmania are then allowed to transform back into promastigotes and grow at 26°C for 2 days, at which point they are counted. The cell number reflects then the ability of the leishmania to survive intracellularly, recover, and to transform and grow again as promastigotes.

Figure 3.13 Effects of c-Myc siRNA and 10058-F4 on leishmania infection and survival.
A: infection rate of siRNA-treated cells after 24 hours infection. B: A parasite rescue assay was performed to assess intracellular survival. Briefly, the cells were lysed using a mild detergent that does not affect leishmania. The leishmania were incubated at 26°C for 3 days, at which point they were counted. C: infection rate of 10058-F4 treated cells after 24 hours infection. D: parasite rescue assay on 10058-F4 treated cells. T-test. (p-values: * < 0.05, ** < 0.01).
Down regulation of c-Myc by siRNAs decreased leishmania counts by half (Figure 3.13 B). Similarly, the inhibition of c-Myc by 10058-F4 led to a ten-fold reduction in parasite counts (Figure 3.13 D). Taken together, these findings show that upregulation of c-Myc is a novel leishmania virulence strategy.

### 3.2.12 c-Myc induction through signalization pathways

We next aimed at understanding what leads to the upregulation of c-Myc in leishmania-infected cells (Figure 3.10 B). We know that the stability of c-Myc is increased in infected cells (Figure 3.10 C). The main pathways that regulate c-Myc stability are the Raf/MEK/ERK pathway and the PI3K/Akt pathway (Zhu et al., 2008). ERK induces stabilization of c-Myc by phosphorylation at Ser62, while Akt activation blocks Myc degradation by inhibiting phosphorylation at Thr58 by Gsk3β. The two pathways play a central role in determining the half life of c-Myc at any given time. Moreover, it has been shown that the PI3K/Akt pathway is activated in leishmania infected cells (Nandan et al., 2012). Another pathway that can modify c-Myc levels is the Wnt/β-catenin pathway, in which β-catenin induces the transcription of c-Myc (Higgs et al., 2013). It has also been shown that β-catenin is induced in leishmania-infected cells (Gupta et al., 2016).

We first assessed the activation of Akt in our model: Western blotting for phospho-Akt (the active form) and Akt was performed on lysates from infected and control cells. The ratio p-Akt/Akt was much higher in infected cells than in control cells, which confirms the increased activation of Akt (Figure 3.14 A). Concerning beta-catenin, we did not observe a significant upregulation of β-catenin (Figure 3.14 B), but we only looked at the total protein amount, as opposed to the active phosphorylated form.
Figure 3.14 Levels of p-Akt/Akt and β-catenin in infected cells, effects of Akti and XAV939 on c-Myc expression

A, B: HMDMs were infected for 24 hours with leishmania. Cell lysates were used for Western blotting for p-Akt, Akt, β-catenin and actin. C,D: HMDMs were treated with 10 µM Akti for 4 hours, washed three times with HBSS, and infected or rested for 24 hours. E,F: XAV939 was used at 10 µM for 24 hours, washed with HBSS, and infected or rested for 24 hours. DMSO was used as control.

We used Akt1/2 inhibitor (Akti) to assess the role of Akt in the upregulation of c-Myc that we observed in infected cells. HMDMs were treated for 4 hours with Akti and then either infected or rested for 24 hours. Akti strongly inhibited the phosphorylation of Akt, both in control and infected cells (Figure 3.14 C,D), but did not change the expression of c-Myc in
control or infected cells (Figure 3.14 C, E). To assess the role of Wnt/β-catenin, we used XAV939, a Wnt/β-catenin inhibitor that increases the protein levels of the axin-GSK3β complex and promotes the degradation of β-catenin (Huang et al., 2009). HMDMs were treated with XAV939 for 24 hours and then infected with leishmania or rested for 24 hours. XAV939 successfully downregulated the levels of β-catenin both in infected and control cells, but did not lower the expression of c-Myc (Figure 3.14 F,G,H). To conclude, neither the PI3K/Akt nor the Wnt/β-catenin pathways are responsible for increasing c-Myc expression in infected cells.

3.3 Discussion

This project was initiated by examining the changes in the expression of host miRNAs in HMDM infected with L. donovani, using the nCounter Human v2 miRNA Expression Assay (Nanostring). Of the 800 miRNAs included in this array, 46 were reproducibly detected in samples from multiple independent donors (Figure 3.1 A). Of this group, a substantial majority (35/46) appeared to be downregulated in infected cells, as compared to controls (Figure 3.1 A, B). Formal statistical analysis indeed revealed that the expression of 19 of these miRNAs (herein named as the “group of 19”) were significantly downregulated by infection (Figure 3.1 C). The Nanostring results were validated by randomly selecting five miRNAs from the group of 19 and examining their expression by RT-qPCR. The results of this analysis (Figure 3.1 D) confirmed the downregulation of miRNAs observed using Nanostring technology (Figure 3.1 E). In this exercise, there were no miRNAs identified to be upregulated by infection. Taken together, these results show that host miRNA expression is broadly downregulated by infection with L. donovani. The phagocytosis of beads did not lead to the same repression of macrophage miRNAs (Figure 3.3), and the general profile of RNAs was the same in infected and control cells (Figure 3.2), altogether suggesting that this repression was specific to leishmania infection, and
to miRNAs.

3.3.1 Understanding the mechanism leading to the repression of host miRNAs

To the best of our knowledge, such widespread downregulation of miRNAs has not previously been reported in the context of infection by any other intracellular protozoan or for that matter, bacterial pathogens. However, global decreases in miRNA expression have been associated with several types of cancers. In these studies, the downregulation was attributed variably to mutations or deletions in the miRNA processing proteins Dicer and Drosha (Wu et al., 2013; Muralidhar et al., 2007; Martello et al., 2010; Rupaimoole et al., 2014), to hypermethylation of miRNA genes (Zhang et al., 2014), to mutations or suppression of the protein Exportin-5 (Melo et al., 2010; Sun et al., 2016), or to transcriptional repression of miRNA genes by oncogenic transcription factors (Chang et al., 2008). In other models, the stability of miRNAs was reduced, for example by murine cytomegalovirus infection (Marcinowski et al., 2012).

