Novel insights into leishmania biology: the role of parasite and host-derived small non-coding RNAs

by

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Abstract

Infection with leishmania parasites causes severe chronic and potentially fatal illness in millions of people annually. Nevertheless, leishmania-host interactions remain understudied, and available treatments are sub-optimal. Pivotal to the establishment of infection, parasite replication and development of clinical disease is the subversion of microbicidal activities of host macrophages by leishmania. The overall aim of this thesis was to enhance our understanding of the modus operandi of macrophage subversion and explore the involvement of parasite- and host-derived small non-coding RNAs in this process.

My first objective was to investigate whether leishmania exosomes act as shuttle vehicles to export and deliver leishmania RNAs to host macrophages, where they may contribute to pathogenesis. We used high-throughput sequencing to characterize the transcriptome of leishmania exosomes and found that leishmania exosomes are selectively and specifically enriched in small RNAs derived almost exclusively from non-coding RNAs such as rRNAs and tRNAs. In depth analysis revealed the presence of tRNA-derived small RNAs, a novel RNA type with suspected regulatory functions. Exosomes protected their RNA cargo from degradation and were competent to deliver RNAs to macrophages. Furthermore, our results demonstrated a remarkably high degree of congruence in exosomal small non-coding RNA content between two distinct leishmania species, which argues for a conserved mechanism for exosomal RNA packaging in leishmania.

My second objective was to investigate whether macrophage miRNA expression is modulated during leishmania infection. Here, I was interested to know whether targeting of the host RNAi machinery is a potential novel mechanism of pathogenesis used by leishmania to control macrophage phenotype and promote chronic infection. I profiled miRNA expression in human macrophages at later stages of infection using two independent technologies. The data showed that leishmania infection induced an overall down-regulation of miRNA expression in macrophages. This down-regulation

was not caused through effects on synthesis or stability of Drosha and Dicer, two essential enzymes involved in miRNA maturation.

Taken together, my findings suggest that both leishmania- and host-derived small non-coding RNAs may contribute to pathogenesis. They open up new avenues of research on small RNA pathways in leishmania infection biology, which may identify novel therapeutic approaches.

Preface

Chapter 1. Figures 1.1, 1.3 and 1.4 are used with permission from applicable sources. Part of the text of Chapter 1 “Immunopathology and molecular mechanisms of leishmania pathogenesis” (section 1.2 including sub-sections and Figure 1.2) has been published as part of a review on virulence factors secreted by leishmania and immune evasion during visceral leishmaniasis, of which I am the lead author. Lambertz U., Silverman J.M., Nandan D., McMaster W.R., Clos J., Foster L.J., and Reiner N.E. (2012) Secreted virulence factors and immune evasion in visceral leishmaniasis. *Journal of Leukocyte Biology*, 91(6):887-99. I selected the topic, prepared Figure 1.2 and wrote all of the manuscript. Silverman, J.M. was involved in design, performance and data analysis of the vaccination experiments and in preparation of Figure 2. Nandan, D. contributed to manuscript revision and editing. McMaster, W.R. was involved in design of vaccination experiments and data analysis. Clos, J. contributed the HSP100 k/o leishmania for the vaccination experiments. Foster, L.J. was involved in experimental design and performance of proteomic analyses. Reiner, N.E. was the supervisory author on this paper and was involved in overall experimental design, conceptual design of the literature review, and editing of the manuscript. The text was modified from the original publication for my thesis. The respective section and image are being republished by permission from *The Journal of Leukocyte Biology*.

Chapter 2. This chapter is based on collaborative work performed in the laboratories of Dr. Reiner (UBC), Dr. Unrau (SFU) and Dr. Myler (Seattle BioMed). A version of Chapter 2 has been published as Lambertz U., Oviedo Ovando M.E., Vasconcelos E.J.R., Unrau P.J., Myler P.J., and Reiner N.E. (2015) Small RNAs derived from tRNAs and rRNAs are highly enriched in exosomes from both old and new world Leishmania providing evidence for conserved exosomal RNA Packaging. *BMC Genomics*, 16:151. I principally designed the study and conceived the experiments, conducted the experiments, interpreted the data and wrote the manuscript for the published paper. Oviedo Ovando, M.E. is listed as an equal contributor on the published paper. She co-designed, optimized and collaboratively conducted the experiments for the high-throughput sequencing library construction and Northern

blotting and contributed to data interpretation. She also wrote the respective parts of the Materials and Methods (sections 2.4.6 and 2.4.8). Vasconcelos, E.J.R. conducted the bioinformatic analyses of high-throughput sequencing data under my supervision. He also wrote the respective part for the Materials and Methods (section 2.4.7). Unrau, P.J. and Myler, P.J. contributed reagents and analysis tools, and helped with experimental design and data interpretation. Reiner, N.E. was the supervisory author on this project and was involved throughout the project in conceptual and experimental design, data interpretation and manuscript revision and editing. The paper was published under Biomed Central's Open Access policy, and is thus licenced under the Creative Commons Attribution Licence 4.0.

Chapter 3 is based on work performed in the laboratory of Dr. Reiner (UBC). I conceived the research theme, designed the study and experiments, performed the experiments, analysed and interpreted the data and composed the manuscript. Dr. Reiner was the supervisory author on this project and was involved throughout the project in conceptual and experimental design, data interpretation and manuscript revision and editing.

All work with hamsters in this study (project title: Mononuclear Phagocyte Cell Signalling) was reviewed and approved by The University of British Columbia Animal Care Committee (protocol licence number: A14-0218). The animal care and use protocol adhered to the standards and regulations provided by the Canadian Council on Animal Care in Science (http://www.ccac.ca/en_/).

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Lists of Abbreviations

AGO	Argonaute
AP-1	activator protein 1
BLAST	basic local alignment search tool
BMDM	bone marrow-derived macrophage
CCL	CC-chemokine ligand
cDNA	complementary DNA
CDS	(protein) coding sequence
CIP	calf intestinal phosphatase
CIR74	chromosomal internal repeats, 74-nucleotide long
CL	cutaneous leishmaniasis
CLASH	cross-linking ligation and sequencing of hybrids
CR3	complement receptor 3
CREB	cAMP response-element binding
DAT	direct agglutination test
DC	dendritic cell
DGCR8	DiGeorge syndrome critical region 8
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsRNA	double-stranded RNA
EBV	Epstein-Barr virus
EF-1α	elongation factor 1-α
ELISA	enzyme-linked immuno-sorbent assay
ERK	extracellular signal-related kinase
ESCRT	endosomal sorting complex required for transport
EV	extracellular vesicle
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GIPL	glycosylinositol phospholipids
GM-CSF	granulocyte-macrophage colony-stimulating factor
GO	gene ontology

GP63	glycoprotein 63 kDa
GPI	glycosylphosphatidylinositol
GSK-3β	glycogen synthase kinase -3β
GTPase	guanosine triphosphate hydrolase
HBSS	Hank's buffered salt solution
HITS-CLIP	high-throughput sequencing of cross-linking immunoprecipitation
HIV	human immunodeficiency virus
HMDM	human monocyte-derived macrophage
hnRNPA2B1	heterogeneous nuclear ribonucleoprotein A2B1
HS	human serum
HSP	heat shock protein
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IRAK1	interleukin-1 receptor-associated kinase 1
IκB-α	inhibitor of nuclear factor kappa B-α
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase
KEGG	Kyoto encyclopaedia of genes and genomes
LD	*Leishmania donovani*
LINE	long interspersed elements
LPG	lipophosphoglycan
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCL	mucocutaneous leishmaniasis
M-CSF	macrophage colony-stimulating factor
MHC-1	major histocompatibility complex-1
miRNA	micro RNA
MKK	mitogen-activated protein kinase kinase
MOI	multiplicity of infection
mRNA	messenger RNA

MRP	myristoylated alanine-rich C-kinase substrate-related protein
MVE	multi-vesicular endosome
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NFκB	nuclear factor kappa B
NK	natural killer (cell)
NO	nitric oxide
nt	nucleotide
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PI3K	phosphoinositide 3 kinase
piRNA	P-element induced wimpy testes (PIWI)-interacting RNA
PKC	protein kinase C
PKDL	post-kala-azar dermal leishmaniasis
PKR	double-stranded RNA-activated protein kinase R
PNK	polynucleotide kinase
PPG	proteophosphoglycan
PPM1A	protein phosphatase, Mg^{2+}/Mn^{2+} dependent, 1A
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PrP	prion protein
pSILAC	pulsed stable isotope labeling by amino acids in cell culture
PTP	protein tyrosine phosphatase
qPCR	quantitative PCR
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease

ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	reverse transcription
SAcP	secreted acid phosphatase
scRNA	small cytoplasmic RNA
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SHP-1	Src homology region 2 domain-containing phosphatase-1
shRNA	short hairpin RNA
SINE	short interspersed elements
siRNA	small interfering RNA
SL	spliced leader
SLACS	spliced leader-associated conserved sequence
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SOCS1	suppressor of cytokine signalling 1
sRNA	small regulatory RNA
srpRNA	signal recognition particle RNA
TAB2	TGF-β activated kinase 1/MAP3K7 binding protein 2
TAM	tumor-associated macrophage
TAP	tobacco acid phosphatase
TAS	telomere-associated sequence
TATE	telomere-associated transposable element
TCPTP	T-cell protein tyrosine phosphatase
TGFβ	transforming growth factor -β
$T_H1/2$	T-helper cell type 1/2
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
TRBP	HIV-1 TAR RNA binding protein

tRF	tRNA-derived RNA fragment
tRNA	transfer RNA
tsRNA	tRNA-derived small RNA
U	unit
UTR	untranslated region
VL	visceral leishmaniasis
vRNA	vault RNA
WT	wild-type

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Für meine geliebten Eltern,

Monika und Klaus

Chapter 1 Introduction

1.1 The leishmaniases

The leishmaniases are a group of vector-borne diseases caused by protozoan parasites of the genus *Leishmania*. They are widely distributed throughout tropical and sub-tropical regions of the world. In addition to causing severe morbidity and mortality, the leishmaniases present additional socio-economic burdens to already impoverished people due to their stigmatizing effects. First documentations of disease symptoms date back as far as 1500-2500 years B.C. [1]. However, the causative parasite, leishmania, was only associated with the disease in the early 1900's when William Leishman and Charles Donovan independently discovered ovoid bodies in spleens of patients in DumDum near Calcutta, India. The disease was accordingly termed "dumdum fever". Another name commonly used until today is "kala-azar" ("kala" is Sanscrit for "black", and "azar" Hindustani for "disease"), referring to visceral leishmaniasis [2,3]. It was not until about two decades later that sand flies were demonstrated to be the transmitting vector for leishmania [3]. Out of 35 different leishmania species known to date, at least 20 can infect humans. In general, three main clinical forms of human leishmaniasis can be differentiated: cutaneous, mucocutaneous, and visceral leishmaniasis, with the latter being the most deadly. Current treatments (discussed in section 1.1.4) have limited efficacy and are often associated with severe toxicity. Given this context, it is not surprising that – in the closing years of the 20th Century – the WHO and Rockefeller Foundation declared the leishmaniases as one of the *Great Neglected Diseases of Mankind*.

1.1.1 Global importance

An estimated 12 million people across 88 countries are currently affected by at least one form of leishmaniasis, with 1-2 million new cases each year, and upwards of 310 million people living at risk of becoming infected [4]. Cutaneous and visceral leishmaniasis are endemic to countries in both Old and New World, whereas mucocutaneous leishmaniasis almost exclusively occurs in the New World. 90% of

visceral leishmaniasis cases are found in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan [4]. Leishmaniasis is often considered a disease of the underprivileged, and poverty is one of the main risk factors associated with contracting leishmaniasis. This is believed to be caused by enhanced sand fly breeding and increased access to humans for blood meals in areas with poor, crowded housing and inadequate sanitary conditions [5]. The natural breeding places for phlebotomine sand flies are microhabitats such as rock cavities, tree roots and water tanks [6], which are more prevalent in areas with poor living conditions. Other major risk factors include malnutrition, population mobility, environmental changes such as deforestation and urbanization, and climate change [5]. The combination of the expansion of endemic areas, co-infection with HIV, emerging drug resistance and lack of an effective vaccine has recently created an urgent need for more effective anti-leishmanial treatments and control measures [7-10]. Therefore, more research on the molecular basis and the biology of the infection is needed to identify new potential drug targets and design novel treatment strategies and vaccines.

1.1.2 Life cycle and transmission

Leishmania are unicellular eukaryotes belonging to the class of Kinetoplastida and are characterized by the presence of an extranuclear DNA structure called 'kinetoplast'. Leishmania parasites are transmitted to humans via the bites of infected female sand fly vectors. Out of 500 different phlebotomine sand fly species known to date, only about 30 have been shown to be vectors for leishmania [4]. In the Old World, these sand flies are of the genus *Phlebotomus*, whereas the genus *Lutzomyia* transmits leishmania in the New World. Leishmania undergo a complex developmental process in the vector, also known as "sand fly stage" (top of Figure 1.1), which is initiated when a sand fly takes a blood meal and ingests macrophages infected with amastigotes, the ovoid-shaped, small (3-5 μm), non-motile form of the parasite. The development takes place within the digestive tract of the sand fly, where amastigotes initially transform into procyclic promastigotes, which are flagellated, weakly motile and replicate by division. These undergo further development and division, and upon migration to the anterior midgut, they transform into non-dividing, spine-shaped metacyclic promastigotes

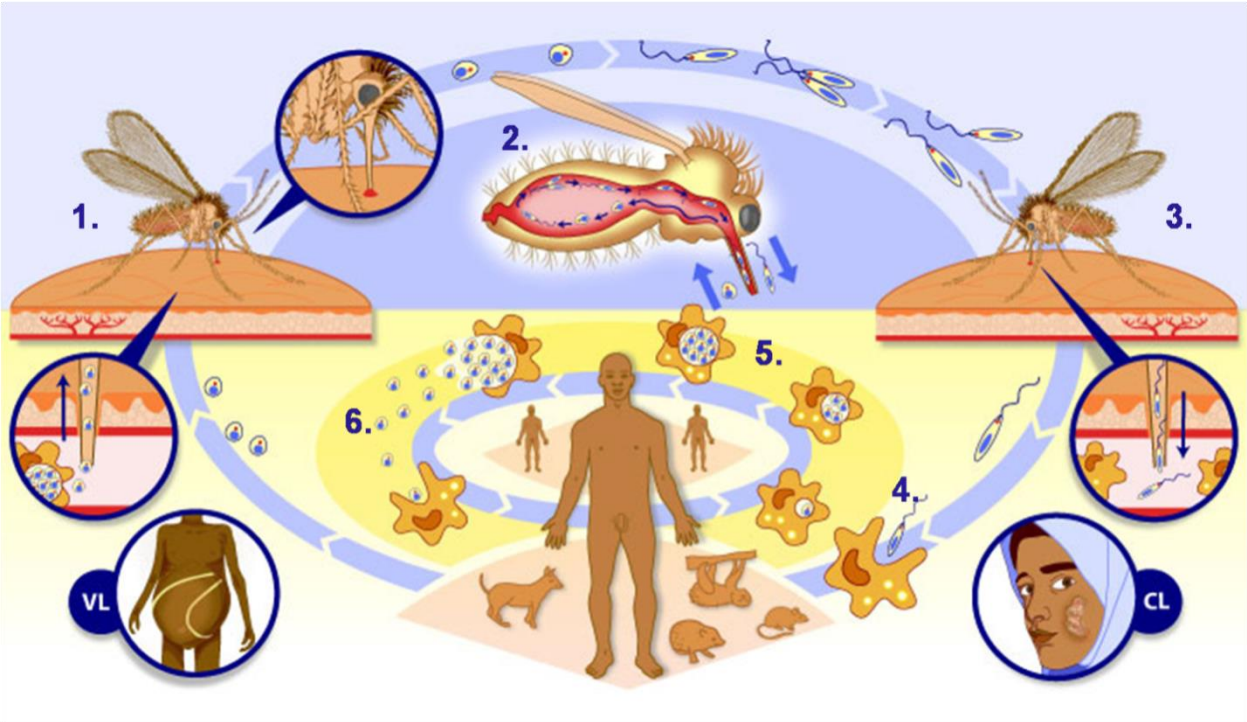

Figure 1.1 The leishmania life cycle.
The sand fly stage (top) and mammalian stage (bottom) of the leishmania life cycle. **1.** A sand fly ingests macrophages infected with amastigotes during a blood meal. **2.** Within the digestive tract of the sand fly, amastigotes transform into promastigotes and replicate by division. **3.** Metacyclic promastigotes are injected into the dermis of the mammalian host. **4.** Promastigotes are ingested by phagocytes, mainly macrophages and neutrophils. Within phagolysosomes of macrophages, they transform into amastigotes. **5.** Amastigotes multiply by division. **6.** Increasing parasite burden ultimately leads to physical rupture of infected macrophages and release of parasites into the extracellular environment, where they are engulfed by uninfected macrophages and multiply further. Infected cells are taken up by sand flies during blood meals, completing the cycle. VL, visceral leishmaniasis, CL, cutaneous leishmaniasis (© 2012 Liquid Jigsaw, WHO and Wellcome Trust, reprinted with permission)

(length 15-30 μm). This is the infective stage that is transmitted to humans and other vertebrate hosts during the next blood feeding. The total time for development within the sand fly is about 10 days [11]. While taking a blood meal, the sand fly injects the flagellated metacyclic promastigotes into the dermis of the host, thereby initiating the "mammalian stage" of the life cycle (bottom of Figure 1.1). Within the puncture wound, promastigotes are ingested by phagocytes, primarily macrophages and neutrophils, and take residence in phagolysosomes of macrophages, where they transform into amastigotes. Amastigotes multiply by division, and increasing parasite burden ultimately leads to physical rupture of infected macrophages and release of parasites into the extracellular environment. Released amastigotes are then engulfed by

uninfected macrophages and multiply further; parasites and infected cells can metastasize within the skin and to visceral organs, leading to the different clinical disease manifestations. The life cycle is completed when amastigotes within phagocytes are taken up by sand flies during blood meals. A variety of animals have been found to serve as natural reservoir hosts for leishmania including both domestic and feral dogs, rodents, foxes, jackals, wolves, racoons, hyraxes, sloths, aardvarks, opossums, and rodents, such as rats and mice, depending on the leishmania species and geographic focus [12]. Therefore, the leishmaniases are also considered as zoonoses. The reservoir hosts are primarily responsible for long term maintenance of leishmania populations and parasite transmission. Humans are generally considered incidental hosts for leishmania, but in some cases humans may also act as reservoirs, in particular for visceral leishmaniasis.

1.1.3 Pathobiology and disease manifestations

The spectrum of clinical manifestations observed in humans infected with leishmania and the development of disease can be attributed to genetics and immune status of the host, the infecting leishmania species, and sand fly factors (dose and route of inoculation, composition of inoculum).

Cutaneous leishmaniasis (CL) is the most common form, with 0.7 to 1.3 million new cases reported annually [13]. A large majority of leishmania species infectious to humans can cause CL, with *Leishmania infantum*, *Leishmania braziliensis*, *Leishmania peruviana*, *Leishmania amazonensis*, *Leishmania mexicana*, *Leishmania guyanensis*, and *Leishmania panamensis* being the main causative species in the New World, and *Leishmania major* and *Leishmania aethiopic*a in the Old World [14]. The typical first sign of an infection is the development of a small area of erythema at the site of the sand fly bite, which ultimately develops into an ulcerative lesion. This process can take 2 weeks to 6 months. In many cases, the lesions heal spontaneously, resulting in lifelong protection against homologous re-infection; but also in a lifelong scar, which may have substantial traumatizing and stigmatizing effects.

Mucocutaneous leishmaniasis (MCL) can be caused by *L. guyanensis*, *L. panamensis*, *L. amazonensis*, *L. infantum*, *L. major*, and *L. tropica*. However, it is most commonly associated with *L. braziliensis*, and therefore is mostly limited to the New World [14]. MCL typically appears 1 to 5 years after CL has healed. It develops when parasites disseminate to mucous membranes through the blood or lymphatic vessels, and typically involves ulceration of the nasal mucosa and perforation of the septum; sometimes, the lips, cheeks, soft palate, larynx or pharynx are also involved. MCL can be severely disfiguring, potentially life-threatening, and is very difficult to treat.

Visceral leishmaniasis (VL) is the most severe form and is characterized by hepatosplenomegaly, high fever, pancytopenia, hypergammaglobulinemia, and co-infection with opportunistic pathogens, and is nearly always fatal if untreated. It is almost exclusively caused by *L. donovani* (Old World) and by *L. infantum* (both Old and New World) [15]. VL affects approximately 300,000 people and causes 20,000 deaths annually [13]. Development of disease can take anywhere from 1 to 20 weeks. Post-kala-azar dermal leishmaniasis (PKDL) occurs in some patients (5-50% depending on geographic region) after VL has been apparently cured by drug treatment [12]. PKDL presents as maculopapular or nodular rash on the face, upper arms, trunk, or other parts of the body and individuals with PKDL are believed to be a potential human reservoir for anthroponotic VL transmission.

1.1.4 Diagnosis, treatment and vaccine status

The different forms of leishmaniasis are usually diagnosed by combining clinical signs with parasitological and molecular tests. Amastigotes can be identified directly by microscopy of lesion smears (CL and MCL) or splenic/bone marrow aspirates (VL) stained with Giemsa or Leishman's stain. In addition, samples for culture may be taken, which can increase diagnostic sensitivity [16]. Serological tests to detect anti-leishmania antibodies, such as enzyme-linked immuno-sorbent assays (ELISA), western blotting, immunofluorescence, antigen-coated dipsticks and direct agglutination tests (DAT) have been developed. Their use for diagnosis of CL and MCL is limited by their sensitivity, as antibodies tend to be undetectable or present in low titres due to

poor humoral responses [17]. The most widely used method for diagnosis of visceral leishmaniasis is the rK39 rapid diagnostic test, which is a recombinant antigen coated dipstick that detects antibodies in serum of patients infected with parasites of the *L. donovani* complex [17]. Recently, highly sensitive and specific polymerase chain reaction (PCR) tests to detect leishmania DNA have become available [18]. However, these require expensive materials and equipment and are therefore currently not practical in the majority of endemic areas. Further limitations of currently available diagnostic methods are availability, specificity, feasibility, sustainability, and field applicability.

A variety of drugs are available to treat the various forms of leishmaniasis [reviewed in [12]]. For decades, pentavalent antimony (Sb) has been considered the gold standard for local and systemic leishmaniasis therapy, and despite its multiple side effects and increasing parasite resistance, Sb is still widely used. Liposomal amphotericin B has been shown to be a highly effective and less toxic treatment, but its cost limits widespread use. Other available drugs and therapies include paromomycin, pentamidine, miltefosine, and topical treatment with imidazole quinolone, cryotherapy, and heat therapy. The failure of currently available treatments to induce sterile cure, poor patient compliance due to route and frequency of administration, high toxicity, and emergence of drug-resistant strains create a continued need for new therapies against the leishmaniases.

Recent advances have been made in the development of vaccines to prevent and/or treat leishmaniasis [reviewed in [19]]. Potential candidates include killed or attenuated parasites, purified immunogenic surface or secreted antigens, recombinant proteins, and DNA vaccines. However, to date there is still no vaccine proven to be efficient and safe for use in humans.

1.2 Immunopathology and molecular mechanisms of leishmania pathogenesis

Upon injection of leishmania promastigotes by the sand fly into the dermis of the human host, an intricate network of molecular parasite-host interactions is initiated. This network involves many factors triggered by the host's innate and adaptive immune responses, including multiple cell types such as macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells, and T-cells. Although leishmania can interact with a variety of host cell types, macrophages and DCs are the most important cells that regulate the outcome of infection [20]. Some evidence has suggested that neutrophils are the first cells to take up newly injected promastigotes [21,22]. These short-lived cells may act as "Trojan horses" to facilitate silent uptake of parasites by macrophages [23]. Once macrophages are recruited to the injection site, they take up free parasites or infected neutrophils, and become the final residence of leishmania, where replication takes place [23].

Development of symptomatic leishmaniasis is due to failure of the host to mount an effective immune response. Clinical cure ensues when macrophages become activated to kill leishmania and this requires an effective T-helper cell type 1 (T_H1) response. It is becoming increasingly evident that mechanisms of disease pathogenesis and host-leishmania interactions vary significantly depending on the infecting species [24]. The next section summarizes the mechanisms of pathogenesis that have been described in infections with leishmania, with a spotlight on the interactions of visceralizing species with macrophages.

1.2.1 Leishmania interfere with macrophage cellular functions

Although more than 100 years have passed since *L. donovani* was identified as a causative agent of VL [2,25], molecular mechanisms of disease have been the focus of intensive investigation only during the past three decades. Despite this relatively brief interval, much has been learned about leishmania pathogenesis. A great deal of evidence supports a model in which leishmania escape host immune responses by actively suppressing critical macrophage activities (see Figure 1.2).

Macrophages play key roles in the immune system's first line of defence and their major functions are microbial killing and initiation of adaptive immune responses to invading microbes. To carry out these functions, macrophages must become activated. Upon ligation of their pattern recognition receptors (or stimulation with interferon (IFN) -γ secreted by NK or T_H1-cells), macrophages undergo activation and produce pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL) -1β, and IL-6, leading to initiation of a local anti-microbial response [26]. In addition, macrophage activation triggers the production of reactive oxygen- and nitrogen intermediates, neutral proteases and lysosomal hydrolases, and anti-microbial proteins and peptides [27,28]. Ideally, ingested pathogens are internalized in phagosomes, which fuse with lysosomes and mature into phagolysosomes, where the intruders are readily digested. Antigens derived from digested pathogens are then presented to T-cells, initiating the adaptive immune response. In summary, intact functional properties of activated macrophages are critical for resistance to intracellular infections as typified by leishmania.

Despite this arsenal of macrophage defence mechanisms, leishmania appear to survive quite well within macrophage phagolysosomes. This implies that these microbes have developed strategies to efficiently evade or subvert macrophage microbicidal effector mechanisms. A strategy that has been studied extensively over the past three decades is interference with macrophage intracellular signalling through effects on host kinases and phosphatases. One of the earliest reports showed that lipophosphoglycan (LPG) purified from *L. donovani* potently inhibited the activity of protein kinase C (PKC) isolated from rat brain [29]. Inhibition of PKC activity in macrophages in response to *L. donovani* or LPG was shown to attenuate the oxidative burst and protein phosphorylation [30,31]. Moreover, *L. donovani* LPG was shown to delay phagosome maturation by inhibiting PKC-dependent depolymerization of periphagosomal F-actin [32,33]. Thus, by modulation of PKC activity, leishmania limits microbicidal activity.

MAP kinases are also targeted by leishmania. For example, in naïve murine bone marrow-derived macrophages (BMDMs), *L. donovani* prevented the activation of the mitogen-activated protein kinases (MAPKs) extracellular signal-related kinase (ERK)

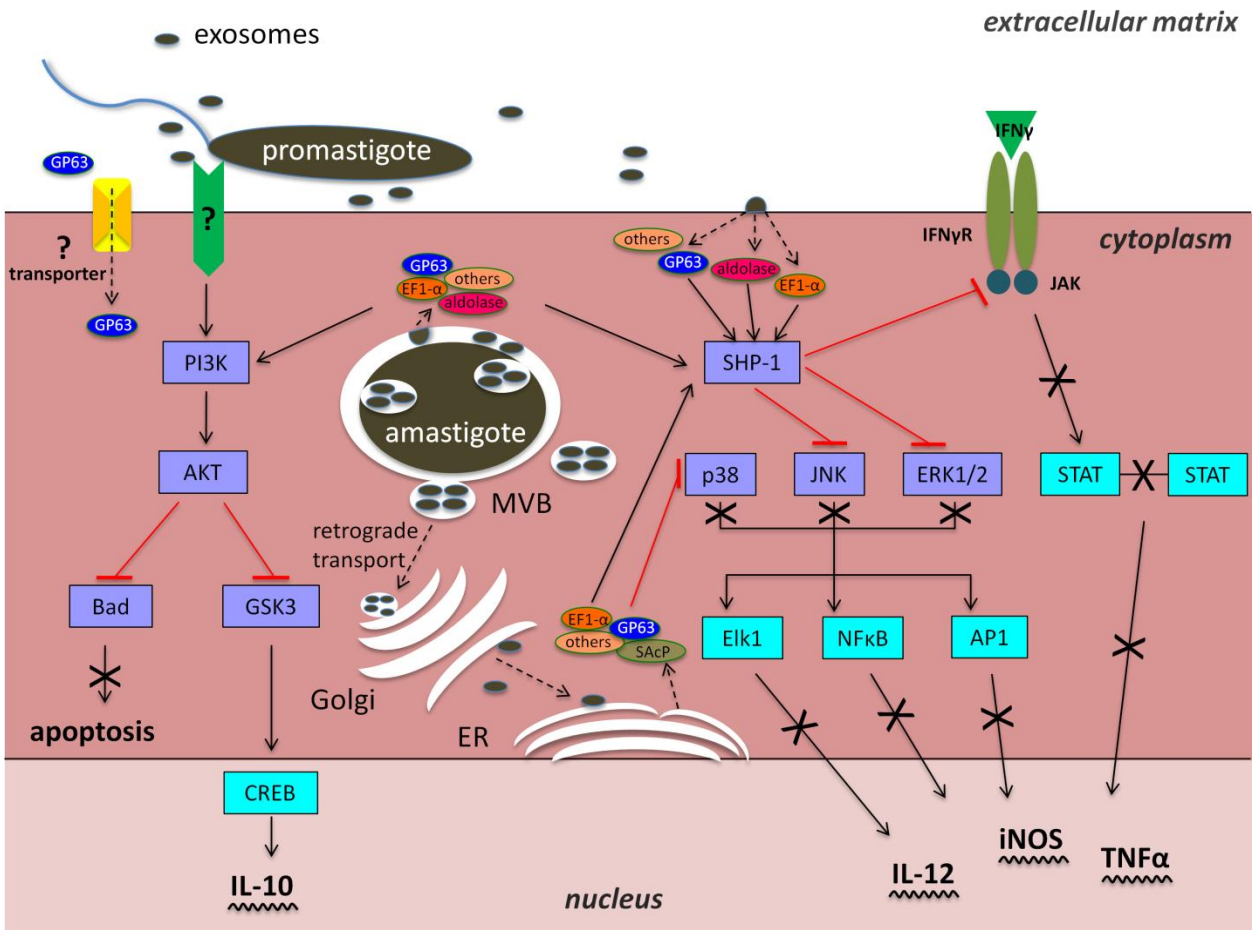

Figure 1.2 Leishmania secreted effector molecules target and disrupt macrophage intracellular signalling.
Leishmania promastigotes enter macrophages through facilitated phagocytosis and inhibit or activate MAPK and PI3K signalling cascades. Molecules released by leishmania promastigotes extracellulary (before uptake by macrophages) or by amastigotes intracellularly (from within the phagolysosome) may access macrophage cytoplasm through membrane transporters. Exosomal cargo proteins may be released into the cytoplasm by formation of multivesicular bodies followed by retrograde transport through Golgi and ER, or by fusion of the exosomal membrane with either the plasma membrane or the membrane of the phagolysosome. Once in the host cytoplasm, leishmania secreted effectors such as EF-1α, GP63, aldolase and SAcP target host kinases and phosphatases such as SHP-1 and PI3K. This prevents full macrophage activation and expression of antimicrobial effector mechanisms. (© 2012 *The Journal of Leukocyte Biology*, reprinted with permission [34])

1/2, p38 and c-Jun N-terminal kinase (JNK), as well as the degradation of inhibitor of nuclear factor kappa B (IκB) -α [35]. Conversely, activation of p38 MAPK was found to attenuate *L. donovani* infection in macrophages [36]. The modulation of MAPK activities by leishmania has been linked to suppression of IL-12 and induction of IL-10

production [37], thus representing an important mechanism by which leishmania inhibits proinflammatory responses.

The double-stranded RNA-activated protein kinase (PKR) has been recently shown to become activated in response to *L. amazonensis* infection, supporting parasite proliferation through upregulation of IL-10 production [38]. The role of PKR in infection with more common visceralizing leishmania has not been studied.

The phosphoinositide 3 kinase (PI3K)/Akt signalling pathway has also been shown to play a role in leishmania pathogenesis. Infection of RAW264.7 macrophages with either *L. major*, *L. pifanoi* or *L. amazonensis* results in a rapid and pronounced phosphorylation of Akt [39]. Activation of PI3K is important for host cell survival as it leads to inhibition of caspase-3 and Bcl-2-associated death promoter (Bad) [39]. Furthermore, activation of PI3K by *L. amazonensis* results in inhibition of macrophage IL-12 production [40], and induction of IL-10 production by *L. donovani* in myeloid cells is mediated through PI3K-dependent inactivation of glycogen synthase kinase-3β (GSK-3β), leading to reciprocal activation of the transcription factor cAMP response-element binding (CREB) [41]. Taken together, these data firmly establish a role for the PI3K/Akt pathway in promoting leishmania pathogenesis.

Regarding targeting of host phosphatases by leishmania, it was initially reported that *L. donovani* infection brings about an increase in protein tyrosine phosphatase (PTP) activity, including the specific activity of Src homology region 2 domain-containing phosphatase-1 (SHP-1) toward MAPK [42]. Moreover, inhibition of host PTPs prior to infection reverses the inhibition of MAPK activity, c-fos, and inducible nitric oxide synthase (iNOS) expression [42]. These findings were confirmed subsequently in another study, which demonstrated that *L. donovani*-infected macrophages display an increase in SHP-1 activity, coinciding with a decrease in IFN-γ induced Janus kinase 2 (JAK2) tyrosine phosphorylation and enhanced binding of JAK2 to SHP-1 [43]. This mechanism explains the defective response to IFN-γ observed in infected cells. More recent investigations have indicated that activation of SHP-1 prevents IFN-γ dependent nitric oxide (NO) production in *L. donovani* infected macrophages not only through

inactivation of JAK2 but also through inactivation of ERK1/2 and prevention of nuclear translocation of nuclear factor kappa B (NFκB) and activator protein 1 (AP-1) [44].

Several other host PTPs have been shown to play a role in leishmania pathogenesis. PTP1B and the T-cell PTP (TCPTP) are activated and post-translationally modified in *L. mexicana* infected macrophages [45]. In addition, gene expression profiling recently revealed that *L. donovani* infection markedly upregulates the expression of MAPK-directed phosphatases MKP1 and MKP3, and PP2A [46]. Upregulation of MKP1 results in p38 deactivation, whereas increased MKP3 and PP2A deactivate ERK1/2, with possible effects on inhibition of iNOS expression [46]. In summary, the modulation of host kinase and phosphatase activities appears to play complex roles in promoting leishmania persistence.

Other mechanisms by which leishmania avoid the activation of macrophages have been described. Phagocytosis of leishmania, a receptor-dependent process, appears to be mediated through complement receptor 3 (CR3) ligation, which does not trigger pro-inflammatory responses [47,48]. Upon internalization, promastigotes were shown to delay maturation of phagosomes into phagolysosomes and associated processes [49,50], enabling the parasite to transform into the amastigote stage, which have their optimal metabolic activity at acidic pH [51]. Furthermore, leishmania amastigotes from within the phagolysosome subvert the generation of reactive oxygen species (ROS) through heme degradation and prevention of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex assembly [52,53]. Another host pathway that was shown to be targeted and exploited by leishmania is the arginase pathway. Macrophage arginase is induced by T-helper cell type 2 (T_H2) cytokines (which induce polarization into an anti-inflammatory, or M2 phenotype) and hydrolyzes L-arginine into L-ornithine, which is needed for synthesis of polyamines that are essential for intra-macrophage growth of leishmania [54]. In summary, leishmania target a wide variety of host processes to allow establishment of chronic infection and avoid parasite killing.

