LEISHMANIA PARASITOPHOROUS VACUOLES- INTERACTION WITH THE MACROPHAGE ENDOPLASMIC RETICULUM

By

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To my mom, daughter and wife

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BFA	Brefeldin A
BLAST	Basic Local Alignment Search Tool
BME	Basal Medium Eagle
˚C	Degrees Celsius
%	Percent
CD+4	Cluster of differentiation 4
CD+8	Cluster of differentiation 8
cm	centimeter
CO_2	Carbon dioxide
Da	Dalton (atomic mass unit)
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EEA-1	Early Endosome Antigen 1 protein
ER	Endoplasmic reticulum
ERS-24	Endoplasmic Reticulum SNARE-24
g	Gram
GFP	Green fluorescent protein
GM-130	Golgi matrix protein
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICBR	Interdisciplinary Center for Biotechnology Research

IFA	Immunofluorescence assay
kDa	Kilo Dalton
kV	kilovolt
L	Liter
L. amazonensis	*Leishmania amazonensis*
L. donovani	*Leishmania donovani*
L. infantum	*Leishmania infantum*
L. mexicana	*Leishmania mexicana*
L. pifanoi	*Leishmania pifanoi*
LAMP-1	Lysosomal-associated membrane protein 1
LB	Luria-Bertani broth
LCM	*Legionella* containing vacuole
M	Molar
MD	Maryland
MES	2-(N-morpholino)ethanesulfonic acid
mg	Milligram
MHC	Major Histocompatibility Complex
min	Minute
NaN_3	Sodium azide
NH4Cl	Ammonium chloride
nm	Nanometer
nM	Nanomolar
Ω	Ohm
OD	Optical density
PBS	Phosphate buffered saline

pCMV	cytomegalovirus plasmid
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PH	potential of Hydrogen
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
RCA II	*Ricinus communis* agglutinin II
RCF	Relative centrifugal force
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription polymerase chain reaction
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SOC	Super Optimal broth with Catabolite repression
EM	Electron Microscopy
TGN	Trans-Golgi network
ug	Microgram
uL	Microliter
um	Micrometer
USDA	United States Department of Agriculture
YFP	Yellow fluorescent protein
w	Weight
WHO	World Health Organization

Abstract of Dissertation Presented to the Graduate School
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Leishmania parasites infect approximately 2 million people every year and cause leishmaniasis. *Leishmania* are transmitted by sand flies to human tissues; where parasites preferentially enter macrophages by phagocytosis. It is well established that *Leishmania* reside and replicate in membrane-bound structures called parasitophorous vacuoles (PVs), which interact with endocytic compartments such as lysosomes. The extent of *Leishmania* PV (LPV)s interaction with host endoplasmic reticulum (hER) is poorly understood. This study presents a comprehensive assessment of hER contributions to LPV biogenesis and maturation, and *Leishmania* development within macrophages. Macrophages expressing either calnexin or ER-associated SNAREs tagged with fluorescence proteins were infected with parasites or ZymosanA. Samples were processed through immuno-fluorescence assays and analyzed by fluorescence microscopy to assess the recruitment of hER molecules to LPVs. We found that more than 90% of PVs harboring *L. pifanoi* or *L. donovani* parasites recruited calnexin and SNAREs to their PV membrane throughout the course of infection. Electron microscopy analysis of infected macrophages expressing Sec22b/YFP confirmed that LAMP-1 and

Sec22b are recruited to LPVs. Unlike PVs, no more than 20% of Zymosan phagosomes recruited calnexin or Sec22b to their phagosomal membrane. Similarly, phagosomes containing dead parasites recruited lower level of ER molecules after infection. To further gain insight into hER-LPV interactions, the intracellular trafficking pathway of ricin was exploited. We established that Raw264.7 macrophages are a suitable system to study ricin trafficking in eukaryotic cells. Ricin was targeted into LPV compartments via hER, and this process could be blocked by BrefeldinA. Ricin accumulation in LPVs and hER was not immediate, suggesting that molecules in the hER are continuously delivered to LPVs by vesicular transport. Finally, we analyzed the *Leishmania* infection in Raw246.7 cells transiently expressing either dominant negative or over-expression constructs of Sec22b, Syntaxin 18, or D12. Our data suggest that hER membrane-associated SNAREs are essential for both *Leishmania* replication and PV development, but not for their entry in macrophages. Altogether, this study demonstrated that *Leishmania* PVs are hybrid compartments composed of both endocytic and host ER components; hER are essential for a successful *Leishmania* survival and replication, and PV development in macrophages.

CHAPTER 1
GENERAL INTRODUCTION

Introduction

This general introduction will contain an overview of the disease, and highlight *Leishmania* parasite development in mammalian cells. We will also discuss the process of phagocytosis, which is the way that these parasites enter their mammalian hosts. In addition, we discuss some aspects of the structure of the endoplasmic reticulum, as well as its interactions with secretory pathways, and molecule trafficking in eukaryotic cells. At the end of this chapter, we provide the rationale and design of this research project.

Leishmaniasis

Leishmaniasis is a vector-borne disease, which exhibits a spectrum of clinical symptoms ranging from a self-healing cutaneous ulcer to a severe mucocutaneous and potentially fatal visceral pathology. Leishmaniasis is endemic in 88 countries mostly in tropical and subtropical regions (Figure1-1), where approximately 350 million people are at risk and 12 million infected. The incidence rate of this disease is estimated at 2 million new cases each year. The overall death rate is estimated at 3%, but the rate is 100% deaths among untreated individuals with visceral leishmaniasis (Desjeux, 2004). Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*, which belongs to the family Trypanosomatidae and order Kinetoplastida (Grimaldi and Tesh, 1993). *Leishmania* parasites are transmitted to mammals by female bloodsucking sandflies of the phlebotomine sub-family. Dogs, rodents and other small mammals serve as natural reservoirs for these parasites, and humans are considered accidental hosts of the parasites (Neuber, 2008).

The incidence of leishmaniasis is increasing, with many endemic areas reporting a 500% increase over the past seven years (Roberts *et al.,* 2000). The incidence rate of leishmaniasis is also increasing in Western world populations including Europeans and North Americans, especially among Afghanistan and Iraq war soldiers and veterans. For example, 35% of Dutch soldiers were infected with cutaneous leishmaniasis within 3 months of establishing a new base in northern Afghanistan (Neuber, 2008). More than 1000 American soldiers were infected with cutaneous leishmaniasis within 2 years after their arrival in Iraq in 2003. The control of this disease is stalled by several factors such as the absence of an efficient vaccine, limitation with current drugs, and the increased transmission as a result of co-infections with HIV, inadequate vector (sandfly) control, and insufficient access to or impetus for developing affordable new drugs (Croft *et al.,* 2006; Murray *et al.,* 2005).

Leishmania **Life Cycle**

Leishmania sp are haemogagellate parasites, which have two distinct morphological forms during their life cycle (Figure 1-2): promastigotes and amastigotes. Promastigotes (Figure 1-3A) are the motile, flagellated, and extra cellular forms that replicate in the sand-fly's gut as procyclic promastigotes. They migrate in their vector's mouthparts to become non-dividing and infective metacyclic promastigotes, which are transmitted to mammals during sand-fly (of *Phlebotomus* and *Lutzomyia* genera) bites. In mammals' tissue site, 20-µm flagellated promastigotes exploit the phagocytosis process to silently enter and to evade triggering host responses (Engwerda *et al.,* 2004; Sacks and Sher, 2002). These parasites preferentially infect professional phagocytes such as macrophages, dendritic cells, and neutrophils; other host cell types can be infected as well (Laskay *et al.,* 2003; Bogdan *et al.,* 2000). Once inside cells, these

metacyclic promastigotes are enclosed in a membrane-bound structure called a parasitophorous vacuole (PV), which interacts with other host compartments and displays lysosomal properties (Courret *et al.,* 2002). After a period of 24 to 72h, promastigotes transform into amastigotes (Figure 1-3B), which are aflagellated and strict intracellular pathogens. Amastigotes have the capacity to survive the host cell defense mechanisms; they replicate in a low oxygen and low pH environment to cause the disease (Besteiro *et al.,* 2007). *Leishmania* lifecycle is completed when the vector bites and ingests blood from infected mammals. In the vector's gut, infected macrophages are lysed, and the free amastigotes transform into procyclic promastigotes, which proceed to divide rapidly and attach to the sand-fly gut. After about 3 days, sand-flies will evacuate the remnants of the blood meal, including unattached parasites. The parasites that were attached by their flagellae to the vector's gut (mid-gut microvillae, the hindgut cuticular surface, the hind gut triangle or pyloric valve) are released. They divide repeatedly and differentiate as 'metacyclic' forms, which are generally unattached, fast-swimming, with small body and long flagellum (Rogers and Bates, 2007).