Previous work has been done on leishmania infection and host miRNAs, looking at different species and different cells types - human and murine, primary cells and cells lines (Lemaire et al., 2013; Frank et al., 2015; Tiwari et al., 2017; Singh et al., 2016; Muxel et al., 2017) (Table 1.2). Each of these studies identified both up- and down-regulated miRNAs, but there was no commonality amongst these studies and no mechanism was identified to explain these changes. Only one previous study looked at human primary macrophages infected with *L. donovani* (Geraci et al., 2015) and the findings revealed both up- and down-regulated miRNAs as well with no consistent pattern or mechanism was described.

Members of the group of 19 are shown in Table 3.1 and inspection of the data shows that they came from diverse regions of the genome and that they represented all types of miRNAs.
The finding that the downregulated miRNAs were diverse in terms of chromosome localization of their genes and loci, and their type, suggested that this likely involved a novel and versatile mechanism of gene repression that is activated in leishmania infected cells. In pursuit of such a mechanism, we found that the downregulation of miRNAs in leishmania-infected cells was not due to any change in either the abundance or subcellular localization of proteins involved in miRNA biogenesis (Figure 3.4, Figure 3.5). Neither could this be accounted for by decreased stability of mature miRNAs (Figure 3.6). On the other hand, levels of both pri- and pre-miRNAs were decreased in infected cells in concert with their cognate mature miRNAs (Figure 3.7). Consistent with these findings, the transcription rates of pri-miRNAs were significantly repressed by leishmania infection (Figure 3.8). These findings raised the specter of the possible role of a transcriptional repressor activated by leishmania and our search for such a candidate led to c-Myc.

3.3.2 Role of c-Myc in miRNA repression

The oncogene c-Myc is upregulated in many cancers (Dang, 2012) and it was shown to participate in the global downregulation of miRNAs observed in lymphoma cells (Chang et al., 2008). Of special interest was our observation that 11 members of the group of 19 (Figure 3.10 A) had been shown previously in other systems to be transcriptionally repressed by c-Myc (Chang et al., 2008; Gao et al., 2009). These findings made c-Myc an excellent candidate for repression of miRNA expression in our system. Indeed, we found that c-Myc expression itself was dramatically increased in leishmania-infected cells (Figure 3.10 B). Furthermore, using either c-Myc inhibitor 10058-F4 or siRNAs to silence c-Myc, we were able to rescue miRNA expression in infected cells and restore them to control levels (Figure 3.11 D, Figure 3.12 E). c-Myc was also responsible for the upregulation of Drosha at 48 h post-infection (Figure 3.11 C,
Figure 3.12 D). These results clearly show that c-Myc is hijacked by leishmania to act as a virulence factor by proxy, leading to broad-based, genome-wide repression of miRNA expression and enhanced parasite survival (Figure 3.13 B, D).

A number of miRNAs are known to be induced by c-Myc (Figure 3.10 A) (Psathas and Thomas-Tikhonenko, 2014), and interestingly, none of these were increased upon leishmania infection. This was the case despite the finding that c-Myc itself was increased (Figure 3.10 B) and suggests that an additional mechanism regulates their expression. Furthermore, while c-Myc acts as a regulator of miRNAs, its expression is modulated in turn by a number of miRNAs, among them miR-34a, which was downregulated in leishmania-infected cells (Figure 3.1), perhaps explaining the reciprocal increase in c-Myc we observed (Figure 3.10 B) (Jackstadt and Hermeking, 2015).

It is informative to consider how this c-Myc-driven strategy otherwise affects cell regulation leading to altered macrophage phenotype. The specific effects of miRNA repression in leishmania infected cells is not known at this time. In cancer cells, it has been hypothesized that the global downregulation of miRNAs contributes to neoplastic transformation by allowing for increased expression of proteins with oncogenic potential (Kumar et al., 2007). The group of 19 has thousands of predicted target genes, suggesting that their downregulation is highly likely to lead to pleiotropic effects on macrophage cell regulation and cell phenotype.

In order to understand the role of individual miRNAs, as well as groups of miRNAs in leishmania pathogenesis, it would be interesting to use miRNA mimics to study the roles of individual miRNAs, as well as in combination. The transfection of gastric cancer cells with a let-7b mimic downregulated c-Myc and reversed multidrug resistance (Yang et al., 2015a). And in a recent study, the systemic delivery of a single c-Myc-repressed miRNA (miR-26) to a mouse
hepatocellular carcinoma model successfully reversed disease progression (Kota et al., 2009). The delivery of Myc-repressed miRNAs therefore represents a therapeutic strategy for cancer, and could be attempted in leishmania infection.

### 3.3.3 Other roles of c-Myc during leishmania infection

A review of the literature searching for c-Myc and infection of human macrophages revealed that c-Myc was found to be upregulated in primary macrophages infected with several mycobacteria species (Bacillus Calmette-Guérin, *M. avium*, *M. cheloneae*, or *M. kansasii*), but this was associated with suppression of *M. avium* survival (Yim et al., 2011), the converse of what we report here for c-Myc and leishmania (Figure 3.13). c-Myc was reported to be increased by infection with a number of other intracellular pathogens, in various cell types (Table 3.2): *Toxoplasma gondii* in human fibroblasts, *Salmonella typhimurium* in mouse macrophages, and *Theileria parva* in human B-lymphocytes (Franco et al., 2014; Seong et al., 2009; Dessauge et al., 2005). Interestingly, c-Myc was thought to be involved in both inducing host cell death during *S. typhimurium* infection and promoting host cell survival in *Theileria parva* infection (Seong et al., 2009; Dessauge et al., 2005). This underlines the role of c-Myc in determining cell fate, being able to induce cell death or promote cell survival depending on the context.

C-Myc is also a marker of M2 macrophages and is required for alternative activation of human macrophages (Pello et al., 2012). In contrast, c-Myc is inhibited by the pro-inflammatory agonists LPS and IFN-γ, which induce classically activated (M1) macrophages (Liu et al., 2016). Although macrophages exist in a continuum between M1 and M2 activation profiles, a clear correlation exists in leishmania infection, in which classical activation is linked to microbicidal functions (production of TNF-α, nitric oxide and reactive oxygen species) and parasite killing, while alternative activation leads to parasite survival and disease progression (Liu and Uzonna,
This suggests the hypothesis that the induction of c-Myc is part of the leishmania-induced alternative activation pathway of macrophages, favoring parasite survival. Conversely, when c-Myc is inhibited by 10058-F4 or siRNAs, this may block alternative activation of macrophages and in turn suppress leishmania survival (Figure 3.13 B, D). Given the role of c-Myc in alternative activation, it would be interesting to look at what happens to microbicidal functions in infected cells when c-Myc is silenced.