1.2.2 Leishmania virulence factors

The mechanisms by which leishmania targets host cellular processes have been the subject of intense invstigations. However, only a small number of leishmania-derived virulence factors have been described and characterized in detail (see Figure 1.2). Several leishmania cell surface molecules have been implicated as virulence factors. The best studied example is lipophosphoglycan (LPG), a glycolipid that coats the surface of *L. donovani* promastigotes. As mentioned above, LPG has been shown to interfere with macrophage signalling through PKC- and MAPK cascades [30,31,55], and generation of NO in response to IFN-γ [56]. LPG was also shown to delay phagosome maturation and affect composition and properties of promastigote-harbouring phagosomes [57]. Other, less well studied components of the leishmania surface coat that were implicated in virulence, are the glycosylinositol phospholipids (GIPLs) [58]. Notably, pre-incubation of macrophages with leishmania GIPLs rendered them unresponsive to treatment with IFN-γ and bacterial lipopolysaccharide (LPS) [59]. In addition, leishmania proteophosphoglycans (PPGs), which exist as surface, filamentous and secreted forms, were shown to play important roles in establishment of infection [58]. Filamentous PPG was found in gel-like plugs in sand fly mid guts, forcing sand flies to increase the number of bites needed for feeding, thereby increasing the chance of transmission [60,61]. Promastigote PPGs were also shown to enhance alternative activation of macrophages and arginase activity in these cells [54].

Several classes of leishmania proteases have been shown to contribute to virulence and macrophage polarization, including cysteine proteases, serine proteases and metallo-proteases [62]. The most studied leishmania protease is the zinc metalloprotease glycoprotein 63 kDa (GP63). It exists as a surface-bound form, anchored to the membrane via glycosylphosphatidylinositol (GPI) anchors [63], and it was also shown to be secreted [64-67]. An important confirmation of GP63 as a virulence factor was a study by Joshi *et al*, in which susceptible Balb/c mice infected with GP63 knock out *L. major* showed delayed onset of lesion development [68]. A variety of functions have been proposed for GP63, many of which can be attributed to its protease activity. These include, for example, the evasion of complement-mediated

lysis by degradation of the complement component C3 [69] and direct breakdown of antimicrobial peptides [70]. GP63 may also directly interact with signalling proteins present in the macrophage cytosol. For example, it was shown to cleave epitopes on major histocompatibility complex-1 (MHC-1) molecules, thereby preventing antigen presentation to T-cells [71]. Furthermore, the PKC-substrate MARCKS-related protein (MRP) is degraded by GP63, providing a mechanism by which leishmania interferes with PKC signalling [72,73]. GP63 from *L. mexicana* was shown to modulate the activities of host PTPs SHP-1, PTP1B and TCPTP by cleavage, independent of internalization of parasites [45]. Importantly, these findings suggest that GP63 can gain access to macrophage cytosol, either by internalization of extracellular GP63 released from promastigotes, and/or by crossing the phagolysosomal membrane after release from intracellular amastigotes. Other leishmania secreted proteins have been detected in the cytosol of infected macrophages including: elongation factor 1-α (EF-1α), fructose-1,6-bisphosphate aldolase and secreted acid phosphatase (SAcP) [74-76]. *L. donovani* EF-1α and fructose-1,6-bisphosphate aldolase were both found to bind and activate macrophage SHP-1 [74,75]. The role of SAcP in both physiology and pathogenesis remains largely unknown.

A global proteomic analysis revealed that *L. donovani* secretes at least 151 proteins [67]. Only two out of the 151 secreted proteins contained a classical N-terminal secretion signal peptide. Additional study of these secreted proteins led to the discovery that leishmania uses an exosome-based secretion mechanism to release and deliver effector molecules, such as GP63, EF-1α, and fructose-1,6-bisphosphate aldolase to host cells and modulate their phenotypes [77,78]. The findings described above identify exosomes as a novel and potentially highly powerful virulence strategy for leishmania.

The mechanisms by which leishmania exosome cargo can access macrophage cytoplasm are still unknown. Considering that leishmania interacts with macrophages both extracellularly or intracellularly, there are at least two possible scenarios for translocation of leishmania molecules to host cytosol: i) extracellular molecules are transported across the plasma membrane, or ii) intracellular leishmania release

molecules into the lumen of the phagolysosome which are then transported across the phagolysosome membrane. In scenario one, internalization of extracellular molecules could occur either passively through transporter/carrier proteins or lipid rafts, as has been shown for GP63 [45], or actively through phagocytosis, macropinocytosis or endocytosis. In the case of exosome encapsulated molecules, fusion of the vesicle membrane with the plasma membrane and release of cargo into host cytosol is a likely possibility. In the case of scenario two, molecules could be transported across the phagolysosome membrane by transporter proteins. Alternatively, leishmania exosomes may either fuse with the phagolysosome discharging their cargo or be reverse endocytosed out of the phagolysosome followed by retrograde trafficking through the Golgi to release molecules into the cytosol.

1.3 Exosomes

Intercellular communication is an essential process that takes place in all organisms studied to date. Recent studies have demonstrated that a large number of molecules with functions in intercellular communication are shuttled through extracellular vesicles, including exosomes and other microvesicles. Evidence suggests that these extracellular vesicles mediate intercellular communication not only within one organism, but also between individuals from the same species, and even across organisms from different species and kingdoms. Thus, extracellular vesicles have gained increasing attention in the scientific community.

1.3.1 Biogenesis and functions

Vesicle-based secretion is a major mechanism of export used by a wide range of cell types. Exosomes are a discrete sub-population of secreted vesicles and are 50-100 nm in diameter. The generation of exosomes is achieved by invagination of endosomal membranes, resulting in formation of multi-vesicular endosomes (MVEs), see Figure 1.3. Release of exosomes into the extracellular space occurs upon fusion of the MVE membrane with the plasma membrane [79]. Hence, exosomes are distinct from other extracellular vesicles such as shed microvesicles released by blebbing, or vesicles shed

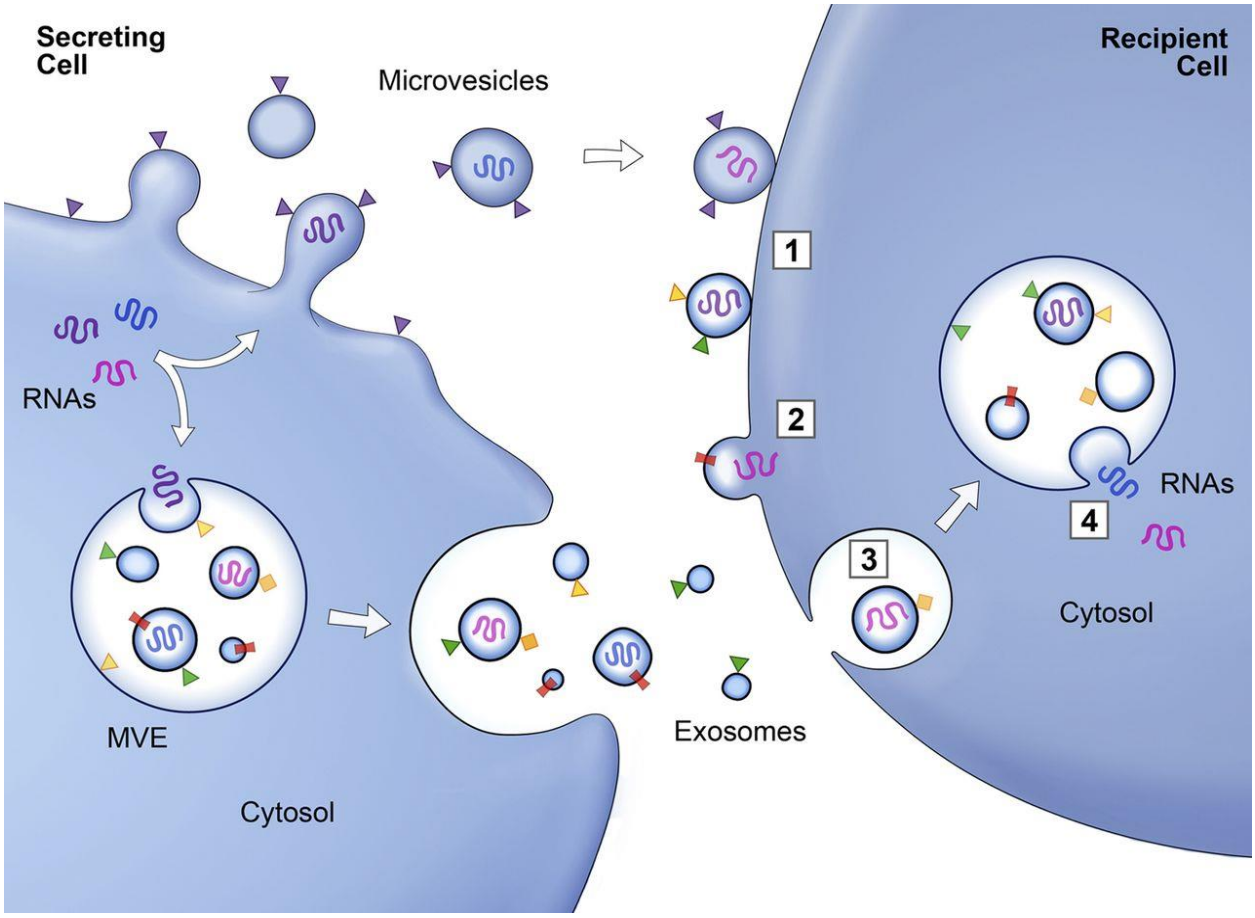

Figure 1.3 Transfer of proteins, lipids and RNA occurs through different types of extracellular vesicles.
Shedding microvesicles bud off the plasma membrane of the secreting cell (top). Exosomes are formed by invagination of the limiting membrane of multivesicular endosomes (MVEs, bottom) and are released when MVEs fuse with the plasma membrane of the secreting cell. Different types of proteins (membrane associated: triangles, transmembrane: rectangles) and RNA can be packaged into shedding microvesicles and exosomes. **1.** Both types of vesicles can dock at the plasma membrane of a recipient cell. Two mechanisms have been proposed for delivery of vesicular cargo to the cytosol of a recipient cell: **2.** fusion of the vesicular membrane with the plasma membrane of the recipient cell, resulting in unloading of cargo; or alternatively, **3.** uptake of vesicles by endocytosis, followed by fusion of the vesicular membrane with the endosomal membrane and release of cargo into the cytosol **(4.)**. (© 2013 *The Journal of Cell Biology*, reprinted with permission [80])

during apoptosis, which is not only reflected by their endosomal origin but also in their specific composition and effector functions.

The exact mechanisms of exosome biogenesis still remain unknown. In mammalian cells, formation of intraluminal vesicles of MVEs was shown to involve several proteins, such as ceramide and CD63 [81,82]. The involvement of the

endosomal sorting complex required for transport (ESCRT) has been suggested but is still being disputed [83]. MVE fusion with the plasma membrane appears to be regulated by small guanosine triphosphate hydrolases (GTPases) of the Rab family [84-86] and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins [87]. These studies were all performed in mammalian cells and little is known about these processes in lower eukaryotes. A study performed in the Reiner Lab indicated that the heat shock protein HSP100 of *L. donovani* may be involved in exosomal protein packaging, as the cargo of exosomes from HSP100 knock out parasites was distinct from wild type (WT) parasites [78]. Thus, exosomes from HSP100 knock out leishmania contained reduced amounts of virulence factors such as GP63 and EF-1α, and heat shock proteins such as HSP90 and HSP70, whereas they were enriched in histone proteins such as H3, H4 and H2b when compared to exosomes from WT parasites [78]. Furthermore, a study comparing the exosomal proteomes of WT and GP63 knock out *L. major* indicated that the metalloprotease may also play a role in exosomal protein sorting [88]. Similarly, knock down of the protein Sec6 using short hairpin RNA (shRNA) in *Cryptococcus neoformans* resulted in release of exosomes that were specifically lacking virulence factors [89]. Futher studies will be needed to fully understand mechanisms of exosome formation and release, in lower as well as higher eukayotes.

The functions of exosomes are varied and depend on their cargo and origin. They have been most extensively studied in mammalian systems. In the context of immune responses, exosomes released by immune cells such as B-cells and DCs were shown to participate in antigen presentation and induction of antigen-specific adaptive immune responses to tumors [83]. Conversely, exosomes secreted by some tumors, placental explants, or prostasomes in semen can have immunosuppressive properties [83]. Furthermore, suggested functions of exosomes include contribution to tissue repair [90], communication within the nervous system [91-93] and shuttling of pathogenic prion proteins involved in neurodegenerative diseases [94,95]. Studies in leishmania and other microorganisms have recently indicated that exosomes and other

extracellular vesicles contribute significantly to the pathogenesis of infectious diseases. See section 1.3.3 for a detailed discussion of this topic.

1.3.2 Exosomal cargo: lipids, proteins and RNA

A great variety of molecules have been found to be present in exosomes and their specific composition largely depends on the cell of origin and its activation state. A main characteristic of exosomes is their phospholipid bilayer membrane, which is composed of plasma membrane lipids, including phospholipids, sphingomyelin and cholesterol [96]. Due to the mechanism of biogenesis of exosomes, their membrane is in the same orientation as the plasma membrane of the cell of origin (inner vs outer leaflet). Certain lipids, such as phosphatidylserine, disaturated phosphatidylcholine, disaturated phosphatidylethanolamine, sphingomyelin, ganglioside GM3, and cholesterol, have been shown to be enriched in exosomes when compared with their parental cells [96]. These enriched lipids are thought to contribute to membrane stability and rigidity. Furthermore, they have been suggested to display biologic activity such as mediating angiogenesis and triggering intracellular signalling pathways in target cells [96].

Proteomic profiling of exosomes showed the presence of hundreds of cell type-specific proteins, both transmembrane and luminal [96]. Considering this relatively large number of cargo proteins for such a small vesicle, it has been argued that the proteins end up in exosomes as a consequence of random cytosolic sampling. However, it is becoming increasingly clear that protein sorting into exosomes is a specific process, and several mechanisms have been proposed. For example, sorting of ubiquinated proteins into exosomes by ESCRT, or co-sorting by protein-protein or protein-lipid interactions [97]. Functions of common exosomal proteins include vesicle structure, biogenesis and trafficking, whereas cell-type specific exosomal proteins are involved in physiologic and pathophysiologic processes. To this end, it was shown that pathogen-derived exosomes contain soluble and transmembrane protein virulence factors, such as Hmp1 in *C. neoformans* exosomes [98], and GP63 and EF1-α in leishmania exosomes [77]. Interestingly, it was observed that overall release and

protein composition of exosomes could be altered in response to external stimuli, such as changes in pH and temperature [77], indicating that the exosomal biogenesis and/or packaging machineries may directly react to such stimuli.

Recent studies have shown that exosomes released by various cell types contain – in addition to proteins – diverse RNA molecules, such as messenger RNA (mRNA), micro RNA (miRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA). This raises the exciting possibility that the transfer of genetic material via exosomes might affect the phenotype and function of recipient cells. The presence of mRNA and miRNA was first demonstrated in exosomes released from mast cells [99]. Interestingly, mRNA in mast cell exosomes was shown to be transferred between cells and successfully translated into proteins in recipient cells [99]. Since this initial discovery, numerous reports of exosomes containing RNA molecules have been published. For example, exosomes containing RNA were shown to be released by prostate cancer cells [100], tracheobronchial epithelial cells [101], the kidney cell lines HEK293 and COS-7 [102], secreted in human saliva [103] and bovine milk [104], and also by eukaryotic pathogens such as *Trichomonas vaginalis* and *Leishmania spp.* [Chapter 2 and [105,106]]. It is becoming increasingly evident that exosomes are specifically enriched in small non-coding RNAs [107]. However, mechanisms for specific packaging of RNAs into exosomes are still unclear. The RNA-induced silencing complex (RISC) has been proposed to participate in miRNA sorting into exosomes [108,109]. Furthermore, several sequence motifs have been identified in exosome associated RNAs, which could control their sorting into exosomes [110,111]. It remains to be established whether there is a conserved mechanism or whether it is cell-type specific.

1.3.3 The roles of exosomes in infectious diseases

Extracellular vesicle (EV) release is now considered to be a widely conserved phenomenon among prokaryotic as well as eukaryotic pathogens. Gram-negative as well as Gram-positive bacteria are well-known to shed microvesicles from their cell surface, which are rich in bacterial membrane proteins, soluble toxins and other signalling molecules [112]. These microvesicles, also known as bacterial outer

membrane vesicles, were shown to be released both *in vitro* during interaction with host cells and *in vivo*, and contribute to pathogenesis [112]. More recently, it was discovered that eukaryotic pathogens (including fungi and parasites) also release at least two types of EVs: shed microvesicles as well as exosomes [113]. Models of stimulation of host cells with fungal EVs from *C. neoformans* and *Histoplasma capsulatum*, have demonstrated that they are immunologically active and have the potential to regulate key pathogenic steps during fungal infections [114,115]. A number of studies have now been published characterizing EVs released by diverse parasites including apicomplexa, kinetoplastids, and helminths (see Table 1.1). However, only a small number of these organisms were speficially shown to release *bona fide* exosomes, including only two protozoans:

Table 1.1 Extracellular vesicles released by human parasites

Pathogen	***Type of EV***[a]	***Cargo analyzed***	***Function***	***Reference***
Protozoa				
Trypanosoma brucei	extracellular vesicles	proteins	N.D.	[116]
Trypanosoma cruzi	extracellular vesicles	proteins, RNA	Life cycle transition, susceptibility of mammalian cells, modulate host gene expression, enhance parasite adhesion, enhance heart parasitism	[117] [118] [119] [120] [121] [122]
Leishmania spp.	exosomes	proteins, RNA	modulate immune responses, enhance parasite proliferation, modulate host gene expression, affect host miRNA pathway	[77] [78] [88] [123] [106]
Trichomonas vaginalis	exosomes	proteins, RNA	modulate immune response, enhance parasite adherence	[105]
Giardia lamblia	extracellular vesicles	proteins	release of cyst wall components	[124]
Helminths				
Echinostoma caproni	extracellular vesicles	proteins	N.D.	[125]
Fasciola hepatica	extracellular vesicles	proteins	N.D.	[125]
Dicrocoelium dendriticum	extracellular vesicles	proteins, miRNA	N.D.	[126]
Heligmosomoides polygyrus	exosomes	proteins, RNA	suppress Th2 responses, modulate recipient cell gene expression	[127]

[a]The type of extracellular vesicle (EV) was determined based on the experiments done in the respective study. “Exosome” refers to *bona fide* exosome, “extracellular vesicle” refers to undefined vesicle population (could be microvesicles or exosomes or a mixture).
N.D. = not determined.

leishmania and *T. vaginalis* [77,105]. In other organisms, e.g. *Trypanosoma spp*., the released EV type is less well defined and may be a mixture of exosomes and other microvesicles [117].

T. vaginalis is an extracellular parasite that causes trichomoniasis, the most prevalent sexually transmitted infection. Proteomic studies of exosomes purified from *T. vaginalis* culture supernatant showed that the vesicles contain tetraspanins, a well-known component of exosomes, and other previously described virulence factors. Moreover, *T. vaginalis* exosomes were shown to deliver their cargo to host cells, to modulate production of host cytokines, and contribute to adherence to epithelial cells [105]. Interestingly, *T. vaginalis* exosomes contain small RNA cargo, which remains to be characterized.

Recently, it was shown that protein secretion by leishmania involves the release of *bona fide* exosomes [77]. Importantly, *L. donovani* exosomes had generally immunosuppressive effects *in vitro*, and promoted a pro-parasitic phenotype *in vivo* [78]. One study has suggested that GP63 on *L. donovani* exosomes targets the host micro RNA processing enzyme Dicer 1 in hepatic cells, resulting in reduction of miR-122 activity, lower serum cholesterol, and increased parasite burden in the liver of infected mice [123]. In addition to *L. donovani*, *L. mexicana*, *L. braziliensis* and *L. major* were also found to release exosomes [77,88,106]. In studies with *L. major* exosomes, it was found that the vesicles modulate host macrophage PTPs and transcription factors in a GP63-dependent manner [88]. Very recently, in our studies with *L. donovani* and *L. braziliensis*, we discovered that their exosomes not only contain lipids and proteins, but also nucleic acids in the form of RNA, which can successfully be transferred to host macrophages [Chapter 2 and [106]]. Importantly, the exosomal RNA cargo was mainly composed of small, non-coding RNAs with potential regulatory functions. The striking overlap between *L. donovani* and *L. braziliensis* exosomal RNA cargo strongly argues for an evolutionary conserved mechanism of RNA packaging. The results of this work are discussed in detail in Chapter 2. In summary, secretion of exosomes appears to be a major mechanism by which leishmania export virulence factors and deliver them to the host, where they subvert immune responses and contribute to pathogenesis.

Interestingly, mammalian cells infected with viruses, bacteria, parasites and prions have been shown to release exosomes, which contain pathogen-derived molecules and have immunomodulatory properties. Nguyen and co-workers provided evidence that human immunodeficiency virus (HIV) budding from primary macrophages occurs through the exosome release pathway, commonly referred to as the Trojan exosome hypothesis [128]. Of high interest, Epstein-Barr virus (EBV)-infected human B-cells were found to contain virus-derived miRNAs that were shuttled to target cells, and these viral miRNAs were shown to repress expression of target mRNAs in recipient cells [129]. The latter report was the first to clearly demonstrate the delivery of pathogen-derived functional genetic elements to bystander cells via exosomes. Exosomes released from *Mycobacterium avium*-infected macrophages were found to contain mycobacterial glycopeptidolipids, and were shown to induce a pro-inflammatory response in resting macrophages [130]. Furthermore, exosomes isolated from *Toxoplasma gondii*, *Eimeria tenella* or *L. major* pulsed DCs were shown to induce a T_H1-mediated, protective immune response against the respective parasite *in vivo* [131-133]. *L. mexicana*-infected macrophages were also shown to release exosomes containing virulence factors [134]. In addition, misfolded proteins associated with proteinopathies such as the prion proteins PrPc and PrPsc, were found to be actively released into the extracellular environment and spread through exosomes [135].

Although the importance of exosomes in infection biology is now starting to be appreciated, much remains to be learned about their biogenesis and release, mechanisms of uptake/delivery of cargo, and the contribution of individual exosomal cargo components to pathogenesis.

1.4 Small regulatory RNAs

One of the most ground-breaking discoveries in genome biology in the last two decades was the finding that short, double-stranded RNA molecules can silence gene expression by interfering with mRNA translation [136]. It is now well-established that small RNAs with gene regulatory functions exist across all kingdoms of life. Numerous types of small regulatory RNAs (sRNAs) have been identified and new types are

continuing to be discovered. Multiple pathways for sRNA biogenesis have been described, and these molecules participate in diverse mechanisms of gene regulation, gaining increasing complexity in higher organisms. Importantly, some sRNAs have been shown to be mobile. Thus, they can function not only inside the cell where they were generated, but also inside recipient cells at local or distant sites, transferred through extracellular vesicles and/or RNA-binding proteins [137].

1.4.1 RNA interference

sRNAs are non-coding, 20-30 nucleotide RNA sequences that participate in regulation of gene expression. Generally, sRNAs have been divided into three groups: small interfering RNAs (siRNAs), micro RNAs (miRNAs) and PIWI-interacting RNAs (piRNAs). Although the biogenesis and mechanism of action differ between these three groups, what they all have in common is that their sequences are complementary to portions of the transcripts they regulate, and that they associate with Argonaute (AGO) proteins. Originally, the term “RNA interference” (RNAi) had been specifically invented to name the mechanism of action of siRNAs; however, it is now commonly used to refer to sRNA-mediated gene silencing.

siRNAs are generated by cleavage of longer, double-stranded RNA (dsRNA) precursors, which can originate from of either RNA virus replication, convergent transcription of genes or transposons, self-annealing of transcripts, or experimental introduction (Figure 1.4 A) [138]. The cleavage is mediated by the RNase III-type endonuclease Dicer, which results in the production of 21-25 nucleotide short, double-stranded products. These siRNA duplexes then associate with the RISC (composed of Dicer, AGO and HIV-1 TAR RNA binding protein (TRBP) in humans), where the guide strand is loaded onto AGO, and the passenger strand is cleaved and ejected. The siRNA-containing RISC then finds and binds target mRNAs whose sequences are perfectly complementary to the siRNA, followed by target degradation, thus preventing mRNA translation into proteins. Originally, endogenous siRNAs were thought to function exclusively in silencing of transposons during development and defence

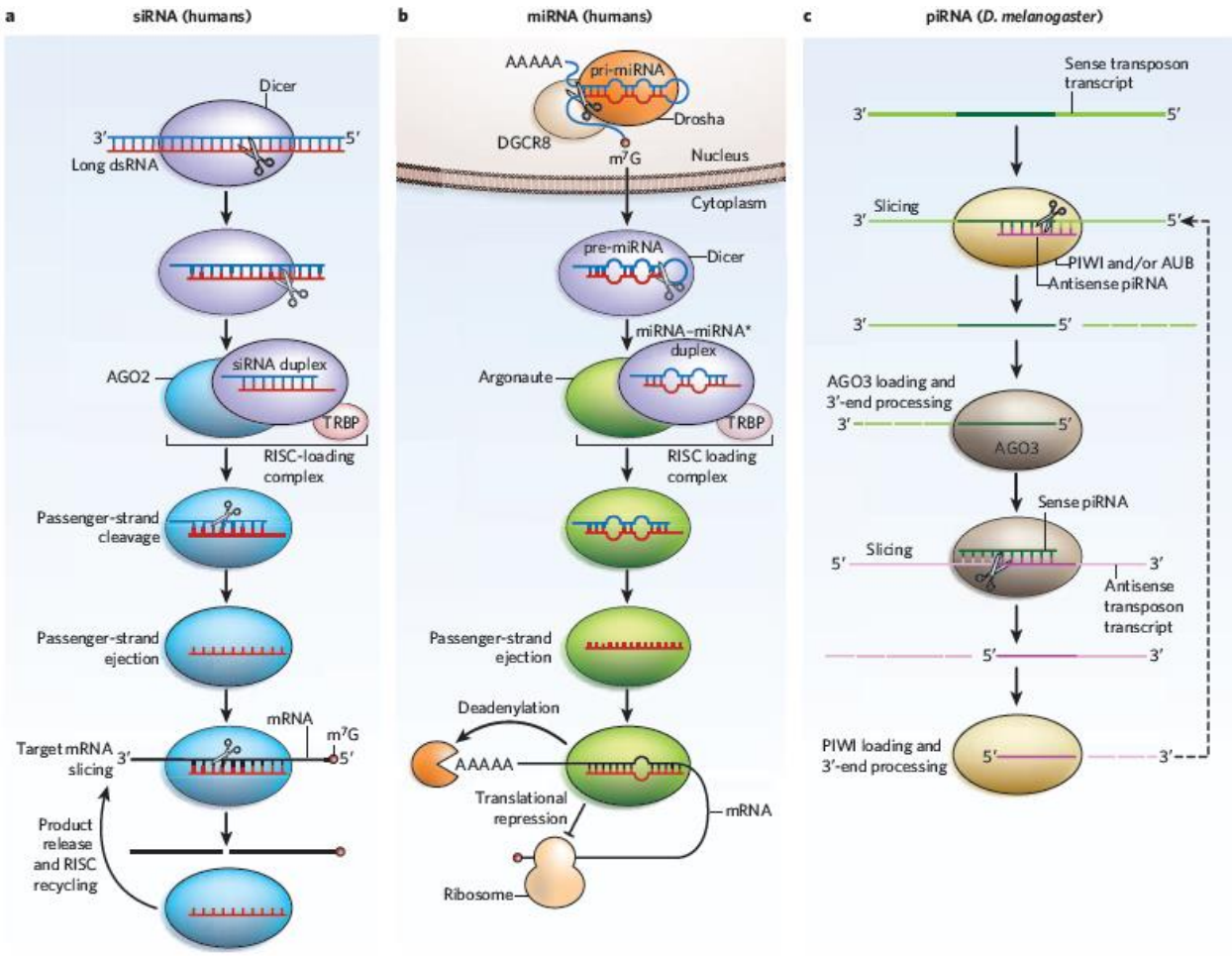

Figure 1.4 Biogenesis and mechanism of action of small regulatory RNAs.
Mechanisms of biogenesis and function of siRNAs (a) and miRNAs (b) in humans, and piRNAs (c) in flies. See text for details. (© 2009 *Nature*, reprinted with permission [138])

against viruses, but it is now becoming increasingly evident that they also function in physiological processes such as regulating synaptic plasticity in the brain [139].

miRNAs are encoded in genomic clusters, either as separate genes or introns of protein coding genes, and are generated by a complex expression and processing system as shown in Figure 1.4 B. After transcription and hairpin loop formation, the resulting primary miRNA (pri-miRNA) product is recognized by the DiGeorge Syndrome Critical Region 8 (DGCR8) protein, which associates with the RNase III-type endonuclease Drosha, forming the so-called microprocessor complex [140-143]. In this complex, the hairpin loop is liberated from the pri-miRNA, resulting in formation of a shorter (~70 nt) structure called precursor miRNA (pre- miRNA). This yields a double-

stranded ~22 nt RNA duplex [144] which is released and the guide strand is loaded onto AGO. The mature RISC complex then uses the seed sequence of the miRNA to recognize complementary mRNA transcripts, typically in the 3' untranslated region (3'UTR) of the mRNA [145], resulting in translational repression. In animals, miRNAs are only partially complementary to their target mRNAs. One miRNA can target multiple mRNAs and conversely, many mRNAs have multiple miRNA binding sites. It is believed that at least 60% of all genes if not more are regulated by miRNAs, making the latter indispensable for a vast majority of cellular processes [146]. Thus, it is not surprising that dysregulation of miRNAs has been associated with various diseases, such as cancer, disorders of the cardiovascular and nervous system and perturbation of immune responses during infection. Notably, miRNAs have also recently been implicated in epigenetic silencing in the nucleus through histone modification and DNA methylation of promoter sites, thereby inhibiting transcription [147].

piRNAs are slightly longer than miRNAs and siRNAs (24-31 nucleotides). Their mechanism of biogenesis is less-well understood, but it is known to be independent of Dicer (Figure 1.4 C). piRNAs associate with PIWI proteins, which are a germ-cell specific subclass of AGO. Their main function is thought to be silencing of transposons during germline development [138]. Furthermore, piRNAs may be involved in epigenetic regulation of gene transcription [148].

In addition to the above mentioned sRNA types, other small non-coding RNAs with potential regulatory functions are continuing to be discovered. It has been found that constitutively expressed, structural non-coding RNAs such as rRNAs, tRNAs, small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs), can give rise to short 5' and 3' end fragments [149,150]. These fragments were found to associate with AGO proteins and may therefore function in RNAi [149]. More research will be needed to dissect the exact mechanism of action and the targets of these unconventional sRNAs.

RNAi has been found to be highly conserved across eukaryotes. Even prokaryotes were recently found to have an analogous system to silence foreign genetic elements mediated by small non-coding RNAs, albeit the involved proteins and the

mechanism of action are different from eukaryotes [151]. Notably, essential protein components of the RNAi pathway appear to have been evolutionarly lost in several subspecies of single-celled eukarytoes, such as the budding yeast *Saccharomyces cerevisiae*, and the protozoans *T. cruzi* and *Leishmania* subgenus *Leishmania* [152,153]. It is believed that this may have happened due to the emergence of an alternative pathway with similar function, or the lack of selective advantage in certain niches [154]. The determination of loss of RNAi has to be done with caution, as the respective genes may have undergone great variations during evolution, leading to low similarities and difficulties in identifying protein homologues. To this end, a non-canonical AGO protein with a functional PIWI domain has been identified in RNAi-deficient *T. cruzi* (TcPIWITryp), and was shown to associate with rRNA- and tRNA-derived small RNAs in this parasite, indicating that this organism may be competent to perform non-canonical RNAi [155].

1.4.2 Pathogen-derived sRNAs and their role in disease

As mentioned above, the existence of sRNAs is conserved across the majority of eukaryotic organisms. It is thus not surprising that several infectious pathogens were recently shown to make functional sRNAs, with previously unappreciated roles in pathogen physiology and infection biology. Infection of plants and invertebrates with either DNA or RNA viruses can lead to the generation of siRNAs from viral dsRNAs. These siRNAs have been shown to target viral mRNAs or viral genomic RNAs, constituting a potent anti-viral response [156]. Whether a similar, siRNA-based anti-viral response also exists in mammalian cells is still being disputed, but seems unlikely [156]. Importantly, DNA viruses, particularly herpesviruses, can encode miRNAs that target either viral or host mRNAs and play roles in viral replication and pathogenesis [156].

Intracellular bacteria, such as *Listeria*, *Salmonella*, and *Chlamydia* have been shown to produce small non-coding RNAs that regulate the expression of bacterial target genes involved in intracellular growth and virulence [157].

In parasites, the existence of sRNAs has been investigated in trematodes, nematodes, arthropods, and protozoa. Protein homologues of Dicer and/or AGO and miRNAs were shown to be ubiquitously present in worms and arthropods, however, their roles in pathogenesis remains largely unknown [158]. In protozoa, on the other hand, the existence of canonical RNAi is less-well conserved. miRNAs with roles in virulence have been reported in *T. gondii*, *T. brucei* and *Giardia spp.* (see Table 1.2) [159-161]. Many other protozoan pathogens were shown to produce diverse types of sRNAs with potential regulatory functions in the parasite and/or the host (see Table 1.2), such as *Toxoplasma gondii, Giardia lamblia, T. vaginalis, Entamoeba histolytica, T. brucei, T. cruzi*, and *L. braziliensis* [159,162-167]. In some of these pathogens, sRNAs were found to associate with the pathogen's RNAi machinery. Thus, the various proposed functions of RNAi in these parasites include retrotransposon control in *T. brucei* and *L. braziliensis* [167,168], gene regulation in *E. histolytica* [164], and control of antigenic variation in *G. lamblia* [161]. However, in other pathogens such as *T. gondii* and *T. vaginalis,* studies on sRNAs have been limited to endogenous sRNA sequencing alone with no function yet reported [159,163]. Interestingly, *T. cruzi* tsRNAs were shown to be transferred to mammalian susceptible cells via extracellular vesicles, and to modify gene expression in recipient cells [120]. The precise mechanisms of action of *T. cruzi* tsRNAs are still unknown. In our studies with leishmania, we recently found that both RNAi-deficient *L. braziliensis* and RNAi-competent *L. donovani* release exosomes containing a select repertoire of small non-coding RNAs with potential regulatory functions, derived from tRNAs and rRNAs. These RNAs were efficiently delivered to macrophages [Chapter 2 and [106]]. It remains to be established, whether the exosomal RNAs contribute to leishmania virulence.

1.4.3 Human miRNAs: their role in innate immunity and defence against pathogens

miRNAs are gaining increasing importance as regulators of immune responses during infections with viruses, bacteria, parasites and fungi. On the one hand, they can act as part of a host response program, initiated for example by ligation of pattern regognition receptors, to achieve pathogen clearance. On the other hand, the

Table 1.2 Small regulatory RNAs produced by protozoan parasites

Pathogen	*RNAi*[a]	*sRNA types*[b]	*Possible functions*[c]	*Reference*
Apicomplexa				
Toxoplasma gondii	Yes	siRNA NAT	heterochromatin formation	[169] [159] [170] [171]
		miRNA tsRNA	gene regulation in parasite and host	[172] [173]
Plasmodium falciparum	No	tsRNA rsRNA	gene regulation	[174] [175]
		centromeric ncRNA NAT	heterochromatin formation	[176] [177]
Cryptosporidium spp	No	N.D.	N.D.	[178]
Babesia bovis	No	N.D.	N.D.	[178]
Theileria spp	No	NAT	N.D.	[179] [178]
Eimeria tenella	No	N.D.	N.D.	[178]
Neospora caninum	Yes	N.D.	N.D.	[158]
Kinetoplastids				
Trypanosoma brucei	Yes	NAT siRNA miRNA tsRNA rsRNA	transposon silencing, regulation of parasite genes	[180] [160] [181] [165] [182]
Trypanosoma cruzi	No	tsRNA rsRNA	virulence, regulation of gene expression in host	[153] [183] [120] [166]
Leishmania Viannia brazilienis	Yes	siRNA tsRNA rsRNA	transposon silencing virulence, regulation of gene expression in parasite and host	[153] [167] [106]
Leishmania Leishmania donovani	No	tsRNA rsRNA	virulence, regulation of gene expression in parasite and host	[106]
Flagellates				
Giardia lamblia	Yes	miRNA NAT tsRNA siRNA	regulation of genes in parasite, regulation of genes for variant-specific surface proteins, differentiation	[162] [184] [161] [185] [186] [187] [188] [189]
Giardia intestinalis	Yes	siRNA miRNA	Transposon silencing Regulation of parasite genes	[190] [163]
Trichomonas vaginalis	Yes	miRNA	Regulation of parasite genes	[163] [191] [187]
Others				
Entamoeba histolytica	Yes	miRNA siRNA NAT	Regulation of parasite genes	[192] [193] [194] [164] [195] [196]

[a]Canonical RNA interference (RNAi) pathway present as determined by the presence of homologues for genes for Dicer and/or Argonaute
[b]small regulatory RNA (sRNA) types: siRNA = small interfering RNA, miRNA = micro RNA, NAT = natural antisense transcript, tsRNA = tRNA-derived small RNA, rsRNA = ribosomal RNA-derived small RNA, ncRNA = non-coding RNA
[c]The majority of possible functions have not been directly experimentally validated and are hypothetical. N.D. = not determined.

expression of miRNAs can be actively modulated by pathogens, preventing anti-microbial responses and facilitating pathogen persistence, replication, and spread.