Leishmania **Structure**

Promastigotes and amastigotes share many ultrastructural characteristics (Figure 1-3). *Leishmania* belong to the order of Kinetoplastida (Figure 1-5), and members of this order are characterized by the presence of a special organelle called, the kinetoplast. Kinetoplasts are bar-like structures, which represent a portion of the single mitochondrion of kinetoplastids (de Souza, 2002). The kinetoplast is located within the mitochondrial matrix, perpendicular to the axis of the flagellum; its DNA is made of several thousand of minicircles of about 0.5 to 2.5 kb in size and few dozen maxicircles,

which vary in size between 20 and 40 kb depending on the species (Liu and Englund, 2007; Liu *et al.,* 2005; Shapiro and Englund, 1995).

Leishmania possess general structural characteristics of eukaryotic cells such as the presence a nucleus, rough and smooth endoplasmic reticulum, Golgi apparatus, lysosomes and a mitochondrion. They also have a contractile vacuole believed to be involved in osmotic regulation, and storage vacuoles. Kinetoplastids including *Leishmania* have a peroxisome like organelle called glycosome, which hosts many metabolic activities such as glycolysis, β-oxidation of fatty acids, ether lipid biosynthesis and purine salvage (Parsons *et al.,* 2005). *Leishmania* possess acidocalcisomes, which enable these parasites to mobilize and store many chemicals such as calcium, sodium, phosphorous and various other cations (Zn+ and Mg+) (Miranda *et al.,* 2004; McConville *et al.,* 2002).

Leishmania Genetics

The sequencing of the genome of three *Leishmania* species: *L. major* (Ivens *et al.,* 2005) and *L. infantum* and *L. braziliensis* (Peacock *et al.,* 2007)*(Peacock, 2007)* has provided a powerful tool to understand the biology of the parasites and to identify new targets for drugs and vaccine development. *Leishmania* genomes seem significantly conserved with only about 200 out 8000 genes that show a differential distribution between the three species (Peacock *et al.,* 2007). Approximately 3-4% of the predicted proteomes may potentially have a role in pathogenicity.

Leishmania are diploid organisms, with a 3.55x 10^7bp genome, which is organized into 34 to 36 chromosome pairs ranging in size from ~250 kilobases (kb) to ~ 4 megabases (Mb) depending on the species. *Leishmania* chromosomes are characterized by the presence of repetitive telomeric sequences (Wincker *et al.,* 1996),

varying degrees of aneuploidy , and extrachromosomal DNA in the form of linear or circular minichromosomes. These minichromosomes are believed to be generated spontaneously, or by *Leishmania* genome sequence amplification or exposure to adverse conditions, such as drug selection or nutritional stress within these parasites (Segovia, 1994; Ouellette and Borst, 1991; Beverley, 1991).

H locus specific genes such as P-glycoprotein-related gene ltpgpA, were shown to be directly involved in arsenite resistance (Papadopoulou *et al.,* 1994a) and a short-chain dehydrogenase reductase gene confers resistance to methotrexate, by reducing pterins (Papadopoulou *et al.,* 1994b). This H locus seems to be quite conserved in different *Leishmania* species, but they do not necessarily favor extra-chromosomal gene amplification in response to drug resistance induction, as shown by a recent experimental study comparing *L. major* and *L. braziliensis* (Dias *et al.,* 2007). H loci were reported among natural antimony-resistant Indian isolates of *L. donovani* (Mukherjee *et al.,* 2007), while no episomal amplification was encountered in antimony-resistant Iranian isolates of *L. tropica* (Hadighi *et al.,* 2007).

Leishmania genome has a GC content of approximately 60% and the typical gene structure includes a 5' untranslated region (UTR) of 197 nucleotides on average, coding region and a 3' UTR, about 1021 nucleotides in length. *Leishmania* genes have no introns, and they exist as single copy, paired or multi-copy genes that are arranged in tandem repeats or may alternate with other repeated genes (Stiles *et al.,* 1999).

Leishmania parasites have an estimated 8311 genes (Dujardin, 2009) distributed in an unusual pattern on all chromosomes, with clusters of genes present as a contiguous unit on one DNA strand with other similar units on the opposite strand

(Smith *et al.,* 2007; Myler *et al.,* 1999). These genes clusters are transcribed as a single unit (Martinez-Calvillo *et al.,* 2003) prior to trans-splicing and polyadenylation, which is consistent with the polycistronic transcription model of kinetoplastids (Pays *et al.,* 1994).

Leishmania directional gene clusters (DGCs) range in size from a few to hundreds of genes stretching over 1 Mb of DNA (Inga *et al.,* 1998). DGCs are separated by AT-rich strand-switch regions considered to contain sites for transcription initiation and termination (Martinez-Calvillo *et al.,* 2003). DGCs do not contain clusters of genes of related function like in prokaryotic operons (Smith *et al.,* 2007), but may contain tandem arrays of genes (like the rRNA, (Inga *et al.,* 1998)) or multigene families (like the gp63 glycoprotein, (Victoir *et al.,* 2005)). Individual monosistronic mRNA are produced from polycistronic transcripts by two RNA processing reactions: 1-trans-splicing and 2-polyadenyation (LeBowitz *et al.,* 1993). The mechanism of gene regulation is not fully understood, however the regulation of transcript abundance appears to be post-transcriptional, dependent on 3'UTR and intergenic sequences with the involvement of labile protein factors (reviewed in (Stiles *et al.,* 1999)).

Differences between the genomes of the three *Leishmania* species include the identification of potentially active retrotransposons and genes implicated in the RNAi pathway in *L. braziliensis* (Peacock, 2007), not present in *L. major* (El-Sayed *et al.,* 2005). It was hypothesized that *L. braziliensis* could have retained RNAi as an antiviral defense mechanism (Smith *et al.,* 2007): indeed RNA viruses were often reported in that species as well as in other species of the subgenus *Viannia* (Widmer and Dooley, 1995). The specific role of these mobile elements and the reason behind their absence in *L. major* are still unknown (Dujardin, 2009). The amastin gene array, largest family of

surface-expressed protein genes in *Leishmania*, is twice as large as in *L. major* than in *L. braziliensis*, while the gp63 gene array is 4 times larger in *L. braziliensis* than in the two other species (Dujardin, 2009).

Leishmania Parasites and Vectors

*Leishmania*sis (Figure 1-4) are caused by 20 species of the *Leishmania* genus, protozoan parasites transmitted by female sandflies, which suck mammalian blood in order to obtain protein necessary to develop their eggs. Species of these phlebotomines belong to two genera, Phlebotomus in the "Old World" (Eurasia, Africa) and Lutzomyia in the "New World" (the Americas). These small flies, 2-3 mm long, can pass through the holes in even fine mosquito netting to bite their hosts. There are around 800 known Phlebotomines species, and only about 70 of them carry *Leishmania (Ashford, 2000)*.

Leishmania promastigotes promote its transmission to mammalian hosts by enhancing the feeding behavior of the sand fly. The metacyclic promastigotes produce a secretory gel containing a *Leishmania* specific filamentous proteophosphoglycan (fPPG). The fPPG impairs vector's mechanoreceptors and also promotes the vector's hunger state, persistence of the fly, or alternatively, increase in the threshold blood volume at which blood-seeking behavior is inhibited (Rogers and Bates, 2007). The *Leishmania* transmission is the product of the physical blockage of the gut with a filamentous proteophosphoglycan (fPPG) that ensures regurgitation of infective forms; and a subsequent exacerbation of infection in the mammalian host through the action of fPPG and vector saliva (Rogers *et al.,* 2004; Stierhof *et al.,* 1999; Jefferies *et al.,* 1991).

*Leishmania*sis Clinical Symptoms

The clinical outcome of *Leishmania* infection is determined by the parasite species, vector virulence factors and host immune responses. *Leishmania* species *are* a

very complex and diverse group of parasites in terms of their numbers and the outcomes of their interactions with mammalian or human host cells. There are five main type of clinical manifestations (Figure 1-4): cutaneous, mucocutaneous, diffuse cutaneous, visceral and post-kala-azar leishmaniasis.

Visceral leishmaniasis

Visceral leishmaniasis (VL)(Figure 1-4D) also known as kala azar is caused by *L. donovani* in the Indian sub-continent, Asia, and Africa, *L. infantum* in the Mediterranean basin, and *L. chagasi* in South America. Other species such as *L tropica* in the Middle East and *L amazonensis* in South America are occasionally viscerotropic (Desjeux, 2004; Desjeux, 2001; Guerin *et al.,* 2002). Newly acquired infection varies from subclinical (no clinical symptoms), to oligosymptomatic, to fully established (kala azar). The kala-azar is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia (occasionally serious). Active visceral leishmaniasis may also represent relapse (recurrence 6–12 months after apparently successful treatment) or late reactivation (recrudescence) of subclinical or previously treated infection (Murray, 2005). The reactivation of the infection from the latent phase can be spontaneous, but is often provoked by changes in T cell (CD4) number or function—corticosteroid or cytotoxic therapy, anti-rejection treatment in transplant recipients, or advanced HIV disease (Murray *et al.,* 2005; Fernandez-Guerrero *et al.,* 2004; Pintado *et al.,* 2001). The estimated annual global burden of VL is 500,000 new cases and more than 60,000 deaths, of which 90% occur in just five countries—India, Bangladesh, Nepal, Sudan, and Brazil (W.H.O., 2009)(Raguenaud *et al.,* 2007; Maltezou *et al.,* 2000).