Table 3.2 Literature review of c-Myc and infection with intracellular pathogens

<table>
<thead>
<tr>
<th>Article</th>
<th>Pathogen</th>
<th>Cell type</th>
<th>c-Myc expression</th>
<th>Other observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yim et al. PNAS 2011</td>
<td>mycobacteria (BCG, <em>M. avium</em>, <em>M. chelonae</em>, and <em>M. kansasii</em>)</td>
<td>HMDMs</td>
<td>increased with infection</td>
<td>c-Myc increase associated with suppression of <em>M. avium</em> survival</td>
</tr>
<tr>
<td>Franco et al. Eukaryot Cell. 2014</td>
<td><em>Toxoplasma gondii</em>, <em>Neospora caninum</em></td>
<td>human fibroblasts (HFFs)</td>
<td>increased by <em>Toxoplasma</em>, but not <em>Neospora</em></td>
<td></td>
</tr>
<tr>
<td>Seong et al. J Microbiol. 2009</td>
<td><em>Salmonella enterica</em> serovar Typhimurium</td>
<td>RAW264.7, mouse peritoneal MP</td>
<td>increased by infection, but not by LPS or heat-killed bacteria</td>
<td>cell death by <em>Salmonella</em> infection is related to c-Myc</td>
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<tr>
<td>Dessauge et al. Oncogene. 2005</td>
<td><em>Theileria parva</em> clone (B2)</td>
<td>human B-cell clone (B2)</td>
<td>increased by infection</td>
<td>survival of host lymphocytes linked to c-Myc</td>
</tr>
</tbody>
</table>

It is also thought that c-Myc promotes apoptosis by sensitizing cells to a number of signals rather than by directly inducing cell death, although the signaling pathways by which c-Myc drives apoptosis are not well understood (McMahon, 2014). Considering that leishmania prevents apoptosis of its host cells (Moore and Matlashewski, 1994), it is worth noting that the upregulation of c-Myc in infected cells did not lead to decreased cell viability (Figure 3.11 A, Figure 3.12 B). Moreover, as described above, c-Myc was reported to play a role in inducing host cells death during Salmonella infection, but to promote host cell survival during Theileria infection (Seong et al., 2009; Dessauge et al., 2005). We can assume that this dichotomy may well be related to other concurrent signals which counteract any c-Myc associated input that
would lead to host cells apoptosis. For example, leishmania could be using the Akt pathway, which is induced in leishmania-infected macrophages (Nandan et al., 2012), to counteract the effects of pro-apoptotic c-Myc by inducing pro-survival signaling, as was observed in mammary tumor cells and Burkitt's lymphoma (Yeh et al., 2013; Spender and Inman, 2014).

Paradoxically, the c-Myc inhibitor 10058-F4 was also found to induce apoptosis in human myeloid leukemia (Huang et al., 2006), making the inactivation of c-Myc in cancer cells a potential treatment. We found no evidence of cell death in our cells treated with c-Myc inhibitor, both control and infected (Figure 3.11 A, Figure 3.12 B), similar to what had been described before in healthy human primary monocytes-derived macrophages (Pello et al., 2012).

### 3.3.4 Upstream of c-Myc

In order to understand what events lead to the increased expression of c-Myc in infected cells (Figure 3.10 B), we first showed that the c-Myc protein is more stable in infected cells, as compared to control cells (Figure 3.10 C). c-Myc is a highly unstable protein the expression of which is controlled mostly through changes in its stability, and to a lesser extent, through transcription (Sears, 2004). The PI3K/Akt pathway, for example, prevents c-Myc degradation by inhibiting GSK-3β, while the MAPK/ERK pathway extends the stability of c-Myc (Zhu et al., 2008) (Figure 3.15). On the other hand, β-catenin promotes the transcription of c-Myc (Higgs et al., 2013). Both the PI3K/Akt pathway and β-catenin are known to be induced in leishmania infection (Nandan et al., 2012; Gupta et al., 2016), which we confirmed in our system (Figure 3.14 A, B). However, they did not play a role in c-Myc induction, as we show here using Akt and β-catenin inhibitors: the inhibition of Akt and β-catenin did not change the expression of c-Myc in either control or infected cells (Figure 3.14 C-H).
Figure 3.15 Signaling pathways that regulate c-Myc synthesis and stability

β-catenin induces the transcription of c-Myc. The Raf/MEK/ERK pathway induces the phosphorylation of Ser62 that stabilizes c-Myc. The PI3K/Akt pathway inhibits GSK-3β which induces Thr58 phosphorylation that sends c-Myc for proteasome degradation. Adapted from (Sears, 2004).

The MAPK/ERK pathway may play a role in inducing c-Myc in infected cells. In human macrophages, L. donovani is reported to modulate the TLR2-stimulated MAPK pathway by repressing p38 and activating ERK1/2 phosphorylation (Chandra and Naik, 2008a). Therefore, it is worth testing whether c-Myc expression is induced by ERK1/2.

3.3.5 Conclusion

In summary, the results of the present study identify c-Myc as novel leishmania virulence factor by proxy. c-Myc is induced by infection and brings about broad-based, genome-wide repression of host miRNAs. c-Myc is a transcriptional repressor for these miRNAs and lowers their transcription rate, while the rest of the miRNA biogenesis machinery is not changed by infection. This broad-based repression of miRNA expression is likely to have big consequences as thousands of these miRNA target genes might see their expression altered. Moreover, this study shows that c-Myc is essential for parasite survival. Given the central role of c-Myc as an oncogene, it has been and continues to be the focus of many attempts to develop inhibitors for therapeutic purposes in cancer (Whitfield et al., 2017). We anticipate that it should be possible to capitalize on this work in the cancer clinic to identify novel therapeutics for leishmaniasis.
3.4 Material and Methods

3.4.1 Purification and culture of human cells

Buffy coats from healthy donors were obtained from Canadian Blood Services Network Centre for Applied Development (netCAD). Monocytes were enriched by a centrifugation in Ficoll gradient (GE Healthcare) followed by thorough washings in PBS. The cells were then incubated for 1 hour at 37°C, 5% CO$_2$ in culture flasks (Corning), and non-adherent cells were washed away with HBSS. Adherent cells were collected using a cell scraper, and monocytes were counted using Türk's solution. The cells were plated at a density of 0.75x10$^6$ cells/ml in multiwell plates in RPMI supplemented with 10% FBS (Gibco), 2 mM L-glutamine (StemCell) and 100 U/ml penicillin/streptomycin (StemCell). Differentiation into macrophages was induced by addition of 10 ng/ml GM-CSF (StemCell). After 3 days, the cells were washed with HBSS and given fresh supplemented RPMI with 10 ng/ml GM-CSF. The cells were washed again on day 6, rested in supplemented RPMI without GM-CSF and used for experiments on day 7.