Several miRNAs have been identified in macrophages and DCs that play a role in regulating intracellular signalling events initiated by toll-like receptor (TLR) activation. For example, TLR stimulation with bacterial LPS caused miR-155 induction in DCs, via both the NFκB and JNK pathways [197,198]. Suppressor of cytokine signalling 1 (SOCS1), a protein negatively regulating the antigen-presenting capacity of DCs, and TGF-β Activated Kinase 1/MAP3K7 Binding Protein 2 (TAB2), a protein which activates MAPK kinases, were both identified as *bona fide* targets of miR-155 [145,199]. miR-146 was also found to be up-regulated after LPS stimulation and this increase in miR-146 brought about reduced expression of interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), both key components of the TLR signalling cascade [200]. Hence, by driving a negative feedback mechanism to attenuate the TLR response, miR-146 may prevent excess inflammation. Expression of both miR-146 and miR-155 was found to be increased in response to infection with various bacterial pathogens, including: *Salmonella enterica*, *Helicobacter pylori*, *Francisella tularensis*, *Listeria monocytogenes*, and mycobacteria [201]. The expression of miR-125 was found to be down-regulated in response to stimulation of macrophages with LPS [198]. One target of miR-125 is TNF-α, suggesting that down-regulation of miR-125 and concomitant upregulation of TNF-α is required to generate a pro-inflammatory response to a microbial agonist. Other miRNAs that appear to play key roles in shaping innate immune responses towards bacterial pathogens include the let-7 family, miR-223 and miR-21 [201], and more are continuing to be identified. The mechanisms by which bacteria are able to directly target the RNAi pathway to modulate host miRNA expression remain to be identified.

Several viruses have been shown to be capable of generally inhibiting RNAi by interfering with components of the host RNAi pathway, thus favouring virus replication. For example, the mammalian Nodamura virus protein B2 was found to inhibit the Dicer cleavage reaction *in vivo* and *in vitro* by binding Dicer substrate RNAs, leading to inhibition of endogenous miRNA generation [202]. Individual miRNAs can also be

induced in response to virus infection or anti-viral interferons and TNF-α, such as miR-155 [197,198]. Viruses are also able to take advantage of host cell miRNAs. For example the liver-specific miR-122, enhances hepatitis C virus replication [203].

A variety of protozoan pathogens have been shown to specifically modulate host miRNAs during infection (see Table 1.3). For instance, infection of cholangiocytes with *C. parvum* resulted in decreased expression of the miRNA let-7 and this was associated with an up-regulation of TLR4 in these cells [204]. Other studies confirmed the specific dysregulation of host miRNAs by *C. parvum*, and demonstrated concomitant reductions in mRNA targets, which have important functions in the immune response to this parasite [205-208]. In addition, infection of epithelial cells with *T. gondii* was shown to specifically increase the expression levels of miRNAs of the miR-17 ~92 cluster [209], but the impact of this on infection was not assessed in this study. In later studies, the increases in miR-17 ~92 were related to the inhibition of apoptosis by *T. gondii* [210,211]. Myocardial tissue samples of patients with chronic Chagas disease cardiomyopathy, caused by *T. cruzi*, showed dysregulated expression of several miRNAs when compared to healthy tissue [212]. In infection with *L. donovani*, it was recently shown that miR-122 was downregulated in the liver of infected mice, resulting in reduction of serum cholesterol and enhancement of parasite persistence [123]. Moreover, two other studies have evaluated the miRNA response to *L. major* and *L. donovani* in human macrophages and dendritic cells [213,214]. Both of these studies examined early infection timepoints only (initial 3-24 hours), and obtained contradictory results with regard to the miRNA expression profiles. In seeking a more comprehensive answer as to which miRNAs are specifically regulated particularly during chronic leishmania infection, I performed a global miRNA expression analysis in human monocyte-derived macrophages infected with *L. donovani* for 24-72 hours. The results, which are discussed in Chapter 3 of this thesis, showed that macrophage miRNAs appear to be generally downregulated in response to *L. donovani*. Notably, this downregulation could not be attributed to effects on Drosha or Dicer protein levels.

Taken together, the findings described above suggest that diverse pathogens are able to modulate the host immune response so as to promote infection through effects

Table 1.3 Protozoan parasites that were shown to modulate host miRNAs

Pathogen	*Host tissue*[a]	*miRNA change*[b]	*Effect*	*Reference*
Cryptosporidium parvum	epithelial cells	decrease let-7i	upregulation of TLR-4	[204]
	epithelial cells	decrease miR-513	upregulation of B7-H1	[205]
	epithelial cells	decrease let-7 and miR-98	upregulation of CIS and SOCS4	[206]
	epithelial cells	increase miR-27b	downregulation of KSRP	[207]
	epithelial cells	decrease let-7i	downregulation of SIRT1	[208]
Toxoplasma gondii	fibroblast	increase miR-17 ~92 and miR-106b ~25	N.D.	[208]
	macrophages	increase miR-17 ~92	downregulation of Bim, anti-apoptosis	[209]
	macrophages	increase miR-30c-1, miR-125b-2, miR-23b-27b-24-1 and miR-17 ~ 92	anti-apoptosis	[210]
	mouse brain	deregulation	N.D.	[211]
	mouse brain	deregulation miR-146a and miR-155	parasite survival	[212]
	neuroepithelioma cells	increase miR-132	decreased expression of dopamine receptors	[213]
Plasmodium spp.	mouse liver	deregulation	development of protective immunity	[215]
	Anopheles	deregulation	parasite development in mosquito	[216]
Leishmania major	macrophages	deregulation	N.D.	[213]
Leishmania donovani	mouse liver	decrease miR-122	lowering of serum cholesterol	[123]
L. major and L. donovani	macrophages and DCs	deregulation	N.D.	[214]
Trypanosoma brucei	PBMCs	deregulation	N.D.	[217]
Trypanosoma cruzi	myocardial tissue	deregulation	deregulation of targets	[212]

[a]Unless otherwise stated, experiments were performed with human cells and tissues
[b]The term "deregulation" designates miRNA profiles with both up- and down-regulated miRNAs
N.D. = not determined.

on expression of host miRNAs. Further detailed study will be required to elucidate the specific functions of miRNAs in infection, how their expression is modulated by pathogens and linked to pathogen persistence, and how these processes may potentially be targeted therapeutically.

1.5 Research questions and objectives

Interactions of leishmania with host macrophages are pivotal to the establishment of infection, parasite replication and development of clinical disease. However, the mechanisms by which leishmania manipulate macrophages to subvert

their microbicidal activities and persist inside these cells are still unclear. The manipulation of gene expression in the host by targeting host small regulatory RNA pathways has recently been discovered as a novel virulence strategy employed by a variety of pathogens. In addition, several pathogens were recently shown to make their own small non-coding RNAs, which have previously unappreciated roles in infection biology. Therefore, the overall goal of this thesis was to examine the role of small non-coding RNAs in leishmania pathogenesis from two different yet complementary perspectives, namely the involvement of both leishmania-derived small non-coding RNAs released within exosomes and host-derived miRNAs acting within infected macrophages. The first objective was to confirm the presence of RNA in leishmania exosomes, to characterize exosomal RNA sequences in detail, and to explore the hypothesis that these sequences can be delivered to, and function in, host macrophages. The second objective was to investigate if leishmania infection has an effect on host macrophage miRNA expression and to determine whether targeting of the host RNAi machinery is a novel leishmania virulence strategy.

Chapter 2 Small RNAs derived from tRNAs and rRNAs are highly enriched in exosomes from both Old and New World leishmania providing evidence for conserved exosomal RNA packaging

2.1 Background

Protozoan parasites of the genus *Leishmania* are highly endemic to tropical and sub-tropical regions of the world. They are transmitted to humans and other mammals by sandfly vectors. After inoculation, promastigotes are engulfed by host mononuclear phagocytes either directly or indirectly as cargo of apoptotic neutrophils [23]. Promastigotes take up residence in macrophage phagolysosomes, where they transform into amastigotes and undergo cell proliferation. Depending on the infecting leishmania species, disease manifestations and symptoms can vary widely from mild self-healing cutaneous lesions to lethal visceral disease. The two species that are the focus of the present study, *Leishmania donovani* and *Leishmania braziliensis*, cause visceral and mucocutaneous leishmaniasis, respectively. While the former is naturally the more serious threat as it can lead to death if left untreated, the latter can have an extremely high impact on the affected individual due to debilitating and disfiguring destruction of critical soft tissue structures.

Despite ongoing research efforts, there is still a large gap in knowledge about the intricate interplay between leishmania and host macrophages. The mechanisms by which leishmania manages to survive within these potent immune cells are just starting to be elucidated. One key strategy employed by leishmania appears to be the subversion of macrophage activation, the latter being a process that is crucial to induce macrophage digestion and killing functions [36,42,74]. At the same time, leishmania are resistant to the harsh conditions of the acidifying phagolysosome [218].

Our group has recently discovered that leishmania use a non-classical secretion mechanism to export a majority of their secreted proteins and communicate with macrophages, which involves the release of small vesicles called exosomes [67,77]. Exosomes are 50-100 nanometre-sized membrane vesicles secreted by a variety of

single- as well as multi-cellular eukaryotic organisms. They are distinct from membrane microvesicles, which are produced by blebbing, since their release occurs through fusion of multivesicular bodies from the endocytic/exocytic pathway with the plasma membrane of the cell [219]. The release of extracellular vesicles such as exosomes and other microvesicles has also been documented in the context of infection. Extracellular vesicles containing pathogen derived factors, may be released either by infected cells, as has been shown following infection with Epstein-Barr virus, mycobacteria, toxoplasma, plasmodia or leishmania [129,134,220-222], or by the pathogen directly, as has been shown for mycobacteria, cryptococci, trypanosoma and leishmania [77,89,117,119,223].

Importantly, in our studies, *L. donovani* exosomes and exosomal proteins were detected in the cytosolic compartment of infected macrophages [77]. Moreover, we showed that *L. donovani* exosomes can modulate mononuclear cell phenotypes *in vitro*, rendering them anti-inflammatory by specifically inhibiting cytokine production. Studies with C57Bl/6 and Balb/c mice provided evidence that treatment with exosomes from *L. donovani* as well as *Leishmania major* prior to infection exacerbated disease *in vivo* [78]. These findings have fundamentally transformed our understanding of how leishmania are able to communicate with the host. Two other studies have since supported a role for exosomes in leishmania pathogenesis. In the first, the authors showed that the metalloprotease GP63 delivered by *L. donovani* exosomes cleaved the microRNA (miRNA) processing nuclease Dicer 1 in murine hepatocytes, resulting in downregulation of miRNA-122 expression, lowering of serum cholesterol and enhancement of murine liver infection [123]. In a second study, another group looking at *L. major* exosomes reported that the vesicles globally affected macrophage gene expression, which was in part GP63-dependent [88]. In summary, these results make a strong case for the importance of exosomes in leishmania pathogenesis.

In addition to their protein cargo, exosomes and microvesicles were recently shown to be carriers of nucleic acids in the form of RNA [99]. Notably, some of these molecules were functional and could transduce signals in recipient cells. Exosomal RNAs have also been implicated in the pathogenesis of a variety of important, chronic

infections. For example, Epstein-Barr virus-infected B-cells were shown to release exosomes containing viral miRNAs which could regulate gene expression in recipient cells [129]. *Toxoplasma gondii*-infected fibroblasts released exosomes containing a set of host mRNAs and miRNAs that was distinct from that of uninfected, serum-starved cells [221]. However, to date only two protozoan pathogens have been found to release RNA-containing extracellular vesicles directly. Thus, *Trichomonas vaginalis* exosomes were reported to contain RNA sequences, the biotype and function of which still remain to be determined [105], and *Trypanosoma cruzi* was shown to release extracellular microvesicles containing a variety of non-coding RNAs including tRNA-derived small RNAs, which have a suspected regulatory nature [118,119].

Based on the evidence that exosomes may serve as biologically important shuttle vectors for RNAs, in the present study, we sought to investigate the RNA content of leishmania exosomes. We indeed found that leishmania exosomes contained RNA cargo which they were able to deliver to host cells *in vitro*. Using high throughput sequencing and bioinformatics analyses, we found that leishmania exosomes were enriched in small RNAs derived from largely non-coding RNAs. Notably, we discovered that these vesicles contained a relatively abundant and highly selective population of small RNAs derived from mature tRNAs. Furthermore, we found a number of novel transcripts, some of which were highly enriched in exosomes. Although exosomes released by both *L. donovani* and *L. braziliensis* had largely similar RNA content, *L. braziliensis* exosomes specifically contained transcripts derived from genes that also code for siRNAs.

Taken together, these findings show for the first time that leishmania exosomes are highly enriched in small non-coding RNAs, particularly tRNA-derived small RNAs with potential regulatory functions. This suggests that these RNAs may have functions in intercellular communication. These findings raise the distinct possibility of a previously unrecognized potential mechanism of leishmania pathogenesis, mediated through the exosomal delivery of small, principally non-coding RNAs to mammalian host cells.

2.2 Results

2.2.1 *L. donovani* and *L. braziliensis* exosomes contain short RNA sequences; and intact vesicles protect their RNA cargo from degradation.

We previously reported that leishmania use an exosome-based secretion mechanism in order to export proteins with potential virulence properties [67,77]. Based on a number of studies in mammalian systems demonstrating the presence of RNA in exosomes, we were encouraged to expand the scope of our research to examine the RNA content of leishmania exosomes. We performed all experiments for this study with exosomes purified from supernatants of *L. donovani* or *L. braziliensis* cultured *in vitro* under infection-like stressors (acidic pH and elevated temperature for 24 hours, see Materials and methods), which induce the cells to transform into amastigotes. We had previously observed that these "early" axenic amastigotes release increased quantities of exosomes enriched in specific virulence factors [77]. Moreover, while undergoing transformation into amastigotes, leishmania modulate critical macrophage processes to allow for establishment of chronic infection. Combined with the fact that amastigotes are literally the only life cycle stage found *in vivo* in the mammalian host once infection is established, we felt that exosomes purified from this life cycle stage were the most relevant to examine.

Exosomes were purified from supernatants of early axenic amastigotes and subjected to RNA extraction with phenol/chloroform. Results depicted in Figure 2.1 A and B show that *L. donovani* axenic amastigote exosomes contained significant amounts of RNA that were detectable with the Agilent Bioanalyzer. Quantification with nanodrop revealed an average yield of 12.5 ng of RNA per µg of exosomal protein (data not shown). Notably, the length profile of exosomal RNA was distinct from that of *L. donovani* total RNA, with the bulk of exosomal sequences being short (25-250 nt). Furthermore, we did not detect full-length ribosomal RNA (rRNA) peaks in exosome RNA profiles. In contrast, these full-length rRNA peaks were prominent in the total RNA profiles. To confirm that the purified nucleic acid was in fact RNA, we incubated exosome RNA with DNase, RNase or KOH. As can be seen in Figure 2.1 C, exosomal

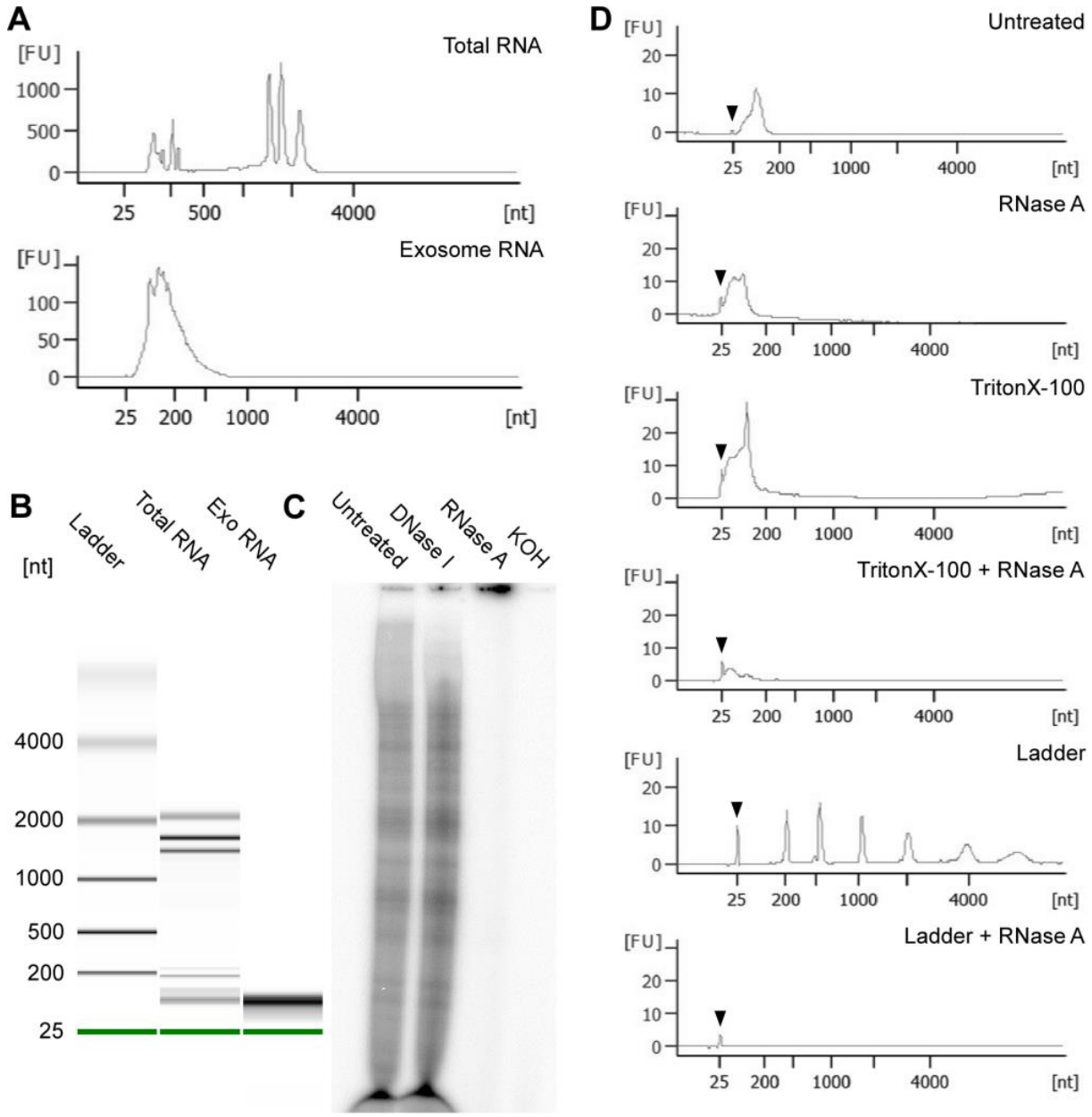

Figure 2.1 *L. donovani* exosomes contain RNA cargo.
Exosomes were purified from *L. donovani* axenic amastigote culture supernatant as described in the Materials and methods. RNA was extracted from exosomes or whole cells by phenol-chloroform extraction and then analyzed. **A.** Agilent Bioanalyzer RNA length profiles of exosome RNA alongside total RNA (~100 ng RNA were loaded for each), **B.** Gel-like image from Agilent Bioanalyzer measurement, **C.** Purified exosome RNA (~250 ng/sample) was either left untreated or treated with DNase I, RNase A or KOH followed by radiolabelling with γ32P dATP and separation on a denaturing 15% polyacrylamide gel, **D.** RNA inside exosomes is resistant to degradation. Prior to RNA extraction, intact exosomes (purified from 400 mL culture supernatant) were either left untreated or treated with RNase A or TritonX-100 or both. As a control for RNase A activity, 1 µL of the Agilent pico ladder was treated with the same concentration of RNase A. Samples were then subjected to RNA extraction and run on the Agilent Bioanalyzer. Arrowhead indicates internal 25 nt marker. nt, nucleotides. All images are representative of at least 3 independent experiments.

RNA was resistant to treatment with DNase, but was completely degraded upon exposure to either RNase or KOH.

To exclude the possibility that RNA was merely co-purified during exosome isolation but was not directly associated with or internal to the vesicles, we treated intact exosomes with RNase in the presence or absence of membrane-permeabilizing detergent. The results in Figure 2.1 D show that when treated with RNase alone, exosome RNA remained intact. In contrast, when exosomes were treated with RNase and TritonX-100 simultaneously, the RNA signal was greatly diminished. These findings suggested that the RNA was confined within the exosomal membrane and thereby protected from degradation. The fact that we still saw a small residual signal after detergent and RNase treatment could indicate that a fraction of the RNA was bound to RNA-binding proteins and was thereby protected.

To investigate whether the release of RNA within exosomes is conserved between leishmania species, we purified and analyzed RNA from exosomes released by *L. braziliensis* early axenic amastigotes using the same procedures as described for *L. donovani*. This analysis showed that *L. braziliensis* exosomes also contain RNA, with similar characteristics to that of *L. donovani* exosome RNA (Figure S2.1 A and B). Taken together, these data represent the first description of RNA released by leishmania within exosomes.

2.2.2 Leishmania exosomes deliver RNA cargo to human macrophages.

In order to investigate the potential delivery of exosomal RNA cargo to host macrophages, we labelled exosomes purified from the supernatant of *L. donovani* early amastigotes with an RNA specific fluorescent dye. Size and homogeneity of exosomes was assessed by Nanosight analysis (see Figure 2.2 A) and the median size was determined to be 120 nm. Fluorescence of labeled exosomes was confirmed by microscopy (Figure S2.2). PMA-differentiated THP-1 cells were incubated for 2 hours with fluorescently labelled exosomes and uptake was assessed by flow cytometry and confocal microscopy. As shown in Figure 2.2 B, we observed a dose-dependent increase in fluorescence of cells, suggesting that macrophages readily take up

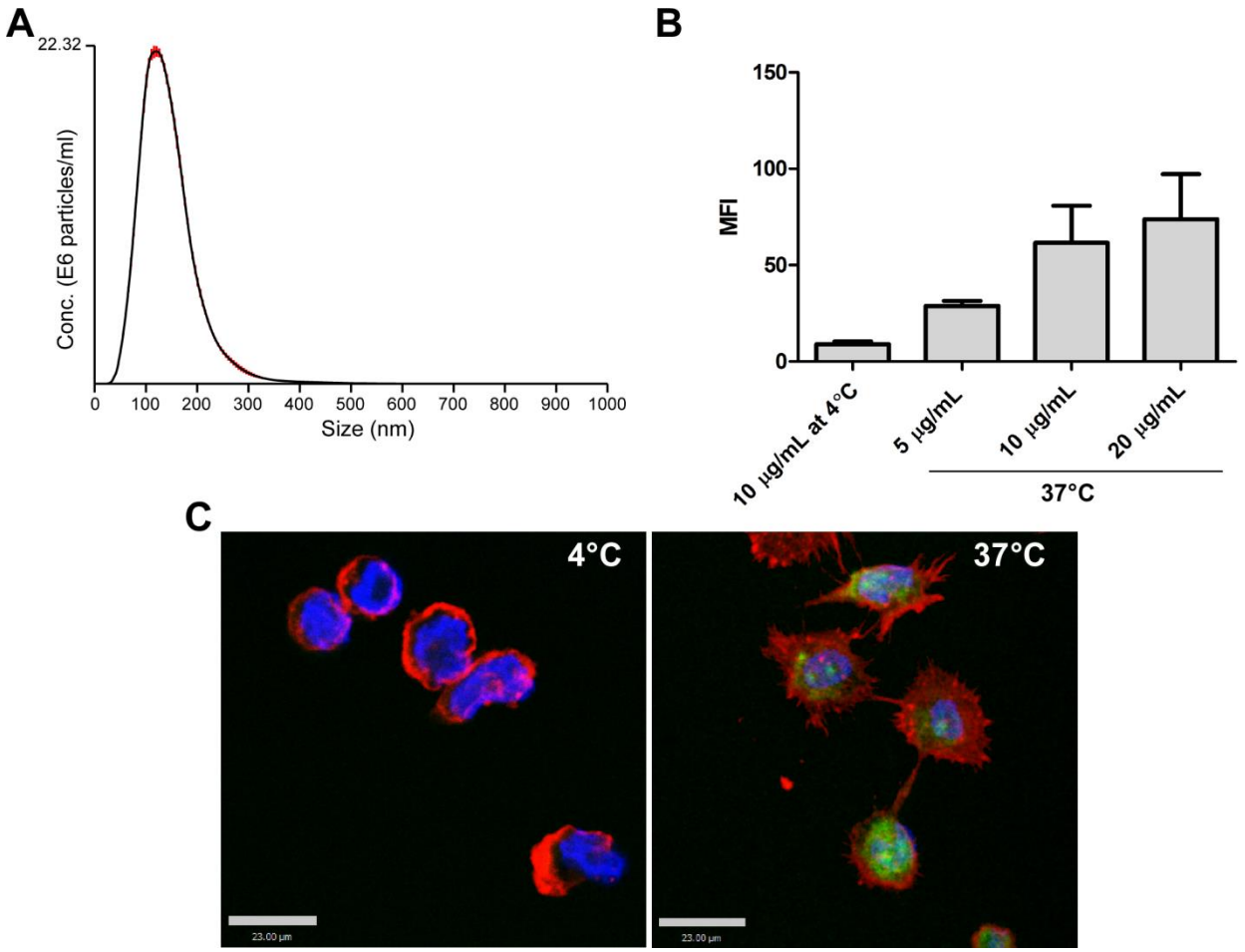

Figure 2.2 Exosomal RNA cargo is delivered to macrophages.
Exosomes were purified from 400-800 mL supernatant of *L. donovani* axenic amastigotes, protein concentration was determined by Micro BCA, and exosomes were stained with a green fluorescent RNA-specific dye. PMA-differentiated THP-1 cells were incubated for 2 hours with labelled exosomes at either 37°C or 4°C to inhibit phagocytosis. **A.** Nanosight size profile of purified exosomes. **B.** Cells were treated with different concentrations of labelled exosomes as indicated and analysed by flow cytometry. Histograms were drawn, median fluorescence intensity (MFI) of cells was recorded, and the mean of 3 independent experiments was calculated (error bars represent standard error of the mean, SEM). **C.** Confocal microscopy of cells incubated with 10 µg/mL exosomes (green) at 4°C (left) or 37°C (right). Cells were stained with phalloidin-Alexa 594 to detect actin (red) and DAPI to detect nuclei (blue). Confocal microscopy was done with a Leica DMIRE2 inverted microscope equipped with a SP2 AOBS laser scanning head. Images were taken with a 63x magnification objective. Images are representative of 3 independent experiments. Scale bars = 23 µm.

exosomes and their RNA cargo. In contrast, control cells incubated at 4°C to inhibit phagocytosis showed only background fluorescence. To exclude the possibility that exosomes were just bound to the macrophage membrane but not internalized after incubation, we examined exosome-treated cells by confocal microscopy. Figure 2.2 C shows that the fluorescence was localized to the cytoplasm of the macrophages and not

to the membrane, indicating that the exosomes containing RNAs were indeed taken up by the cells. These results confirm that leishmania exosomes and their RNA cargo can be internalized by host cells and can access their cytoplasm.

2.2.3 Characterization of leishmania exosome RNA cargo: exosomes are enriched in small non-coding RNAs derived from tRNAs and rRNAs

In order to assess the global transcriptome present in leishmania exosomes, we constructed complementary DNA libraries for high-throughput sequencing. We chose to compare RNA purified from exosomes released by early axenic amastigotes of *L. donovani* and *L. braziliensis* for three reasons: a) these two organisms cause distinct disease manifestations and hence can be expected to differ in their mechanisms of pathogenesis; b) they are spread through different vectors: *L. donovani* is transmitted by sandflies of the genus *Phlebotomus* in the Old World, whereas *L. braziliensis* is transmitted by *Lutzomyia* in the New World; and c) *L. braziliensis* was found to have a functional RNA interference pathway, which was not the case for *L. donovani* [153]. We hypothesized, therefore, that these two organisms could differ in their composition of exosomal RNA and chose to examine this directly. We used a strategy for library construction that was optimized for sequencing of small RNAs, as we had observed by gel electrophoresis that the exosomal RNA sequences were mainly short (Figures 2.1 and S2.1). We also incorporated a series of enzymatic treatments including dephosphorylation with calf intestinal alkaline phosphatase (CIP), 5' cap removal with tobacco acid phosphatase (TAP) and 5' re-phosphorylation with polynucleotide kinase (PNK) into the library construction procedure in order to pick up all sequences present in the exosomal transcriptome regardless of their 5' modification (see Materials and methods). Sequencing of the libraries by paired end 150 bp MiSeq Illumina sequencing resulted in ~1.4 million paired reads for *L. donovani* and ~1.1 million paired reads for *L. braziliensis* (Table 2.1). After adapter trimming and adjustment of the orientation of all reads to correspond to that of the original RNA sequence, reads were collapsed into unique reads prior to further analysis. As shown in the histograms in Figure 2.3 A, read length distributions of reads were clearly skewed towards shorter reads with the mean

read length being 55 nt for *L. donovani* and 57 nt for *L. braziliensis* (medians 37 nt and 49 nt, respectively).

Table 2.1 Sequencing statistics

	***L. donovani* library**	***L. braziliensis* library**
Total paired reads	1,435,277	1,062,571
Collapsed/unique reads[a]	688,524	538,034
Unique and single copy	574,049	421,086

[a]Reads were combined into unique reads by collapsing all identical reads to one read for downstream analysis.

To get a general overview about what types of RNA transcripts were represented in our libraries, we aligned the reads of the *L. donovani* and the *L. braziliensis* libraries with reference genomes, respectively LdBPK (*Leishmania donovani* strain BPK282A1) and LbrM (*Leishmania braziliensis MHOM/BR/75/M2904*) using Bowtie 2 and the very-sensitive-local option which sets the seed length to 20 nucleotides, allowing for only one mismatch within the seed alignment (see Materials and methods section 2.4.7). We were able to align 58.61% of reads from the *L. donovani* library with the LdBPK reference genome and 22.87% of reads from the *L. braziliensis* library with the LbrM reference genome (see Table S2.1 for full datasets). The comparatively low alignment rates especially in case of the *L. braziliensis* library is likely a result of incomplete assembly of the reference genome or the fact that we used a different *L. braziliensis* strain (a clinical isolate from the Peruvian Amazon region) than the strain used to generate the reference genome. This is supported by the fact that we were able to align significantly more reads (52.88%) from the *L. braziliensis* library with the *L. major* reference genome (*Leishmania major* MHOM/IL/81/Friedlin, LmjF, see Table S2.1). Other possible causes for low alignment rates could be sequencing errors, misinterpretation of modified nucleosides by the sequencer or RNA editing of sequences prior to packaging into exosomes, making it difficult to compare our transcriptomic data with the available reference genomes derived from DNA sequencing. RNA editing is a well-described process in leishmania and other trypanosomatids [224,225].

In order to ensure that our libraries were not contaminated with unrelated nucleic acids, we performed a BLAST search of all reads that failed to align with either the LdBPK, the LbrM, or the LmjF reference genome, against the NCBI nucleotide collection database (NCBI-NT). The results of this analysis showed that 28.1% of reads from the *L. donovani* exosome library and 36.3% of reads from the *L. braziliensis* exosome library aligned to sequences in the NCBI-NT database (see Table S2.2). Of these, 4.93% of *L. donovani* and 4.17% of *L. braziliensis* aligned with other leishmania genomes. The rest aligned with a promiscuous group of >6000 different plant, fungi, helminth and bacteria species, several of which were plant pathogens or soil inhabitants. Based on the observation that there was no enrichment of any particular species and that overall, the majority of reads from both libraries aligned with leishmania genomes (in total 63.54% of reads of the *L. donovani* library and 57.05% of the *L. braziliensis* library, see summary of alignment statistics in Table S2.3), we concluded that we did not have a contamination issue that would impugn our data. We think that many, if not all, of the reads mapping to bacteria or helminth genomes are likely false positive hits, i.e. they are not actually sequences derived from these organisms present in our samples. Rather, we think that the unaligned reads are actually sequences originating from leishmania that have been either misinterpreted by the sequencer or are naturally very diverse from the reference genomes we used for alignment. Thus, even though our alignment rates were somewhat lower than we might have expected, we think that our datasets are valid and large enough to draw meaningful conclusions about the exosomal RNA content.

When categorizing reads into RNA biotypes based on reference genome annotations, we saw that for both libraries, the majority of reads were aligning with rRNA and tRNA genes, in the sense orientation (Figure 2.3 B). In addition, a large number of reads mapped to non-annotated (intergenic) regions of the reference genomes (42.47% for *L. donovani* and 34.46% for *L. braziliensis*), which could potentially be novel transcripts. Interestingly, we saw less than 4% of reads mapping to protein coding genes (CDS) or spliced leader (SL) RNA genes. These results indicated that the majority of sequences present in the leishmania exosome transcriptome are

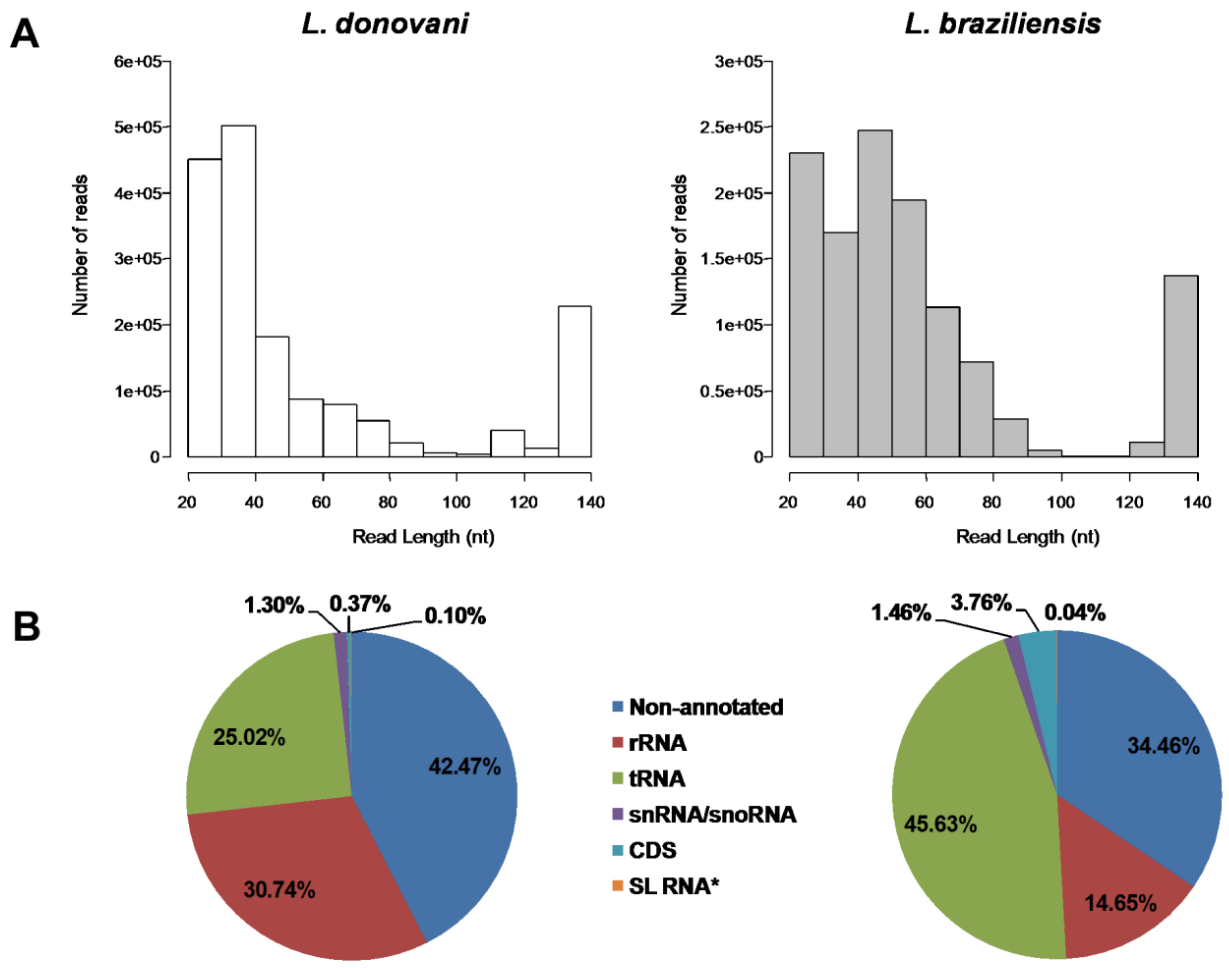

Figure 2.3 Sequencing of leishmania exosomal RNA reveals conserved RNA cargo composed mainly of sequences derived from non-coding RNA.
Exosome RNA from *L. donovani* and *L. braziliensis* was purified and processed for high-throughput sequencing as described in Materials and methods. **A.** Sequence length distribution of reads obtained from sequencing *L. donovani* and *L. braziliensis* exosome libraries. **B.** Categorization of reads according to their alignment with genomic features annotated in the *L. donovani* and *L. braziliensis* reference genomes. CDS, coding sequence; *SL RNA, spliced leader RNA. Numbers for reads mapping to SL RNA genes were obtained from alignment with the *L. major* reference genome, as these genes have currently only been annotated in this genome.

derived from non-coding RNAs and intergenic regions, whereas sequences derived from mRNAs are underrepresented.