Post-kala-azar dermal leishmaniasis (PKDL)

PKDL is a cutaneous manifestation of visceral leishmaniasis (VL), characterized by skin lesions, nodules or papules, frequently on the face(Figure 1-4E). It often appears 2-7 years after the apparently successful treatment of VL with pentavelent antimonial drugs (Croft, 2008), amphotericin B and miltefosine (Kumar *et al.,* 2009). The incidence of PKDL varies according to regions, for example 5–15% in India (Ramesh and Mukherjee, 1995) and about 60% in Sudan. So far, little is known about the factors that drive the parasite to cause a shift in the site of infection from viscera to dermis, and thereby the clinical manifestation of the disease (Croft, 2008).

Cutaneous leishmaniasis (CL)

CL accounts for more than 50% of new cases of leishmaniasis, and 90% of the cases are reported from eight countries, six in the Old World (Afghanistan, Iran, Iraq, Algeria, Saudi Arabia and Syria) and two in the New World (Peru, Brazil). CL is caused by *L.major*, *L. tropica*, and *L. aethiopica* in the Old World, and by the *L. mexicana* species complex and the *Vianna* subgenus in the New World. The disease starts as papules which progress into nodules and ends as ulcerative lesions with a central depression and indurated border that results in atrophic scars over time (Figure 1-4A) (Ashford, 2000). CL usually heals spontaneously in the Old World, but less frequently in New World, because species of the *L. viannia* subgenus (*L viannia* braziliensis*, L viannia guyanensis*, and *L viannia panamensis*) tend to disseminate in mammalian host (Lawn *et al.,* 2004; Choi and Lerner, 2001; Herwaldt, 1999). In the United States, CL is endemic in south-central Texas, and recently some autochthonous cases of cutaneous leishmaniasis have been reported in northern Texas (Wright *et al.,* 2008), suggesting an

increase in transmission and distribution of leishmaniasis in the United States (McHugh, 2010).

Diffuse cutaneous leishmaniasis (DCL)

DCL is caused by normal CL species, L*. aethiopica* and *L. tropica* (in the Old World), *L. amazonensis* and *L. mexicana* (New World), mostly in anergic hosts with poor immune responses. Infection is characterized by disseminated, flaking, and non-ulcerated nodular lesions on the skin that resemble lepromatous leprosy (Figure 1-4B). DCL is considered to an hypersensitivity condition in which most parasites are eliminated but the infection is not completely cured (Ashford, 2000).

Mucocutaneous leishmaniasis (MCL) or *espundia*

MCL is primary caused by parasites of the *L. braziliensis* complex in the New World, and occasionally by *L. d. infantum* in the Old World, especially in Sudan. The disease begins as simple skin lesions or “oriental sore” that can metastasize via the blood stream or lymphatics. Lesions can lead to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues (Figure 1-4C). Generally, less than 5% of patients with simple cutaneous leishmaniasis will develop MCL, which can occur while the primary lesion is still active or several years after the primary lesion has healed (Ashford, 2000).

MCL can be either ulcerative or non-ulcerative; the ulcerative form is characterized by a rapid and extensive mutilation of soft tissue and cartilage. The non-ulcerative is marked by local edema and hypertrophy, particularly of the upper lip; but if not treated, the disease will progress and lead to severe pathology and deformity (Ashford, 2000). The parasite tropism for macrophages of the oronasopharyngeal region is a poorly

understood phenomenon and may involve several factors including sensitivity of the parasite to temperature, as well as permissiveness of the various macrophage populations (Lodge and Descoteaux, 2005).

Diagnosis of leishmaniasis

In active endemic areas, all forms of leishmaniasis can be diagnosed with some reliability by clinical examination, with an additional blood count to show anemia and leucopoenia in the specific case of visceral leishmaniasis. The confirmation of leishmaniasis diagnosis is generally done by demonstrating the presence of the amastigote parasites in stained microscopical preparations or cultures from skin biopsies (CL) or aspirates from bone marrow or spleen specimens (VL).

VL displayed a very strong serological response, to the extent that the albumin/globulin ratio is reversed. The raised serum proteins are used diagnostically in a non-specific formol-gel test. Various DNA and monoclonal antibody probes have been developed, but none of these has reached routine practice. The formol-gel test, which consists of the addition of a drop of formalin to 1 ml of the patient's serum, is generally used, as/because a positive reaction is indicated by the rapid coagulation of the serum. Although this test is insufficiently specific to be recommended, it is still widely used in endemic areas. For MCL, parasites are difficult to find in lesions, therefore the patient's history of CL and serology (ELISA, IFA) are used as diagnostic tools (Ashford, 2000).

Treatment

Pentavalent antimony, such as sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), are the core or first-line of treatment for visceral leishmaniasis, CL, and mucosal leishmaniasis. These drugs are prescribed at a dose of

20 mg/kg/d parenterally (by piercing the skin or the mucous membrane) for a 3- to 4-week period (Blum *et al.,* 2004; Berman, 1996; Herwaldt and Berman, 1992). Limitations of these drugs, which include a high cost, difficulty of administration, toxicity, length of treatment, and drug resistance or relapses in up to 25% of cases with some *Leishmania* strains, have lead to the search for new anti-*Leishmania*sis compounds (Schwartz *et al.,* 2006).

Pentamidine isethionate is generally prescribed as an alternative for individuals, who are intolerant to antimonial treatment, or in cases of antimonial resistance. It is the first line of treatment of cutaneous leishmaniasis (3 mg/kg/d IM every other day for 4 injections) in French Guiana where more than 90% of infections are caused by *L guyanensis* (Desjeux, 2004).

Paromomycin sulfate is also a topical anti- leishmaniasis agent to treat Old World CL (*L major*, *L tropica*, *L aethiopica*). Injectable paromomycin has been experimentally used to treat visceral leishmaniasis (primarily caused by *L donovani*) (Davis and Kedzierski, 2005), and only *L mexicana* infections in the New World CL (Lawn *et al.,* 2004; Blum *et al.,* 2004). A less costly combination of 15% paromomycin/12% methylbenzethonium ointment to treat *L mexicana* infection is accepted (Mitropoulos *et al.,* 2010; Soto *et al.,* 1998).

Amphotericin B preparations have typically been used in treating visceral and mucosal leishmaniasis unresponsive to antimonial therapy. Amphotericin B is associated with infusion-related toxicity–and thus needs to be administered slowly–and nephrotoxicity (Mitropoulos *et al.,* 2010).

Miltefosine, a phosphocholine analogue, was initially developed as an anti-neoplastic agent. It was found to have anti-leishmanial activity in vitro and in vivo, probably via effects on cell-signaling pathways and membrane synthesis (Kuhlencord *et al.,* 1992; Croft *et al.,* 1987). Miltefosine is administered orally at a dosage of 2.5 mg/kg/d for 28 days, to cure visceral leishmaniasis in India and CL in Pakistan rates comparable with pentavalent antimony. The assessment of the miltefosine efficacy on New World CL is still an ongoing process (Mitropoulos *et al.,* 2010).

Imiquimod is an imidazoquinoline amine, which induces interferon-α, tumor necrosis factor-alfa, interleukin (IL)-1α, lL-6, and IL-8 (Wagner *et al.,* 1997), and has been shown to effectively stimulate leishmanicidal activity in macrophages (Buates and Matlashewski, 1999).

Allopurinol (4-hydroxypyrazolo[3,4-d] pyrimidine) is a drug traditionally used for the treatment of gout. It has been shown to inhibit the growth of *Leishmania* in vitro at concentrations that are attainable in human tissues and body fluids. This compound is believed to act by prohibiting the de novo synthesis of pyrimidines, probably through the formation of allopurinol ribotide, which leads to the inhibition of protein synthesis in the *Leishmania* parasite (Pfaller and Marr, 1974). Oral allopurinol (20 mg/kg/d for 15 days) is mainly recommended as pentavalent antimonial adjunct to treat New World CL (Martinez and Marr, 1992; Guderian *et al.,* 1991). Other experimental drugs that have demonstrated leishmanicidal activity comprise some plant extracts, chalcones, alkaloids, terpenes, and phenolics (El-On, 2009). Cryotherapy, heat application, curettage, electrodessication, and surgical excision have also been implemented in the treatment of early, small, cutaneous lesions in leishmaniasis. These physical

approaches constitute an alternative to costly and toxic proposed first-line agents, or when systemic therapy is contraindicated, such as in cases of pregnancy, heart conditions, and impaired renal or hepatic function (Alkhawajah, 1998).

Vaccines

To date, there is no FDA-approved vaccine; however a live *Leishmania* prophylactic vaccine in Uzbekistan and a dead *Leishmania* vaccine, as an adjunct to antimony therapy, have been registered for clinical use against human disease in Brazil (Palatnik-de-Sousa, 2008). Three main approaches are generally pursued in the development of a potential vaccine against leishmaniasis. The leishmanization consists of inducing the first infection by injecting live virulent parasites in an aesthetically acceptable site of the body in healthy individuals; this first natural infection with *L. major* is highly protective against subsequent infections (Belkaid *et al.,* 2002). Leishmanization adverse side effects including the development of large persistent lesions, complications in immuno-compromised individuals, and the fact that this technique is not suitable for large-scale use, have led to the discontinuation of this technique in many countries(Hepburn, 2003).