3.4.2 Parasite culture

*Leishmania donovani* Sudan strain S2 was obtained from Dr. Kwang Poo Chang (at the time Rockefeller University, NY, USA). Parasite virulence was maintained by regular passages through Syrian Golden Hamsters. Promastigotes were routinely cultured in the lab, passaged every 3 days for a maximum of 20-25 passages. Promastigotes were cultured at 26°C in M199 (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Gibco), 10 mM HEPES (Stemcell), 6 µg/ml hemin (Sigma-Aldrich), 10 µg/ml folic acid (Sigma-Aldrich), 2 mM L-Glutamine (Stemcell), 100 U/ml penicillin/streptomycin (Stemcell) and 100 mM adenosine (Sigma-Aldrich).
3.4.3 Infection and phagocytosis experiments

Infection was performed with day 5 stationary phase promastigotes. Briefly, the cells were counted, washed in HBSS, resuspended in HBSS and used to infect human cells, at a MOI of 20:1. When infected for more than 24 hours, the cells were washed at 24 hours post-infection to remove uninternalized leishmania, and fresh complete RPMI was added to the wells before returning them to the incubator. As a phagocytosis control, human macrophages were given 2 µm non-functionalized polybead microspheres (Polysciences). To parallel the MOI of 20:1 used for leishmania, 20 beads were added per human cell.

Infection and phagocytosis rates were assessed using cells grown on coverslips and subsequently infected. 24 hours post-infection, the coverslips were washed three times with PBS and fixed with 2% paraformaldehyde in PBS for 15 minutes at room temperature. The coverslips were washed in PBS and mounted on microscopy slides using ProLong Diamond Antifade Moutant with DAPI (ThermoFischer). Fluorescence microscopy images were taken using an Axioplan II epifluorescence microscope (Zeiss), and AxioCam MRm Camera (Zeiss) and the AxioVision software Version 4.8.2 (Zeiss). At least 10 images were collected for each coverslip, at 40X magnification. DAPI stained the macrophage nuclei and the leishmania nuclei and kinetoplast, which allowed us to count the total number of human cells, the number of infected cells, and the number of leishmania in each image. The infection rate (number of infected cells/total number of cells x 100) and the parasite load (number of parasites / number of infected cells) were calculated on each image, as well as the average of the 10 images.

3.4.4 Parasite rescue and transformation assay

Human cells were also subjected to parasite rescue and transformation assays (Jain et al., 2012): differentiated macrophages were infected with stationary phase leishmania for 24 hours.
The wells were washed three times with HBSS. The macrophages were lysed by incubating them in 0.01% sodium dodecyl sulfate (SDS) in HBSS for 30 minutes at 37°C, 5% CO2. The lysis of macrophages was confirmed by visually checking under the microscope. 300 µl of complete M199 was added to each well. The plates were then incubated at 26°C for 2 days to allow the released leishmania to transform and grow as promastigotes. After 2 days, the leishmania were counted.

3.4.5 Western blotting

Frozen pellets of cells were lysed on ice using protein lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton-X-100, 1 mM EDTA, 2.5 mM sodium orthovanadate, 1 mM b-glycerophosphate, 1 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin). Lysates were incubated on ice for 5 minutes, then passed 10x through a 27 gauge needle for mechanic disruption. Lysates were centrifuged at 13,000 g for 5 minutes, before addition of 4x Laemmli loading buffer and boiled for 7 minutes. Samples were ran on SDS-PAGE gels (7.5 to 15%), followed by semi-dry transfer to nitrocellulose membranes. Immunoblotting was performed with primary antibodies against Drosha (Abcam ab183732), DGCR8 (Abcam ab191875), Dicer (Santa Cruz sc-30226), TRBP (Abcam ab42018), Ago2 (Abcam ab186733), c-Myc (Cell signaling 5605) and Actin (Sigma Aldrich). The blots were incubated with secondary antibody anti-rabbit HRPO (ABM), followed by developing with Supersignal West Femto Chemoluminescence Substrate (Thermo). Densitometric analysis was performed in ImageJ.

3.4.6 Confocal immunofluorescence microscopy on infected cells

Human macrophages grown on coverslips were infected with Leishmania donovani for 24 hours and 48 hours (in which case they were washed at 24 hours post-infection to remove unbound parasites) or kept uninfected as control. Intracellular immunofluorescence staining was
performed following Abcam's guidelines: fixation was done with 2% polyformaldehyde in PBS and permeabilization with 0.3% Triton X-100 in PBS. Anti-Drosha (Abcam ab183732) was used at 1:100 and anti-DGCR8 (Abcam ab191875) was used at 1:1000, while the secondary antibody, anti-rabbit Alexa Fluor 594 (Life Technologies), was used at 1:250. The coverslips were mounted on slides using ProLong Diamond Antifade Mountant with DAPI (ThermoFischer). The slides were then analyzed using a Zeiss LSM 780 confocal microscope (Carl Zeiss, Thornwood, NY). Controls for staining were performed using only the secondary antibody, as well as full staining with both primary and secondary antibodies on uninfected macrophages, both of which led to no detectable fluorescence. ImageJ was used to determine the fluorescence intensity in the nucleus versus the cytoplasm: DAPI positive regions were selected as "nucleus" and the intensity of the DGCR8 or Drosha staining was measured in the DAPI regions, while the staining in the rest of the image was measured for cytoplasmic localization.