When working with the LdBPK and LbrM reference genomes, we had to take into account that both are limited in their annotations. Hence, it was not surprising that we found a large number of reads in our libraries mapping to intergenic regions. Whereas

the annotations of CDS are thought to be comprehensive in these genomes, the assignments of SL RNAs as well as structural non-coding RNAs such as rRNAs, tRNAs, snRNAs and snoRNAs are clearly lacking in completeness. Consequently, it has to be considered that the large number of reads mapping to intergenic regions may not necessarily all be novel transcripts, but could also have resulted from incomplete annotation of non-coding RNA types in these regions. Keeping this in mind and still trying to dissect what types of RNA sequences are highly represented in exosomes, we decided to inspect in greater detail the alignment of the most abundant exosomal sequences manually using the Artemis genome browser software [226]. For this purpose, reads were clustered into unique regions of alignment and the regions were ranked by abundance (number of reads found per region). Considering that *L. major* is the species with the best assembled genome to date and presents the most complete annotation of non-coding RNAs, we also performed alignments of genomic sequences of *L. major* annotated non-coding RNAs with the LdBPK and LbrM reference genomes, in order to identify non-annotated, non-coding RNA loci in the LdBPK and LbrM genomes. The results of the screening using Artemis showed that the top 20 most abundant reads from both libraries mapped to three RNA classes in the sense orientation: rRNA, tRNA and snRNA (Table 2.2).

The high abundance of reads mapping to rRNA genes observed in both libraries is congruent with other recent reports on RNA types found in exosomes. Upon closer inspection we saw that the majority of reads mapping to rRNA genes were shorter fragments (median length 39 nt for the *L. donovani* library and 52 nt for *L. braziliensis*, see Figure S2.3). We then looked for enrichment of specific rRNA genes within our pool and saw that the majority of reads from both libraries mapped to 28S and 18S rRNA genes (>90%, Supplementary Table S2.4). Furthermore, we investigated the position of alignment of reads within the various rRNA genes, and found that for both libraries, reads aligned along the entire length of these genes (Table S2.4). It was of particular interest to find a large number of reads mapping to tRNAs in both libraries, as tRNA-derived small RNAs have recently been discovered in *T. cruzi* [166,183], and

Table 2.2 Top 20 most abundant clusters of transcripts present in leishmania exosomes

L. donovani						*L. braziliensis*					
Chr	***Genomic coordinates***		***Annotation***	***Reads***	***RNA type***	***Chr***	***Genomic coordinates***		***Annotation***	***Reads***	***RNA type***
27	1014367	1019133	LdBPK_27rRNA3	344191	28S rRNA	**6**	334041	334897	LmjF.27.rRNA.31	58906	28S rRNA
			LdBPK_27rRNA4						LmjF.27.rRNA.34		
			LmjF.27.rRNA.13								
			LmjF.27.rRNA.22								
			LmjF.27.rRNA.29								
			LmjF.27.rRNA.31								
			LmjF.27.rRNA.33								
			LmjF.27.rRNA.42								
27	1019947	1021495	LdBPK_27rRNA6	132730	18S rRNA	**00**	463646	464099	LbrM.27.rRNA1	11166	18S rRNA
15	312758	313248	LmjF.15.TRNAASP.01	65737	tRNA-Asp	**15**	324587	325394	LbrM.15.tRNA1	18229	tRNA-Asp
			LmjF.15.TRNAGLU.01		tRNA-Glu				LbrM.15.tRNA2		tRNA-Glu
			LmjF.09.5SrRNA.02		5S rRNA				LbrM.15.rRNA1		5S rRNA
			LmjF.05.5SrRNA.01								
			LmjF.15.5SrRNA.01								
24	715730	715801	LdBPK_24tRNA5	43207	tRNA-Asp	**24**	659346	659417	LbrM.24.tRNA5	30583	tRNA-Asp
17	328838	328909	LdBPK_17tRNA1	42601	tRNA-Asp	**17**	296223	296604	LbrM.17.tRNA1	30523	tRNA-Asp
									LbrM.17.tRNA2		tRNA-Ser
									LbrM.17.tRNA3		tRNA-Ala
24	658796	658976	LdBPK_24tRNA2	35611	tRNA-Gln	**24**	600448	600615	LbrM.24.tRNA2	8749	tRNA-Gln
09	429809	430355	LdBPK_09tRNA6	29246	tRNA-Glu	**09**	395278	395779	LbrM.09.tRNA3	4467	tRNA-Val
			LmjF.09.TRNAARG.01		tRNA-Arg				LbrM.09.tRNA4		tRNA-His
			LmjF.09.TRNAVAL.02		tRNA-Val				LbrM.09.rRNA1		5S rRNA
			LmjF.05.5SrRNA.01		5S rRNA				LmjF.05.5SrRNA.01		
			LmjF.11.5SrRNA.03						LmjF.11.5SRRNA.03		
			LmjF.21.5SrRNA.01						LmjF.21.5SrRNA.02		
						09	403494	403565	LbrM.09.tRNA5	5226	tRNA-Glu
31	495812	496115	LdBPK_31_tRNA3	18528	tRNA-Glu	**31**	582437	582738	LbrM.31.tRNA2	4719	tRNA-Gly
									LbrM.31.tRNA3		tRNA-Glu
27	1019543	1019804	LdBPK_27rRNA5	18473	5.8S rRNA						
11	156707	157038	LmjF.11.TRNAALA.01	15493	tRNA-Ala	**11**	63421	63678	LmjF.33.TRNAALA.01	3041	tRNA-Ala
			LmjF.36.TRNALEU.01		tRNA-Leu				LmjF.11.TRNALEU.02		tRNA-Leu
27	1014054	1014340	LmjF.27.rRNA.47	14260	28S rRNA						
			LmjF.27.rRNA.48								
23	230438	230509	LdBPK_23tRNA9	13814	tRNA-Gly	**23**	216842	216916	LbrM.23.tRNA9	3679	tRNA-Gly
36	1630332	1630403	LdBPK_36tRNA2	11529	tRNA-Gln						
05	360707	361335	LdBPK_05snRNA1	9971	snRNA	**05**	349991	350587	LbrM.05.rRNA1-1	6306	5S rRNA
									LbrM.05.ncRNA1-1		ncRNA
33	104560	104930	LdBPK_33tRNA1	9352	tRNA-Ala	**33**	105787	105859	LbrM.33.tRNA1	5730	tRNA-Arg
			LdBPK_33tRNA2		tRNA-Arg						
			LdBPK_33tRNA3								
23	229645	229857	LdBPK_23tRNA5	8487	tRNA-Leu	**35**	2472707	2472788	LbrM.35.tRNA4	2592	tRNA-Leu
			LdBPK_23tRNA6		tRNA-Thr						
16	445957	446028	LdBPK_161tRNA1	7916	tRNA-Gln	**16**	442089	442160	LbrM.16.tRNA1	8695	tRNA-Gln
23	230585	230656	LdBPK_23tRNA10	7804	tRNA-Trp	**23**	216992	217063	LbrM.23.tRNA10	3657	tRNA-Trp
						21	430678	430798	LbrM.21.rRNA1	3273	5S rRNA

Table 2.2 on previous page.
Reads were clustered into genomic loci based on Bowtie 2 alignments with reference genomes (as described in Materials and Methods) to identify the RNA types that were most abundant in exosomes. The details of the top 20 clusters with the highest numbers of reads falling into them are listed. Clusters of reads in the *L. donovani* library are listed in descending order of abundance, with the homologous cluster of reads in the *L. braziliensis* library given in the same row.
Chr = chromosome number
Annotation = annotation in reference genomes (LdBPK = *L. donovani*, LbrM = *L. braziliensis* or LmjF = *L. major*) followed by the gene name
Reads = number of reads found in sequencing data that were mapping to this genomic region
RNA type = type(s) of RNA that is annotated in the reference genomes in this region.

these novel small RNAs are thought to participate in regulation of gene expression [155,227] (see below for a more detailed analysis of tRNA-derived small RNAs).

Notably, and to our surprise, the overlap of the RNA profiles for *L. donovani* and *L. braziliensis* was striking. Thus, these parallel and independent RNA-seq replicates provide direct evidence for the reproducibility of our data.

2.2.4 Exosomes carry putative novel transcripts

To make sure we did not miss any important information amongst the group of less abundant reads, we inspected their alignments with the reference genomes manually. Interestingly, we discovered several reads mapping to intergenic regions at different genomic loci (Table 2.3). These intergenic regions were neither annotated at those loci in any of the sequenced leishmania or trypanosome genomes, nor did they share homology to any known trypanosomatid gene (as assessed by performing BLAST searches on TriTrypDB and NCBI). These findings suggested that the sequences mapping to these regions corresponded to *bona fide* novel transcripts. Notably, we found homologous novel transcripts in both libraries, providing evidence that they are both conserved between species as well as packaged into exosomes. When overlaying our sequencing data from the *L. donovani* exosome RNA library with a recently sequenced *L. donovani* spliced leader (SL) RNA library (P. Myler, unpublished data), we observed that the genomic loci giving rise to our identified novel transcripts had SL sites in the 5' region upstream of them (see Figure 2.4 for two examples). This indicates that they might be processed by *trans*-splicing and are hence likely to be functional mature transcripts rather than promiscuous transcriptional by-products.

Table 2.3 Intergenic regions coding for putative novel transcripts in exosomes.

L. donovani					*L. braziliensis*				
Name[a]	***Coordinates***		***#***	***ORF***	***Name***	***Coordinates***		***#***	***ORF***
LdBPK_301180 _leftof	379397	380435	363	7	LbrM.30.1240 _leftof	394563	397304	317	13
LdBPK_291610 _leftof	706385	707360	181	9	LbrM.29.1600 _leftof	663702	664543	103	7
LdBPK_360420 _leftof	109068	109733	139	3	LbrM.35.0480 _leftof	132138	133299	185	5
LdBPK_363000 _leftof	1183324	1183522	132	1	LbrM.35.3080 _leftof	1176690	1176833	36	1
LdBPK_362290 _leftof	872006	872406	89	5	LbrM.35.2400 _leftof	892711	895894	29	28
LdBPK_313190 _leftof	1452630	1452719	83	1	LbrM.31.3490 _leftof	1508257	1510948	0	N.A.
LdBPK_040550 _leftof	225336	230737	80	8	LbrM.04.0610 _leftof	229925	233378	111	25
LdBPK_131560 _leftof	555895	556192	75	2	LbrM.13.1200 _leftof	433509	433481	0	N.A.
LdBPK_364270 _leftof	1570740	1570991	57	1	LbrM.35.4310 _leftof	1563654	1563940	29	2
LdBPK_366120 _leftof	2270195	2272110	49	13	LbrM.35.6160 _leftof	2242345	2244214	153	14
LdBPK_330560 _leftof	173136	173362	40	0	LbrM.33.0550 _leftof	184438	184751	19	1
LdBPK_366590 _leftof	1903364	1906720	0	N.A.	LbrM.35.6630 _leftof	2438694	2438825	155	0

[a]Names are derived from the annotated genes adjacent to the intergenic region plus the designation "_leftof", indicating that the intergenic region is on the left side of the annotated gene on the same strand, regardless of transcriptional direction.
# = number of reads mapping to the respective region
ORF = open reading frame
N.A. = not applicable.

We also searched for open reading frames (ORF) within the sequences of the novel transcripts to see whether they have the potential to code for a protein or peptide and found potential ORFs for the majority of them (Supplementary Table S2.5). However, when we translated the ORFs and looked for homologies to known proteins in the NCBI database using Blastp, we did not obtain any hits.

Based on the hypothesis that these novel transcripts could have a role in regulation of gene expression in either the mammalian or insect host or both, we performed Bowtie2 alignments to the human and the vector (lutzomyia and phlebotomous) genomes to search for potential targets based upon complementarity. We obtained 60 hits for all of the 1288 reads representing novel transcripts in the *L. donovani* library when searching against the human genome and 15 hits when searching against the phlebotomus genome (Table S2.6A). For the 1137 reads that comprise the novel transcripts in the *L. braziliensis* library, 25 hits were observed when

searching against the human genome and 6 hits when searching against the lutzomyia genome (Table S2.6B). However, nearly all of these hits were determined to be in non-annotated regions of the respective genomes, implying – at least for the human genome – that there are no genes that could be regulated by the novel transcripts, based on the parameters applied in our analyses (perfect complementarity). Of note, the annotation of the vector genomes is a very recent effort and far from complete. It is quite possible, therefore, that there are as yet non-annotated protein coding genes in the genomic regions where the novel exosomal transcripts aligned, which would have been missed, resulting in false negative findings. Moreover, in most animals, regulatory RNAs such as miRNAs have incomplete homology with their target sequences [228] and, therefore, our predictions based on perfect complementarity may have missed some potential targets in the host genomes, again leading to false negative results. Unfortunately, as the tools to predict RNA-RNA interactions at the level of potential regulatory RNA-mRNA target pairs are fairly limited (generating a massive amount of ambiguous results when analysing large datasets), we were unable to carry out a comprehensive host mRNA target prediction with the novel transcripts that was informative. We also performed an alignment search of the novel transcripts against the databases of human and mouse miRNAs (http://www.mirbase.org), but failed to detect homologous sequences. All of the novel transcripts we identified were present in the sense orientation of transcription in leishmania, which implies that they are unlikely to be present in exosomes as double strands, which is a characteristic of canonical siRNAs and miRNAs.

To validate the presence of the identified novel transcripts in exosomes and compare their expression in exosomes with total leishmania RNA, we designed probes for Northern blotting. We selected the two most abundant novel transcripts identified in the *L. donovani* exosome library, one of which was positioned in between the genes 1170 and 1180 on chromosome 30 (LdBPK_301180_leftof) and the other in between the genes 1600 and 1610 on chromosome 29 (LdBPK_291610_leftof) for probe design. The regions that are complementary to the probes used are indicated in Figure 2.4 A (sympol “P”). We loaded equal amounts of *L. donovani* total and exosome RNA into a

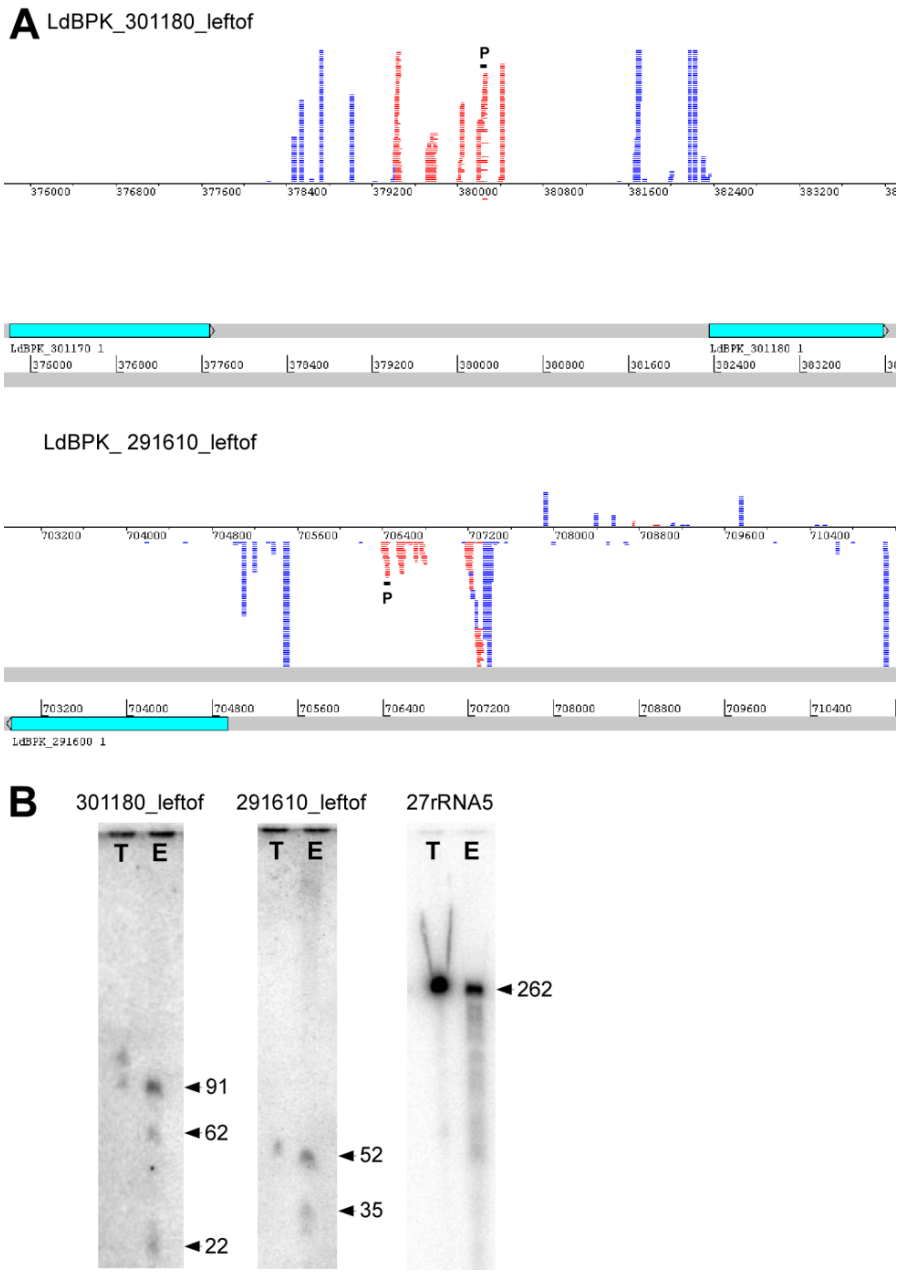

Figure 2.4 Novel transcripts are found in leishmania exosomes.
A. Artemis genome browser alignments of the *L. donovani* exosome library and a *L. donovani* spliced leader sequence library (P. Myler, unpublished) with the *L. donovani* reference genome. Shown are two regions with reads mapping to them. Top: intergenic region on chromosome 30 (LdBPK_301180_leftof), bottom: intergenic region on chromosome 29 (LdBPK_291610_leftof). Light blue boxes on grey tracks are annotated genes. In dark blue are the reads from the spliced leader library, in red the reads from the exosome libraries. "P" designates the regions that were used for designing probes for Northern blotting. **B.** Northern blots with probes designed for novel transcripts found in exosomes, corresponding to the genomic regions shown in panel A (301180_leftof and 291610_leftof), plus an additional probe against a 5.8S rRNA (27rRNA5). *L. donovani* total (T) and exosome (E) RNA were probed on the same membrane. Equal amounts of RNA (3 µg) were loaded in each lane. Sizes of bands as indicated are in nucleotides (nt) and were calculated based on 262 nt, 150 nt and 21 nt size markers.

polyacrylamide gel, along with 21 nt and 150 nt size markers. The results from the Northern blots showed that both novel transcripts could be detected in total and exosome RNA (Figure 2.4 B). Of interest, we detected bands of larger size that were present in both total and exosome RNA (which likely represent the primary transcript), but where the signal appeared to be stronger in the exosomal RNA lane. In addition, we detected bands of smaller size in the exosome RNA, that were completely absent in the total RNA. For comparison, we also incubated a blot with a probe for 5.8S rRNA (LdBPK_27rRNA5), which appeared to be much more abundant in total than in exosome RNA. These results confirm the presence of the novel transcripts identified by sequencing in exosomes and indicate that fragments of these transcripts with specific lengths are uniquely present in exosomes, consistent with selective packaging.

2.2.5 *L. braziliensis* exosomes carry a low abundance of sequences derived from siRNA-coding regions

As discussed above, *L. braziliensis* can regulate gene expression through the RNAi pathway and produce siRNAs [167]. We were interested in exploring the possibility of finding siRNAs as part of *L. braziliensis* exosomal RNA cargo. The main classes of *L. braziliensis* siRNAs are derived from the spliced leader-associated conserved sequence (SLACS) retroposon [229], the telomere-associated transposable element (TATE) [229], the *L. braziliensis*-specific telomere-associated sequence (TAS) [230] and the chromosomal internal repeats, 74-nucleotide long (CIR74) [167]. We generated a BLAST database from a FASTA file with 41 SLACS/TATEs extracted from TriTrypDB (file name: TriTrypDB-5.0_LbraziliensisMHOMBR75M2904_Annotated Transcripts.fasta) comprising the nucleotide sequences of the SLACS and TATEs genetic elements and performed a BLAST search with our libraries against this database. In the *L. braziliensis* library, we found 4471 reads mapping to these elements (Table S2.7). Interestingly, about 50% of these reads were both sense and antisense, suggesting that the sequences were present in exosomes as double-stranded RNAs. The lengths of reads were somewhat heterogeneous, ranging from 20 nt to 70 nt, whereas *L. braziliensis* mature siRNAs (*Lbr*AGO1-bound) are believed to be 20-25 nt in length [167]. For comparison, we also performed the same BLAST analysis with the *L.*

donovani library, where we only found 353 reads mapping to the siRNA-coding genetic elements (Table S2.7). The fact that we found some reads in the *L. donovani* library mapping to these elements could be due to settings used for the BLAST search that were not stringent enough (cut off 80 % identity and 70% query coverage), possibly resulting in false positive alignments. Despite this, it is clear that there were >10 times more reads in the *L. braziliensis* library mapping to siRNA-coding genetic elements, indicating that the results are specific and providing evidence that *L. braziliensis* may export siRNAs or their precursors in exosomes. However, we cannot rule out the possibility that the sequences we found in exosomes may originate from regions of the SLACS/TATEs genes other than the ones giving rise to siRNAs.

2.2.6 Leishmania exosomes contain an abundance of specific tRNA-derived fragments

Remarkably, we found a large number of reads in both the *L. donovani* and *L. braziliensis* exosome RNA libraries that mapped to tRNA genes. A few recent studies characterizing the RNA content of mammalian exosomes had reported the presence of tRNAs or their fragments in these vesicles. For example, reads mapping to tRNAs were found in sequencing libraries made with RNA from exosomes released from neuronal cells (13.5%) [231], immune cells (~7%) [232] and plasma exosomes (1.24%) [233]. Strikingly, in our datasets, 351,919 reads (36.4 %) and 135,149 reads (21.1%) from *L. donovani* and *L. braziliensis*, respectively, mapped to tRNA genes when aligned to the *L. major* MHOM/IL/81/Friedlin (LmjF) reference genome (which has the best curation on tRNA annotation amongst leishmania species). These frequencies exceeded by some measure those reported for mammalian exosomes in the studies cited above. Close inspection of the genome alignments revealed that a high percentage of these sequences were covering only parts of the respective tRNA genes (Figure 2.5), consistent with the occurrence of tRNA-derived small RNAs (tsRNAs), which has recently been recognized as a specific phenomenon.

In light of these findings we decided to characterize the reads mapping to tRNAs in more detail. In the case of both libraries, the vast majority (99.8 %) of reads were in

the sense direction of transcription. Looking at their length profiles, we found the mean read length to be slightly different between the two libraries, 38 nt for *L. donovani* and 46 nt for *L. braziliensis*, however, the median read length was similar (33 nt and 34 nt, respectively) (Figure 2.5 A). For both leishmania libraries, tsRNAs derived from tRNA-Asp, tRNA-Gln, tRNA-Glu and tRNA-Leu were most abundantly present (Figure 2.5 B and Table 2.4). To make a case that these tsRNAs were specific cleavage products that are selectively packaged into exosomes and not just a by-product of tRNA turnover that is disposed by the cell, we calculated the Pearson's correlation of the predicted cellular amino acid usage and the relative expression of our tsRNAs as assessed by sequencing. The results showed that there was no correlation (r = 0.163 for *L. donovani* and r = 0.114 for *L. braziliensis*), indicating that the tsRNAs were unlikely to be random degradation products. Strikingly, we observed the same rank order frequency of tRNA isotypes as origins of tsRNAs in both libraries (Figure 2.5 B and Table 2.4), indicating that the formation of specific tsRNAs and their appearance as exosomal cargo is an evolutionary conserved phenomenon in leishmania.

Next, we investigated whether the tsRNAs were derived from the 3' or 5' end of mature tRNAs, and found that the most abundant $tsRNA^{Asp}$ and $tsRNA^{Gln}$ were derived from the 5'end or mid-5' end in both libraries (Table 2.4). Notably, this was not the case for all of the tsRNAs, as some appeared to be derived from the 3' end, and two were not derived from the same end in the two different species ($tsRNA^{Glu}$ and $tsRNA^{Gly}$) (Table 2.4). Furthermore, we saw tsRNAs of different lengths, some of them corresponding to the length of tRNA-halves (~35 nt, e.g. most abundant read length for $tsRNA^{Asp}$), and others to the length of tRNA-derived RNA fragments (21 nt, e.g. most abundant read length for $tsRNA^{Leu}$) (Figure 2.5 C).

Based on the hypothesis that leishmania tsRNAs may act as miRNAs or siRNAs in the mammalian or invertebrate host, we performed additional Bowtie2 alignments with all reads mapping to leishmania tRNAs against the human and vector genomes looking for complementarity to find possible targets for these potential regulatory RNAs. This search yielded a very large number of hits (~20,000), the majority of which fell into

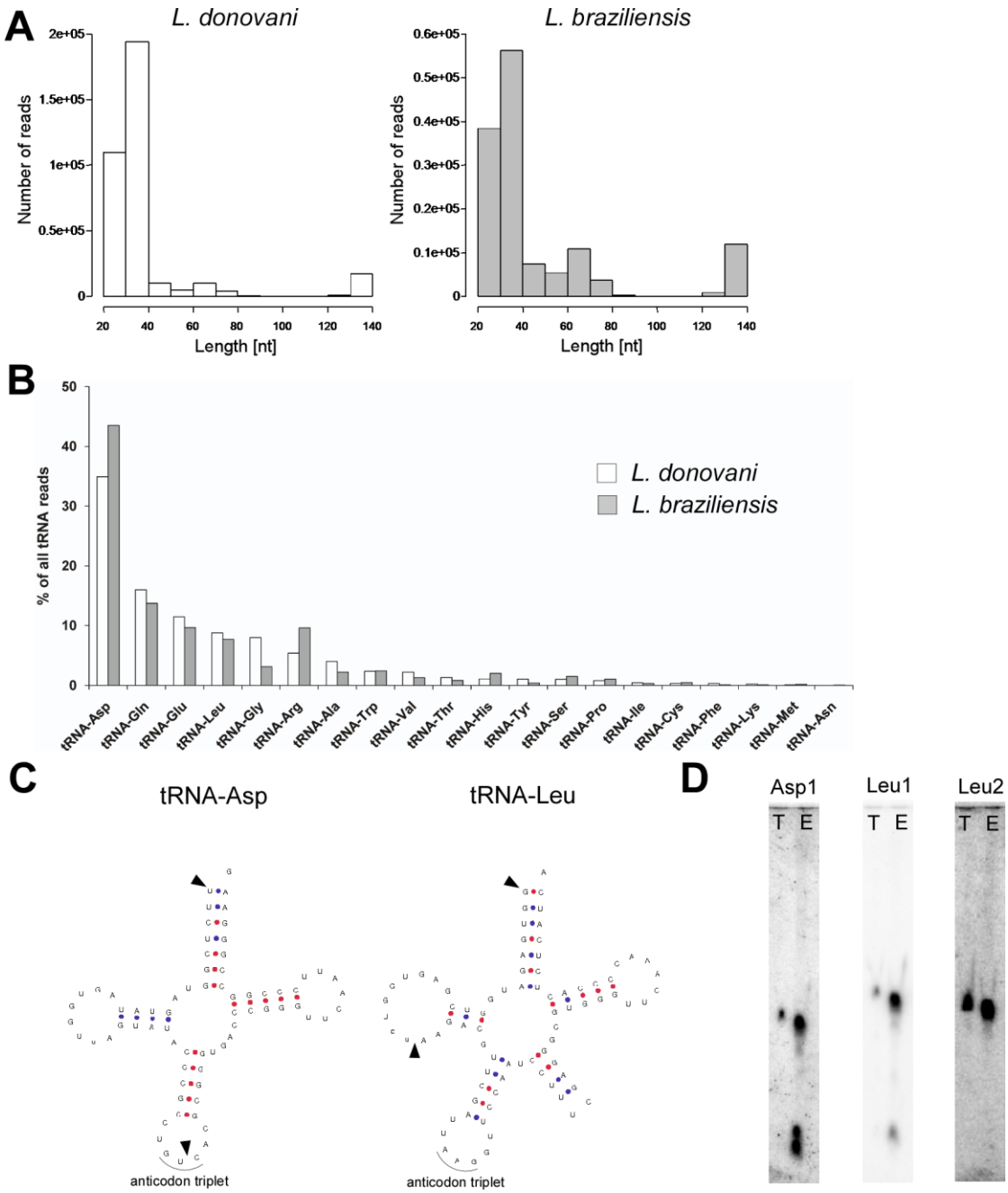

Figure 2.5 tRNA-derived fragments are cargo of leishmania exosomes.
A. Length distributions of reads mapping to tRNAs in *L. donovani* and *L. braziliensis* exosome RNA sequencing libraries. **B.** Bar graph showing percentages of reads from *L. donovani* (white bars) and *L. braziliensis* (grey bars) mapping to the respective tRNA isoacceptors. **C.** tRNA secondary structures for leishmania tRNA-Asp and tRNA-Leu (downloaded from [234]). Arrowheads indicate major 5' end cleavage products as observed in the sequenced libraries. **D.** Northern blots with probes designed against tRNA-Asp (Asp1) and tRNA-Leu (Leu1 and Leu2). *L. donovani* total (T) and exosome (E) RNA were probed on the same membrane. Equal amounts of RNA (3 µg) were loaded in each lane. Asp1 and Leu1 probes were designed to specifically detect full length tRNA as well as t-RNA-derived small RNAs seen in sequencing libraries, whereas Leu2 was designed against the mid region (anticodon loop) of tRNA-Leu and hence only detects the full length tRNA.

Table 2.4 Reads mapping to tRNAs

	L. donovani			*L. braziliensis*		
tRNA	***% of total tRNA reads***	***Length [nt]***[a]	***Position***[b]	***% of total tRNA reads***	***Length [nt]***[a]	***Position***[b]
Asp	34.91	47	5'	43.51	58	5'
Gln	16.00	32	mid-5'	13.73	36	mid-5'
Glu	11.48	38	3'	9.65	46	5'
Leu	8.80	29	5'	7.66	31	5'
Gly	8.02	37	3'	3.11	43	5'
Arg	5.40	31	mid-5'	9.62	28	mid-5'
Ala	4.03	34	3'	2.21	34	3'
Trp	2.38	37	mid-3'	2.42	40	mid-3'
Val	2.24	36	5’	1.28	41	5’
Thr	1.33	31	3'	1.49	35	3'
His	1.08	39	mid-3'	1.99	42	mid-3'
Tyr	1.06	31	3'	0.38	28	3'
Ser	1.02	34	3'	0.82	39	mid-3'
Pro	0.80	31	3'	1.02	34	3'
Ile	0.44	25	3'	0.31	31	3'
Cys	0.34	25	mid	0.48	26	mid
Phe	0.32	36	3'	0.07	36	3'
Lys	0.24	36	3'	0.09	37	3'
Met	0.10	29	3'	0.15	33	3'
Asn	0.01	33	mid-5’	0.01	38	mid-3'

[a]Average read length
[b]Most abundant read position (of all readsmapping to the respective tRNA)
Sorted according to abundance in *L. donovani* library. nt = nucleotide. N.A. = not applicable

non-annotated regions of the host genomes (Table S2.6). Analogous to our target prediction analysis the “novel transcripts” described in section 2.2.4, we were unable to perform a more comprehensive (based on more complex RNA-RNA interactions rather than simple complementarity alone) analysis of potential leishmania tsRNA-host target mRNA interactions due to the lack of appropriate tools for large datasets. We also performed a miRNA homology search against the human and mouse miRNA databases, and found only one miRNA (miR-135b-5p) that shared 88% identity with one of the tsRNAs present in both libraries ($tsRNA^{Arg}$) (Table S2.8). These results indicate that leishmania tsRNAs are not highly similar to canonical mammalian miRNAs.

In order to validate the presence of the identified tsRNAs in exosomes and to compare their abundance in exosomes with total RNA, we performed Northern blotting with probes specific for $tsRNA^{Asp}$ (Asp1) and $tsRNA^{Leu}$ (Leu1). Probes were designed to be complementary to the 5’ end of each tRNA and hence recognize both full length tRNA and 5’ tsRNA. We also included a probe that was designed against the middle

region (anticodon loop) of tRNA-Leu for comparison (Leu2). When hybridizing blots of RNA isolated either from *L. donovani* total cells or from exosomes with these probes, we detected a common band corresponding to full length tRNA in total and exosome RNA for the probes Asp1 and Leu1 (72 nt and 82 nt respectively) (Figure 2.5 D). In addition, we detected two smaller bands in the exosome RNA lane of the blot probed with Asp1 which were absent in the total RNA lane. Similarly, we detected a smaller band in the exosome RNA lane of the blot probed with Leu1 which was absent in the total RNA lane. These results demonstrate that 5' tsRNAs are produced from tRNA-Asp and tRNA-Leu and that these tsRNAs are highly enriched in exosomes. In the blot probed with Leu2 we only detected a band corresponding to the full length tRNA in both total and exosome RNA, confirming the absence of fragments that are derived from the anticodon loop of this tRNA. In summary, these findings are the first to show that leishmania produce tRNA-derived small RNAs and that tsRNAs are specifically and selectively enriched in exosomes.

2.3 Discussion

2.3.1 Leishmania exosomes contain specific RNA cargo

It has been firmly established that exosomes released by various mammalian cell types can serve as shuttle vehicles to deliver RNA molecules to recipient cells, thereby influencing gene expression. To date, however no protozoan pathogen has been shown to release *bona fide* exosomes containing RNAs with gene regulatory or other sequence-specific properties. Leishmania have recently been shown to secrete exosomes that contain a plethora of protein virulence factors capable of affecting the phenotype of host mononuclear phagocytes [77,78]. Considering the enormous potential impact of exosome-mediated delivery of regulatory RNAs to either recipient leishmania or mammalian host cells or both, we sought to investigate whether leishmania exosomes also carry RNA cargo. Here, we provide unambiguous evidence that leishmania parasites of two distinct species, namely *L. donovani* and *L. braziliensis*, release exosomes containing RNA sequences. These RNA sequences are heterogeneous, but overall short in length (25-250 nt). The enrichment of short RNA

sequences in leishmania exosomes is concordant with the majority of reports on exosome RNA in other organisms published thus far [105,232], with the exception of a few reporting the presence of longer RNAs such as full length ribosomal RNAs [235] and mRNAs [99]. The RNA cargo of exosomes is largely dependent on the cell of origin and appears to be affected by environmental conditions such as infection or nutritional stress [221], which likely explains the observed differences.