The first-generation vaccine approach uses fractions of the parasite or whole killed *Leishmania* with or without adjuvants; and second-generation vaccines using live genetically modified parasites, or bacteria or viruses containing *Leishmania* genes, recombinant or native fractions (Khamesipour *et al.,* 2006; Selvapandiyan *et al.,* 2006). The monophosphoryl lipid A in a stable emulsion (MPL-SE), multi-antigen vaccine, is the only second-generation vaccine against leishmaniasis that has reached human trials. MPL-SE is being developed for prophylaxis, a therapy based on studies in mice,

and preliminary trials in human beings on a compassionate basis (Mitropoulos *et al.,* 2010; Khamesipour *et al.,* 2006).

Synthetic anti-*Leishmania*l vaccines, which involve elements of the immune response such as $CD4^+$ and $CD8^+$ T cells, have been developed (Gurunathan *et al.,* 2000). It is believed that CD8 cells also play a significant role in the maintenance of long-term vaccine-induced immunity (Belkaid *et al.,* 2002). The capacity to induce both $CD4^+$ and $CD8^+$ cell responses has, traditionally, been known to be a feature of DNA vaccines. However, some protein-based vaccines have demonstrated the same capacity (Rhee *et al.,* 2002; Reed, 2001). More than thirty nonapeptides, which specifically trigger IFN-gamma secretion by human CD8+ cells through the MHC class system have been identified, and have been of interest in many vaccine projects (Basu *et al.,* 2007). Recombinant BCG expressing *Leishmania* antigens. Recombinant BCG (*Mycobacterium bovis* bacillus Calmette–Guerin) has been used in vaccination against leishmaniasis (Gicquel, 1995). It is being used in clinical trials (Basu *et al.,* 2007)as an adjuvant in vaccine preparations with dead *L. mexicana* or with *L. braziliensis* promastigotes, but its efficacy is still controversial (Khamesipour *et al.,* 2006; Noazin *et al.,* 2008; Convit *et al.,* 2004; Handman, 2001).

Vaccines based on sand fly salivary antigens. Although most vaccines strategies are generally directed against antigens of the infectious agent transmitted by the vector, other approaches are focused on the arthropod itself (Grimaldi and Tesh, 1993). It is known that saliva of sand flies enhances the infectivity of *Leishmania* parasites in their mammalian hosts (Lima and Titus, 1996). Vaccines have been designed against components of saliva or insect gut antigens that can protect from infection and decrease

the viability and reproducibility of the insect (Titus *et al.,* 2006; Milleron *et al.,* 2004). These vector components include the protein MAX or MAXADILAN (Brodie *et al.,* 2007) and the SP15 antigen obtained from *Phlebotomus papatasi* and have been shown to induce substantial resistance in mice to *L. major* infection (Valenzuela *et al.,* 2001).

Leishmania versus Mammalian Host Defense

Important leishmanial virulence factors. Virulence factors that enable *Leishmania* to establish and maintain the infection in mammals differ according to parasite species and stages. In the sandfly vector, the metacyclogenesis process that transforms log-phase procyclic promastigotes to stationary-phase metacyclic promastigotes is critical in equipping *Leishmania* parasites with infective capacities for mammalian. Metacyclic promastigotes selectively increase the expression of virulence factors, such as the surface lipophosphoglycan (LPG) and the metalloprotease gp63 (Yao *et al.,* 2003). LPG has been identified as a virulence factor for *L. major* and *L. donovani* (Spath *et al.,* 2000; McNeely and Turco, 1990; Turco, 1990) but not *L. mexicana* (Ilg, 2000), while gp63 has been recognized as a vital virulence factor for *L. major*, *L. mexicana*/*L. amazonensis*, and *L. donovani* (Joshi *et al.,* 2002; Joshi *et al.,* 1998; Chen *et al.,* 2000; Seay *et al.,* 1996; Wilson *et al.,* 1989).

The glycoprotein of 63 KDa (GP63). GP63, also known as leishmanolysisn or major surface protease (MSP), is the most abundant surface protein of *Leishmania* promastigotes, and may account for approximately 1% of the total protein content (Bahr *et al.,* 1993). It is not highly expressed in amastigotes (Yao *et al.,* 2003; Hsiao *et al.,* 2008). Gp63 on parasites' surface membrane inhibits complement-mediated lysis by cleaving and converting C3b to C3bi, and this will favor parasite uptake via host

macrophage CR3 (Brittingham *et al.,* 1995). Extracellular release of gp63 (McGwire *et al.,* 2003) may also facilitate the promastigote's migration and dissemination through tissue by degrading extracellular matrix components such as fibronectin. Other functions attributed to extracellular gp63, which may serve to subvert or circumvent immune responses, include cleaving major histocompatibility complex (MHC) class I molecules (Garcia *et al.,* 1997) and limit CD4 T-cell responses (Hey *et al.,* 1994). Gp63 may deactivate macrophages by down modulating MARCKS-related (myristoylated alanine-rich C kinase substrate) protein (Corradin *et al.,* 2002; Corradin *et al.,* 1999). The role of gp63 in promoting amastigote pathogenesis remains unclear, as its expression is significantly down regulated in the intracellular amastigote stage.

Lipophosphoglycan (LPG). LPG is the most predominant glycoconjugate of infective metacyclic promastigotes, at approximately 5 million copies per cell (Lodge and Descoteaux, 2005), and distributed along the entire cell surface and the flagellum. Its main structure consists of a polymer of Galβ1-4Man- PO4 units, attached to the cell surface via a glycophosphatidylinositol (GPI) anchor. LPG is present in all *Leishmania* species, but its structures and role vary among species (McConville, 1995; Turco, 1992). Gene knock-out studies have demonstrated that LPG is a virulence factor in *L. major* and *L. donovani* (Spath *et al.,* 2000). LPG forms a compact glycocalyx that act as a first line of the parasite defense, and also contributes to the evasion of complement mediated lysis (Puentes *et al.,* 1988). LPG can bind to the C3b component of the complement cascade, and in conjunction with GP63-mediated conversion to C3bi, promotes parasite internalization via the macrophage C3 receptor (C3R) (Brittingham *et*

al., 1995). The LPG has been demonstrated to be critical in a number of biological/virulence mechanisms (Turco *et al.,* 2001; Ilg *et al.,* 2001; Moore *et al.,* 1994; Descoteaux *et al.,* 1991) important to *Leishmania* survival. LPG modulates nitric oxide (NO) production (Proudfoot *et al.,* 1996), inhibition of apoptosis (Moore and Matlashewski, 1994)(Moore *et al.,* 1994), delay in phagolysosome maturation (Holm *et al.,* 2001), and inhibition of macrophage signal transduction (Descoteaux *et al.,* 1991). LPG-deficient *L. mexicana* organisms are as virulent and infective to their murine hosts as wild type organisms, suggesting that LPG is not a critical virulence factor for *L. mexicana*, as it is for both *L. major* and *L. donovani* parasites (Turco *et al.,* 2001; Ilg *et al.,* 2001). Therefore, *L. mexicana* may use alternate ligands or mechanisms to enter cells and evade host immune system.

The A2 gene locus. It encodes a family of amastigote stage-specific 42–100 kDa proteins, localized to the parasite cytoplasm (Charest and Matlashewski, 1994). The A2 gene locus has been demonstrated to be important for the virulence of *L. donovani* (Zhang *et al.,* 2003; Zhang and Matlashewski, 2001); *L. donovani* organisms made genetically deficient for A2 fail to be infective to macrophages and mice (Zhang and Matlashewski, 1997). Although the A2 genes appear to be required for the virulence of *L. donovani*, they are not expressed in *L. major*. Further, the transformation of *L. major* to express the A2 proteins (Zhang *et al.,* 2003; Zhang and Matlashewski, 2001) results in increased visceralization and/or survival of *L. major* in the spleen but diminished parasite survival in cutaneous tissue. The diminished infection in the skin appears to be due, in part, to increased migration of *Leishmania*-infected cells (dendritic cells and

macrophages). Thus, the loss or gain of A2 expression in *Leishmania* appears to contribute to the species-specific tissue 'tropism'. However, the mechanisms involved remain to be elucidated and may be complex and involve other species-specific parasite genes (Zhang *et al.,* 2003).