3.4.7 RNA extraction and RT-qPCR, Nanostring assay

For mature miRNAs study, RNA was collected from cell pellets using the miRCURY RNA isolation kit Cell and Plant (Exiqon), known to conserve small RNAs, following the manufacturer's instructions. The RNA was eluted in nuclease-free water, and the concentration of RNA was determined by nanodrop. A DNase treatment was performed with DNase I (Thermo Scientific), either on column or in solution. On column, 12.5 Kunitz Units DNase I was used per column, incubated for 15 minutes at room temperature. In solution, 1 ug of eluted RNA was treated with 1 U of DNase I in a total volume of 15 ul, and incubated for 30 minutes at 37°C, before addition of 1 ul of 50 mM EDTA and incubation at 65°C for 10 minutes.10 ng of RNA (extracted with the Exiqon kit) were used for cDNA synthesis using the Universal cDNA synthesis kit II (Exiqon) according to the manufacturer's instructions. The obtained cDNA was
diluted 80x in nuclease-free water and ROX reference dye (Life Technologies) was added, at a dilution of 50x. Diluted cDNA was used as input for qPCR, using the EXILENT SYBR Green master mix kit (Exiqon) and miRNA-specific primers (Tables 3.3 and 3.4). Real-time PCR amplification was performed using the StepOne Plus System (ABI).

**Table 3.3 Commercially available primers used for RT-qPCR**

<table>
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<th>Company</th>
<th>Product</th>
<th>Reference</th>
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<tbody>
<tr>
<td>U6</td>
<td>Exiqon</td>
<td>microRNA LNA™ PCR primer sets</td>
<td>203907</td>
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<tr>
<td>miR-98-5p</td>
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<td>pre-miR-15a</td>
<td>Qiagen</td>
<td>MiScript precursor assay</td>
<td>MP00001043</td>
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**Table 3.4 Custom primers used for RT-qPCR**

<table>
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<th>Gene</th>
<th>Sequences: forward (Fwd) and reverse (Rev)</th>
<th>Reference</th>
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<tr>
<td>GAPDH</td>
<td>Fwd: ACCACAGTCCATGCCATCAC Rev: TCCACCACCTGTTGCTGTA</td>
<td>(Nothelfer et al., 2016)</td>
</tr>
<tr>
<td>18S</td>
<td>Fwd: CAAGACGGACGAGCGAAA Rev: GCGGGGTATGGGAATAAC</td>
<td>(Wisskirchen et al., 2011)</td>
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<tr>
<td>β-actin</td>
<td>Fwd: CCACCGCGAGAAGATGA Rev: TCCATCAGTGCCAGTG</td>
<td>(Rzymski et al., 2008)</td>
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<tr>
<td>c-Myc</td>
<td>Fwd: AATGAAAGGCCCCCAAGGTAGTTATCC Rev: GTCTTTCGCAACAAAGTCTCTTCTC</td>
<td>(Guo et al., 2015)</td>
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<tr>
<td>pri-let-7a</td>
<td>Fwd: CCTGGATCTTTCCTTCACTG Rev: GCCGGAAGATGGATCTTC</td>
<td>(Jiang et al., 2005)</td>
</tr>
<tr>
<td>pre-let-7a</td>
<td>Fwd: AGGTAGTTAGGTGTTATGTTTATGG Rev: TAGGAAGACAGATGGATTTGTTGTTG</td>
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<td>pri-miR-34a</td>
<td>Fwd: CCTCCAAGGCTGTCAGTTG Rev: TGCACCTTCGCAAATGCTCGC</td>
<td>(Suzuki et al., 2009)</td>
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<td>pre-miR-34a</td>
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<td>pri-miR-15a</td>
<td>Fwd: CTAGCAGGCAATATGTTGTTG Rev: GTAGCAGCACAATATGTTGTTG</td>
<td>(von Brandenstein et al., 2012)</td>
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</table>

Total RNAs were extracted to study primary and precursor miRNAs using GeneJET RNA purification kit (Thermo), following the manufacturer's instructions. RNAs were treated with DNAse as described previously, and 10 ng of RNA (extracted with the Thermo kit) were
used for cDNA synthesis using the OneScript cDNA Synthesis Kit (ABM) according to the manufacturer's instructions. The cDNA was diluted 80x in nuclease-free water and used as input for qPCR, using the EvaGreen 2x qPCR MasterMix-ROX. The primers for pre-miRNAs, pri-miRNAs and control genes (GAPDH and 18S) are listed in Tables 3.3 and 3.4.

Purified RNA (using the Exiqon kit) was diluted to a concentration of 50 ng/µL with nuclease-free ddH₂O and sent to Nanostring (Seattle, WA) for profiling of miRNA expression with the Nanostring nCounter Human v2 miRNA Expression Assay, which measures the expression of 800 human miRNAs (plus controls) from miRBase v.18. Nanostring miRNA expression data were analysed using the nSolver2.0 Analysis software (Nanostring). Background subtraction was done using the mean of the negative controls +2 standard deviations. Data were normalized by computing a normalization factor using the geometric mean of all genes except housekeeping genes. This resulted in 46 miRNAs that were detectably expressed in all samples (average >50 counts). Agglomerative clustering (heat map) of the 46 expressed miRNAs was generated using nSolver’s built-in analysis feature, applying linkage type “average” and metric type “Euclidean distance”. miRNA expression ratios of infected over uninfected samples were plotted as bar graphs in EXCEL.

3.4.8 Click-iT labeling of nascent RNA

Differentiated macrophages were infected for 24 hours, and uninfected cells were used as control. Uninternalized parasites were removed by washing the cells thoroughly. Fresh complete RPMI containing 0.5 mM Ethynyl Uridine (EU, abcam) was added to the wells. Non-EU treated cells were collected as baseline. After 15, 30 and 60 minutes, the cells were collected and lysed for total RNA extraction using GeneJET RNA purification kit (Thermo).