One important property of exosomes is their capacity to act as both short and long distance messengers. RNA-containing exosomes have been detected in a variety of human body fluids such as plasma, saliva and semen [236-238], which supports a role in long distance communication. As RNases are ubiquitously present in all organisms, RNAs travelling long distances need to be protected from degradation. In our *in vitro* experiments, we were able to show that leishmania exosomal RNA cargo is protected from degradation by exogenous RNase. When we incubated PMA-differentiated THP-1 cells *in vitro* with exosomes purified from axenic amastigotes of *L. donovani*, we saw that the exosome RNA cargo was readily taken up by host cells. This finding suggests that it should be possible for leishmania-derived RNAs to gain access to host cells through exosomes *in vivo*.

Numerous studies on exosome RNA have reported the presence of small regulatory RNAs such as miRNAs in these vesicles. It was of interest, therefore, to examine the leishmania exosome RNA content in detail by high throughput sequencing. We elected to sequence exosome RNA from *L. braziliensis* and *L. donovani* in parallel in order to compare the exosome RNA transcriptome of an RNAi-competent organism with an RNAi-deficient one. When sequencing exosomal RNAs from these two leishmania species, we found that they both contained a variety of small non-coding RNA species, the majority of which appeared to be cleavage products derived from longer known non-coding RNAs such as rRNA, tRNA, snoRNA and snRNA. We also saw a small number of reads mapping to protein coding genes. In addition, we discovered a number of novel transcripts that were conserved in both libraries, and *L. braziliensis* exosomes uniquely contained transcripts derived from siRNA-coding putative mobile elements and repeats, such as SLACS and TATEs [167]. Other studies

which examined mammalian exosome RNA content by deep sequencing reported a similar composition of the exosomal transcriptome, with a dominant fraction of sequences being derived from rRNA and other non-coding RNA [232,233,235,239]. Conspicuously, sequences derived from protein coding genes seem to be underrepresented in exosomes, regardless of their origin. Thus, it appears that exosomes from many diverse organisms selectively package non-coding RNAs, the exact function of which still needs to be determined.

Importantly, our study is the first to purify *bona fide* exosomes from a protozoan parasite and provide a comprehensive analysis by high-throughput sequencing of exosomal RNA cargo. By virtue of comparing two distinct (Old and New World) species of leishmania, we have made the serendipitous discovery that the packaging of specific RNA sequences into exosomes appears to be a conserved phenomenon in leishmania. At the present time it remains unclear whether our findings are illustrative of what happens in other eukaryotic pathogens. However, there is some evidence indicating that the release of RNA within vesicles might occur in other parasitic organisms as well. In particular, there have been two articles published by independent groups that demonstrate the release of RNA within extracellular vesicles shed by the protozoan *T. cruzi* [118,119]. However, these vesicles were not characterized or classified as *bona fide* exosomes. The distinction between exosomes and other extracellular vesicles is important, as their origin, mechanism of biogenesis and thus loading of cargo differs substantially [240]. Three other articles have been published which examined the RNA cargo of exosomes released by parasitic pathogens; one of them a protozoan (*T. vaginalis*) and the other two helminths (*Heligmosomoides polygyrus* and *Dicrocoelium dendriticum*). However, all of these studies have significant limitations in their experimental design and RNA analysis when compared with the present study. The work on *T. vaginalis* only showed a size profile of RNA purified from exosomes measured by bioanalyzer, but no sequencing data on exosomal RNA [105]. The article on *D. dendriticum* described the analysis of exosomes by high-throughput sequencing, however, data analysis was focused on looking for miRNAs and the authors did not report on other types of RNA in the exosomes [126]. Lastly, a very recent report on *H.*

polygyrus reported sequencing data of libraries that were generated with RNA obtained from parasite secretions and a vesicular fraction collected by ultracentrifugation, but not RNA from *bona fide* exosomes that were purified with specific exosome purification protocols [127]. The limitations of these studies do not allow for a direct comparison with our data and do not definitively answer the question whether the release of specific types of RNA within exosomes is a widespread phenomenon among protozoa and parasites more generally. While the data available suggest that this may certainly well be the case, further research will be needed to confidently answer this question.

Results from a number of studies [241-244] led to the suggestion that fragments derived from non-coding RNA species such as rRNA, snoRNA, vault RNA (vRNA) and tRNA can act as regulatory RNAs similar to miRNAs in RNAi. This hypothesis was based upon the finding that some of these fragments were shown to bind to AGO proteins and formed RISCs, which regulated expression of target mRNAs. *L. donovani* does not have the canonical proteins that are required for functional RNAi including AGO. However, an AGO/PIWI-like protein homolog, has been found in RNAi-deficient leishmania and other trypanosomes [245]. The function of this AGO homolog is currently unknown, however, one study suggested that it is not involved in the biogenesis or stability of siRNAs [246]. The presence of an alternative pathway to regulate gene expression in RNAi-deficient trypanosomatids is likely, since these organisms carry out transcription polycistronically and hence regulation of expression of individual genes can only take place at the post-transcriptional level. A number of studies have indicated that post-transcriptonal regulation of gene expression in trypanosomes may involve *cis*-acting regulatory motifs within the 3' UTRs of mRNAs and *trans*-acting RNA-binding proteins [247-249]. Other evidence for the presence of an alternative RNAi pathway in RNAi-deficient trypanosomatids comes from a recent series of studies in *T. cruzi*. The authors identified a unique AGO/PIWI protein termed TcPIWI-tryp that is expressed in all life cycle stages of the parasite and localizes to the cytoplasm [250]. Interestingly, they found that TcPIWI-tryp bound to a repertoire of RNAs distinct from siRNAs, namely small RNAs derived from rRNAs and tRNAs [155].

While these findings are intriguing, it remains to be established whether these complexes function in regulation of gene expression.

A large portion of reads in both our libraries mapped to rRNA genes in the reference genomes and they appeared to be shorter fragments. As mentioned above, the presence of rRNA-derived sequences in leishmania exosomes is in agreement with other recent reports on exosome RNA cargo [232,233,235,239]. At this point it is unknown whether rRNA fragments have any specific function. Some limited evidence has been presented to show that rRNA fragments are produced by specific cleavage rather than random degradation in humans and mice [150]. These specific products were characterized by termini specific processing and asymmetric stabilization. However, in our data, no bias for either 5' or 3' processing was observed (Table S2.4), but mapping of reads was rather scattered along the entire length of the rRNA gene. Moreover, we did not see enrichment of specific rRNA fragments derived from a subset of genes. Further study will be needed to elucidate whether rRNA fragments in leishmania exosomes are specifically enriched or selectively packaged.

Our finding that leishmania exosomes are overall enriched in non-coding RNA fragments which are taken up by host mononuclear cells, raises the interesting possibility that these RNA fragments may interfere with gene expression in the host, possibly by binding to host AGO. This type of epigenetic regulation of gene expression across kingdoms has been proposed, but so far little consistent and conclusive evidence has been presented. One elegant study recently showed that small RNAs from the plant fungal pathogen *Botrytis cinera* could silence Arabidopsis and tomato genes involved in plant immunity by binding host AGO [251]. Regarding human pathogens, some evidence has been presented to show that *T. cruzi* tRNA-derived small RNAs (tsRNAs) can modify the expression of specific host genes [120]. It remains to be established, how these tsRNA-induced changes of host gene expression were brought about and if this process involves hijacking of host RNAi pathways. In what follows below, we discuss potential roles of the non-coding, small RNA species found in leishmania exosomes which we believe are most likely to have regulatory functions, namely novel transcripts, siRNAs and tRNA-derived small RNAs.

2.3.2 Novel transcripts

When examining reads that were less abundant in the libraries to make sure we did not miss any important information amongst this group of reads, we discovered 12 distinct genomic loci (Table 2.3 and Figure 2.4) that apparently gave rise to transcripts which have not been previously described. None of these transcripts had homology to any annotated gene in TriTrypDB or GenBank. BLAST search, however, confirmed that these non-annotated genomic sequences are conserved amongst most leishmania species. Of considerable interest, we found that all of these novel transcripts had a spliced leader site upstream of their 5' end (see Figure 2.4 for two examples) implying that they are processed alongside other transcripts during *trans*-splicing. The fact that the majority of these novel transcripts contained one or more ORFs suggests that they may be translated into peptides or proteins. However, we were not able to find any homologous protein in any other species.

Two of the twelve novel transcripts that were most abundant in the group were further examined by quantifying their expression in exosomes in comparison to total cells by Northern analysis (Figure 2.4 B). Both of these transcripts produced shorter processing products that were uniquely present in exosomes. This indicates that cleavage products of these transcripts may be specifically targeted for packaging into exosomes for release from the cell. Alternatively, they may be cleavage products arising only within exosomes. Of note, the 3'-nucleotidase/nuclease (LmjF31.2310), which hydrolyzes single-strand nucleic acids [252], was found to be present in exosomes [77], which may be involved in cleavage of RNA sequences within exosomes. The absence of signals for these shorter products in Northerns of total RNA may also explain why these transcripts have not been reported in any of the previous studies on sequencing the leishmania transcriptome. Another possibility for why they have not previously been found is that they could be differentially expressed in the different life cycle stages (as we focussed only on axenic amastigotes). One question that remains to be answered is what type of RNA these novel transcripts represent (protein coding, structural, regulatory) or whether they represent novel type(s) of RNA.

Further studies will be needed to properly address the functions of these novel transcripts.

2.3.3 siRNAs

We detected a number of sequences in the *L. braziliensis* exosome RNA library that mapped to siRNA coding loci such as SLACS and TATEs in the *L. braziliensis* genome. Even though the functional significance of endogenous siRNAs in *L. braziliensis* is still unclear, they are thought to be a genome defence mechanism to control the spread of potentially harmful nucleic acids, such as mobile elements, repeats and viruses [167]. Although these sequences were detectable in our library, they were generally in low abundance when compared to fragments of rRNA or tRNA. The fact that half of these sequences were each sense and antisense support their tentative identity as siRNAs, given that one cardinal feature of siRNAs is that they are double stranded. Moreover, these were the only type of sequences in our libraries where this phenomenon was observed, as the vast majority of the other sequences were present only in the sense direction. The lengths of these putative siRNA sequences did not correspond exactly to what has been reported for *L. braziliensis* mature siRNAs (*Lbr*AGO1-bound) [167]. This might be due to different library construction strategies (size selection), it may be that what we detected were siRNA precursors, or that the sequences we detected were derived from distinct regions within the SLACS and TATEs loci. The finding that *L. braziliensis* packages these putative siRNA sequences as cargo of exosomes is of significant interest. It implies that these RNAs may function not only in the cell where they originated, but that they may also act in intercellular communication when taken up by other leishmania or by host cells or both. To our knowledge, no other parasite has been shown to release pathogen-derived siRNAs within vesicles directly. Further studies will be needed to confirm the identity of the sequences as siRNAs and delineate their function in parasite biology and in host-pathogen interaction.

2.3.4 tRNA-derived small RNAs

A striking finding of the present study was the abundance of tRNA fragments principally originating from a small subset of tRNA isoacceptors (Figure 2.4 B) that were highly conserved in the *L. donovani* and *L. braziliensis* exosome transcriptomes. Only recently has tRNA fragmentation been recognized as a specific process. tRNA fragments, also called tRNA-derived small RNAs (tsRNAs), have been found in all domains of life and can be divided into several categories, depending on the cleavage site: cleavage within the anticodon loop gives rise to 5' and 3' tRNA halves (30-35 nt), and cleavage within the D-arm (5') or T-arm (3') gives rise to smaller tRNA-derived RNA-fragments (tRFs) (13-20 nt) [253]. Each of these fragments appears to be generated through distinct pathways and have distinct functions. tRNA halves are known to be produced in response to stress, whereas the smaller tRFs, on the other hand, can be generated at any point of tRNA processing, by Dicer-dependent or –independent mechanisms [253,254]. In human cells, tRNA halves were found to inhibit protein translation by specifically targeting the translation initiation machinery and displacing elongation initiation factors [255]. tRFs, on the other hand, were shown to be involved in regulation of translation by directly binding to the small 30S ribosomal subunit and interfering with peptidyl transferase activity in archaea [256]. Furthermore, a similar mechanism was observed in human cells, where a tRF was shown to inhibit translation by affecting peptide bond formation [257]. In addition to these direct effects on translation, tRFs have also been shown to function in post-transcriptional silencing. Haussecker *et al* showed that tRFs can associate with Argonaute proteins, however, they associated more effeciently with the non-silencing AGO3 and AGO4 [243]. Furthermore, they saw that tRFs can affect the silencing activities of miRNAs and siRNAs, indicating a potential broad based role in regulating RNA silencing. In another study it was found that tRFs can function like miRNAs in RNAi. Thus, a tRNA-derived fragment designated CU1276 was generated in a Dicer-dependent manner, associated with AGO and inhibited the expression of RPA1 (a protein involved in DNA repair) by binding to the 3'UTR of its mRNA [258].

A small number of studies have looked at the presence of tsRNAs in protozoan parasites. In *Plasmodium berghei* and *T. gondii*, tRNA-halves were detected, and a relation between tRNA-half production and growth rate was observed [173]. However, the precise function of these parasite-derived tRNA-halves remains unknown. As mentioned above, tsRNAs were recently discovered in leishmania's close relative *T. cruzi*. Despite the fact that the lengths and origins of tsRNAs differed slightly from study to study, their production was convincingly demonstrated in both trypomastigotes and epimastigotes in a number of reports [166,183,259]. Importantly, these tsRNAs were bound to the *T. cruzi* AGO homologue TcPIWI-tryp [155], and the majority were derived from the 5' halves of tRNA-Glu. TcPIWI-tryp-tsRNA complexes were also found in vesicles shed from *T. cruzi.* The authors proposed that these vesicles may have a role in life cycle transition from epimastigote to trypomastigote as well as contribute to infection susceptibility of mammalian cells [119]. These findings provide evidence that tsRNAs in *T. cruzi* may participate in non-canonical regulatory pathways and raise the question as to whether a similar phenomenon may be operative in leishmania.

In the present study, we provide evidence for the first time that leishmania also produce specific tsRNAs, and that these potential regulatory RNAs are released by the intracellular amastigote stage within *bona fide* exosomes, competent for delivery to mammalian cells. The major fraction of tsRNAs found in both *L. donovani* and *L. braziliensis* exosomes were 5' tRNA halves, however, we also found shorter tsRNAs derived from the D-arm or T-arm of the tRNA, corresponding to 5'tRFs and 3'tRFs. As mentioned above, tRNA-halves are produced in response to stress [253,254]. Thus, the production and presence of tRNA halves in exosomes from leishmania amastigotes might be a result of the elevated temperature and acidic pH to which the parasites are exposed. We found that the vast majority of tsRNAs in leishmania exosomes were derived from tRNA-Asp, tRNA-Gln, tRNA-Leu, tRNA-Glu and tRNA-Gly (Table 2.4 and Figure 2.5) and this was highly conserved between *L. donovani* and *L. braziliensis*. Although we did not carry out a comprehensive and quantitative analysis of the frequencies of all tsRNAs in leishmania whole cells, strikingly, we found by Northern blotting that tsRNAs from tRNA-Asp and tRNA-Leu were highly enriched in exosomes,

with no detectable amounts in leishmania total RNA. This indicates that these tsRNAs are preferentially and quantitatively packaged into exosomes to be released from the cell rather than even partial retention in the whole cell RNA pool.

At this point, the mechanism of biogenesis and function of tsRNAs in leishmania is unknown. We are cannot, therefore, be 100% certain that the same classification of tRNA halves and tRFs that has been used in the literature [253,254,260] applies to our data. However, as many of the characteristics (length, cleavage site, isoacceptor origin) correspond to what has been reported in other organisms, we conclude that the phenomenon of specific tsRNA generation is evolutionarily conserved in leishmania as well. Based upon our initial functional predictions it appears clear that leishmania tsRNAs are not highly similar to canonical mammalian miRNAs or siRNAs. Further detailed investigations will be needed to delineate the functions of tsRNAs in leishmania biology, what roles they play in parasite-parasite, parasite-vector or parasite-host interactions, whether this involves their association with the host RNAi machinery and how they are targeted for exosomal packaging.

2.4 Materials and methods

2.4.1 Cell culture

L. donovani Sudan strain 2S promastigotes were routinely cultured in M199 (Sigma-Aldrich) with 10% heat inactivated fetal bovine serum (FBS, Gibco), 20 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 µM adenosine (Sigma-Aldrich) at 26°C. Every 3 days the organisms were subcultured 1:10 in fresh medium and were kept in culture for a maximum of 20-25 passages. Fresh parasites were obtained by purification of amastigotes from spleens of infected Syrian Golden hamsters followed by *in vitro* transformation into promastigotes by culturing for 7 days at 26°C in promastigote media. *L. braziliensis* (clinical isolate from the Peruvian Amazon region) promastigotes were routinely cultured in the same media as above

except for supplementation with 20% FBS. *L. braziliensis* promastigotes were subcultured 1:5 every 3 days in fresh media and kept at 26°C.

2.4.2 Purification of exosomes

Exosomes were purified from *L. donovani* and *L. braziliensis* axenic amastigote culture supernatant as described previously [77,78]. Briefly, 400-800 mL of day 5 promastigotes (at a concentration of 5x10E7 cells/mL) were washed 2x with Hank's buffered salt solution (HBSS, Sigma-Aldrich) followed by incubation in serum-free buffered exosome collection media at pH = 5.5, RPMI1640 supplemented with 1% D-glucose, 20 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 25 mM MES (all from Sigma-Aldrich), at 34°C for *L. braziliensis* and 37°C for *L. donovani*. After 24 hours of incubation, exosomes were purified from the 400-800 mL culture supernatant under endotoxin-free conditions by a series of centrifugation and filtration steps, followed by flotation on a sucrose cushion, as described in [78]. After a final pelleting step at 100,000xg for 1 hour, purified exosomes were resuspended in 50-100 µL of PBS and processed immediately (in case of RNA extractions) or stored at 4°C for a maximum of 5 days (for macrophage uptake experiment and Nanosight analysis).

2.4.3 Nanosight particle tracking analysis

The size and concentration of the isolated exosomes were analysed using the NanoSight™ LM10-HS10 system (Malvern Instruments). For analysis, a monochromatic laser beam (405nm) was applied to the diluted exosome solution (1:100 in 0.02 µm filtered PBS) that was injected into a LM12 viewing unit using a computer controlled syringe pump. NanoSight™ tracking analysis (NTA) software version 2.3 was used to produce the mean and median vesicle size together with an estimate of particle concentration. Samples were measured 3 times to confirm reproducibility.

2.4.4 Extraction and biochemical characterization of RNA

RNA was purified from leishmania exosomes by phenol/chloroform extraction using all RNA-grade reagents. For this purpose, 150 uL of LETS buffer (0.1M LiCl,

0.01M Na_2EDTA, 0.01M Tris-Cl pH=7.4, 0.2% SDS, all Sigma-Aldrich) was added to 50 µL of exosomes resuspended in PBS followed by addition of 200 µL Ultra-Pure buffer-saturated phenol pH = 7.4 (Life Technologies). The mixture was vortexed vigorously and centrifuged for 2 minutes at 13,000xg in a microcentrifuge at room temperature. The upper aqueous phase was collected and the phenol extraction was repeated once more followed by two extractions over 200 µL chloroform each (Fisher Scientific). RNA was precipitated by addition of 0.3 M NaCl, 2 µg/mL glycogen (Ambion) and 75% EtOH, and incubation at -20°C over night. RNA was pelleted by centrifugation at 13,000xg for 30 minutes at 4°C. RNA pellets were washed with ice-cold 75% ethanol and resuspended in 10-20 µL ddH_2O. RNA concentration was determined by measuring the OD260 with the nanodrop (Thermo).

To look at length profiles of exosome-derived RNA, 2 µg of purified leishmania total RNA and 1 µg of exosome RNA were first treated with 5 units DNase I (Thermo) to remove potential DNA contamination. After incubation for 30 minutes at 37 °C, DNase was inactivated by addition of 2.5 mM EDTA and incubation at 65°C for 10 minutes followed by phenol-chloroform extraction and ethanol precipitation as above. RNA was resuspended in 4 µL ddH_2O and RNA length profiles were obtained with the Agilent Bioanalyzer using the RNA 6000 Pico kit according to the manufacturer's instructions (Agilent). To confirm identity of nucleic acid purified from exosomes as RNA, 1-2 µg of phenol/chloroform extracted RNA was treated with DNase (as above), followed by treatment with either 0.4 mg/mL RNase A (Thermo) for 15 mins at 37°C or hydrolysis with 50 mM KOH (Sigma-Aldrich) for 15 min at 95°C. Samples were then 5' end labeled according to the manufacturer's instructions using polynucleotide kinase (PNK) (New England Biolabs, NEB) and γ^{32}PdATP (Life Technologies) and run on 15% polyacrylamide gels followed by imaging with a Typhoon phosphor-imager (GE Healthcare).

To assess whether the exosomal membrane was protecting the vesicular RNA content from degradation by exogenous RNases, intact exosomes resuspended in PBS (from 400 mL culture supernatant, split into 4 samples) were treated with 0.4 mg/mL RNAse A for 15 mins at 37°C in the presence or absence of 0.1% Triton X-100 (Sigma-

Aldrich). As a control for RNase activity, 1 µL of prepared RNA pico ladder (Agilent) was treated with RNase A under the same conditions. After incubation, samples were extracted with phenol/chloroform 2x each and RNA was precipitated with ethanol as above. Samples were then treated with DNase, again phenol/chloroform extracted and ethanol precipitated, resuspended in 4 µL ddH_2O and run on the Agilent Bioanalyzer to determine whether or not RNA had undergone degradation.

2.4.5 Vesicle delivery of RNA cargo to macrophages.

Exosomes were purified from 400 mL culture supernatant of *L. donovani* axenic amastigotes as described above. Pelleted exosomes were resuspended in 100 µL PBS. Protein concentration in the exosome preparation was determined using the Micro BCA Protein Assay kit (Pierce). Exosomes were then stained with the membrane-permeant, RNA-specific dye SYTO RNASelect (Life Technologies) according to the manufacturer's recommendations. For this purpose, the SYTO dye was diluted in DMSO and added to the resuspended exosomes at a final concentration of 10 µM, followed by 20 minutes incubation at 37°C. Excess unbound dye was removed by washing twice with 1 mL PBS, pelleting the exosomes at 100,000xg for 1 hour at 4°C. Exosomes were then resuspended in the original volume of PBS (100 µL). Labelling efficiency was assessed by fluorescence microscopy using an Axioplan II epifluorescence microscope equipped with 63×/1.4 Plan-Apochromat objective (Carl Zeiss Inc). Images were recorded using an AxioCam MRm Camera coupled to the AxioVision software Version 4.8.2 (Carl Zeiss Inc.).

To investigate the exosome-mediated delivery of RNA to host macrophages, THP-1 cells were differentiated over night with 10 ng/mL phorbol-12-myristate 13-acetate (PMA), followed by washing and resting cells for 24 hours. Differentiated cells were then treated with labeled exosomes for 2 hours at 37°C. As a negative control, cells were treated with labelled exosomes and incubated at 4°C for 2 hours, preventing phagocytosis. For quantification of exosome RNA uptake, exosome-treated THP-1 cells were washed 3x with PBS to remove non-internalized exosomes. Cells were then resuspended in PBS, transferred to eppendorf tubes, pelleted by centrifugation at 500xg

for 5 minutes, fixed with 2% paraformaldehyde (Sigma) in PBS for 15 minutes at room temperature. After fixation, cells were again washed with PBS and then analyzed by flow cytometry (FACS Calibur, BD). To verify that exosomes were in fact internalized and not just bound to the cell membrane, the same experiment was performed with THP-1 cells grown on coverslips to be analysed by confocal microscopy. After incubation, cells on coverslips were washed and fixed as above, permabilized with 0.1% Triton X-100 in PBS for 5 minutes, and stained with Alexa Fluor 594 phalloidin (Life Technologies) for 1 hour at room temperature in the dark. After 3x washing with PBS, coverslips were mounted with Prolong Gold antifade mounting media containing DAPI (Life Technologies) to detect macrophage nuclei. Confocal microscopy was done with a Leica DMIRE2 inverted microscope equipped with a SP2 AOBS laser scanning head. Pictures were taken with a 63x magnification oil immersion objective.

2.4.6 Library construction and sequencing

We used 1-2 µg of RNA extracted by phenol/chloroform extraction from *L. donovani* and *L. braziliensis* exosomes (from one individual exosome preparation each, from 800 mL supernatant) as starting material. RNA was first treated with DNase I (as described above) to remove potentially contaminating DNA. This material was then used for library construction using the steps shown in Figure 2.6. To remove 5' phosphates on the RNA, we first performed a calf intestinal alkaline phosphatase (CIP) treatment using 1 unit of CIP (Roche) per 10 µL reaction and incubation for one hour at 37°C. Once incubation was completed, samples were phenol-chloroform extracted twice and ethanol precipitated as described in section 2.4.4. In order to monitor the efficiency of the CIP treatment, a parallel reaction was spiked with a 24 nt long radiolabelled RNA, and pre and post incubation with CIP were loaded in a 20% denaturing polyacrylamide gel to follow the disappearance of counts. Next, the CIP treated RNA sample (resuspended in 10 µL ddH2O) was treated with tobacco acid phosphatase (TAP) to remove 5' caps. Half of the CIP treated RNA sample was combined with 2.5 units of TAP (Epicentre), 1X TAP buffer, brought to a final volume of 10 µL with ddH2O and incubated for one hour at 37°C. In order to control for the efficiency of 5' cap removal, a parallel reaction was spiked with γ^{32}PdATP and pre-and

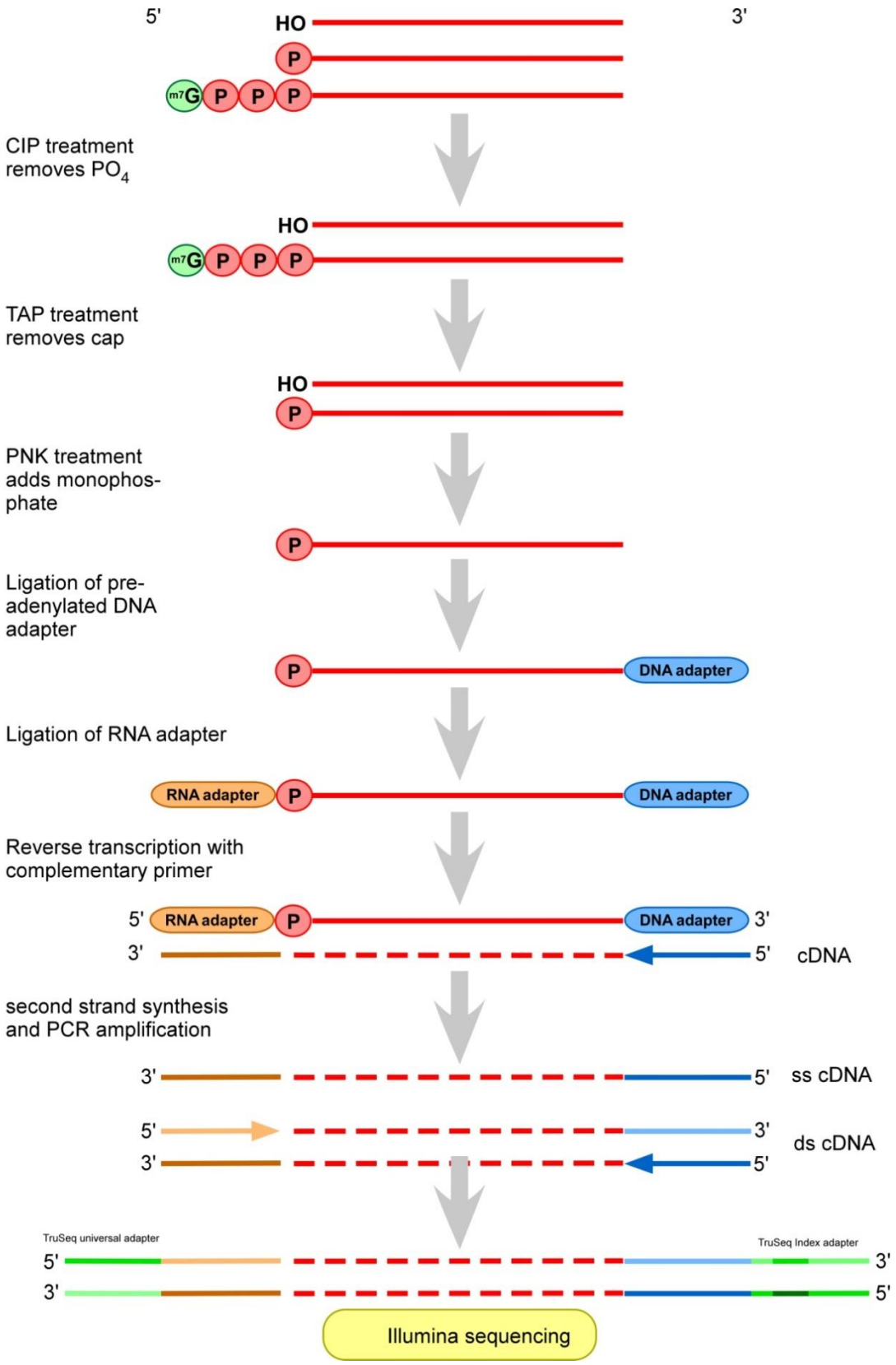

Figure 2.6 Flow scheme for library construction procedure
See text section 2.4.6 for details.

post ligation samples were loaded in a 20% plyacrylamide gel to monitor the disappearance of counts. RNA was ethanol-precipitated and resuspended in 10 µL ddH2O. The CIP and TAP treated RNA were then labeled with polynucleotide kinase (PNK) to have the same 5' phosphate in all RNA molecules about to be ligated. Ten units of PNK (NEB) were used along with 1X PNK buffer (NEB), and γ^{32}PdATP in a 10 µL reaction and incubation for one hour at 37°C. Next, ~ 10% of the sample was loaded onto a denaturing 15% polyacrylamide gel. The remainder of the PNK reaction was taken to a 30 µL volume with ddH2O and run through a dye terminator removal (DTR) cartridge (EdgeBio) following manufacture's indications in order to remove ions and unincorporated γ^{32}PdATP. The sample was ethanol precipitated and resuspended in 5 µL of ddH2O. The next step was to ligate a custom adenylated AppDNA adaptor (Table 2.5) to the 3' end of RNA molecules. This adaptor was slightly modified so that the 3' end was blocked in order to prevent self ligation. Half of the pre-treated exosomal RNA sample was combined with T4 RNA ligase buffer (50 mM HEPES, pH 8.3, 10 mM MgCl2, 3.3 mM DTT, 10 g/ml BSA and 8.3% glycerol; [261], 2.5 U of T4 RNA Ligase (Epicentre), and 20 µM AppDNA adaptor. Reactions were incubated at room temperature for one hour, and then the enzyme was denatured by heating at 65°C for 20 minutes. Samples were gel purified on 10% polyacrylamide gels to remove un-ligated adaptor. A second ligation reaction was set up to attach an RNA adaptor (Table 2.5) to the 5' end of the RNA-DNA hybrid. Conditions were the same as described above for the first ligation reaction with the only difference being that γ^{32}PdATP (final concentration of 0.4 mM) was added. The ligation reaction was gel purified from a denaturing 10% polyacrylamide gel and the recovered material was used as a template in a reverse transcription (RT) reaction. For this purpose, half of the recovered sample was combined with 100 µM RT primer (Table 2.5), ddH2O and incubated at 80 °C for two minutes. After cooling samples down slowly, 1X First Strand Buffer (Life Technologies), 0.8 µM dNTP and 200 U of Superscript II Reverse Transcriptase (Life Technologies) were added. Controls with no enzyme and no template in the reaction were prepared in parallel. Reactions were incubated for 1 hour at 48 °C. The RNA template was hydrolyzed by heating in the presence of 100 mM KOH followed by neutralization with 1 M Tris-HCl pH=5 (to a final pH=8), and the resulting cDNA was

isolated on a 10% denaturing polyacrylamide gel. 20 cycles of PCR amplification were performed in the presence of 5 mM MgCl2, 100 µM dNTPs, 1 µM each forward primer (Table 2.5) and reverse primer (same as RT primer), 1X Taq buffer and 2.5 Units Taq polymerase (UBI). PCR products were then gel purified and quantified by Qubit (Life Technologies) and used as input material for ligation of TruSeq adapters (Illumina) according to the manufacturer's recommendations. 150 base pair, paired-end sequencing was performed using an Illumina MiSeq instrument (Illumina) at the Epigenomics core of Albert Einstein College of Medicine, NY.

Table 2.5 Oligos used for library construction and Northern blotting

Oligo Name	*Nucleotide sequence*
Library construction (2.4.6)	
3' adenylated DNA adaptor	5' App-GAA GAG CCT ACG ACG A
5' RNA adaptor	5' rAUC GUA GG CAC CUG AAA
RT primer	5' TCG TCG TAG GCT CTT C
Forward primer	5' ATC GTA GGC ACC TGA AA
Northern blotting (2.4.8)	
LdBPK_291610_leftof probe	5' AAG GCG TCC CCA TGA TAA CG
LdBPK_301180_leftof probe	5' GAC CTC AAG TAT CTA CGG GAG A
tRNA-Asp probe ASP1	5' GGC GGG TAT ACT AAC CAC TAT AC
tRNA-Leu probe LEU1	5' AGA CCA CTC GAC CAT CTC A
tRNA-Leu probe LEU2	5' TGG AAC CTT AAT CC AAC GTC TT
5.8S rRNA probe [262]	5′ ACG GGG ATG ACA CAA TAG AGC TTC TCC

2.4.7 Sequencing data analysis

After completion of Illumina paired-end sequencing and read quality control checking by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), both *L. donovani* and *L. braziliensis* exosomal RNA reads had their adapters trimmed by cutadapt version 1.0 (http://journal.embnet.org/index.php/embnetjournal/article/view/200/479). For each library, the output files from the trimming were separated into RNA adapter-trimmed reads and DNA adapter-trimmed reads, and the former was used to guide the assignment of correct orientation for all reads sequenced. We ran FLASh (settings: -M 100 –x 0.2) [263] and FASTX-Collapser (http://hannonlab.cshl.edu/fastx_toolkit) to respectively combine the mates, for the cases where DNA inserts were

shorter than twice the length of reads, and then collapsed identical sequences into single ones to facilitate handling the data in subsequent specific analyses. Bowtie2 version 2.1.0 (settings: very-sensitive-local –N1) [264] was used to align the collapsed reads (cReads) from both libraries against their respective reference genomes (LdBPK (*Leishmania donovani* strain BPK282A1) and LbrM (*Leishmania braziliensis MHOM/BR/75/M2904*) from TriTrypDB version 6.0), as well as against the species with the best assembled and annotated genome (LmjF, *L. major* MHOM/IL/81/Friedlin, TriTrypDB version 6.0). The very-sensitive-local setting of Bowtie 2 uses a seed length of 20 nt for the alignment, and the –N1 command allows for only one mismatch on that seed alignment. The alignments with LdBPK and LbrM were used to categorize the exosomal RNAs for the respective species, relying on htseq-count script [265] and the GFF files provided by TriTrypDB v6.0. The alignment with LmjF was done mainly to refine the analyses of reads mapping onto tRNAs. Of note, right after bowtie2 execution, samtools version 0.1.18 [266] was applied to generate sorted bam files, which were then used as input to cufflinks (settings: -u --min-intron-length 3 --3-overhang-tolerance 25 --overlap-radius 10 --min-frags-per-transfrag 1) [267] for the assembly of reads mapping on the same locus into individual “transcripts” or clusters. Artemis genome browser software [226] was used to manually inspect in greater detail and visualize the alignment of exosomal sequences with the reference genomes.