The *Leishmania* homolog of receptors for activated C kinase (LACK). LACK antigen promotes IL-4 production through the activation of $V\beta4^{+}V\alpha8^{+}CD4^{+}$ T cells (Malherbe *et al.,* 2000; Julia *et al.,* 1996) and is critical for the susceptibility of BALB/c mice to *L. major* infection. Further, studies indicate that LACK is required for parasite persistence within macrophages and the mammalian host (Kelly *et al.,* 2003). Although LACK-responsive T cells develop during *L. mexicana* infection, susceptibility to *L. mexicana* is independent of LACK induction of IL-4 (Torrentera *et al.,* 2001). In fact, LACK-tolerant BALB/c-LACK transgenic mice are resistant to *L. major*, and yet these mice are fully susceptible to *L. mexicana*. The development of 'healing' versus 'non-healing' for *L. major* has been related to LACK-peptide T-cell receptor affinity. However, the LACK epitope involved is identical in both the *L. major* and the *L. mexicana* LACK proteins, suggesting that additional parasite molecules contribute to the developing pathogenesis in BALB/c mice during cutaneous infection caused by *L. mexicana*.

Cathepsin L-like cysteine protease B (CPB) enzymes. CPB enzymes are expressed in high abundance and localized to the megasome organelles unique to strains of *L. mexicana* complex, and are considered to be a critical for virulence (Denise *et al.,* 2003; de Araujo Soares *et al.,* 2003; Alexander *et al.,* 1998a). CPB-deficient *L. mexicana* amastigotes have reduced ability to induce lesion growth in BALB/c mice (Denise *et al.,*

2003; Alexander *et al.,* 1998a), and the lesions of other susceptible strains of mice (C3H and C57BL/6) heal (Buxbaum *et al.,* 2003). The virulence of *L. mexicana* for BALB/c mice has been associated with the ability of CPB (Denise et al., 2003; Alexander et al., 1998a) to induce IL-4 production and a Th2 response. CPB-deficient parasites fail to induce IL-4, resulting in a T-helper 1 cell (Th1) response and inhibition of lesion growth. Genetic restoration of CPB expression restores virulence together with the ability to induce BALB/c IL-4 production (Denise et al., 2003).

Sand fly saliva. It induces vasodilatation, inhibition of coagulation and immunomodulatory effects (Sacks and Kamhawi, 2001); and attraction of PMNs as well as macrophages (Anjili et al., 2006; Zer et al., 2001). The parasite itself also produces a chemoattractant protein called *Leishmania* chemotactic factor, which can attract PMNs (van Zandbergen *et al.,* 2002). Two hours after saliva injection, an intense and diffuse inflammatory infiltrate comprising PMNs, eosinophils and macrophages is induced only in mice pre-exposed to saliva (Silva *et al.,* 2005). PMNs are the first cells to arrive at the site of *Leishmania* infection (Muller *et al.,* 2001). Human PMNs infected with *Leishmania* secrete chemokines such as IL-8 (CXCL8) (Laufs *et al.,* 2002) that are essential in attracting more PMNs to the site of infection. Upon experimental infection with *L. major*, MIP-2 and keratinocyte-derived cytokine (KC; also known as CXCL1), the functional murine homologs of human IL-8 (Modi and Yoshimura, 1999), are rapidly produced in the skin (Muller *et al.,* 2001). *In vitro* studies have also shown that *L. major* promastigotes induce rapid and transient expression of KC by murine macrophages (Racoosin and Beverley, 1997) and of IL-8 by human macrophages (Badolato *et al.,* 1996). All of these chemokines are chemo-attractants for PMNs (Baggiolini, 2001).

PMNs can function as phagocytic cells, taking up and killing *Leishmania* (Lima *et al.,* 1998), and they have been implicated in early parasite control.

Chemokines have different roles in *Leishmania* infection including the recruitment of sentinel immune cells (DCs, macrophages and γδ T lymphocytes) to the site of parasite delivery. They also play an important role in the adaptive immunity, macrophage activation and parasite killing (Teixeira *et al.,* 2006). These cells possessed Toll-like receptors (TLRs) (Muzio *et al.,* 2000) and phagocytic receptors (Ross, 2000), which enable them to detect pathogen-associated molecular patterns (Gordon, 2002) and uptake pathogens and opsonized particles. Sentinel cells also express various receptors for cytokines, which induce the production of chemokines to initiate the innate responses (Spellberg and Edwards, 2001; Spellberg, 2000). *L. major*-infected mice induce overall upregulation of CCL5, also known as regulated on activation normal T cell expressed and secreted (RANTES), MIP-1α, CXCL10 and CCL2 in the footpads and LNs (Antoniazi *et al.,* 2004).

The role of PMNs in the context of the early response to *Leishmania. Leishmania* extends the lifespan of PMNs (Aga *et al.,* 2002) and can survive within these cells for hours or days after infection (van Zandbergen *et al.,* 2004). *Leishmania* Infected PMNs induce the release of MIP-1β, which recruit macrophages to the site of infection (van Zandbergen *et al.,* 2004). Infected PMNs taken up by macrophages do not activate macrophage microbicidal function (van Zandbergen *et al.,* 2004). In macrophages containing apoptotic PMNs, proinflammatory cytokine production is inhibited through mechanisms involving transforming growth factor-β, prostaglandin E_2 and platelet-activating factor (Ribeiro-Gomes *et al.,* 2004; Fadok *et al.,* 1998a; Fadok *et al.,* 1998b).

These events contribute to a ‘silent’ entry of *Leishmania* into macrophages, its main host cell type (Laskay *et al.,* 2003).

Macrophages are the second wave of cells that enter the site of *Leishmania* infection, and are considered the ultimate host cells for *Leishmania* parasite development. They perform antigen-presentation and also produce cytokines that will modulate the T cell-mediated immune response. Moreover, after appropriate activation by Th1 cells, they serve as effector cells for intracellular parasite killing (Teixeira *et al.,* 2006). This suggests that *Leishmania* parasites, especially the amastigotes form have evolved mechanisms to survive in macrophages.

Monocytes are attracted in the early stages of infection by products of sand fly saliva (Zer *et al.,* 2001; Anjili *et al.,* 1995) and, two to three days later, by chemokines such as MIP-1β (van Zandbergen *et al.,* 2004). *Leishmania* can also induce other monocyte-attractant chemokines. For example, *L. major* promastigotes induce rapid and transient expression of JE, a protein inducible by platelet-derived growth factor, in murine macrophages (Racoosin and Beverley, 1997) and of its homolog CCL2 in human macrophages (Badolato *et al.,* 1996). Besides attracting monocytes and macrophages, CCL2 can attract other cells such as NK cells and DCs that are positive for the chemokine receptor CCR2 (Ritter and Moll, 2000). In human Leishmaniasis, CCL2 and MIP-1α seem to be responsible for macrophage activation in the skin lesions. In *L. mexicana* localized CL, synergistic action of CCL2 and IFN-γ kill parasites within the macrophage (Muzio *et al.,* 2000). But if the parasite stimulates the production of IL-4, which can suppress CCL2 expression and increase the MIP-1α level, the lesion will progress into non-healing DCL lesions (Ritter *et al.,* 1996).

***Leishmania* affect host cell signaling.** Host invasion by pathogens is frequently associated with the activation of nuclear factor kappaB (NF-kappaB), which modulates the expression of genes involved in the immunological response of the host. However, pathogens may also subvert these mechanisms to secure their survival. *L. amazonensis* has developed an adaptive strategy to escape from host defense by activating the NF-kappaB repressor complex p50/p50. The activation of this specific host transcriptional response negatively regulates the expression of iNOS, favoring the establishment and success of *L. amazonensis* infection. NF-kappaB-mediated repression of iNOS expression in *Leishmania amazonensis* macrophage infection {{798 Calegari-Silva,T.C. 2009}}. *L. donovani* induces less CD2 on the surface of CD4+ T-cells, which once activated orchestrate the protective IFN-gamma dominant host defense mechanism via PKC-mediated signal transduction and cell cycle(Calegari-Silva *et al.,* 2009) (Bimal *et al.,* 2008).

Pathogenesis study of experimental cutaneous and visceral Leishmaniasis using mannose receptor R-deficient [MR-knockout (KO)] C57BL/6 mice, has revealed that the host mannose receptor MR is not essential for blocking IFN-gamma/LPS-induced IL-12 production and MAPK activation by *Leishmania*. Mannose receptors are not essential for host defense against *Leishmania* infection or regulation of IL-12 production (Akilov *et al.,* 2007).

***Leishmania*–macrophage interactions.** These interactions are complex because *Leishmania* species modulate macrophage signaling pathways and metabolism to different extents. For example, activation of *L. major*-infected macrophages with low

levels of IFN-γ is sufficient to clear intracellular amastigotes, while incubation of *L. amazonensis*-infected macrophages with IFN-γ promote intracellular amastigote growth.

IFN-γ treatment stimulated arginine uptake, but did not stimulate NOS2 activity or decrease arginase activity in the infected macrophages (Wanasen *et al.,* 2007). *Leishmania amazonensis* amastigotes therefore appear to induce a unique activation state in the host cell in which cytosolic levels of arginine are elevated but not further catabolized. Modulation of host cell responses to *L. major* amastigote infection has also been observed in human THP-1 macrophages (Dogra *et al.,* 2007). Interestingly, apoptotic cells can induce a similar activation state in macrophages in which NOS2 activity is not down regulated, but arginase II activity is elevated and the flux into the polyamine pathway increased). *Leishmania* amastigotes appear to mimic apoptotic cells and could direct subsequent host cell responses by engagement of specific receptors and associated signaling pathways that limit cell activation. Alternatively, there is increasing evidence that some amastigote proteins can be exported to the host cytoplasm and directly modulate host cell signaling pathways, although the mechanisms underlying this process are poorly defined (Kima, 2007).