The click-iT Nascent RNA Capture Kit (ThermoFisher) was used according to the
manufacturer’s instructions. Briefly, 1 µg of RNA was biotinylated using 0.5 mM biotin azide. The RNA was then precipitated overnight in ethanol. 400 ng of biotinylated RNA was bound to 12 µl of streptavidin beads, which were washed thoroughly. cDNA synthesis was done using the RNA captured on the beads as template, and the SuperScript VILO cDNA synthesis kit (ThermoFisher). The cDNA was then released from the beads and used for qPCR (called "nascent"). In parallel, total RNA from the same samples was reversed transcribed and used for qPCR as normalization. Calculation for newly synthesized RNA were based on the ΔΔCt method as follows (Livak and Schmittgen, 2001):

\[
\Delta C_t = \text{nascent C}_t - \text{total C}_t \\
\Delta \Delta C_t = \Delta C_t \text{ [EU sample]} - \Delta C_t \text{ [t=0]} \\
\text{Fold change} = 2^{-\Delta \Delta C_t}
\]

3.4.9 siRNA transfection

siRNAs - three c-Myc siRNAs duplexes (-A, -B, -C) and one scrambled negative control - were obtained from ORIGENE (SR321047). After 6 days of treatment with GM-CSF, differentiated macrophages (in 24-well plates) were washed 3 times with HBSS, and given 250 µl of fresh complete RPMI. Transfection mixes containing (per well:) 117.5 µl of serum-free RPMI, 3.75 µl of the transfection reagent HiPerfect (Qiagen) and siRNAs at a final concentration of 200 nM were incubated for 15-20 minutes at room temperature. 125 µl of transfection mix was added to each well. Cells were incubated with siRNAs for 6 hours, and then 125 µl of complete RPMI was added to each well. After 48 hours of siRNA treatment, the cells were washed with HBSS and infected or rested in culture media for 24 hours.

3.4.10 10058-F4, Akti, XAV939 treatment

The c-Myc inhibitor 10058-F4 was purchased from Sigma (reference F3680). Cells were treated with 25 µM or 50 µM of 10058-F4 for 24 hours, at which point the media was removed
and fresh 10058-F4 was added, as well as leishmania for infected samples. DMSO was used as control.

Akt1/2 kinase inhibitor (Akti, A6730) and XAV939 (X3004) were purchased from Sigma. Macrophages were treated with 10 µM Akti for 4 hours, washed with HBSS, and infected or rested for 24 hours. XAV939 was used at 10 µM for 1 hour, washed with HBSS, and infected or rested for 24 hours. DMSO was used as control.

3.4.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism v.5 and v.6. p-values are represented as stars: (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001).
Chapter 4: Discussion

4.1 Scientific significance for the field

The overall goal of my research was to study different aspects of the host-pathogen interaction between *Leishmania donovani* and human macrophages, in particular how leishmania manipulates its host macrophages to establish the infection and replicate intracellullarly, leading to the development of clinical manifestations. My thesis investigates two potential actors of pathogenesis, each on one side of the host-pathogen interaction: chapter 2 describes the role of the leishmania protein CPN10, while chapter 3 identifies the mechanism leading to an alteration of host cell miRNA expression during infection. I showed that both the leishmania protein CPN10 and the host transcription factor c-Myc contribute to leishmania survival in the macrophage phagolysosome.

In chapter 2, I studied the role of the leishmania protein CPN10, in parasite biology and during infection. I generated *Leishmania donovani* strains that express less or more CPN10 as compared to WT (respectively CPN10+/- and CPN10+++ Ld). Although CPN10 seems essential to leishmania, only half the amount of protein (as displayed by CPN10+/- Ld) was sufficient to have a similar growth curve, morphology and resistance to heat shock and a variety of other stresses as WT Ld (Figure 2.6, Figure 2.7, Figure 2.8). However, CPN10+/- Ld were more susceptible to tunicamycin-induced stress (Figure 2.8), underlying a potential role for leishmania CPN10 in the unfolded protein response, which had not been described before. The most striking observations were that leishmania CPN10 is required for parasite intracellular survival, while also repressing the internalization of promastigotes (Figure 2.12, Figure 2.13). Figure 4.1 shows
**Figure 4.1 Model of macrophage infection with WT versus CPN10+/- Leishmania donovani**

WT leishmania are taken up by macrophages through phagocytosis, differentiate into amastigotes. They inhibit the macrophage microbicidal activity to allow for parasite survival and intracellular replication. The macrophage is lysed and the amastigotes are released and go on to reinvade other macrophages. **CPN10+/-** leishmania are taken up more readily by macrophages. We hypothesize that the macrophage activation and microbicidal activity are increased compared to infection with WT Ld. This leads to increased parasite killing and lower parasite survival, and finally less released parasites that can infect other macrophages.

This work is the first description of a leishmania HSP modulating the uptake of leishmania by macrophages. Indeed, many leishmania HSPs have been shown to play a role in pathogenesis and leishmania survival inside phagolysosomes (Table 1.3), but none has been shown to alter the internalization stage of infection. My results underline the importance of the uptake stage in establishment of the infection: a higher internalization rate does not necessarily mean a higher infection rate nor that the parasites will be more successful at surviving in phagolysosomes. On the contrary, CPN10 plays a role in negatively regulating the rate of parasite uptake, which we hypothesize allows leishmania to enter the macrophages more
"silently", without activating the host cells, which is ultimately beneficial for leishmania intracellular survival. This hypothesis is corroborated by the observations made on the proteomics analysis of infected macrophages: cells infected with CPN10+/− Ld show higher abundance of several proteins involved in the innate immune response as well as membrane organization and apoptotic processes (Figure 2.14, Table 2.2). One or more of these might contribute to the clearance of the parasite, as opposed to its survival. Altogether, these findings provide insights into an aspect of infection that is often overlooked: early events surrounding and following the phagocytosis of pathogens largely determine whether internalization will lead to efficient killing of the microbe or successful establishment of an intracellular infection. Similar examples of modulation of the mode of entry that affect parasite survival include the use of neutrophils as "Trojan horses" to enter macrophages without activating them (van Zandbergen et al., 2004). A second example concerns the use of different macrophage receptors: Leishmania infantum metacyclic promastigotes enter macrophages using the CR3 receptors, which does not trigger NAPDH oxidase activation, as opposed to avirulent promastigotes that also use mannose receptors, which promote inflammatory responses (Ueno et al., 2009; Linehan et al., 2000; Sehgal et al., 1993).