As mentioned above, we used reads mapping to *L. major* tRNAs for a better categorization of potential tRNA-derived small RNAs present within the exosomes. An ad-hoc PERL script was written to calculate the cReads position within each tRNA feature they mapped onto: 5’end (cReads mapping entirely on the 5’end half of the tRNA gene), mid-5’ (cReads starting on the first 1/3 and ending before the last 1/3 of the tRNA gene length), 3’end (cReads mapping entirely on the 3’end half of the tRNA gene), mid-3’end (cReads starting after the first 1/3 and ending within the last 1/3 of tRNA gene length), mid (cRead overlaps both halves of the tRNA gene and not within the 1/3 extremity regions). The same method was used to calculate the cReads position within each rRNA feature for rRNA fragments found in exosomes.

The discovered 12 novel transcribed loci had their nucleotide sequences translated by the getorf program from the EMBOSS package [268] with the following paramaters: -minsize 33 -find 1 –noreverse, which sets a 10 amino acids minimum ORF length, translates solely from ATG to STOP codons and only on the three possible frames from the same strand where the exosome RNA reads mapped to, respectively. In order to check whether the putative ORFs outputted by the getorf program have similarity to any already known protein, we ran sensitive blastp against nr-NCBI (-word_size 2 -num_descriptions 5 -num_alignments 5 -evalue 1e-3) and no hits were found for any of them.

To determine whether there were transposable elements-derived RNA fragments within leishmania exosomes and also discard any possibility of cross-contamination between the libraries, we performed a BlastN search [269] for *L. braziliensis*-specific SLACS/TATEs elements (extracted from TriTrypDB-6.0_Lbraziliensis MHOMBR75M2904_AnnotatedTranscripts.fasta downloadable file at http://www.tritrypdb.org). The following thresholds were applied during this screen: e-Value <= 1, identity >= 80% and query (cRead) coverage >= 70%.

To search for any sequences homologous to mammalian miRNAs within the leishmania exosomal RNA libraries, we ran blastn from the BLAST Plus package [270] version 2.2.29+ querying the top thousand most abundant cReads on each library against the whole human and mouse miRNA dataset (hairpin and mature) available at miRBase (http://www.mirbase.org). The blastn+ parameters were the following: -dust no -word_size 4 -evalue 1 -outfmt 6, and we also established a cutoff of 70% identity and 70% sequence coverage (ad-hoc PERL script). In a parallel approach to identify host genes that could potentially be targeted by putative regulatory RNAs in leishmania exosomes, we aligned the cReads from both libraries against human (hg19, NCBI) and vector (*Lutzomyia longipalpis* and *Phlebotomus papatasi,* https://www.vectorbase.org/) reference genomes. Bowtie2 version 2.1.0 (settings: --very-sensitive-local –N1) [264] and htseq-count script (http://www-huber.embl.de/HTSeq/doc/overview.html) (using the option –s reverse, which reports reads mapping to annotated features on a reverse complement fashion) were used for this purpose.

2.4.8 Northern blotting

Aliquots of ~3 µg RNA per lane (*L. donovani* axenic amastigote total RNA from a single culture, or exosome RNA pooled from 4 separate exosome preparations) were loaded onto 8% denaturing polyacrylamide gels. Gels were stained with SYBR Green and imaged, then the samples were blotted onto Hybond N^+ nylon membrane (GE Healthcare). The membranes were UV cross-linked using a Stratalinker (1200 µJ for 30 seconds), blocked, probed and washed according to [271]. Twenty-one and 150 nt long *in vitro* transcribed RNAs were used as size markers. For hybridization, 5'end labelled DNA probes were used (see Table 2.5). For 5' end labelling, the PNK labelling reaction was carried out as suggested by the manufacturer (NEB). The efficiency of γ^{32}PdATP incorporation was determined by running a small fraction of the PNK reaction on a native 20% polyacrylamide/urea gel and typically ranged between 80-95%, resulting in probes with high specific activity. The blotted membranes were placed in glass bottles containing a minimal amount of hybridization buffer (6X SSPE, 1% SDS, 2X Denhart's solution, 100 µg/mL of salmon sperm DNA; [272]) in a Hybaid™ mini oven MKII and pre-hybridized with constant rotation for 4 hours at 37°C. After pre-hybridization, approximately 10 µCi of labelled probe was added and the membrane was hybridized for at least 18 hours at 37°C. The next day, the membrane was washed twice with a high stringency solution (2X SSPE and 0.1% SDS), and twice with a low stringency solution (0.2X SSPE and 0.1% SDS) for 15 minutes each at room temperature. The radioactive signal from the membranes was detected using a Storm 820 phosphorimager. Quantification of signals was performed in Imagequant.

2.4.9 Statistical analysis and graphs

R environment version 3.0 was used to generate read length distribution histograms for each library (calculating their mean and median values), as well as to perform the Pearson's Correlation analysis regarding the tRNA-derived small RNA reads abundance and the amino acid usage frequency of the respective predicted proteomes. Other graphs were generated with GraphPad Prism 4.0 and EXCEL.

Chapter 3 Leishmania silence macrophage miRNAs as a potential, novel virulence strategy

3.1 Background

Micro RNAs (miRNAs) have recently emerged as essential regulators of gene expression in mammals and other organisms. They are ~22 nucleotide short non-coding RNA sequences that are encoded in genomic clusters and are generated from longer precursors by an elaborate biogenesis and processing pathway [273]. Mature miRNAs associate with Dicer and Argonaute (AGO) proteins to form the so-called RNA-induced silencing complex (RISC), which exerts its gene-regulatory function in the cell cytoplasm, where it finds and binds target messenger RNAs (mRNAs), and prevents their translation into proteins. The key to binding of a given miRNA to its mRNA target is the seed sequence, which is located within the 5' end of the miRNA [145]. One miRNA can bind to and regulate hundreds of different mRNA targets and conversely, one mRNA can be targeted by multiple different miRNAs. Thus, the miRNA-mRNA regulatory network is very intricate, and additional complexity is added through positive and negative regulatory feedback loops. Approximately 1900 miRNAs have been identified in humans (miRBase v.21), and it is estimated that more than 60% of all human protein-coding genes are regulated by miRNAs [146]. Hence, the fine-tuning of gene expression by miRNAs is important in a majority of cellular processes.

It has become clear that miRNAs play important roles in shaping innate and adaptive immune responses towards pathogens. Moreover, several pathogens have been found to specifically exploit host miRNA expression as a virulence strategy to manipulate host cellular pathways. For example, the intracellular pathogenic bacteria *Listeria monocytogenes*, *Salmonella enterica* and *Mycobacterium tuberculosis* have all been shown to affect the expression levels of specific host miRNAs, resulting in de-regulation of target mRNAs and in turn affecting various host processes such as cell cycle and apoptosis, immune responses and autophagy (reviewed in [274]). Several viruses have been shown to inhibit miRNA-mediated gene silencing by interfering with components of the RNAi pathway, thus favouring virus replication. For example, the

mammalian Nodamura virus was found to inhibit Dicer cleavage of pre-miRNAs *in vitro*, leading to complete abrogation of endogenous miRNA generation [275]. The protozoan pathogen *Plasmodium falciparum* was found to have improved survival in its vector host *Anopheles gambia* when miRNA maturation was impaired in the mosquito [276]. Infection of cholangiocytes with *Cryptosporidium parvum* resulted in decreased expression of the host miRNA let-7 and this was associated with up-regulation of TLR4 in these cells [204]. In addition, infection with *Toxoplasma gondii* was shown to increase the expression levels of specific miRNAs in primary human cells [209]. Taken together, these findings suggest that diverse pathogens are able to modulate the host immune response through effects on expression of host miRNAs.

In infection with *L. donovani*, it was recently shown that miR-122 was downregulated in the liver of infected mice, resulting in reduction of serum cholesterol and enhancement of parasite persistence [123]. Moreover, the miRNA response to *L. major* and *L. donovani* in human macrophages and dendritic cells *in vitro* has been assessed [213,214]. However, these studies examined early infection timepoints only (initial 3-24 hours), and obtained contradictory results with regard to the miRNA expression profiles. Thus, the published reports are somewhat limited by the model organism or host cell type studied, by the quality of the data, or by their focus on only a select number of miRNAs. Consequently, the effects of leishmania infection on host miRNA expression and their role in pathogenesis remain unknown. Despite the limitations of these published studies, there is a general trend that can be observed, biased towards an overall downregulation of miRNAs in response to leishmania infection. Intrigued by these findings and in seeking a more comprehensive answer as to which miRNAs are specifically regulated particularly during chronic leishmania infection, we sought to investigate specifically the effect of *L. donovani* infection on miRNA expression by human primary macrophages, the main host cell of leishmania, at later stages of infection (24 hours and beyond). We were particularly interested in looking at later stages of infection, as this is when full transformation into the amastigote life cycle stage has occurred, chronic infection is established and parasite replication is taking place. We assessed the expression of 800 miRNAs with the sensitive and

unbiased Nanostring nCounter assay, and validated our results by qPCR. Strikingly, our findings showed that *L. donovani* infection induced an overall down-regulation of miRNA expression in macrophages. This effect reached its full effect at 24 and 48 hours post infection. To examine the possibility that *L. donovani* down-regulates host miRNAs by targeting essential components of the miRNA biogenesis pathway, we assessed protein levels of Drosha and Dicer, two ribonucleases involved in miRNA maturation. Western blot results showed that Drosha and Dicer were both abundantly expressed in infected cells, at levels even higher than in uninfected cells. This argues against the possibility that *L. donovani* targets the synthesis or stability of Drosha and Dicer directly, but does not exclude that the functions of these proteins may be compromised in infected cells.

Our findings suggest that inhibition of miRNA expression may be a novel mechanism of pathogenesis used by leishmania to control macrophage phenotype and promote chronic infection. Further study will be needed to decipher the mechanism by which *L. donovani* inhibits macrophage miRNA expression.

3.2 Results

3.2.1 *L. donovani* strain 1S promastigotes potently infect human monocyte-derived macrophages

Initially, we needed to establish a reproducible *in vitro* model system for infection of human primary macrophages with *L. donovani*. We chose to enrich monocytes from buffy coats of healthy donors by plastic adherence. The enriched monocytes were differentiated into human monocyte-derived macrophages (HMDMs) for 6-7 days and used for infection experiments. We varied a number of parameters to optimize the infection rates in HMDMs, which were determined by fluorescence microscopy, using DAPI staining to detect host nuclei and leishmania nuclei and kinetoplasts (Figure 3.1 A). First, we tested the infectivity of promastigotes of two different *L. donovani* strains, 1S and 2S, at multiplicities of infection (MOIs) ranging from 10:1 to 40:1, over a time course of 24, 48, and 72 hours, in GM-CSF differentiated HMDMs. As shown in

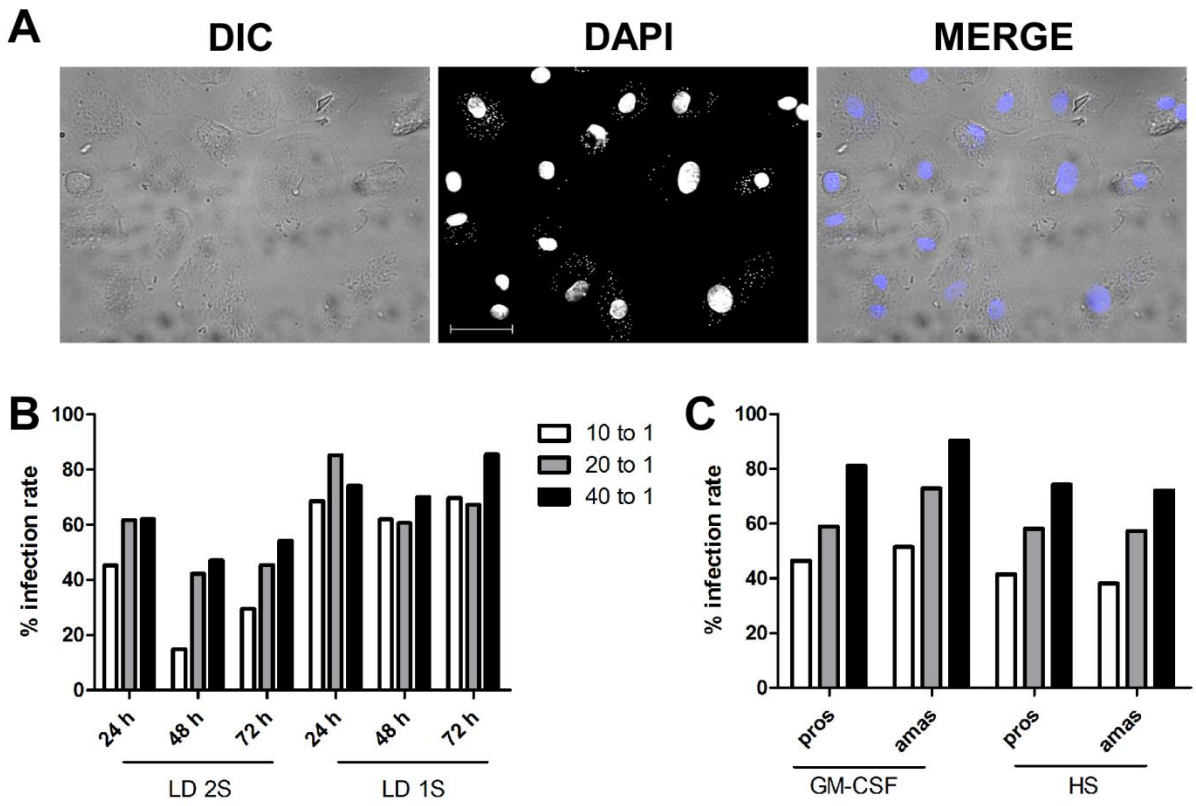

Figure 3.1 Infection rates of human monocyte-derived macrophages infected with *L. donovani*.
Human monocytes were purified from buffy coats, differentiated into macrophages and infected with *L. donovani* as described in Materials and methods. Infection rates were assessed by imaging of cells stained with DAPI to detect host nuclei and leishmania nuclei and kinetoplasts by fluorescence microscopy. The results are representative of 2 independent experiments. **A.** Image of GM-CSF differentiated HMDMs infected with *L. donovani* 1S promastigotes at MOI 40 to 1 for 72 hours, acquired with an Axioplan II epifluorescence microscope (Carl Zeiss Inc) at 40x magnification. Scale bar = 50 µm. **B.** Comparison of infection rates of GM-CSF differentiated HMDMs infected with promastigotes of *L. donovani* strains 1S or 2S at indicated MOIs and time points. **C.** Comparison of infection rates of HMDMs differentiated in the presence of either GM-CSF or human serum (HS) and infected with either promastigotes or axenic amastigotes of *L. donovani* strain 1S for 24 hours at indicated MOIs.

Figure 3.1 B, *L. donovani* strain 1S was more infectious at all MOIs and time points tested, with infection rates from 60 to 90%. Neither the MOI, nor the infection time appeared to have a major effect on the infection rate of strain 1S in this analysis.

We next tested whether there was a difference in infectivity of promastigotes and axenic amastigotes of *L. donovani* strain 1S at different MOIs, and whether HMDMs differentiated in the presence of GM-CSF or human serum (HS) showed a difference in susceptibility to infection. Treatment of macrophages with GM-CSF has previously

been linked to enhanced leishmanicidal activities of these cells [277], hence we needed to examine whether the method of differentiation had an effect on the infection rate. The results (Figure 3.1 C) showed that at 24 hours post infection, HMDMs differentiated with GM-CSF had a slightly higher infection rate than HMDMs differentiated with HS when infected with either promastigotes or amastigotes. There was no major difference between infections with the different life cycle stages, however, the infection rate strongly increased with increasing MOI. Based on these results, we chose to use GM-CSF differentiated HMDMs and infection with *L. donovani* 1S promastigotes at a MOI of 20:1 or greater for all the following experiments.

3.2.2 *L. donovani* infection represses miRNA expression in human macrophages

We used the Nanostring nCounter Human v2 miRNA Expression Assay to analyze the expression of 800 miRNAs in uninfected and infected HMDMs. Optimized infection conditions based on results described in section 3.2.1 were used to infect HMDMs from healthy donors with *L. donovani.* RNA was extracted from HMDMs and assessed for concentration and purity, infection rates of cells were counted in parallel in cells grown and infected on coverslips under identical conditions. Table 3.1 summarizes the specifications of the 12 samples (from 3 donors) that were selected for miRNA expression analysis.

Table 3.1 Samples submitted for Nanostring miRNA assay

Sample	*Infection rate [%]*	*RNA [µg/mL]*	*OD260/280*	*OD260/230*
Donor 1				
Uninfected (48 h)	0	205.5	1.75	2.27
LD 24 h	91.0	626.5	1.97	2.34
LD 48 h	78.6	478.9	1.91	2.33
LD 72 h	86.8	282.4	1.85	2.35
Donor 2				
Uninfected (48 h)	0	312.5	2.06	2.01
LD 24 h	78.8	380.9	2.09	2.1
LD 48 h	71.3	389.1	2.09	2.13
LD 72 h	74.8	301.5	2.08	2.08
Donor 3				
Uninfected (48 h)	0	113.1	2.04	1.56
LD 24 h	54.6	178.5	2.05	1.88
LD 48 h	50.0	261.0	2.08	2.02
LD 72 h	70.0	163.5	2.06	1.79

After background subtraction and normalization of Nanostring miRNA expression data, 46 miRNAs were reproducibly detected (average >50 counts) in our samples. As shown in the heatmap in Figure 3.2 A and the dot plot in Figure 3.2 B, the majority of miRNAs (35/46) were downregulated in *L. donovani* infected cells when compared to uninfected cells at 24 and 48 hours post infection. Several of these miRNAs had previously been implicated in inflammation and immune responses towards pathogens, such as miR-98, miR-155-5p, and miR-146a-5p. It appears that for a number of these downregulated miRNAs, their expression increased back to levels close to uninfected cells at 72 hours, though this was only the case for 2 out of 3 donors (donors 1 and 3, see Figure 3.2 A). The remaining 11/46 miRNAs did not show a consistent expression pattern across the 3 donors. Statistical tests revealed that 19 miRNAs were significantly downregulated at one or more of the infection time points (Figure 3.2 C). Notably, the magnitude of decrease in expression was at least 0.5 fold at 24 and 48 hours in infected cells. In summary, our data indicate that the expression of host macrophage miRNAs is overall downregulated in response to *L. donovani* at times when infection is firmly established.

To validate the results from the miRNA expression profiling, we randomly selected 5 miRNAs that were detected by Nanostring and assessed their expression in HMDMs from 3 new donors in response to *L. donovani* infection by qPCR. The same timepoints as in the previous experiments were taken (24, 48, and 72 hours), and an additional early infection timepoint of 2 hours was included. The results from the qPCR analysis confirmed our previous observations, as all 5 measured miRNAs were downregulated at 24 and 48 hours post infection (Figure 3.3). Statistical testing showed that this downregulation was only significant in the case of miR-98-5p and miR-148b-3p (Figure 3.3 A and C), which is likely due to the small sample size (n = 3). Nevertheless, there was a clear correlation between the Nanostring and the qPCR data (Pearson coefficient = 0.89, Figure 3.3 F). Notably, the qPCR data revealed that the expression of the 5 miRNAs tested was also downregulated when compared to uninfected cells at 2 hours post infection, though at a slightly lower magnitude than at 24 hours. This indicates that the repression of miRNA expression by *L. donovani* is initiated early

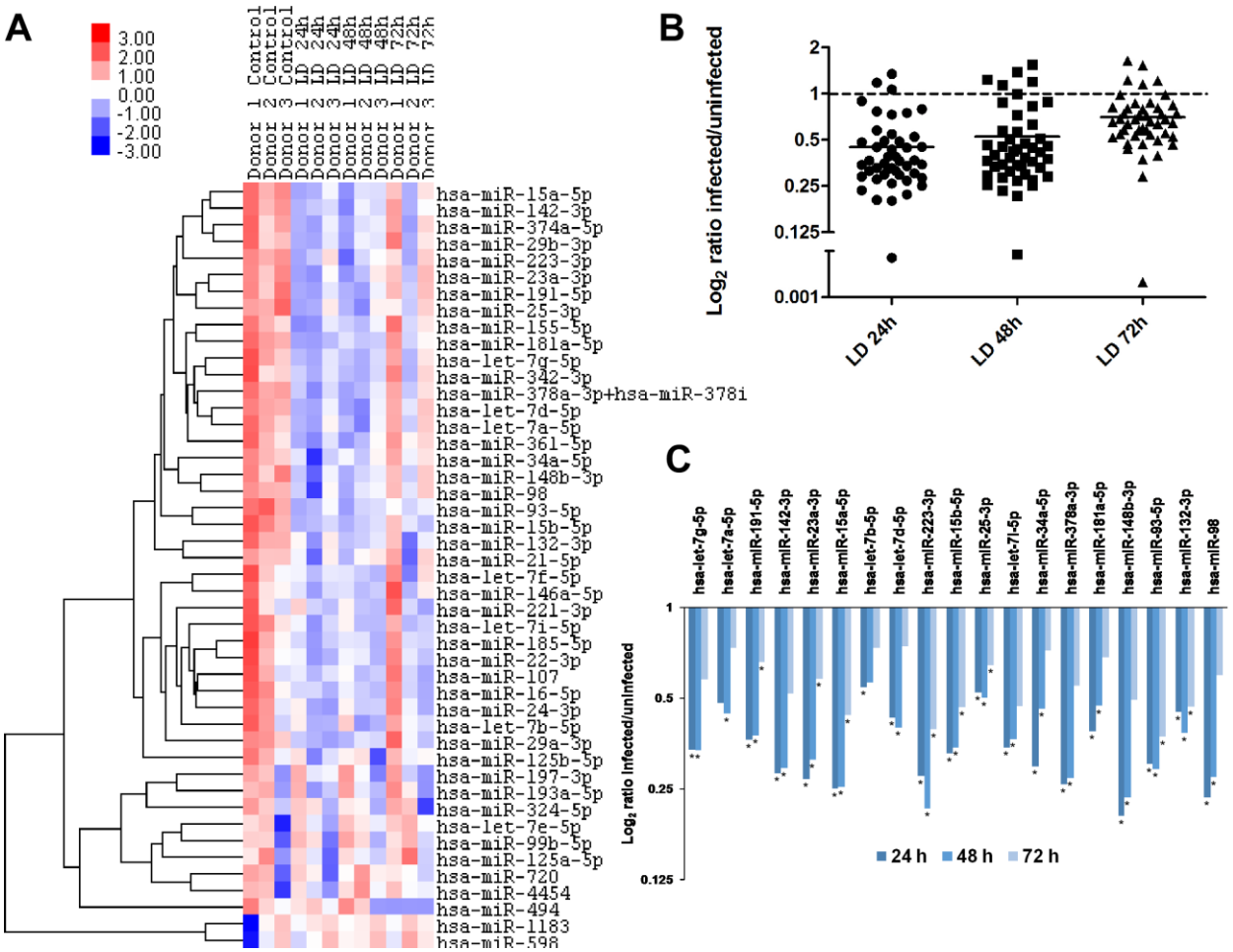

Figure 3.2 miRNA expression profiles of uninfected and *L. donovani* infected human macrophages.

Human monocytes were purified from buffy coats of 3 healthy donors, differentiated into macrophages in the presence of GM-CSF, and infected with *L. donovani* strain 1S promastigotes (LD) at MOI 40:1, or left uninfected (control). Time points were taken at 24, 48 and 72 hours 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.** Log_2 ratios of all 46 expressed miRNAs in infected over uninfected cells at different time points, miRNA expression values were averaged for the 3 donors. **C.** Log_2 ratios of significantly changed miRNAs in infected over uninfected cells at different time points (all 19 downregulated). Asterisk indicates $p < 0.05$.

during infection. In order to explore how the observed downregulation in miRNA expression could contribute to an infected macrophage phenotype, we searched for potential mRNA targets of the downregulated miRNAs. Initially, the validated target module of the miRWalk 2.0 algorithm available online [278] was used to retrieve all validated target genes potentially corresponding to the group of 19 miRNAs we found to

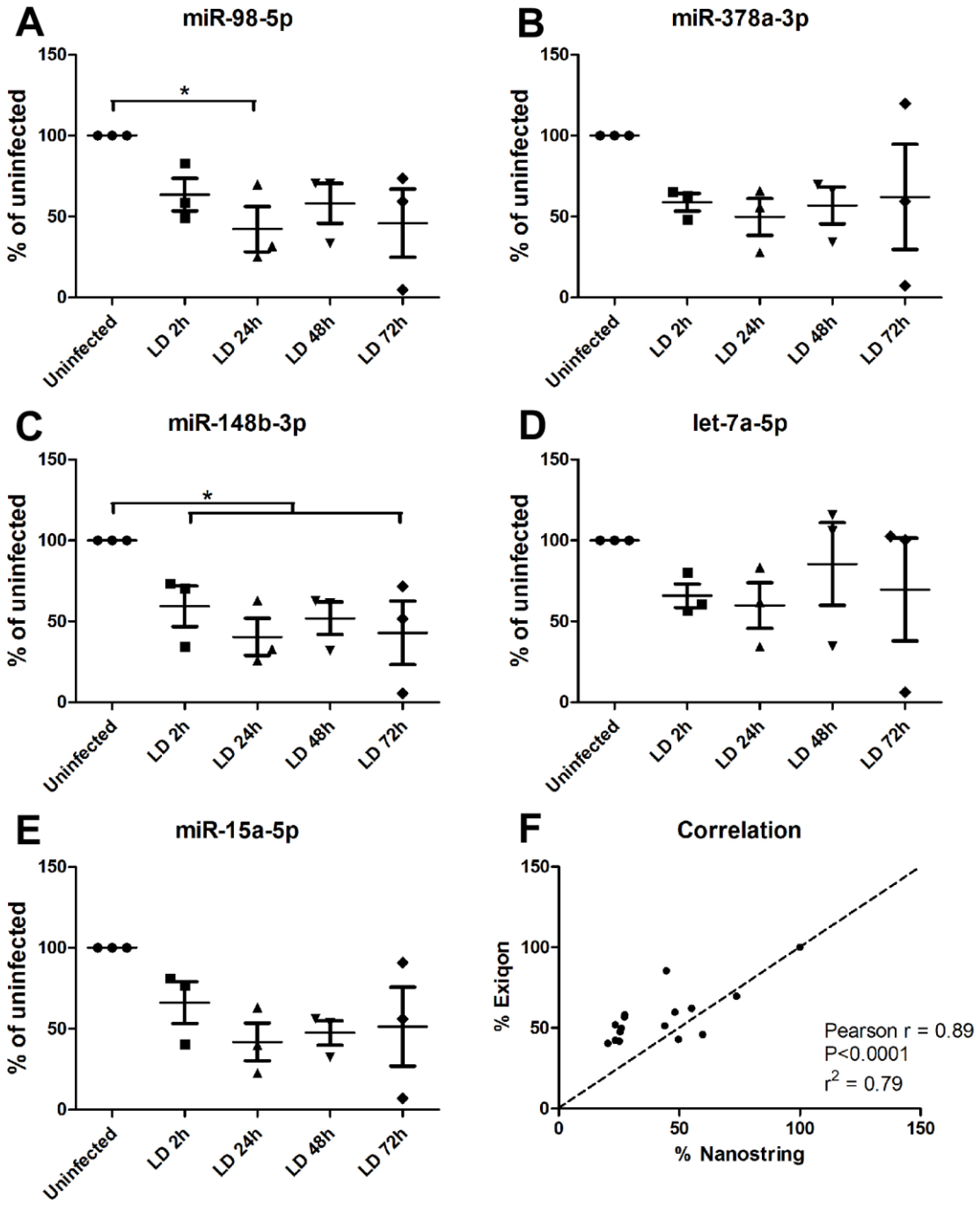

Figure 3.3 qPCR analysis of miRNA expression in *L. donovani* infected HMDMs
Monocytes were purified from 3 donors, differentiated into macrophages in the presence of GM-CSF and infected with *L. donovani* strain 1S promastigotes (LD) at MOI 20:1 for indicated time points or left uninfected. RNA was 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 percent of uninfected. One way ANOVA (A, B, C, D and E) and Pearson correlation (F) were calculated in GraphPad Prism v.5. Asterisk indicates $p < 0.05$.

be significantly downregulated at at least one time point in leishmania infected cells (see Figure 3.2 C). The rationale for choosing this target prediction algorithm was that it specifically retrieves and catalogues targets experimentally verified in previous studies (using various technologies including: reporter assays, Western blot, Northern blot, qPCR, microarray, pSILAC, and high-throughput sequencing). Thus, we expected a finite and specific list of targets for downstream analysis. Surprisingly, the retrieval generated a list of 9697 candidate targets (from a total of 20022 genes in the miRWalk 2.0 database). This number seemed extremely high, as it implied that the 19 miRNAs of interest could potentially target almost 50% of all genes present in the database. Clearly this result did not assist us in prioritizing miRNA-target interactions for follow-up analysis.

A major limitation of the miRWalk database is that it considers large-scale studies based on high-throughput sequencing as “experimentally validated” (e.g. [279], an analysis done by cross-linking ligation and sequencing of hybrids, CLASH), even though these types of analyses are considered “low confidence” and require further validation. Furthermore, it has become increasingly common in the recent literature to use a combination of different programs for miRNA target prediction to overcome differences in results obtained with individual programs. Consequently, we decided to carry out an independent search for targets using TargetScan [146]. TargetScan is a widely recognized algorithm, which predicts biological targets of miRNAs by searching for evolutionary conserved 8mer and 7mer binding sites within putative targets that show complementarity to the seed region of each miRNA of interest. To predict targets for the group of 19 significantly downregulated miRNAs with TargetScan, we used a conservative parameter so that targets were only retrieved if they contained a minimum of one 8mer binding site. This analysis yielded 2950 predicted targets, still a generous number, but considerably smaller when compared to the 9697 targets obtained with miRWalk (overlap: 2100 genes).

These target genes were then used to perform KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathway enrichment and GO (gene ontology) term analyses using the Web-based Gene Set Analysis Toolkit (WebGestalt) [280]. All 2950 target

Table 3.2 KEGG pathway enrichment analysis of target genes of 19 significantly downregulated miRNAs

KEGG pathway	*Number of genes*	*Adjusted p-value*
Pathways in cancer	96	2.47×10^{-33}
MAPK signalling pathway	79	1.26×10^{-27}
Regulation of actin cytoskeleton	57	8.9×10^{-18}
Prostate cancer	36	9.48×10^{-18}
Axon guidance	43	1.67×10^{-17}
Chronic myeloid leukemia	31	3.02×10^{-16}
Neurotrophin signalling pathway	41	3.02×10^{-16}
Focal adhesion	52	4.85×10^{-16}
Renal cell carcinoma	30	5.14×10^{-16}
Endocytosis	52	5.14×10^{-16}

The KEGG pathway enrichment analysis of 2950 target genes was performed in WebGestalt using the homo sapiens genome as a reference gene set, the hypergeometric statistical method with the Benjamin & Hochberg multiple test adjustment, retrieving the top 10 enriched pathways with a minimum of 5 genes per category.

genes were mapped to genes in the WebGestalt database. The results of the analysis for the top 10 most significantly enriched KEGG pathways (Table 3.2) highlighted a number of cellular pathways previously implicated in leishmania pathogenesis. Thus, the “MAPK signalling pathway”, “regulation of actin cytoskeleton” and “endocytosis”, were significantly enriched amongst the target genes. Assuming there is a negative correlation between the expression of miRNAs and their target proteins (i.e. downregulation of miRNAs results in upregulation of their targets); these pathways could be up- or downregulated during infection, depending on whether the positive or negative regulators are mainly targeted by miRNAs.

When changing the parameters of the KEGG pathway enrichment analysis to include not only the top 10 most significant, but all pathways that are enriched with a p-value smaller than $p < 0.01$ and a minimum of 5 genes per category, a total of 96 pathways were significantly enriched among the target genes (see Table S3.1). These included many more biological pathways that have previously been implicated in leishmania pathogenesis, such as “mTOR signalling pathway” (rank 19/96), “TGF-β signalling pathway” (rank 21/96), “Calcium signalling pathway” (rank 28/96), “Jak-STAT

signalling pathway" (rank 31/96), "Fc gamma R-mediated phagocytosis" (rank 41/96), and "Chemokine signalling pathway" (rank 43/96). Of note, we also performed this KEGG enrichment analysis for the 9697 target genes predicted with the miRWalk program for comparison (data not shown). Even though this analysis resulted in a larger number of pathways (194), there was a 96% overlap of enriched pathways, with minor differences in rank orders. This suggests that both miRWalk and TargetScan target prediction programs yielded similar potential targets for the group of 19 significantly downregulated miRNAs that are involved in the same cellular pathways, providing additional confidence for our results.

We next performed a GO term enrichment analysis (GO-slim) to investigate more broadly the biological functions of the target genes (obtained with TargetScan) of the 19 miRNAs that were significantly downregulated in response to *L. donovani* infection. Figure 3.4 shows the results for the enriched biological processes, cellular compartments and molecular functions. The most enriched biological processes (Figure 3.4 A) were "biological regulation", "metabolic process", and "response to stimulus", closely followed by "multicellular organismal process" and "cell communication", which make intuitive sense when considered in the context of a response to an infection. With regard to cellular compartments (Figure 3.4 B), interestingly "membrane" and "nucleus" were the most enriched. Membrane proteins have been shown to be involved in uptake of leishmania and also in initiation of a signalling response. Furthermore, nuclear transcription factors such as NFκB and AP-1 have previously been shown to be affected by leishmania infection. "Protein binding", "ion binding", and "nucleic acid binding" were the most enriched molecular functions among the miRNA target genes (Figure 3.4 C). Again, we also performed the GO-slim analysis with the targets obtained with miRWalk, yielding almost identical results (not shown).

Taken together, our results clearly show that infection with *L. donovani* profoundly affects host macrophage miRNA expression, which presents as an overall decrease in miRNA levels. The results also indicate that this is a general effect that is likely caused by manipulation of components of the miRNA maturation pathway (post-

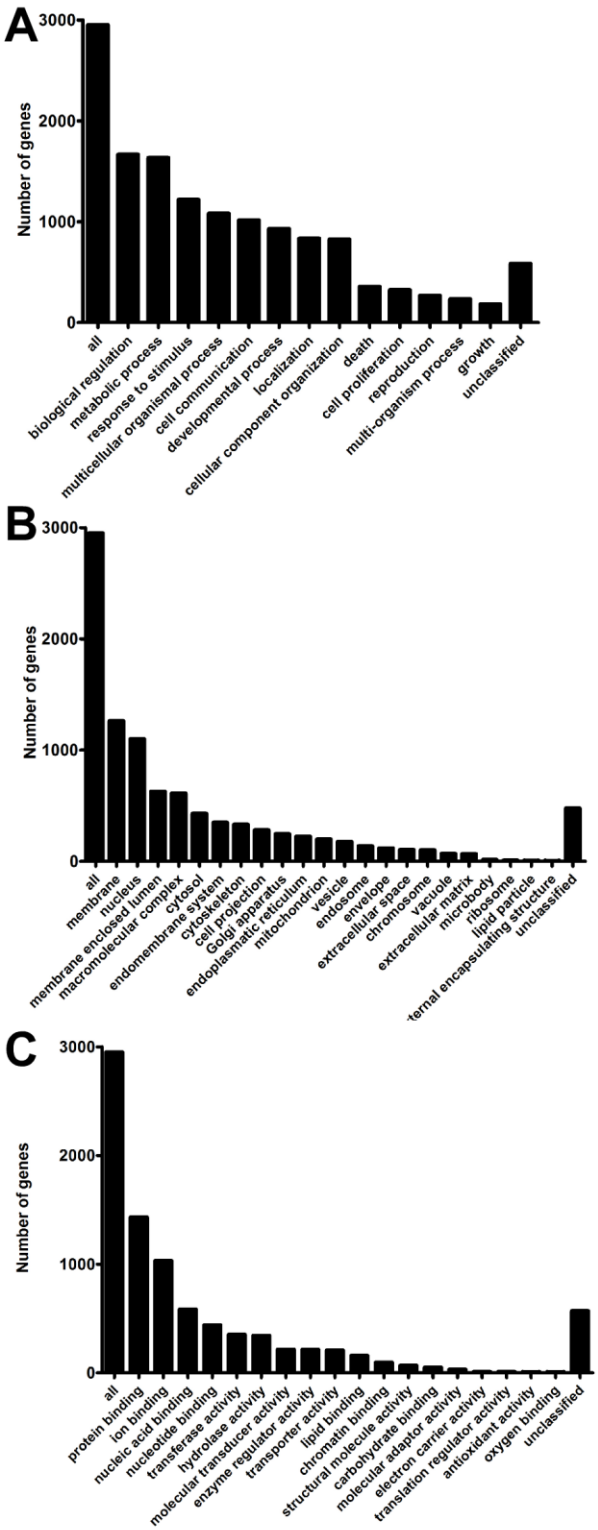

Figure 3.4 GO-slim analysis of target genes of the 19 significantly downregulated miRNAs.