Leishmania versus the Host Adaptative Immune Responses

The development of *Leishmania* infection depends in part on the parasite species, parasite and species-specific factors, and importantly, the host immune response (Murray *et al.,* 2006). In complex organisms such as *Leishmania*, a susceptible phenotype can be associated with one or multiple genes/genetic loci (Havelkova *et al.,* 2006). The innate and acquired immunity play a significant role in controlling *Leishmania* infection. IFN-γ / IL-12 driven T-cell activation results in the polarization of a

T Helper 1 (Th1) -response and control of the disease (Mattner *et al.,* 1996); whereas a predominantly IL-4-driven polarization and IL-10-dependent maintenance of a T Helper 2 (Th2) response (Alexander *et al.,* 1998b; Nylen *et al.,* 2007) will inevitably result in disease progression and a non-curing phenotype (Sacks and Noben-Trauth, 2002; Naderer *et al.,* 2008).

Early studies particularly on *L. major* largely defined the Th1/Th2 paradigm of resistance/susceptibility to infection and the role of IL-12 and IL-4 respectively in driving Th1 and Th2 cell development (Reiner and Seder, 1995). While more recent studies using in particular gene deficient mice have largely substantiated the beneficial activity of IL-12 and the Th1 response in controlling infection, the previously proposed paramount role for IL-4 in disease exacerbation has been significantly questioned with other mediators being identified as playing influential roles (Sacks and Anderson, 2004; Scott and Farrell, 1998) . As the major *Leishmania* species complexes diverged some 40–80 million years ago (Stevens *et al.,* 2001), it is unsurprising that different virulence factors have been identified for different species (McMahon-Pratt and Alexander, 2004) and consequently the growth of the different species is subject to different immunological controls (Alexander and Bryson, 2005).

Thus while IL-4 has been implicated in the non-healing response of BALB/c mice to infection with *L. mexicana* and *L. amazonensis* (Alexander *et al.,* 2002) it has not been shown to promote chronic disease following visceral infection with *L. donovani* (Satoskar *et al.,* 1995) and indeed may play a protective role (Stager *et al.,* 2003a). Moreover, IL-4 under certain circumstances can potentiate IL-12 production (Stager *et*

al., 2003a; Stager *et al.,* 2003b) and a type-1 response and Th-2 cell development can take place independently of IL-4(Mohrs *et al.,* 2000) .

Resolution of Infection and the Th1 response

It is now well established that a protective immune response against both cutaneous *Leishmania*sis, caused by *L. major, L. mexicana* or *L. amazonensis* as well as visceral *Leishmania*sis caused by *L. donovani* is dependent on the development of potent type-1 immunity (Figure 1-6A) (Alexander *et al.,* 1999). The general consensus is that IL-12 from antigen presenting cells (APC), macrophages and dendritic cells, possibly augmented by cytokines such as IL-1α (Von Stebut *et al.,* 2003), IL-18 (Dinarello and Abraham, 2002), IL-23 (Langrish *et al.,* 2004) and IL-27 (Artis *et al.,* 2004b), drives the differentiation and proliferation of T helper 1 cells.

MHC Class II antigen presentation alone is not sufficient to stimulate T cell responses and ligation of co-stimulatory molecules, B7-1/B7-2 and CD40 on the APC with CD28 and CD40L on the T cell respectively is also a prerequisite (Bogdan, 1998; Bogdan *et al.,* 1997). Ligation of CD40/CD40L enhances APC IL-12 production (Heinzel *et al.,* 1998) as can tumor necrosis factor [TNF] related activation induced cytokine (TRANCE)-receptor activator of NF-κB (RANK) interactions (Padigel *et al.,* 2003b). IFN-γ from Th1 cells, and probably to a lesser extent $CD8^+$ T cells as part of the acquired immune response, but also from IL-12 activated NK cells as part of the innate response, mediates macrophage activation, nitric oxide production and parasite killing (Cunningham, 2002).

Macrophage leishmanicidal activity induced by IFN-γ has been shown to be enhanced by other cytokines such as TNF-α (Mannheimer *et al.,* 1996), migration

inhibition factor (MIF) (Juttner *et al.,* 1998; McSorley *et al.,* 1998) and type-1 interferons (Diefenbach *et al.,* 1998) as well as CD40/CD40L interactions (Campbell *et al.,* 1996; Kamanaka *et al.,* 1996; Soong *et al.,* 1996). Interestingly a recent study demonstrated that C57BL/6 mice can control *L. mexicana* independently of IL-12 (Buxbaum *et al.,* 2003). Nevertheless immunity in this study was dependent on the transcription factor STAT4, which is required for IL-12 signaling, a type-1 response and IFN-γ and inducible nitric oxide synthase expression. Recently, the IL-12 related cytokine IL-27, signaling via WSX-1, has been shown to counter-regulate the early disease exacerbatory effects of IL-4 in this mouse strain during *L. major* infection (Artis *et al.,* 2004a) by promoting a Th1 response. In the absence of WSX-1 signaling early lesion growth is greatly exacerbated although healing under the influence of IL-12 occurs late in infection.

Could the IL-12 independent control of *L. mexicana* described above occur via this alternative mechanism of Th1 induction? Significantly a further study on *L. mexicana* (Aguilar Torrentera *et al.,* 2002) demonstrated early but not late resistance to this parasite in C57 BL/6 mice in the absence of IL-12 although the mechanisms involved remain uncharacterized. Collectively these studies would suggest elements of redundancy in the induction of type-1 responses. Following resolution of patent infection concomitant immunity is dependent upon antigen-specific natural $CD4^+$ $CD25^+$ T-regulatory cells producing IL-10 that moderate the activity of T effectors to maintain latent infection (Belkaid *et al.,* 2002). In the absence of persistent infection protective immunity is significantly reduced.

Non-healing *L. major* infection: Th2 dependent or defective Th1 response

The Th1/Th2 paradigm of resistance/susceptibility to intracellular infection is largely based on investigations using *L. major* (Figure 1-6A). Initial studies suggested

that the resolution or progression of disease was dependent on distinct $CD4^+$ T cell subsets Th1 and Th2 producing the counter regulatory and signatory cytokines IFN-γ and IL-4, respectively (Heinzel *et al.,* 1989). Furthermore, the exquisite sensitivity of BALB/c mice was related to the LACK antigen dependent expansion of vβ4 Vα8 $CD4^+$ T cells producing IL-4 which rendered T cells unresponsive to IL-12 (Launois *et al.,* 1995) by suppressing expression of IL-12 Rβ2 (Himmelrich *et al.,* 1998). Thus a strong case for the predominant role of IL-4 and a Th2 response in non-healing disease was clearly established. Nevertheless, early IL-4 production was not necessarily be an indicator of susceptibility to *L. major* as resistant C57BL/6 mice also produced this cytokine early in infection yet proceeded to resolve infection as a Th1 response is developed (Scott *et al.,* 1996)(Scott *et al.,* 1996). In addition, the use of IL-4 and IL-4Rα gene deficient mice did not definitively establish the role of IL-4 in the disease process, as a series of apparently contradictory reports have been published (Mohrs *et al.,* 2000; Noben-Trauth *et al.,* 1996a; Kopf *et al.,* 1996) with some but not others indicating a disease exacerbatory role for IL-4. While further studies have shown that these observed differences may be either due to the strain of parasite used (Noben-Trauth *et al.,* 1996b) or even due to the age of the mice (Kropf *et al.,* 2003), they also posed two significant questions: firstly could other regulatory cytokines be substituting or compensating or indeed responsible for the immunosuppressive activity previously attributed to IL-4 and/or secondly could the well-documented defective Th1 responses of BALB/c mice be playing a major role in progressive disease (Sacks and Anderson, 2004).

The inability to mount a Th1 response irrespective of a Th2 response has been attributed to an inability to produce or respond to IL-12 (Belkaid *et al.,* 1998; Kropf *et al.,*

1997; Guler *et al.,* 1996). While IL-4 is able to downregulate IL-12 production and expression of IL-12Rβ2 this can also be mediated by IL-4 independent mechanisms (Jones *et al.,* 1998). Consequently intrinsic defects in BALB/c antigen presenting cell function or in T cell development may underlie the susceptibility of BALB/c mice for *L. major*.

Defective antigen presenting cell (APC) function attributable to an inability to produce IL-12 is initially hard to reconcile with the ability of the parasite to suppress host cell IL-12 equally as well with cells from resistant as well as susceptible mice (Belkaid *et al.,* 1998). However, two recent studies have demonstrated BALB/c dendritic cell populations to be comparatively deficient in IL-1 production (Von Stebut *et al.,* 2003; Filippi *et al.,* 2003) compared with *L. major* resistant strains. Furthermore, inoculation of IL-1 or IL-1β into BALB/c mice at the onset of infection enhanced protective responses while IL-1 type 1 receptor deficient mice develop enhanced Th2 responses (Satoskar *et al.,* 1998). Significantly, not only is the ability of IFN-γ to inhibit Th2 production dependent on IL-1 but IL-1 also stimulates dendritic cells to upregulate IL-12 production as well as MHC Class II co-stimulatory molecule expression (Eriksson *et al.,* 2003).