In chapter 3, I showed that Leishmania donovani infection increases the expression of the host transcription factor c-Myc in human macrophages (Figure 3.10). Figure 4.2 shows a schematic representation of my findings. c-Myc transcriptionally represses many host miRNAs, leading to a broad-based downregulation of miRNAs (Figure 3.11, Figure 3.12), while also playing a role in leishmania intracellular survival. Indeed, when c-Myc was inhibited or downregulated, the intracellular survival and recovery of leishmania was strongly decreased (Figure 3.13), leading us to identify it as a virulence factor by proxy, or a host factor promoting
parasite survival and pathogenesis. A number of virulence factors have been identified in leishmania - such as LPG, gp63, and several HSPs - but a number of host factors have also been identified as playing roles in pathogenesis. For example, NRAMP1 regulates susceptibility to visceral leishmaniasis (Bucheton et al., 2003). Interestingly, most of the host determinants identified as playing a role in leishmania pathogenesis are immunity-related, including for example cytokines/chemokines (TNF-α, IL-4, TGF-β), their receptors (CXCR2, IL-2 receptor), and actors of innate immunity (mannose-binding lectin) [reviewed in (McCall et al., 2013)]. In contrast, c-Myc is a protein central to many normal cell processes, such as cell cycle, cell growth and differentiation, metabolism, protein synthesis, cell adhesion, migration and apoptosis (Meyer and Penn, 2008). Leishmania it appears alters the expression of this common host protein to favor parasite survival. A similar observation has been reported in infection with Salmonella typhimurium and Theileria parva (Seong et al., 2009; Dessauge et al., 2005), where the pathogens use c-Myc to alter their host cell's survival. This does not seem to be the case in leishmania-infected macrophages, where cell viability is not altered by infection or c-Myc inhibition (Figure 3.11, Figure 3.12). Here, we hypothesize that the induction of c-Myc by leishmania plays a role in pathogenesis by modifying the activation and effector functions of host macrophages. This is supported by the fact that c-Myc is required for alternative activation of human macrophages, and is a marker of M2 macrophages (Pello et al., 2012). This is particularly relevant to leishmania infection, where alternative activation is associated with parasite survival and disease progression, while classical activation correlates with microbicidal activity and parasite killing (Liu and Uzonna, 2012). In the model I propose, therefore, leishmania infection induces c-Myc in order to ensure the alternative activation of their host macrophage, thereby shifting the macrophage effector functions from microbicidal activities that kill parasites to
promoting leishmania survival and disease progression.

Figure 4.2 Schematic model of miRNA repression by c-Myc in macrophages infected with Leishmania donovani.
Leishmania infection increases c-Myc expression, which in return promotes leishmania intracellular survival. c-Myc also inhibits the transcription of multiple miRNAs, leading to a broad-based downregulation of miRNAs. This can result in the modulation of the expression of thousands of targets of these miRNAs.

c-Myc upregulation in leishmania infection strongly alters host cell miRNA expression, leading to a genome-wide repression of miRNAs (Figure 3.1, Table 3.1). RNA interference is central to many essential aspects of cellular function, and the group of 19 downregulated miRNAs may result in alterations in the expression of thousands of target genes. Such a phenomenon had not been shown before in leishmania infection, or any other protozoan or bacterial infection model. Interestingly, such a global miRNA repression has been reported in several types of cancer tissues (Chang et al., 2008; Rupaimoole et al., 2014; Kumar et al., 2007), where it was associated with tumorigenesis and poor prognosis. Although several mechanisms
have been reported to explain the global downregulation of miRNAs in cancer cells, such as a decreased expression of Dicer or Drosha (Dedes et al., 2011; Muralidhar et al., 2007), or an upregulation of c-Myc similar to what we describe here in leishmania-infected cells (Chang et al., 2008), the role in increasing tumor proliferation was reported regardless of the mechanism leading to the downregulation. The global repression of miRNAs could similarly play a role in leishmania pathogenesis.

4.2 Challenges and limitations

All of my experiments, in chapters 2 and 3 were done with in vitro infection models, using either the monocytic cell line THP-1 or human primary monocytes differentiated into macrophages using an external stimulus (the cytokine GM-CSF). They constitute a convenient and good model of infection, but do not necessarily reflect what happens when a whole organism is infected. It would be ideal to confirm my findings using experimentally infected animals. In the case of the alteration of miRNA expression, it would be even better to use clinical samples from patients with leishmaniasis. Moreover, in vitro infection models allow us to only look at early infection events, since the half-life of macrophages in culture is very limited. In vivo infection models would allow us to understand the role of these factors in long-term infection.

In the case of CPN10, the single allele knockouts turned out to be very informative, but having an add-back positive control in the single allele knockout would have improved rigorous conclusions about the functions of CPN10. Moreover, my experiments do not allow us to decipher whether the modulations of uptake and intracellular survival that we see in CPN10+- and CPN10+++ leishmania are the results of a direct effect of CPN10, or an indirect one. I showed that leishmania CPN10 was delivered to the cytosol of infected macrophages, but we still do not know if it interferes with macrophages signaling there (Figure 2.7). Considering that
the delivery of recombinant leishmania CPN10 to the macrophage did not change the infection rate the way overexpressing CPN10 does (Figure 2.11), it suggests that CPN10 does not interact directly with the host cell, but this method of delivery is artificial. Moreover, CPN10 knockdown led to the modification of the expression of many leishmania proteins involved in metabolic processes and cellular processes (Figure 2.10, Table 2.1), and anyone or more of these may be responsible for the change in phenotype. For example, the decrease in CPN10 might downregulate the expression of a marker at the leishmania surface that interacts with receptors on the macrophages that are more likely to induce phagocytosis without activating the macrophage.

A limitation of my work in host miRNA expression during leishmania infection is that I do not know thus far what role miRNAs play in leishmania pathogenesis, especially survival. I proved that c-Myc is involved both in miRNA repression and in leishmania survival, but I did not make the connection between miRNA repression and leishmania survival.

4.3 Future perspectives

A question that remains is how CPN10+/− leishmania are killed by macrophages: we know that CPN10+/− leishmania are internalized in high number but that they do not survive intracellularly as well as WT leishmania (Figure 2.11, Figure 2.12, Figure 2.13). Testing the activation and microbicidal activity (nitric oxide, oxidative burst, cytokine production) of macrophages infected with either WT or CPN10+/− Ld might allow us to understand why CPN10+/− Ld show lower intracellular survival than WT Ld.

With regard to CPN10, I would also like to investigate whether CPN10 interacts directly with any component of the host cell - by looking at the subcellular localization of leishmania
CPN10 inside infected macrophages, and by performing a co-immunoprecipitation to identify partners to leishmania CPN10 in the host cell. For example, leishmania CPN10 could be able to interact with host CPN60 and block it, since it is highly similar to human CPN10. Another very interesting point would be to study the expression of markers at the surface of leishmania that might modify its uptake by macrophages - such as molecules that interact with macrophage surface receptors.