GO-slim analysis of 2950 target genes was performed in WebGestalt using default settings. **A.** Biological process, **B.** Cellular compartment, **C.** Molecular function.

transcriptional processing of miRNA precursors and components of RISC) rather than a separate regulation of expression of individual miRNAs at the transcriptional level.

3.2.3 Effects of *L. donovani* infection on Drosha and Dicer protein levels

To explore the mechanism by which *L. donovani* downregulates miRNA expression in macrophages, we chose to examine the protein levels of two essential ribonucleases that are part of the canonical miRNA biogenesis pathway: Drosha and Dicer. Drosha is a component of the microprocessor complex in the nucleus that processes primary miRNAs (pri-miRNAs) into precursor miRNAs (pre-miRNAs). Dicer is responsible for cleavage of pre-miRNAs to form mature miRNAs in the cytoplasm. We hypothesized, therefore, that one possible mechanism by which leishmania could decrease miRNA levels might involve downregulating Drosha and/or Dicer. The results from our Western blot analysis (Figure 3.5) showed that protein levels of Drosha were increased in response to *L. donovani* infection. This increase was significant at 24 and 48 hours post infection. In the case of Dicer, though not statistically significant, our data also clearly indicated an increase in protein levels at 2 and 24 hours post infection. At later stages of infection (48 and 72 hours), Dicer levels were similar to uninfected cells. Of note, the basal levels of both proteins stayed constant during the 72 hours incubation period in uninfected cells (not shown). These results argue against our original hypothesis and conversely show that *L. donovani* infection induces an increase in Drosha and Dicer protein expression in host macrophages.

3.3 Discussion

It has been well established that leishmania affect many macrophage cellular processes during infection, and this is essential for the transformation, replication, and survival of the parasites inside these cells. The manipulation of macrophage processes by leishmania involves changes in the levels of many proteins, but the underlying mechanisms by which leishmania can alter host protein expression are not very well understood. miRNAs are important regulators of gene expression that can prevent the translation of mRNA into proteins, thereby fine-tuning cellular protein levels. Hence, the

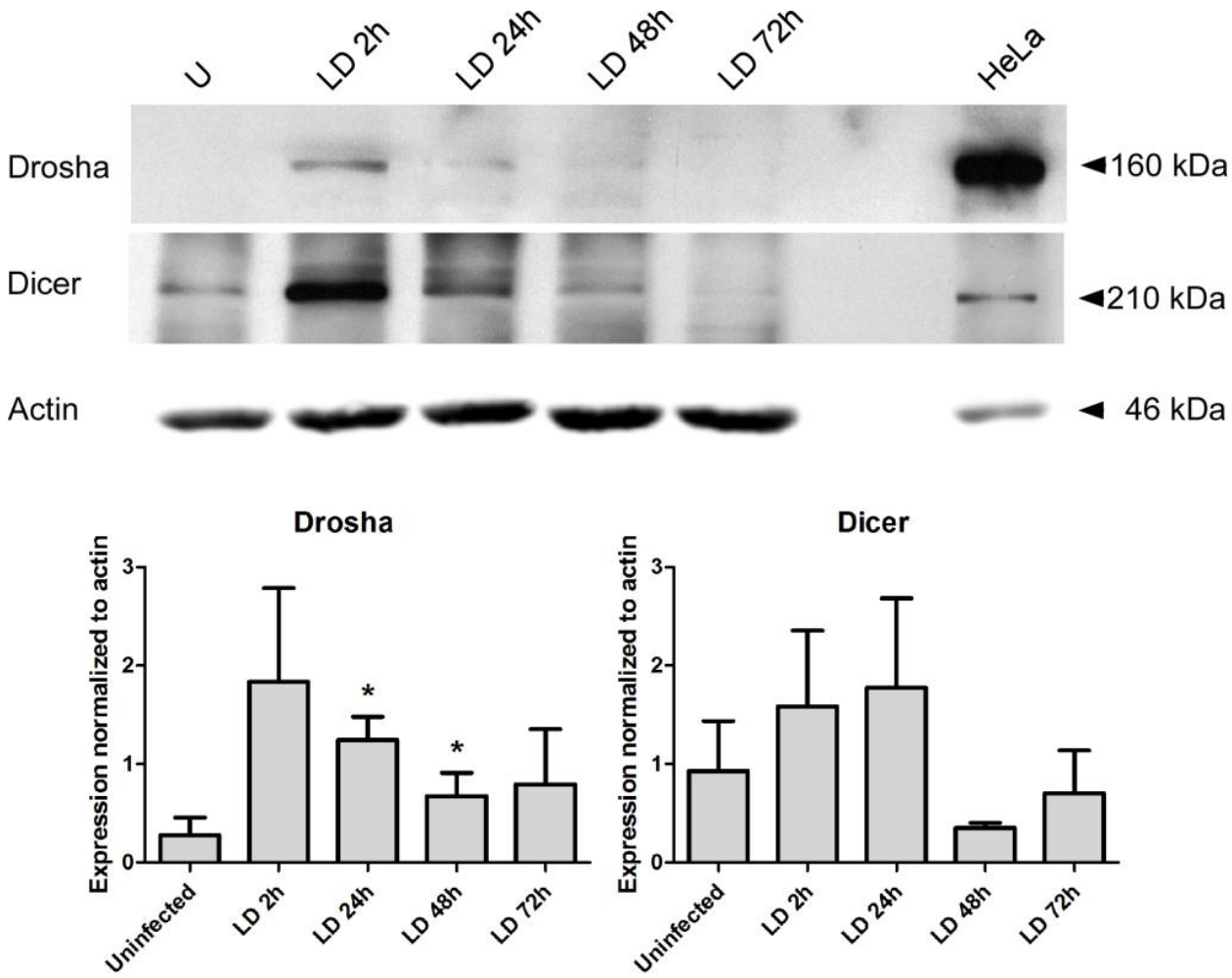

Figure 3.5 L. donovani increase the levels of Drosha and Dicer in HMDMs. Monocytes were purified, differentiated into macrophages in the presence of GM-CSF and infected with *L. donovani* strain 1S promastigotes (LD) at MOI 20:1 for indicated time points (or left uninfected, U). Protein lysates were separated by SDS-PAGE, and Western blotting with antibodies against Drosha, Dicer and Actin as a housekeeping gene were performed (HeLa cell lysate was included as a control for antibody specificity). One representative Western blot is shown for each protein. Bar graphs are densitometric analyses of Western blots (n=3), showing the mean + SEM. Asterisk indicates $p < 0.05$ difference to uninfected sample (t-test).

targeting of miRNAs could potentially be a powerful mechanism for modifying protein levels. Here, we investigated the hypothesis that changes in macrophage protein levels observed during infection with *L. donovani* could be brought about by modulation of themiRNAs that regulate the expression of these proteins. Our results clearly show that the expression profile of miRNAs is considerably altered in human macrophages infected with *L. donovani* when compared to uninfected cells. Strikingly, all miRNAs that were consistently dysregulated in all three donors tested were decreased at 24 and

48 hours post infection (Figure 3.2). These findings were confirmed in a secondary analysis, which additionally revealed that the downregulation of miRNAs is likely initiated early during infection (Figure 3.3).

Previous studies have suggested that leishmania may affect host miRNA expression and that this may contribute to pathogenesis (see Table 3.3 for a comparison) [123,213,214,281]. In a study performed by Lemaire *et al* it was shown that infection with *L. major* resulted in alterations of miRNA levels in human macrophages during early infection (3-24 hours) [213]. However, in their dataset, most miRNAs were differentially regulated at the different infection time points, i.e. the same

Table 3.3 Comparison of studies on the role of host miRNAs in leishmania infection

Study	***Host/model organism studied***	***Infecting Leishmania spp.***	***miRNAs assessed***	***Result***
Lemaire *et al* 2013	Human *In vitro*, monocyte-derived macrophages	*L. major* 3,6,12 and 24h infection	qPCR array (365 miRNAs)	64 miRNAs deregulated (up and down depending on donor and timepoint)
Kelada *et al* 2013	Mouse *In vivo* and *in vitro*, Treg cells	*L. major,* length not specified	High-throughput sequencing	17 miRNAs deregulated (16 down)
Ghosh *et al* 2013	Mouse and human, *in vivo* and *in vitro*, murine liver and human Huh7 hepatic cells	*L. donovani* infection *in vivo* 15, 30 and 60 days, *in vitro* exposure 24h	Only miR-122 (qPCR)	miR-122, downregulated
Geraci *et al* 2015	Human, *in vitro*, monocyte-derived macrophages and DCs	*L. major* and *L. donovani* 8h infection	High-throughput sequencing	104 miRNAs deregulated **DCs**: general suppression of miRNAs in response to *L. major* and upregulation in response to *L. donovani* **Macrophages**: more miRNAs downregulated in response to both *L. donovani* (19/31) and *L. major* (24/34)
Reiner Lab	Human *In vitro*, monocyte-derived macrophages	*L. donovani* 24, 48 and 72h infection	Nanostring array (800 miRNAs)	46 miRNAs detected, 35 consistently downregulated (19 significant)

miRNA was upregulated at some and downregulated at other time points. Furthermore, large inconsistencies between donors were observed, making the results overall inconclusive.

A recent, comprehensive study by Geraci *et al*, which was published while we were finalizing our data analysis, reported that *L. major* and *L. donovani* have differential effects on miRNA levels in macrophages and dendritic cells (DCs) [214]. DCs displayed a general suppression of miRNAs in response to *L. major* and general upregulation in response to *L. donovani.* In macrophages, however, more miRNAs were down- than upregulated in response to both *L. donovani* (19/31) and *L. major* (24/34). Strikingly, 23 of the 31 total miRNAs they detected in macrophages infected with *L. donovani* overlapped with the miRNAs detected in our profiling (we detected a total of 46 miRNAs). The results from Geraci *et al* are generally in line with our observation that the majority of miRNAs are downregulated in macrophages in response to *L. donovani* infection. The fact that Geraci *et al* saw an upregulation of some miRNAs in *L. donovani* infected macrophages could be due to three reasons. First, they examined an earlier infection time point than us (8 hours versus our 24 hours and beyond). Although we did examine the levels of 5 selected miRNAs by qPCR during early infection (2 hours) and confirmed their downregulation, we did not perform a comprehensive analysis at this time point and, therefore, cannot exclude the possibility that other miRNAs may be upregulated after short term (2 hours) infection as well. Second, they used a different stimulus for differentiation of macrophages (M-CSF, whereas we used GM-CSF). It is known that differentiation of macrophages in the presence of either GM-CSF or M-CSF results in differentially polarized cells [282]. Differentiation in the presence of GM-CSF yields type 1 macrophages (M1) with a pro-inflammatory cytokine signature, which produce high levels of IL-23 and IL-12 in response to secondary stimuli [282]. In contrast, M-CSF differentiated macrophages are characterized by an anti-inflammatory, type 2 (M2) phenotype, and secrete high levels of IL-10 [282]. Moreover, differentially polarized macrophages have been shown to vary in their miRNA expression profiles [283], likely reflecting the different conditions used for polarization. This suggests the possibility that the effects of *L. donovani*

infection on miRNA expression in differentially polarized macrophages may differ. Third, Geraci *et al* used high-throughput sequencing for measuring miRNA expression, whereas we used Nanostring nCounter gene expression analysis. The advantage of high-throughput sequencing is that the number of miRNAs that can be measured is not limited. However, this method requires extensive manipulation of RNA material during sequencing library construction, including enzymatic reactions and PCR amplification cycles, which can introduce bias, especially for quantitative analyses, as some sequences can be preferentially amplified over others [284]. The Nanostring nCounter analysis method is limited to measuring the expression of 800 miRNAs. However, extracted raw RNA is directly used as input material without further processing, plus the nCounter technology is highly sensitive [285]. Based upon these considerations, we think that our Nanostring data is of superior quality.

Two other studies (Table 3.3) have looked at miRNA expression in response to leishmania infection, albeit at a smaller scale and looking at other, not directly comparable infection models [123,281]. Despite these substantial differences, the results from both these studies agree with our observation, as they showed predominant downregulation of host miRNA expression induced by leishmania.

When predicting candidate gene transcript targets for the miRNAs that were significantly downregulated in our analyses, we found a large number of genes and enriched KEGG pathways that have previously been shown to play a role in leishmania pathogenesis. Notably, of the top 10 most enriched pathways in our analysis, 8 also appeared amongst the top 10 enriched pathways of predicted miRNA targets in the Geraci *et al* study (downregulated miRNAs in macrophages response to *L. donovani* and *L. major* combined) [214]. Among these enriched biological pathways were “endocytosis”, “focal adhesion”, “MAPK signalling pathway” and “regulation of actin cytoskeleton”.

Endocytosis is required for leishmania uptake and establishment of infection; hence it makes intuitive sense that the parasites would manipulate miRNA expression to achieve upregulation of genes associated with this pathway. Focal adhesion is

important in macrophage adhesion to the extracellular matrix or connective tissues, during activation, maturation and migration. A small number of studies have suggested that leishmania inhibits macrophage adhesion, and amastigote replication was enhanced in non-adherent cells [286,287]. Thus, the upregulation of focal adhesion suggested by our data is surprising and further investigation will be needed to confirm this observation. MAPK signalling has long been known to be modulated in response to leishmania infection. For example, in naïve BMDMs, *L. donovani* prevented activation of the MAP kinases ERK1/2, p38 and JNK [35]. The modulation of MAPK activities by leishmania has been linked to suppression of IL-12 and induction of IL-10 production [37], thus representing an important mechanism by which leishmania inhibits proinflammatory responses. Regulation of the actin cytoskeleton within macrophages is important in phagosome assembly during leishmania uptake. For example, the GTPases Rac1 and RhoA act as important components of cytoskeleton rearrangement and have been found to associate with phagosomes harbouring leishmania [288,289].

Interestingly, a very recent global analysis of the proteome in *L. donovani*-infected THP-1 monocyte-derived macrophages revealed that the expression of proteins involved in key metabolic pathways, including glycolysis and fatty acid oxidation, is upregulated in response to infection [290]. Furthermore, an increased abundance of proteins involved in gene transcription, RNA splicing, DNA repair, replication, cell survival and signal transduction were specifically observed at 24 hours post infection. The authors suggested that this could be an outcome of selective pressure to amplify gene expression in infected cells, in order to cater to the need of increased metabolic events [290]. These results correlate well with our observations of enriched biological pathways and molecular functions among miRNA targets and suggest that *L. donovani* infection may induce a global reprogramming of cell metabolism. It is tempting to speculate that the upegulation of proteins observed in infected cells as described above results from the downregulation of miRNAs that target these proteins.

The results of the miRNA target prediction followed by KEGG pathway enrichment and GO slim analyses obtained in this study form the basis for further

investigations. In cases where interactions have not previously been convincingly demonstrated, experimental validations of interactions of miRNAs with their predicted targets will need to be performed. In the context of leishmania infection, it will be crucial to investigate whether the downregulation of miRNAs actually leads to upregulation of the respective targets, and whether there is a functional/phenotypic consequence for the macrophage. A good starting point for example could be to focus on miRNAs in conjunction with their predicted targets that are part of the MAPK signalling pathway. As mentioned above, the involvement of the MAPK signalling pathway in leishmania infection has been well described and it was also the second most enriched KEGG pathway in our analysis. A major negative regulator of the MAPK signalling pathway that came up in our analysis of predicted targets was protein phosphatase, Mg^{2+}/Mn^{2+} dependent, 1A (PPM1A, also known as PP2CA). PPM1A is a negative regulator of mitogen-activated protein kinase kinases (MKK) 3 and 7, which are activators of p38 and JNK, respectively. As mentioned above, leishmania has been shown to prevent activation of p38 and JNK [35], thus one potential mechanism behind this could be upregulation of PPM1A through downregulation of its repressing miRNA(s). PPM1A was a predicted target for 3 of the 19 significantly downregulated miRNAs in this analysis: miR-23a-3p, miR-34a-5p and miR-93-5p (8mer conserved binding site).

A recognized strategy for experimental validation of miRNA-target interactions involves cloning of a luciferase miRNA target expression vector, which is transfected into cells together with or without a miRNA mimic, followed by a luciferase assay [291]. In the example being discussed here, the miRNA binding site for PPM1A would be cloned and transfected into cells along with miRNA mimics for either miR-23a-3p, miR-34a-5p or miR-93-5p and appropriate controls. Inhibition of luciferase activity as a readout should establish whether PPM1A is directly regulated by miR-23a-3p, miR-34a-5p and/or miR-93-5p. In parallel, protein levels of PPM1A in cells transfected with the same miRNA mimics alone can be measured for example by Western blotting, which will support further the functionality of miRNA-mediated repression. After positive confirmation of a specific miRNA-target interaction, the role of this can be studied in the context of leishmania infection by verifying by qPCR that the given miRNA is

downregulated in response to infection (as originally shown), followed by assessing protein levels of PPM1A in infected cells (expected to be upregulated). In turn, the importance of inhibition of miRNA-mediated repression of PPM1A in pathogenesis could be further studied by loss-of-function and gain-of-function experiments. Thus, if our hypothesis is correct, PPM1A knock-down in macrophages would result in reduced infectivity of leishmania. Likewise, overexpression of the specific miRNA of interest – as established above – in macrophages followed by leishmania infection should result in reduction of PPM1A protein levels and reduced infection rates. This strategy should be broadly applicable to validate the miRNA-target interactions that have been suggested by the findings in this thesis and to explore their role in leishmania pathogenesis.

The results from our study are noteworthy, as this type of general response of miRNA "underexpression", to our knowledge, has never previously been reported in infection with any other protozoan or bacterial pathogen. Thus, our observation raises a number of important questions: 1) how is global downregulation of miRNAs by leishmania brought about, 2) how does leishmania benefit from miRNA repression, and 3) is this a widespread phenomenon that is relevant to infections with other microbes.

Global decreases in miRNA expression have been associated with several types of cancer, which can be attributed to mutations or deletions in miRNA processing enzymes such as Drosha and Dicer [292-294], hypermethylation of miRNA genes [295], mutations in the protein Exportin-5 [296], or transcriptional repression of miRNA genes by oncogenic transcription factors [297]. Virus-mediated global miRNA repression has been described, and is likely a mechanism by which viruses inhibit anti-viral RNAi. For example, the mammalian Nodamura virus protein B2 was found to inhibit the Dicer cleavage reaction *in vivo* and *in vitro* by binding Dicer substrate RNAs, leading to inhibition of endogenous miRNA generation [202]. One study has indicated that repression of miRNAs can be beneficial for the survival of *Plasmodium falciparum*, albeit in its invertebrate host, as knockdown of Dicer1 or AGO in *Anopheles gambiae* resulted in increased susceptibility to infection [276]. Importantly, in a recent report investigating infection with *L. donovani*, it was suggested that Dicer1 was downregulated in hepatic cells *in vivo* in infected mice, and *in vitro* in a human hepatic

cell line, and that this contributed to increased infectivity and reduced serum cholersterol [123]. Conversely, overexpression of Dicer reduced murine liver parasite load and restored serum cholesterol levels. The authors also suggested that Dicer downregulation in hepatic cells was mediated by GP63 delivered through *L. donovani* exosomes. However, the only miRNA that was investigated in this study was miR-122, which was downregulated in response to infection. It remains to be investigated if other miRNAs are also affected by the decreased Dicer levels in this experimental system.

In search for a mechanism by which *L. donovani* downregulates miRNA expression in human macrophages, we assessed the expression of two proteins that are essential for miRNA biogenesis: Drosha and Dicer. Unexpectedly, our results showed that protein levels of both Drosha and Dicer were increased in response to *L. donovani* infection (Figure 3.5). The increase in Dicer levels was surprising since, as mentioned above, this protein has previously been reported to be downregulated in response to *L. donovani* infection [123]. This difference in results could be due to the different cell types investigated (hepatic Huh7 cells versus primary human macrophages) or to different infection time points examined (time point in Ghosh *et al* study unknown). Furthermore, the quality of the Dicer Western blots in the Ghosh *et al* study is limited such that the results are not very convincing and remain questionable.

In summary, it appears that the global downregulation of miRNAs that we observed in our experiments is not caused by a reduction in Drosha or Dicer protein levels. In fact, as Drosha and Dicer are both among the predicted targets of downregulated miRNAs in our study, their upregulation may be due to the decreased inhibition by the miRNAs that normally target them. We cannot exclude the possibility, however, that the specific activities of Drosha or Dicer are impaired during *L. donovani* infection. Interestingly, phosphorylation of Drosha by glycogen synthase kinase 3 β (GSK-3β) was shown to be required for its nuclear localization and activity [298,299]. It was previously shown by Nandan *et al* from our laboratory that *L. donovani* infection results in phosphorylation of Akt-2 in a PI3K-dependent manner, followed by phosphorylation and inactivation of GSK-3β [41]. Thus, *L. donovani* could potentially inhibit Drosha through inactivating GSK-3β and this is currently being studied.

Other possible mechanisms by which leishmania could interfere with miRNA expression are targeting of other components of the miRNA biogenesis pathway such as AGO, DGCR8 or Exportin 5, hypermethylation of miRNA genes, effects on transcription factors and destabilization of miRNAs. Further experiments will be required to answer the questions of how leishmania-induced repression of miRNA expression in macrophages is achieved, and how the underexpression of miRNAs contributes to an altered macrophage phenotype and to leishmania pathogenesis.

3.4 Materials 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 from buffy coats by centrifugation over a Ficoll-Paque PLUS gradient (GE Healthcare) followed by plastic adherence. For this purpose, buffy coats were diluted 1:2.5 with sterile PBS (HyClone, GE Healthcare) containing 2 mM EDTA, distributed over 50 mL tubes and 10 mL Ficoll was underlayed. Tubes were centrifuged for 20 min at 500xg at room temperature, with brake off. The lymphocyte layer was collected, and platelets were removed by repeated washing with 45 mL PBS (3-4 washes), decreasing the centrifugal force with each wash (500-100xg). After the last wash, pellets were resuspended in prewarmed RPMI 1640 (HyClone) supplemented with 10% heat-inactivated FBS (Gibco) and 2 mM L-glutamine (Stemcell Technologies). Cell suspensions were transferred to 150 cm^2 culture flasks (Corning) and incubated for 1 hour at 37°C and 5% CO_2. After incubation, supernatant including non-adherent cells was removed and the adherent cell layer was washed 3 times with warm HBSS. Adherent cells were scraped into supplemented RPMI, a sample was taken and mixed 1:1 with Tuerk's solution for counting. Enriched monocytes were then seeded into 6 well plates (1.5x10E6 cells per well in 2 mL) or 24 well plates containing sterilized glass coverslips (0.2x10E6 cells per well in 0.5 mL), and differentiated into human monocyte-derived macrophages (HMDMs) for 6 days in the presence of 10 ng/mL rh-GM-CSF (Stemcell) [282]. Alternatively, cells were differentiated in the presence of 10% normal

human AB serum (no FBS) [300]. On day 4, cells were washed 3 times with warm HBSS and fresh, supplemented RPMI containing rh-GM-CSF (or 10% human AB serum) was added. On the day prior to infection (day 6), cells were washed again 3 times with HBSS, fresh supplemented RPMI without cytokine was added, and cells were rested for 24 hours. Differentiation into macrophages was assessed by quantifying the expression of cell surface markers CD14 and CD11b using FITC-conjugated antibodies (Molecular Probes) according to the manufacturer's instructions and detection by flow cytometry (FACSCalibur, BD Biosciences).

3.4.2 Parasite culture and infections

L. donovani Sudan strains 1S and 2S promastigotes were routinely cultured in M199 (Sigma-Aldrich) with 10% heat inactivated FBS (Gibco), 20 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 uM adenosine (Sigma-Aldrich) at 26°C. Every 3 days the organisms were subcultured 1:10 in fresh medium and were kept in culture for a maximum of 20-25 passages. Fresh parasites were obtained by purification of amastigotes from spleens of infected Syrian Golden hamsters followed by *in vitro* transformation into promastigotes by culturing for 7 days at 26°C in promastigote media. For *in vitro* differentiation into axenic amastigotes, day 5 promastigotes (at a concentration of 5x10E7 cells/mL) were washed 2 times with HBSS, followed by incubation in RPMI 1640 supplemented with 1% D-glucose (Sigma-Aldrich), 20 mM HEPES (Stemcell), 2 mM L-glutamine (Stemcell), 100 U/mL penicillin/streptomycin (Stemcell) and 25 mM MES (Sigma-Aldrich) to lower the pH to 5.5, at 37°C for 48 hours. Day 5 stationary phase promastigotes or *in vitro* transformed amastigotes were counted, washed 2 times with HBSS, resuspended in HBSS and used to infect HMDMs at indicated multiplicities of infection (MOIs). Infection experiments were incubated at 37°C for 24 hours, then harvested or washed with HBSS to remove unbound parasites and incubated for an additional 24 hours (48 hour timepoint) or additional 48 hours (72 hour timepoint), at 37°C before harvesting. When taking timepoints for RNA extraction or preparation of protein lysates, infected or uninfected HMDMs grown in 6 well plates were washed 3 times with PBS, scraped into

PBS and pelleted at 500xg for 5 minutes at room temperature (cell pellets were stored at -70°C until further processing).

Infection rates were routinely assessed in infected HMDMs grown on coverslips. When taking time points, coverslips were washed 3 times with PBS, followed by fixation with 2% paraformaldehyde in PBS for 15 minutes at room temperature. After fixation, coverslips were washed again 3 times with PBS and mounted onto glass slides using Prolong Gold antifade mounting media containing DAPI (Life Technologies) to detect macrophage nuclei and leishmania nuclei and kinetoplasts. Fluorescence microscopy was performed using an Axioplan II epifluorescence microscope (Carl Zeiss Inc). Images were recorded using an AxioCam MRm Camera coupled to the AxioVision software Version 4.8.2 (Carl Zeiss Inc.). Infection rates were determined by taking at least 10 images at 40x magnification per sample, counting the number of infected cells per total cells per frame, and calculating the average.

3.4.3 RNA extraction and Nanostring assay

RNA was extracted from HMDM pellets using the miRCURY RNA isolation kit Cell & Plant (Exiqon) according to the manufacturer's instructions. An on-column DNase treatment was performed using 12.5 Kunitz Units DNase I (Thermo Scientific) per column, and incubating for 15 minutes at room temperature. RNA was eluted in 50 µL nuclease-free ddH_2O and OD_{260} was determined by nanodrop (Thermo).

Purified RNA was diluted to a concentration of 50 ng/µL with nuclease-free ddH_2O 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 [285].

3.4.4 Nanostring data analysis and mRNA target prediction

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.

Target prediction was performed for the miRNAs that showed a significantly ($p<0.05$) different expression in infected cells. The validated target module of the miRWalk 2.0 algorithm [278] and TargetScan [146] (min. one 8mer binding site, only targets with conserved binding sites) were used to predict targets for the 19 significantly downregulated miRNAs. The list of target genes was further analyzed with the WEB-based Gene Set Analysis Toolkit (WebGestalt) [280] to look for enrichment of KEGG pathways and gene ontology (GO) terms. The KEGG pathway enrichment analysis was performed using the homo sapiens genome as a reference gene set, the hypergeometric statistical method with the Benjamin & Hochberg multiple test adjustment, retrieving the top 10 enriched pathways with a minimum of 5 genes per category. This analysis was expanded by setting the p-Value < 0.01 as a threshold. The GO slim analysis was performed using WebGestalt's default settings.

3.4.5 Quantitative real-time PCR

qPCR was performed to measure the expression of 5 selected miRNAs plus the reference gene U6 in uninfected and infected HMDM. Cells were infected and RNA was exracted as described above, with the exception that DNase I treatment was performed in solution (according to the manufacturer's instructions, Thermo). 10 ng of RNA were used as input material for cDNA synthesis using the Universal cDNA synthesis kit II (Exiqon) according to the manufacturer's instructions. The synthetic RNA spike-in UniSp6 provided with the kit was used as internal control, along with no enzyme and no template controls. cDNA was diluted 80x in nuclease free ddH_2O, and ROX reference dye (Life Technologies) was added at a 50x dilution to the cDNA dilution. Diluted cDNA was used as input for qPCR reactions using the ExiLENT SYBR

Green master mix kit (Exiqon) and miRNA-specific primers according to the manufacturer's protocol. Real-time PCR amplification followed by melting curve analysis was performed using the StepOne Plus system (ABI). Manual baseline (3-15 cycles) and threshold (0.75) settings were applied to obtain Ct values. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in miRNA expression using U6 as a reference gene.

3.4.6 Western blotting

Pellets of infected and uninfected HMDMs as prepared above (section 3.4.2) 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 β-glycerophosphate, 1 mM PMSF, 5 µg/ mL aprotinin, 5 µg/mL leupeptin). Lysates were incubated on ice for 5 minutes, followed by mechanical lysis through a 27 gauge needle (10x). Lysates were then centrifuged at 13,000xg for 5 minutes, followed by addition of 4x Laemmli loading buffer and boiling for 7 minutes. Samples equivalent to ~50 µg of protein were loaded onto 7.5% SDS-PAGE gels and run for 1.25 hours at 100 V, followed by wet transfer to nitrocellulose membranes (35 V over night at 4°C). Immunoblotting was performed with primary antibodies against Drosha, Dicer and Actin (Santa Cruz Biotechnologies) diluted 1:500 in 3% BSA in TBS with 0.1% Tween-20, incubating over night at 4°C. After washing, blots were incubated with secondary antibodies (anti-rabbit-HRP from ABM) for 1 hour at room temperature, washed again and developed with Supersignal West Femto Chemoluminescence Substrate (Thermo). Densitometric analysis was performed in ImageJ.

3.4.7 Statistical analysis

Significant differences between uninfected and infected samples for all experiments were calculated in GraphPad Prism v.5, using one-way ANOVA (repeated measures ANOVA) and Tukey's post test, or one-tailed Sudent's t-test. P-values equal to or less than 0.05 were considered statistically significant. Pearsson's correlation of miRNA expression data measured with Nanostring and qPCR was also calculated in GraphPad Prism v.5.

Chapter 4 Discussion

4.1 Scientific significance for the field

The overall goal of my research was to investigate the role of small non-coding RNAs in the biology of infection with leishmania. I chose to do this from two different yet complementary perspectives, namely the involvement of leishmania-derived RNAs released within exosomes and the roles of host-derived miRNAs acting within leishmania-infected macrophages. Significant progress towards this goal has been made as the findings reported in Chapters 2 and 3 of this thesis clearly indicate a role for small non-coding RNAs, be they leishmania- or host-derived, in the biology of infection with this important pathogen.

First, I established the presence of RNA in leishmania exosomes, characterized exosomal RNA sequences in detail and showed that these sequences can be delivered to host macrophages (Chapter 2). Recently, it had been discovered that leishmania use an exosome-based mechanism to secrete and deliver proteins and thereby communicate with the host. One important question that arose from this discovery was whether leishmania exosomes also carry other, non-protein cargo, which participates in communication with the host. My findings presented in Chapter 2 unambiguously demonstrate that exosomes released from *L. donovani* and *L. braziliensis* are particularly enriched in a conserved subset of small non-coding RNAs derived from rRNAs and tRNAs (Figure 2.3, Table 2.2). Moreover, these exosomes contain a number of novel transcripts, albeit in relatively low abundance (Figure 2.4, Table 2.3). The RNAi-proficient *L. braziliensis* appears to package putative siRNAs or their precursors into exosomes, whereas RNAi-deficient *L. donovani* does not. My data indicate that specific RNA sequences are selectively packaged into exosomes (Figures 2.4 and 2.5). This conclusion is supported by the highly biased distribution of sequences detected in exosomes over only a subset of genes in the leishmania genome combined with a striking paucity of transcripts derived from protein coding genes which are otherwise abundant in total cellular RNA (Figure 2.3). Importantly, my findings provide at least three lines of evidence arguing for the presence of an

evolutionarily conserved mechanism for packaging small non-coding RNAs into exosomes in leishmania: 1) the high degree of overlap between the top 20 most abundant sequences found in *L. donovani* and *L. braziliensis* exosomes (Table 2.2), 2) the vast majorities of identified novel transcripts were present in exosomes from both species (Table 2.3), and 3) the most abundant tRNA-derived small RNAs found in exosomes were derived from a highly biased subset of the same tRNA isoacceptors in both species (Figure 2.5, Table 2.4).

The finding that leishmania exosomes carry a select and biased repertoire of small non-coding RNA cargo that is conserved across old and new world leishmania species is of special interest. This is particularly so since during the last decade; progress in the identification and functional analysis of small, non-coding RNAs has begun to reveal previously unappreciated roles for these molecules in pathogen cell regulation and in infection biology. For example, small non-coding RNAs from the plant fungal pathogen *Botrytis cinera* were shown to act as effectors to block the immune systems of *Arabidopsis* and tomato [251]. This involved hijacking the host RNAi machinery by binding of pathogen small non-coding RNAs to plant AGO proteins, leading to silencing of host defense genes. In another elegant example, human adenovirus was shown to block the host RNAi pathway by saturating the host RISC with virus-associated (VA) small non-coding RNAs [301,302]. VA small non-coding RNAs have also been suggested to act as miRNAs to suppress host mRNA translation [303]. These fascinating examples of cross-kingdom RNAi beg the questions as to how widespread this phenomenon is in nature and whether small non-coding RNA effectors are also operative in major human eukaryotic pathogens such as leishmania. As described in Chapter 1, the occurence of small non-coding RNAs has been described in a number of human protozoan pathogens such as *T. gondii, G. lamblia, T. vaginalis, E. histolytica, T. brucei, T. cruzi* and *L. braziliensis* [159,162-167]. My findings presented in Chapter 2 expand on this knowledge and add three important novel dimensions: 1) not only RNAi-competent (*L. braziliensis*), but also RNAi-deficient leishmania species (*L. donovani*) produce small non-coding RNAs with potential regulatory functions, 2) leishmania produce small non-coding RNAs derived from rRNAs and tRNAs, and 3)

small non-coding RNAs are selectively packaged and released from leishmania within exosomes. Based upon these findings, it seems highly plausible that leishmania exosome-derived small non-coding RNA effectors may target the host translational machinery, RNAi machinery, or both, thereby creating an environment that is permissive to infection (see model Figure 4.1).