The defective Th1 cell development attributed to BALB/c mice was originally identified as an inability to respond to IL-12 (Guler *et al.,* 1996) which was associated with a down regulation of $CD4^+$ T cells IL-12 Rβ2 expression (Hondowicz *et al.,* 1997). While it is well established that IL-12Rβ2 expression can be down regulated by IL-4 (Himmelrich *et al.,* 1998), this can also be an IL-4 independent mechanism as it also occurs in IL-4Rα deficient mice (). However, this is not necessarily a Th2 independent process as IL-4R$\alpha^{-/-}$ *L. major* infected mice continue to mount an unimpaired Th2

response. However, as transgenic IL-12Rβ2 BALB/c mice exhibit a non-healing phenotype despite IL-12 signaling the importance of this defect remains questionable (Nishikomori *et al.,* 2001). Nevertheless BALB/c $CD4^+$ T cells have been demonstrated as having an intrinsic IL-4Rα independent Th2 bias (Bix *et al.,* 1998). This could be a consequence of the recently described defective co-polarization of the TCR and IFN-γ receptor complex of naïve $CD4^+$ T cells at the immunological APC/T precursor synapse in BALB/c mice compared with C57Bl/6 mice (Maldonado *et al.,* 2004). This would significantly favor a commitment to Th2 development. Nevertheless, IL-4 signaling via STAT6 also serves to block this co-localization indicating that both intrinsic T cell defects as well as immune regulatory mechanisms can serve to impair Th1 responses in BALB/c mice.

Studies using gene-deficient and transgenic mice have clearly identified other cytokines in addition to IL-4 as playing major roles in the non-healing response of BALB/c mice to *L. major*. IL-4Rα deficient mice were found to be more resistant than IL-$4^{-/-}$ mice suggesting at least a compensatory role for IL-13 whose receptor also utilizes the IL-4Rα subunit (Mohrs *et al.,* 1999; Noben-Trauth *et al.,* 1999). In addition, a study using transgenic mice over expressing IL-13 on the resistant C57BL/6 background demonstrated that this cytokine was a key factor determining susceptibility (Matthews *et al.,* 2000). IL-13 was found to act independently of IL-4 and studies using IL-$13^{-/-}$, IL-$4^{-/-}$ and IL-4/IL-$13^{-/-}$ mice suggested the effects of IL-4 and IL-13 to be additive. However, as *L. major* infected IL-4R$\alpha^{-/-}$ BALB/c mice failed to upregulate IL-12Rβ2 expression compared with wild-type mice and continued to be more susceptible than C57 BL/6 mice this suggested other factors could be contributing to susceptibility.

Recent studies using gene deficient mice would suggest that IL-10 is at least as influential as IL-4/IL-13 at promoting *L. major* disease progression in BALB/c mice (Noben-Trauth *et al.,* 2003) and this could be generated via either Fcγ mediated uptake of antibody coated amastigotes (Kane and Mosser, 2001), or produced by Th2 cells, or $CD4^+$ $CD25^+$ T regulatory cells (Scott and Farrell, 1998). T regulatory cells are also significant producers of TGF-β (Mills, 2002), which also is partly responsible for suppressing protective responses (Gumy *et al.,* 2004; Jones, 2000; Reed, 1999; Letterio and Roberts, 1998). Paradoxically T regs producing IL-10 are responsible not only for persistence of parasites in C57BL/6 resistant mice but in maintaining concomitant immunity and in the absence of IL-10 sterile immunity accrues and concomitant immunity is lost (Figure 1-6) (Belkaid *et al.,* 2002; Belkaid *et al.,* 2001).

Cutaneous (*L. mexicana*/*L. amazonensis)* infections and IL-4 dependent

The dichotomy in evolutionary terms between Old World and New World Cutaneous leishmaniasis as well the visceral disease is some 40–80 million years (Stevens *et al.,* 2001), which is comparable with the divergence of the mammalian orders. Therefore, it is hardly surprising that different *Leishmania* species have evolved specific adaptations to insect vectors and disparate vertebrate hosts. Consequently different virulence factors have been identified for different *Leishmania* species and while the vast majority of mouse strains are resistant to *L. major* infection most develop non-healing lesions when infected with *L. mexicana* or *L. amazonensis* (McMahon-Pratt and Alexander, 2004). Furthermore, although BALB/c mice are susceptible to both Old and New World cutaneous leishmaniasis and the *L. mexicana* LACK antigen is recognized by $CD4^+$ T cells producing IL-4, LACK tolerant BALB/c mice while resistant to *L. major* remain susceptible to *L. mexicana* (Aguilar Torrentera et al., 2002).

Consequently the virulence of the *L. mexicana* complex for most mouse strains must be a result of additional factors and cysteine peptidases expressed specifically by amastigotes have been identified as likely candidates (Mottram *et al.,* 2004).

Studies examining IL-4, IL-4Rα (Satoskar *et al.,* 1997) and IL-10 (Padigel *et al.,* 2003a) deficient BALB/c mice infected with *L. mexicana* have indicated IL-4 to be the major contributor to non-healing disease phenotype with lesser though significant complementary roles for IL-13 and IL-10. Experiments utilizing severe combined immunodeficiency disease (SCID) BALB/c mice reconstituted with IL-$4^{-/-}$, IL-$4R\alpha^{-/-}$ or wild-type splenocytes demonstrated that a non-lymphocyte source of IL-4 could initiate lesion growth but only in the presence of lymphocytes able to respond to IL4/IL-13. However, in the absence of IL-4 producing lymphocytes (SCID mice reconstituted with IL-$4^{-/-}$ splenocytes) lesions healed. The Cathepsin-L like cysteine peptidase, CPB, has been identified as the likely *L. mexicana* virulence factor inducing IL-4 (Alexander *et al.,* 1998b; Pollock *et al.,* 2003).

While studies on *L. mexicana* clearly identified a major disease exacerbatory role for IL-4 in susceptible mice other than the BALB/c strain similar studies on the closely related parasite, *L. amazonensis*, suggested an insignificant role for IL-4 and signaling via STAT6 in non-healing infection (Jones, 2000). At the footpad site in C57BL/6 and C3H mice studies, a comparison of cysteine peptidases B (CPB) deficient mutants and wild-type parasites has identified the cathepsin-L like cysteine peptidase to be responsible for the inhibition of Th1 responses (Buxbaum *et al.,* 2003). This could be directly related to the recently identified ability of wild-type but not CPB deficient amastigotes to proteolytically degrade the NFκB family of signaling proteins in infected

bone marrow derived macrophages (Cameron *et al.,* 2004). Thus *L. mexicana* amastigote cysteine peptidases have been associated with both promoting IL-4 production and a Th2 response and inhibiting a Th1 response independently of IL-4 production. The relative importance of these different mechanisms of inducing progressive disease is probably in part mouse strain dependent. As the parasite cysteine peptidases also degrade MHC Class II molecules within the parasitophorous vacuole (De Souza Leao *et al.,* 1995; Prina *et al.,* 1993), this probably compounds the inability of most mouse strains to resolve infection with parasites of this complex.

Non-curing visceral *Leishmania*sis and Th2 independent

A disease exacerbatory role for IL-4 and the Th2 response during the course of *L. donovani* infection has yet to be demonstrated (Figure 1-6B). Early studies in both mice (Kaye *et al.,* 1991) and humans (Kemp *et al.,* 1993) suggested that cure was independent of the differential production of Th1 and Th2 cytokines. Consequently studies in B6/129 (Satoskar *et al.,* 1995) and BALB/c mice have shown that IL-4 and IL-4Rα deficient animals are in fact more susceptible to disease than their wild-type counterparts (Stager *et al.,* 2003a)(Stager *et al.,* 2003b). Furthermore while cure in susceptible BALB/c mice is IL-12 dependent this cytokine was also demonstrated to promote the expansion of the Th2 as well as the Th1 response (Stager *et al.,* 2003a)(Stager *et al.,* 2003a). In addition, not only is IL-4 and IL-4Rα signaling essential for optimal clearance of *L. donovani* from the liver and limiting infection in the spleen (Stager *et al.,* 2003a) following primary infection but also for effective T cell dependent chemotherapy (Alexander *et al.,* 2002) and vaccine induced resistance mediated by $CD8^+$ T cells (Stager *et al.,* 2003a). In the absence of IL-4, type 1 responses and IFN-γ production fail to be maintained following chemotherapy or fail to be induced by

vaccination. Studies in mice (Murphy *et al.,* 2001) and humans (Karp *et al.,* 1993) indicate that IL-10 is the major immunosuppressive cytokine in visceral leishmaniasis although TGF-β induced from a latent form by a parasite cathepsin-B like cysteine peptidase (Somanna *et al.,* 2002) has also significant disease promoting activity (Wilson *et al.,* 1998).