In order to understand the role of each individual miRNA or a group of miRNAs in infection and especially in manipulating the host cell phenotype, in vivo complementation experiments could be attempted, by delivering miRNA mimics to infected cells. miRNA mimics have been used successfully in cancer cells and in a mouse cancer model (Yang et al., 2015a; Kota et al., 2009), and could be attempted in leishmania-infected cells. Moreover, miRNA-based therapeutics are of considerable interest, and a few are currently the object of preclinical and clinical studies. Finally, messenger RNA expression could be analyzed by RNA-seq in parallel to miRNA expression, in the context of infection, to understand how repression of miRNAs in infected cells influences the expression of proteins, and beyond that might alter the host cell and the outcome of the infection. Of note, other teams have published the results of RNA-seq comparing leishmania-infected and control macrophages (Fernandes et al., 2016; Guerfali et al., 2008): these data could be used here, but it would be ideal to perform the sequencing of miRNAs and mRNAs in parallel in our system.

Inhibiting c-Myc reversed the repression of miRNA expression seen in infected cells (Figure 3.11, Figure 3.12), but it is unclear whether c-Myc acts directly as a transcriptional repressor to these miRNA genes, especially since the mechanism of transcriptional repression by c-Myc is still not completely understood (Wanzel et al., 2003). Therefore, it would be interesting
to test whether c-Myc binds to the promoter sequences of the miRNAs that are downregulated in infected cells, for example using chromatin immunoprecipitation. Finally, to understand better the extent of c-Myc repression of miRNAs it might be informative to analyze miRNA expression in infected and control cells with and without c-Myc inhibitor.

I showed that the upregulation of c-Myc in infected cells was not due to the PI3K/Akt or the Wnt/β-catenin pathways (Figure 3.14), but I did not show what signaling pathway is responsible for c-Myc induction. The Ras/Raf/MEK/ERK pathway might be responsible, and it should be tested using specific ERK inhibitors. Another aspect worth investigating is how c-Myc inhibition lowered leishmania intracellular survival (Figure 3.13). As stated before, we hypothesize that c-Myc plays a role in inducing the alternative activation of host macrophages, which favors parasite survival. To test this hypothesis, macrophage activation and microbicidal activity should be assessed in infected macrophages treated with c-Myc inhibitors. The production of cytokines (IL-10, TGF-β, IL-12, TNF-α) should be tested, as well as the release of nitric oxide and reactive oxygen species. This would allow us to understand what role c-Myc plays in the infected macrophage, and how leishmania intracellular survival is decreased when c-Myc is inhibited.

My findings show that both the leishmania protein CPN10 and the host protein c-Myc contribute to parasite intracellular survival in vitro (Figure 2.13, Figure 3.13). Using inhibitors in in vivo infection models would allow us to confirm that they are virulence factors and play a role in leishmaniasis pathogenesis. This could open the door to developing drugs that target them directly to treat leishmaniasis. In the case of CPN10, two aspects are especially interesting: (1) it is essential for leishmania, and (2) when its expression is only lowered, it leads to an increase in uptake and killing of leishmania by macrophages. Moreover, drugs targeting GroES/GroEL in
bacteria are currently being developed as potential new antibiotics (Johnson et al., 2014; Abdeen et al., 2016a). Similarly, inhibitors have been used against *Trypanosoma cruzi* HSP60/HSP10 (Abdeen et al., 2016b), which share 85% and 83% identity with *Leishmania donovani* CPN60 and CPN10 respectively, so these compounds might also work against *Leishmania* species and warrant further study.

Due to its central role in many -if not all- human cancers, c-Myc has been the object of intense research, as a target for cancer therapy. Unfortunately, it has for many years been considered "undruggable", because of its nuclear localization, and essential role in normal cell homeostasis (Dang et al., 2017). However, small molecule inhibitors as well as small inhibitory RNAs targeting c-Myc are still being developed and tested (Whitfield et al., 2017). siRNAs targeting c-Myc have been used successfully in a phase I study in patients with advanced solid tumors, although later trial data led the company to terminate the clinical trials (Tolcher et al., 2015). Targeting host c-Myc could be promising in leishmaniasis, as the interest in host-directed therapies is increasing for many other diseases, such as cancer and autoimmunity. There are many advantages to targeting the immune system in parallel with antiparasitic drugs to reduce parasite loads and improve long-term immunity, but also too reduce adverse side effects. Most of these host-targeted therapies in leishmaniasis are immune-based: for example, human recombinant IFN-γ was shown to accelerate parasite clearance and clinical improvement (Badaro et al., 1990; Squires et al., 1993; Sundar et al., 1995). Other approaches consisted of the use of small molecules inhibitors of key signaling pathways: the PI3K/Akt inhibitor wortmannin (Neves et al., 2010), or the SHP-1 inhibitor bpV-phen (Olivier et al., 1998; Matte et al., 2000). With immune dysfunction being central to visceral leishmaniasis, any strategy that could promote host immunity is promising in terms of therapy.
4.4 Concluding remarks

Leishmaniasis was classified as one of the Great Neglected Diseases of Mankind by Kenneth Warren and the Rockefeller Foundation in 1977 (Kenneth Warren and the great neglected diseases of mankind programme, 2017). However, to this day, leishmaniases continues to affect millions of people living in developing countries. The lack of interest in the Western world, combined with the lack of financial incentive for drug companies should not conceal the gravity of this disease, not only from a public health point of view, but also a socio-economic perspective. Leishmaniases are disfiguring, debilitating and lethal, and cause a strong socio-economic burden in highly endemic areas.

In addition to strictly enhancing knowledge about macrophage-leishmania interaction and leishmania biology, understanding how leishmania interacts with its host cells has strong potential in term of developing novel vaccines and drugs. In my work, leishmania CPN10 and host c-Myc appear to be confirmed as good drug targets, and as such open new perspectives in term of treatments. But more broadly, these new concepts can also have implications in understanding host-pathogen interactions with other intracellular pathogens, immunological responses, and parasite molecular biology.

The goal of my research was to understand the roles of two processes in the host-pathogen interaction during leishmania infection. I showed in this work that both the leishmania protein CPN10 and the host protein c-Myc constitute virulence factors for leishmania, contributing to the parasite intracellular survival. I hope that my findings will be of use for further research on molecular parasitology and host-pathogen interactions, but also that it may help in some way the people afflicted by the leishmaniases and potentially other protozoan diseases.
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