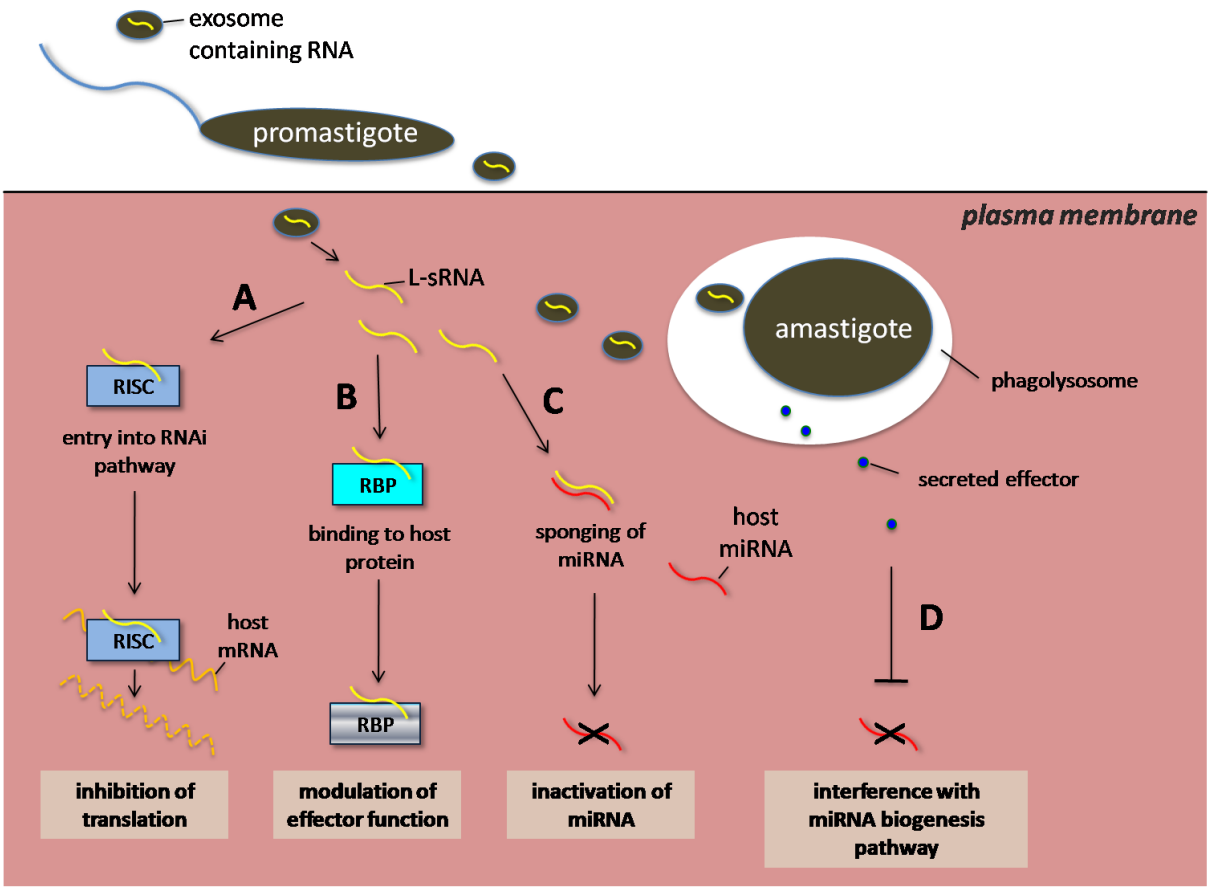

Figure 4.1 Hypothetical model for possible interactions of leishmania and host-derived small non-coding RNAs during infection.
Extracellular promastigotes and intracellular amastigotes release exosomes containing leishmania small non-coding RNA (L-sRNA), which are delivered to macrophage cytosol. The three different potential modes of action of L-sRNAs are: **A.** L-sRNAs are loaded onto the host RNA-induced silencing complex (RISC), thereby entering host RNAi. In turn, they target host mRNAs in a miRNA or siRNA like fashion, thereby inhibiting translation. **B.** L-sRNAs bind to other, non-RNAi related host RNA binding proteins (RBPs) and modulate their activity and/or function. **C.** L-sRNAs act as inactivators of host miRNAs by complementary base pairing, similar to miRNA sponges or antagomiRs. Another possible mechanism for interference with the host miRNA pathway is **D.** the secretion of leishmania protein effectors into the cytoplasm of infected cells, where they interact with components of the host miRNA pathway, leading to repression of miRNA expression.

Taken together, my findings support an emerging paradigm in which parasite-derived small non-coding RNAs are an evolutionarily conserved phenomenon. Furthermore, the fact that small non-coding RNAs are released from leishmania axenic amastigotes within exosomes and are delivered to macrophages (Figure 2.2) strongly suggests that they may actively interact with the host, likely with the purpose of modifying host cell phenotype to support chronic infection. Studies to establish unambiguously that leishmania small non-coding RNAs are novel effectors contributing to disease pathogenesis are of great topical importance and should be forthcoming.

The second major discovery of my thesis is that leishmania have a profound effect on host macrophage miRNA expression, indicating that targeting of the host RNAi machinery is a novel leishmania virulence strategy. My findings reported in Chapter 3 firmly establish that *L. donovani* infection results in a global downregulation of miRNA expression in macrophages *in vitro* that takes its full effect at 24 hours post infection (Figure 3.2). RNA interference mediated through miRNAs is a layer of gene regulation that is crucial to most aspects of cellular functions during health and disease, most notably cell differentiation, proliferation and metabolism. Previous studies have hinted at an involvement of host miRNAs in leishmania pathogenesis [123,213,214,281], but no conclusive evidence had been provided. The broad-based inhibition of miRNA expression in response to leishmania infection observed in my experiments was striking; as such a general response had never been previously reported during infection with any other protozoan or bacterial pathogen. Importantly, though still controversial, global downregulation of miRNAs is a phenomenon that has been described in many types of cancer tissues that have been profiled and is associated with poor prognosis and disease outcome [304,305]. Considering that immune responses in leishmania infection and cancer progression have several important similarities, it is perhaps not surprising that I observed a comparably repressed miRNA expression profile in leishmania infection. Much like leishmania, malignant tumors have developed various mechanisms to evade immune responses and manipulate a variety of immune cells, which include tumor-associated macrophages (TAMs). TAMs are M2 or ‘alternatively activated’ macrophages, which are induced by IL-4, IL-10, IL-13 and

glucocorticoid hormones and express high levels of IL-10 and low levels of IL-12. Furthermore, TAMs are ineffective antigen-presenting cells and they produce CC-chemokine ligand 22 (CCL22), which attracts T regulatory cells that produce IL-10 and inhibit T-cell activation. Secretion of prostaglandin E2 (PGE2) and TGFβ by TAMs further contributes to immune suppression [306]. Hence, the phenotype of TAMs is very similar to that of *L. donovani*-infected macrophages. The appreciation of miRNA deregulation specifically in TAMs remains limited, as the majority of miRNA profiling studies sampled whole-tumor tissues, which comprise not only cancer cells but also variable proportions of cancer-associated fibroblasts, endothelial cells and various immune cell types [307]. Nevertheless, it is likely that TAM miRNA profiles are in line with the general underexpression observed in cancer tissues. Taken together, my findings support the hypothesis that a global downregulation of miRNAs reflects an immunosupressed activation state of macrophages. Further studies on macrophage miRNA expression profiles in other pathologic conditions with an immunosupressed signature should confirm whether this is a “true” biological phenomenon.

An obvious and compelling question that arose from my findings in Chapter 3 is, what is the mechanism by which leishmania downregulates host macrophage miRNA expression? The fact that the vast majority of miRNAs measured were affected in a similar fashion (i.e. decreased) indicates that the miRNA biogenesis and processing pathway is likely modulated by the infection in a way that generally inhibits the generation or affects the stability of mature miRNAs. A small number of studies on other pathologic conditions hint towards possible mechanisms: In cancer, globally decreased miRNA expression has been attributed to mutations, deletions or decreased expression of miRNA processing enzymes such as Drosha and Dicer [292-294], hypermethylation of miRNA genes [295], mutations of the nuclear export protein Exportin-5 [296], or transcriptional repression of miRNA genes by oncogenic transcription factors [297]. In infection with Nodamura virus, host miRNA generation was inhibited by the viral protein B2, which binds double-stranded RNA and prevents pre-miRNA processing by Dicer [202]. My results in Chapter 3 indicate that the protein levels of Drosha and Dicer are not a limiting factor in miRNA generation during

leishmania infection (Figure 3.5). However, it is unknown whether the activities or sub-cellular localizations of these enzymes are compromised. A very exciting hypothesis, which directly connects my findings in Chapters 2 and 3, is that leishmania small non-coding RNAs, delivered to macrophages via exosomes, could interfere with host RNAi, and thereby induce miRNA repression (see model Figure 4.1). This could for example occur through saturation of host RISC components by leishmania small non-coding RNAs, as has been observed in the case of VA small non-coding RNAs mentioned above. Other possible scenarios are that leishmania small non-coding RNAs could bind to and destabilize host miRNAs by acting as miRNA sponges, miRNA-decay elements or antagomiRs, as has been shown in infection with herpesvirus saimiri and cytomegalovirus [308,309]. Though my analyses in Chapter 2 failed to demonstrate sequence homologies or complementarities of leishmania small non-coding RNAs with mammalian miRNAs (sections 2.24, 2.26 and Table S2.8), this hypothesis remains plausible, as these types of RNA-RNA interactions are rarely based on perfect complementary base pairing. Further studies will be needed to decipher the mechanism by which leishmania represses host macrophage miRNA expression.

Importantly, an increasing number of articles, most notably in the field of cancer research, have started to exploit the potential of miRNAs as diagnostic tools, prognostic and predictive biomarkers, and therapeutic targets [310]. Much of this work has focused on individual miRNAs or miRNA families, however, targeting components of the miRNA biogenesis pathway as a therapeutic intervention using by small molecule inhibitors or activators, has recently been gaining momentum [311]. A very compelling idea is that therapeutic intervention to restore miRNA expression to normal levels could be a novel strategy to treat leishmaniasis. Assessing the feasibility of such an approach will first require clarity around the mechanism of host miRNA repression in leishmaniasis.

4.2 Challenges and limitations

A major challenge in exosome research is the fact that exosomal cargo is highly complex. As discovered in the research for my thesis, exosomes released by

leishmania, like exosomes released by many other cells and organisms, contain not only lipids and proteins, but also RNA. It will be a challenging task to decipher not only the individual functions of all exosomal components, but also how they act in concert to exert their functions on recipient cells. An ideal experimental strategy by which one could investigate specifically the contribution of exosomal RNA to exosome-induced effects would be the inhibition of packaging of RNA into exosomes. In mammalian cells, specific sequence motifs have been implicated in sorting of RNAs into exosomes, which is mediated by the RNA-binding protein heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) [111]. However, this study focused on miRNAs and it is unknown whether the loading of other types of RNA into exosomes is dependent on the same sequence motifs, and whether this mechanism of exosomal RNA loading is conserved across other cell types and organisms, such as leishmania. Despite extensive analysis of our high-throughput sequencing data in Chapter 2, we were not able to find enrichment of any particular motif in leishmania exosomal RNA sequences. Homology searches indicated that there is a leishmania homologue for hnRNPA2B1 (LmjF.29.1340, 30% identity, e-value 7×10^{-14}), which is a putative RNA-binding protein with unknown function. Understanding the mechanism of RNA loading into exosomes by leishmania is of significant interest and interfering with this process should be a useful tool in deciphering the contribution of exosomal RNA to leishmania pathogenesis.

Another challenge in exosome research is the low recovery of these vesicles and their cargo during purification. This fact has already had a major impact during analysis of exosomal proteins *in vitro*, and large volumes of culture supernatant are required to obtain measurable amounts of protein (400 mL of supernatant of 2×10^{10} leishmania cells yields ~100 µg of exosomal protein). An order of magnitude less of RNA is recovered from this same volume of cell culture supernatant (5-10 µg of exosomal RNA). Consequently, the majority of work in Chapter 2 of this thesis had to be done with high sensitivity detection methods, involving radioactive labelling with $\gamma^{32}P$, which is not ideal. This is a major limitation during experimental exosomal RNA analysis *in vitro* and also raises the question as to whether the low recovery reflects a low abundance of exosomes and their protein/RNA cargo in nature or whether it is a result of inefficient

purification. If the former scenario is the case, then it is questionable how much impact this low amount of exosomal RNA could have in an *in vivo* situation. Under *in vivo* conditions where the total number of parasites present in a given infected tissue is much lower than what we use for *in vitro* cultures, we would predict the total amount of released exosomal RNA to be much lower as well. However, one has to keep in mind that *in vivo*, amastigotes reside within phagolysosomes of host macrophages; therefore, released exosomes only have to cross the phagolysosomal membrane to gain access to host cell cytoplasm. Despite the fact that the mechanism by which leishmania exosomal cargo is delivered to macrophage cytoplasm is unknown, it has clearly been demonstrated that exosomal proteins released from amastigotes within phagolysosomes, and exosomal proteins and RNAs via extracellular exposure to exosomes, can gain access to host cell cytoplasm ([77] and Chapter 2). Together with the fact that exosomal RNA cargo appears to be extremely stable (Figure 2.1), it seems plausible that even minuscule amounts of RNA can be successfully delivered to their target subcellular locale and have a significant impact on pathogenic processes *in vivo*. Of note, the low amounts of exosomal RNA can potentially make *in vivo* studies very challenging.

A limitation of my work on macrophage miRNA expression in response to leishmania infection is certainly the fact that my studies were done in *in vitro* infection models with macrophages differentiated in response to an experimentally introduced stimulus (rhGM-CSF). It would be ideal to confirm my findings in samples from experimentally infected animals, or better yet, clinical samples from leishmaniasis patients. The miRNA profile in infected tissues *in vivo* could potentially be more complex, as cell types other than macrophages would also be involved. Furthermore, it would be interesting to observe the miRNA expression profile over a longer time course, as the inflammatory profile changes during the course of an infection. Again, this would have to be done *in vivo*, as the half-life of *in vitro* differentiated macrophages is very limited. Another interesting experiment would be to compare my observations of infection with *L. donovani* to infections with other leishmania species as they greatly differ in the induction of inflammatory responses and clinical phenotype.

4.3 Future perspectives

I hope and expect that the results I am reporting in this thesis will form the ground work for many future studies that have great potential to fundamentally transform our knowledge of leishmania pathogenesis. The discovery of a conserved and select RNA repertoire in leishmania exosomes opens up a whole new avenue of research, as each individual RNA sequence could potentially carry out a specific function. Therefore, ongoing studies in the Reiner Lab are focused on exploring the functions of leishmania small non-coding RNAs. Initially, it will be essential to narrow down the number of potential effectors to be investigated in more detail, which could for example be done by comparing RNA sequences in exosomes from promastigotes and amastigotes, and prioritize the ones that are specifically enriched in amastigotes for further study, as these are more likely to play a role in interactions with the human host.

Next, it will be of utmost interest to identify the host targets of leishmania small non-coding RNAs and assess their impact on macrophage phenotype. Potential experiments could include the identification of host RNA binding proteins that interact with leishmania small non-coding RNAs, for example using synthetic RNAs delivered to host cells using liposomal delivery systems, followed by identification of RNA binding protein partners. Additionally, the effect of leishmania small non-coding RNAs on the host transcriptome and translatome could be examined. A limitation of these experiments will be the selection of candidate leishmania small non-coding RNAs to examine. Moreover, in this type of approach, only the effect of a single RNA would be examined, whereas leishmania exosomes contain a multitude of RNA sequences (plus proteins and lipids), which may act in concert in *in vivo* infection. Investigations of direct interactions of leishmania small non-coding RNAs with components of the host RNAi pathway could also be highly informative, and have the potential to uncover a novel, cross-kingdom RNAi-based mechanism of pathogenesis. Recent advances in the development of tools for small non-coding RNA functional studies, such as synthetic RNAs, reporter assays, and high throughput methods for investigating RNA-binding protein-RNA interactions (e.g. HITS-CLIP), should be of great aid in unravelling leishmania exosomal RNA functions.

Although my experiments have shown that extracellular leishmania exosomes and their RNA cargo can be internalized by host cells and can acess their cytoplasm, it remains to be investigated whether intracellular amastigotes, from within the phagolysosome, release RNA-containing exosomes, and whether the RNA is transported across the phagolysosomal membrane into host cytoplasm. Studying the release of exosomes from intracellular amastigotes *in situ* within macrophages still remains a great challenge as it is impossible to separate leishmania exosomes from host exosomes with the currently available purification methods. A potential approach to examine the presence of leishmania small non-coding RNAs in host cell cytoplasm would be *in situ* hybridization in infected cells using labeled, complementary RNA oligos to previously identified leishmania exosomal RNA sequences (Chapter 2). In this way, the sub-cellular localization of leishmania small non-coding RNAs within infected cells can be visualized.

Another important aspect to consider is the mechanism by which small non-coding RNAs are produced in leishmania. The fact that leishmania are a special case with regard to evolutionary retention of RNAi makes this a particularly interesting case to study. The mechanism of biogenesis of siRNAs has been studied in *L. braziliensis* [153,167] and other RNAi-competent trypanosomatids (i.e. *T. brucei*, reviewed in [312]). How other types of small non-coding RNAs – such as tRNA or rRNA-derived small RNAs as described in Chapter 2 of this thesis – are generated in RNAi-competent or RNAi-deficient trypanosomatids remains unknown. A number of enzymes have been implicated in the production of different types of tRNA-derived small RNAs in other organisms. For example, angiogenin in humans and other metazoans, Rny1p in yeast, and PrrC, colicin D and colicin E5 in bacteria were all shown to be involved in generation of tRNA halves [253,260]. The biogenesis of the shorter tRNA-derived fragments was shown to be mediated by Dicer in mammalian cells, but other enzymes such as angiogenin, RNase A and RNase Z have also been proposed to function in Dicer-independent processing [253]. An understanding of small non-coding RNA biogenesis in leishmania will likely have important implications for gene regulation in protozoans in general and should, therefore, be prioritized for future investigations.

With regard to repression of host miRNAs, it will be highly interesting and worthwhile in the near future to identify the mechanism(s) by which leishmania bring about a global decrease of host miRNAs and how it contributes to pathogenesis. Current experiments in the Reiner Lab are focussed on examining the functions of host Drosha and Dicer in the context of leishmania infection. It will be crucial to also examine the potential involvement of other components of the miRNA biogenesis pathway such as DCGR8, TRBP and AGO. Other studies on the mechanism of leishmania-mediated repression of host miRNAs could involve investigating the effects of leishmania infection on miRNA transcription or stability. Regarding the former, it would be worthwhile to investigate whether leishmania affects host transcription factors that transcribe miRNAs. Ten transcription factors have recently been proposed to be master regulators of miRNA expression [313]. Interestingly, at least two of these (AP-1 and c-Jun) have previously been shown to be degraded upon leishmania infection [314,315]. A potential strategy could be to measure the protein levels of these ten transcription factors in response to infection, and to assess whether overexpression can rescue the expression of miRNAs in infected cells. With regard to miRNA stability, it would be interesting to pursue the hypothesis that leishmania-derived small non-coding RNAs can bind to and destabilize host miRNAs by acting as miRNA sponges, miRNA-decay elements or antagomiRs. This could be explored by incubating macrophages with biotinylated synthetic leishmania small non-coding RNAs, followed by streptavidin pull-down and detection of potential host miRNA binding partners by sequencing or Northern blotting. Not only do these proposed studies have the potential to identify targeting of the host miRNA machinery as a novel leishmania virulence strategy, but they may also provide insight into whether reversal of this downregulation can be exploited as novel treatment strategy.

4.4 Concluding remarks

Despite the lack of public awareness in the Western world, neglected tropical infectious diseases such as the leishmaniases continue to have a disastrous impact on countless millions of people living in developing countries. Not only do they cause debilitating disease and death, they can lead to social exclusion and thus, further

aggravate impoverishment. The socio-economic burden of the leishmaniases reaches far beyond the bounds of the endemic areas in which they occur. There is an urgent need for improved disease prevention and control measures; hence, research to enhance our understanding of disease mechanisms is imperative.

Aside from the hope that enhanced knowledge on disease mechanisms will lead to development of novel drugs and vaccines, discerning the interactions of leishmania with host cells also has the potential to teach us important lessons. These can be as far-reaching as general concepts of co-evolution of pathogens and their hosts, parasite molecular biology, and immunological responses to infectious diseases. One remarkable example was the first *in vivo* demonstration of the relevance of the T helper 1/T helper 2 cell balance to the regulation of disease outcome, which arose from studies in the *L. major* mouse model [316].

The original goal of my research was to break new ground by focussing on the roles of small non-coding RNAs in leishmania biology. Importantly, the findings reported in this thesis provide us with fresh new insights into exosomes as delivery vehicles of leishmania RNA, on production of small non-coding RNAs by leishmania and on the effects of leishmania infection on gene regulatory systems in the human host. Moreover, my results bring us one step closer to confirming the fascinating concept of cross-kingdom gene regulation, as a true and widespread biological phenomenon. Ultimately, I hope that my work has unlocked at least a few doors to new avenues of research on molecular parasitology and pathogen-host interactions.

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Appendix A Supplementary material to Chapter 2

In total, 8 tables and 3 figures are available with the online version of this manuscript at http://www.biomedcentral.com/1471-2164/16/151.

Table S2.1 Sequences found in *L. donovani* and *L. braziliensis* exosomes and results of alignments with leishmania reference genomes.

File 1 'LD_Collapsed_reads.tab': Sequences obtained from Illumina sequencing of *L. donovani* exosomes. The table contains collapsed read IDs including full nucleotide sequences.
File 2 'LD_vsLdoB-bowtie2-HTseq.tab': Results of the Bowtie2 alignment of the *L. donovani* exosome sequencing library against the LdBPK reference genome.
File 3 'LD_vsLmjF-bowtie2-HTseq.tab': Results of the Bowtie2 alignment of the *L. donovani* exosome sequencing library against the LmjF reference genome.
File 4 'LB_Collapsed_reads.tab': Sequences obtained from Illumina sequencing of *L. braziliensis* exosomes. The table contains collapsed read IDs including full nucleotide sequences.
File 5 'LB_vsLbrM-bowtie2-HTseq.tab': Results of the Bowtie2 alignment of the *L. braziliensis* exosome sequencing library against the LbrM reference genome.
File 6 'LB_vsLmjF-bowtie2-HTseq.tab': Results of the Bowtie2 alignment of the *L. braziliensis* exosome sequencing library against the LmjF reference genome.
"cRead ID" refers to the identifier of the collapsed read, which is composed of the cRead number, followed by the number of copies. "Strand" refers to the strand the reads were aligning with, plus = top strand, minus = bottom strand. These tables are in .tab format and can be downloaded as zip folder.

Table S2.2 Other hits found in *L. donovani* and *L. braziliensis* exosomes.

File 1 'LD_blastN-results-vsNT.tab': Results of the blastN search of the unaligned reads (i.e. not aligned to LdBPK or LmjF) from the *L. donovani* exosome sequencing library against the NCBI nucleotide collection database.
File 2 'LD_GBhits_description.tab': GenBank description of hits.
File 3 'LD_reads_per_species.tab': Summary of reads per species.
File 4 'LB_blastN-results-vsNT.tab': Results of the blastN search of the unaligned reads (i.e. not aligned to LbrM or LmjF) from the *L. braziliensis* exosome sequencing library against the NCBI nucleotide collection database.

File 5 'LB_GBhits_description.tab': GenBank description of hits.

File 6 'LB_reads_per_species.tab': Summary of reads per species.

These tables are in .tab format and can be downloaded as zip folder.

Table S2.3 Overall alignment statistics.

This table shows the overall sequencing statistics of the *L. donovani* and *L. braziliensis* exosome libraries, plus the results (total read numbers and percentages) of the alignments against leishmania genomes and NCBI-NT. This table is in .docx format.

Table S2.4 Mapping of reads to rRNA genes based on Bowtie 2 alignments with the LmjF reference genome.

This table shows the details of sequences from *L. donovani* and *L. braziliensis* exosomes mapping to ribosomal RNA genes in the LmjF reference genome. It also shows the location of alignment within the rRNA gene (3', 3'mid, 5', 5'mid and mid). This table is in .xlsx format.

Table S2.5 Putative ORFs found in the novel transcripts.

This table shows the putative open reading frames found in the novel transcripts discovered in *L. donovani* and *L. braziliensis* exosomes, including the predicted peptide sequence. This table is in .xlsx format.

Table S2.6 Results of blast search of all reads from *L. donovani* and *L. braziliensis* exosome libraries against human and vector genomes.

A. *L. donovani*, B. *L. braziliensis*. These tables are in .xlsx format and can be downloaded as zip folder.

Table S2.7 Results of blast search of all reads from *L. donovani* and *L. braziliensis* exosome libraries against a SLACS and TATEs database.

This table is in .xlsx format.

Table S2.8 Hits against miRbase.

Top 1000 most abundant reads in both libraries were blast searched against human and mouse miRNAs as retrieved from http://www.miRBase.org. This table is in .xlsx format.

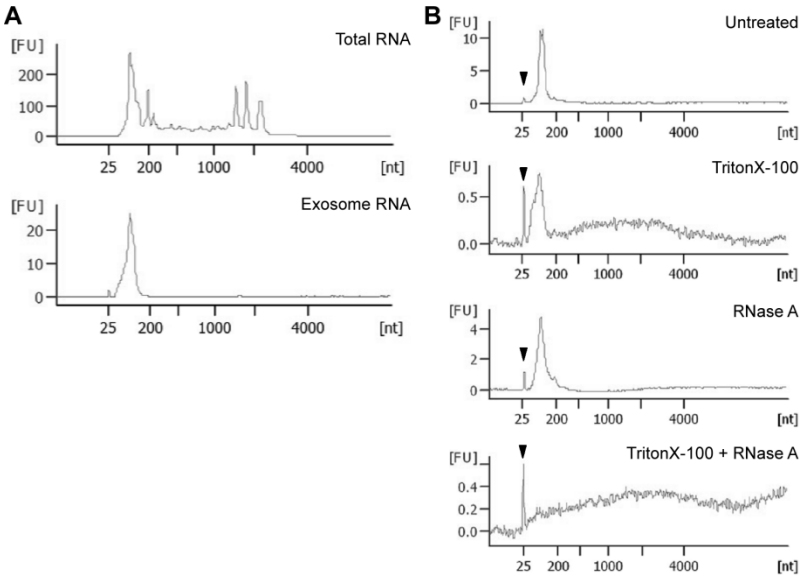

Figure S2.1 *L. braziliensis* exosomes contain RNA.

Exosomes were purified from *L. braziliensis* axenic amastigote culture supernatant as described in Materials and methods. RNA was extracted from exosomes with phenol-chloroform and then analyzed. **A**. Agilent Bioanalyzer RNA length profiles of exosome RNA alongside total RNA, **B**. RNA inside exosomes is resistant to degradation. Prior to RNA extraction, intact exosomes were left untreated or treated with either RNase A or TritonX-100 or both. Samples were then subjected to RNA extraction and run on the Agilent Bioanalyzer. Arrowhead indicates internal 25 nt marker. nt = nucleotides.

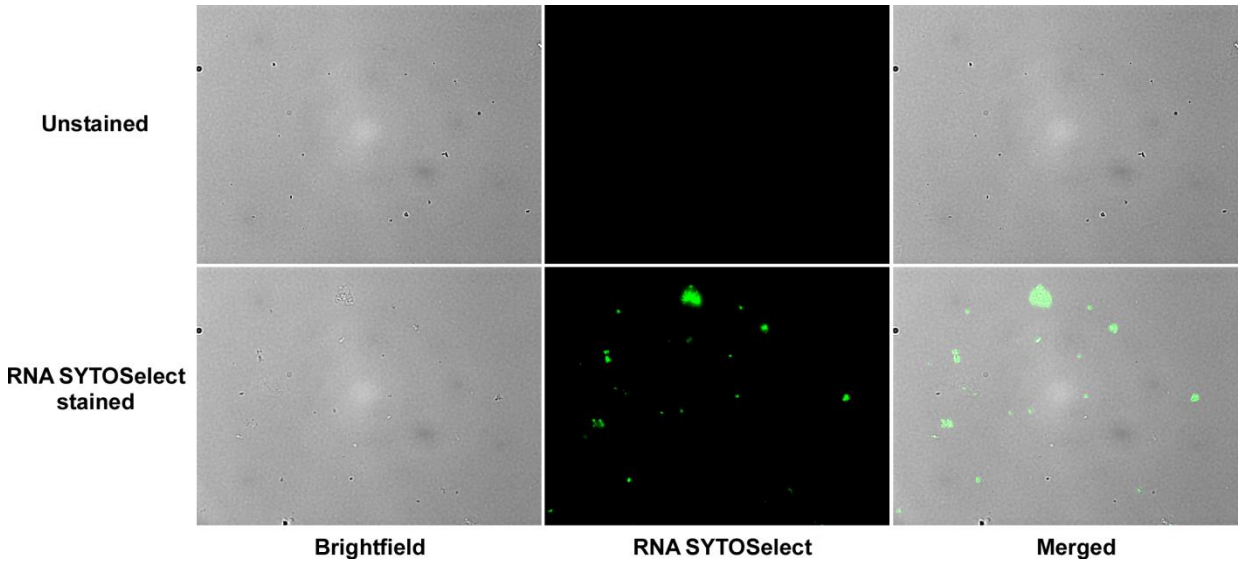

Figure S2.2 *L. donovani* exosomes can be efficiently stained with green fluorescent SYTO RNASelect dye.

Exosomes were purified from 400 mL supernatant of *L. donovani* axenic amastigotes and stained with a membrane permeant, green fluorescent RNA-specific dye (as described in Materials and methods). A sample each of stained and unstained *L. donovani* exosomes were then examined by microscopy using an Axioplan II epifluorescence microscope equipped with 63×/1.4 Plan-Apochromat objective (Carl Zeiss Inc). Images were recorded using an AxioCam MRm Camera coupled to the AxioVision software Version 4.8.2 (Carl Zeiss Inc.).

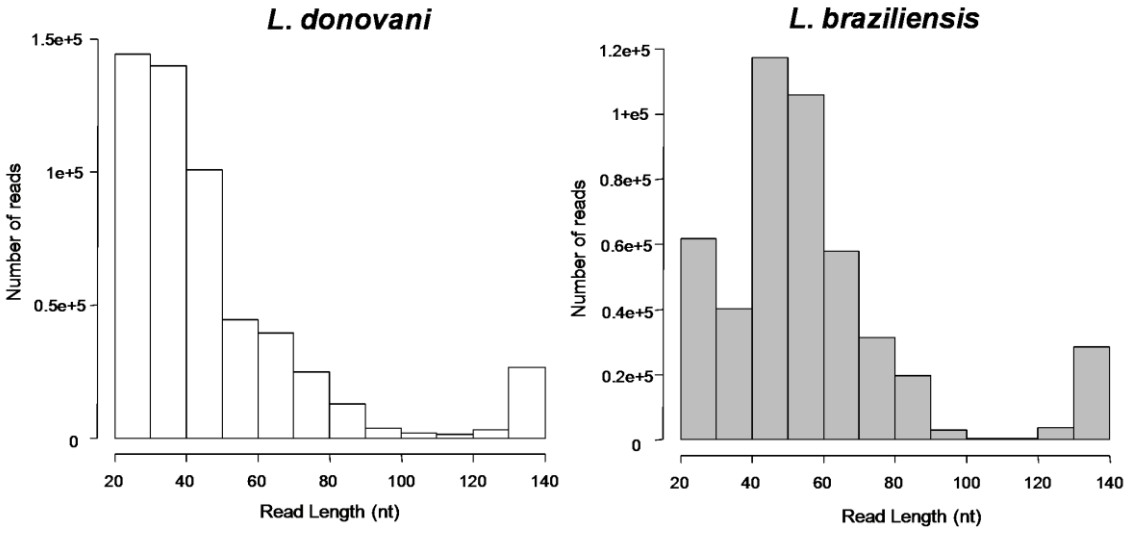

Figure S2.3 Length histograms of reads mapping to rRNA genes.
Exosome RNA from *L. donovani and L. braziliensis* was purified and processed for high-throughput sequencing as described in Materials and methods. Bar graphs show sequence length distributions of reads obtained from sequencing *L. donovani* and *L. braziliensis* exosome libraries aligned against rRNA genes in the *L. major* (LmjF) reference genome.

Appendix B Supplementary material to Chapter 3

Table S3.1 Extended KEGG pathway enrichment analysis of target genes of 19 significantly downregulated miRNAs

KEGG pathway	*Number of predicted target genes*	*adjusted p-value*
Pathways in cancer	96	2.47E-33
MAPK signalling pathway	79	1.26E-27
Regulation of actin cytoskeleton	57	8.90E-18
Prostate cancer	36	9.48E-18
Axon guidance	43	1.67E-17
Chronic myeloid leukemia	31	3.02E-16
Neurotrophin signalling pathway	41	3.02E-16
Focal adhesion	52	4.85E-16
Endocytosis	52	5.14E-16
Renal cell carcinoma	30	5.14E-16
ErbB signalling pathway	32	8.80E-15
p53 signalling pathway	28	1.69E-14
Pancreatic cancer	28	3.82E-14
Insulin signalling pathway	39	1.19E-13
Adherens junction	28	1.19E-13
Non-small cell lung cancer	23	1.78E-12
Melanoma	26	3.29E-12
Colorectal cancer	24	5.35E-12
mTOR signalling pathway	22	5.35E-12
Endometrial cancer	22	5.35E-12
TGF-beta signalling pathway	28	5.35E-12
Tight junction	35	1.40E-11
Glioma	24	1.57E-11
Acute myeloid leukemia	22	4.32E-11
Small cell lung cancer	26	2.49E-10
Dorso-ventral axis formation	14	2.65E-10
Melanogenesis	28	5.63E-10
Calcium signalling pathway	38	1.21E-09
Wnt signalling pathway	34	2.21E-09
T cell receptor signalling pathway	28	2.77E-09

KEGG pathway	***Number of predicted target genes***	***adjusted p-value***
Jak-STAT signalling pathway	34	5.18E-09
Protein processing in endoplasmic reticulum	35	7.45E-09
Ubiquitin mediated proteolysis	31	8.00E-09
Adipocytokine signalling pathway	21	1.09E-08
B cell receptor signalling pathway	22	1.32E-08
Cell cycle	29	1.54E-08
Toxoplasmosis	30	1.66E-08
Metabolic pathways	130	1.66E-08
Gap junction	24	1.92E-08
Bladder cancer	16	2.35E-08
Fc gamma R-mediated phagocytosis	24	4.53E-08
Apoptosis	23	4.53E-08
Chemokine signalling pathway	36	6.60E-08
Cell adhesion molecules (CAMs)	29	6.81E-08
Osteoclast differentiation	28	1.08E-07
Phosphatidylinositol signalling system	21	1.21E-07
Fc epsilon RI signalling pathway	21	1.50E-07
GnRH signalling pathway	24	1.78E-07
Hepatitis C	28	2.78E-07
Chagas disease (American trypanosomiasis)	24	3.10E-07
Oocyte meiosis	25	3.34E-07
Long-term depression	19	4.07E-07
Progesterone-mediated oocyte maturation	21	6.48E-07
Mucin type O-Glycan biosynthesis	12	7.28E-07
Amyotrophic lateral sclerosis (ALS)	16	7.41E-07
Cytokine-cytokine receptor interaction	42	8.16E-07
Hypertrophic cardiomyopathy (HCM)	20	1.45E-06
Glycerophospholipid metabolism	19	3.43E-06
Arrhythmogenic right ventricular cardiomyopathy	18	4.38E-06
Salivary secretion	20	4.50E-06
Dilated cardiomyopathy	20	5.33E-06
Type II diabetes mellitus	14	5.73E-06
Basal cell carcinoma	15	6.31E-06
Long-term potentiation	17	7.84E-06

KEGG pathway	***Number of predicted target genes***	***adjusted p-value***
Hedgehog signalling pathway	15	7.84E-06
Inositol phosphate metabolism	15	9.81E-06
Circadian rhythm - mammal	9	1.50E-05
Vascular smooth muscle contraction	22	2.38E-05
Thyroid cancer	10	2.72E-05
Aldosterone-regulated sodium reabsorption	12	3.33E-05
Neuroactive ligand-receptor interaction	38	4.64E-05
Amoebiasis	20	6.00E-05
Gastric acid secretion	16	6.40E-05
VEGF signalling pathway	16	8.92E-05
Shigellosis	14	9.50E-05
Bacterial invasion of epithelial cells	15	0.0001
Viral myocarditis	15	0.0001
ECM-receptor interaction	17	0.0001
Pancreatic secretion	18	0.0004
Glycosphingolipid biosynthesis - lacto and neolacto series	8	0.0004
Toll-like receptor signalling pathway	18	0.0004
Glycosaminoglycan biosynthesis - keratan sulfate	6	0.0005
NOD-like receptor signalling pathway	12	0.0009
Lysosome	19	0.001
Phagosome	22	0.0013
Natural killer cell mediated cytotoxicity	20	0.0016
RIG-I-like receptor signalling pathway	13	0.0016
N-Glycan biosynthesis	10	0.0024
Epithelial cell signalling in Helicobacter pylori infection	12	0.0034
Type I diabetes mellitus	9	0.0035
Glycosphingolipid biosynthesis - ganglio series	5	0.0039
SNARE interactions in vesicular transport	8	0.0039
Allograft rejection	8	0.0047
Protein digestion and absorption	13	0.005
Huntington's disease	23	0.0052
Glycerolipid metabolism	9	0.0093

The KEGG pathway enrichment analysis was performed was performed as described in Materials and methods 3.4.4, cutoff p-value of <0.01 and a minimum of 5 genes per category.