Phagocytosis and *Leishmania*

Inert particle phagocytosis

Phagocytosis (Figure 1-7) is a process by which phagocytes engulf and degrade large (0.4-25μ diameter) particles or pathogens, and prime the host immune systems. It begins when ligands on the foreign particle's surface interact with host cell membrane receptors (mannose, complement, Fc-, integrins, phosphatidyl serine or Toll-like receptors). Some phagocytosis processes occur in a "Zipper mode", where phagocytes develop pseudopod-like projections to surround and engulf the particle (Cossart and Sansonetti, 2004). The particle engulfment is only completed when late and recycling endosomes fuse to the PM through a process called focal exocytosis (Bajno *et al.,* 2000) or direct fusion of endoplasmic reticulum to the PM at the site of nascent phagosomes (Gagnon *et al.,* 2002). Finally, the particle is enclosed in a membrane-bound vacuole called a phagosome. Alternatively, phagocytosis can involve mannose receptors, and the particle will sink into phagocytes without initiating pseudopod protrusions (Le Cabec *et al.,* 2002). Interactions with phagocytic receptors trigger different signaling cascades (involving Rho-family GTPases, PI3Kinases, phospholipase C, Tyr and Ser/Thr-kinases). These signaling cascades have been shown to influence downstream host cellular functions such as cytoskeleton rearrangement (actin or tubulin polymerization), release of cytokines, oxidative stress, and calcium mobilization. Many

mechanisms related to this initial particle-host cell interaction (ligands/receptors, signaling cascades, cytoskeleton rearrangement, membrane fusion or fission) are still controversial or not fully characterized (Haas, 2007; Touret *et al.,* 2005b).

ER-mediated phagocytosis

By convention, plasma membrane and endosomes seem to be the only source of membrane for nascent phagosome during phagocytosis. However, recent studies point at the endoplasmic reticulum as an alternative source of internal membranes for forming early phagosomes. In 2001, gene knock-out experiments involving two markers of endoplasmic reticulum, calnexin and calreticulin, resulted in a significant impairment of the process of phagocytosis in Dictyostellum sp. organisms (Muller-Taubenberger *et al.,* 2001). More interesting, proteomic analyses, in combination with electron microscopy and glucose-6-phosphatase cytochemistry, have led to the suggestion that during particle engulfment the ER fuses with the plasma membrane at the base of the phagocytic cup (Gagnon *et al.,* 2002). The establishment of continuity between these two membranes may establish a pathway whereby the target particle could "slide" into the lumen of the ER, and scission of the ER and resealing of the plasmalemma was envisaged to complete the phagocytic event (Gagnon *et al.,* 2002; Gagnon *et al.,* 2005)(Gagnon *et al.,* 2002).

A contribution of the ER to phagosome formation and maturation is attractive in several respects. First, as the largest single intracellular compartment, the ER can potentially provide enormous amounts of membranes to satisfy the need for entrapment of multiple large particles. Second, by delivering foreign particles to the ER, this mode of phagocytosis could favor antigen cross-presentation (the presentation on class I

histocompatibility complexes of antigens internalized by endocytosis). However, because antigens require proteasomal degradation before class I presentation, direct exposure to the ER lumen would not simplify the loading procedure; the antigens would purportedly be retrotranslocated across the ER membrane to the cytosol, where proteasomal degradation would ostensibly occur, followed by re-uptake of the resulting peptides into the ER by the TAP transporter.

However attractive, the notion that the phagosome is composed largely of ER-derived membranes is inconsistent with a plethora of earlier biochemical and immunostaining data and is seemingly incompatible with at least some of the established physical attributes of phagosomes. In dendritic cells and macrophages, the lumen of the nascent phagosome becomes acidic shortly after sealing. This acidification has been attributed to the inward pumping of protons by V-ATPases, which are thought to be acquired through fusion with endosomes. The acidification becomes more accentuated as phagosomes age, and this correlates with the graded acidification of the compartments of the endocytic pathway that fuse sequentially with maturing phagosomes. It is difficult to envisage how acidification would develop in a phagosome composed largely of ER, which is believed to be devoid of V-ATPases and is inherently permeable to protons (Paroutis *et al.,* 2004).

Moreover, results from another independent study employing a variety of quantitative methods, including biochemical, immunological, fluorescence imaging, and electron microscopy techniques, did not support the fusion of the ER with plasma membrane during phagocytosis or a contribution of the ER to phagosome maturation (Touret *et al.,* 2005b). Instead, the limiting membrane of phagosomes was confirmed to

derive largely from the plasma membrane, becoming subsequently modified through a series of fusion reactions with sub-compartments of the endocytic pathway. Independent evidence in support of this model was provided by targeting the SNARE proteins of the ER. In one report, trapping antibodies to ERS24/Sec22b inside macrophages depressed the efficiency of phagocytosis (Becker *et al.,* 2005). A related study re-evaluated the role of ERS24/Sec22b and additionally analyzed two other SNARE proteins of the ER: syntaxin 18 and D12. Interference with the function of syntaxin 18 and D12 produced a modest reduction in the phagocytic activity, whereas impairment of ERS24/Sec22b had no discernible effect (Hatsuzawa *et al.,* 2006). Altogether, it appears that phagocytosis is a complex process, which involves a wide range of mechanisms that lead to the internalization of multiple particles or particles as large as the size of the phagocyte. Although, these mechanisms are still controversial or not fully understood, more evidence suggests that phagocytosis requires additional intracellular membranes, which are contributed by host organelles such as endosomes or ER.

Phagosome maturation

After phagocytosis, nascent phagosomes interact with the host endocytic pathway in an orderly and sequential manner involving respectively early endosomes, sorting endosomes, late endosomes and lysosomes either by direct fusion (Figure 1-7) (Braun *et al.,* 2004; Hackam *et al.,* 1998) or fission in "kiss and run" type interactions (Desjardins *et al.,* 1997; Desjardins, 1995; Desjardins *et al.,* 1994). Through these interactions or exchange, the phagosome will acquire a membrane-embedded proton-pump adenosine triphosphatase (ATPase) complex, (vacuolar ATPase) that mediates acidification or lowering of the pH (4-5) of the phagosome lumen.

The phagosome also acquires microbiocidal factors such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex to produce superoxide radicals from molecular oxygen, and a nitric oxide (NO) synthetase to make NO radicals from arginine. In addition, acidic hydrolases, lysozymes, defensin peptides and other non-oxidative tools will make the phagosome a biocidal milieu. Antigens generated from degraded particles or pathogens are presented by major histo-compatibility class II (MHCII) molecules at the surface of infected cells to prime or activate an efficient immune response against the invading entities.

There is evidence that the port of entry used by the particle greatly influences the microbicidal pathways that are activated; for example the engagement of Fcγ-receptors will stimulate an increased production of superoxide radicals, as compared to the involvement of complement or other receptors. Although still very controversial (Touret *et al.,* 2005b), the transient interaction of ER organelles with phagosome can also enable foreign antigens cross-presentation by MHC class I molecules (Gagnon *et al.,* 2002; Garin *et al.,* 2001). In general, microbiocidal and immunological factors make the mature phagosome also called phagolysosome, a very harsh environment or killing machinery for foreign particles or pathogens (Haas, 2007). To date, information about phagocytosis and phagosome biology has come mainly from studies based on dead particles such as Zymosan, latex and magnetic beads because vacuoles containing live parasites are delicate and very hard to isolate. The results with inert particles cannot easily be applied to live organisms because the interactions of live organisms with host cells are more dynamic and complex.

Leishmania phagocytosis

Phagocytosis of parasites appears to follow the same path as that of dead particle, but with an unexpected outcome; at the end of the process, internalized parasites are generally not destroyed. In addition, phagocytosis of *Leishmania* also yields PVs with distinct morphological features. *Leishmania* parasites of the *L. donovani* complex (*L. donovani, L. infantum, L. chagasi*) tend to live in tight individual primary PVs and, after binary replication, daughter parasites form their own vacuoles called secondary PVs. Whereas strains responsible for cutaneous and diffuse cutaneous leishmaniasis of the *L. mexicana* complex (*L. mexicana, L. amazonensis, L. pifanoi*) generally replicate and dwell in a communal PV that distends as the number of parasites increases (Kima, 2007; Chang *et al.,* 2003; Chang, 1978).

The mechanism of how *Leishmania* parasites control different features of their PVs biology such as the source of membranes to form primary and secondary PVs in mammalian cells are still poorly understood. Given the complexity of *Leishmania* parasites, it is not surprising that these pathogens have adapted a wide range of mechanisms including virulence factors, selection of specific host receptors, and use of "Trojan horse" to successfully enter and develop in macrophages (review in (Lodge and Descoteaux, 2005; Bogdan, 2008).

Leishmania parasites express many specific molecules that would help them to complete their life cycle. The selection and use of these molecules also called virulence factors or pathogen associated molecules vary with the strain of parasites involved. An example of such molecules is the lipophosphoglycan, which enables *Leishmania major* and *L. donovani*, but not *L. mexicana* (Ilg *et al.,* 2001), to resist the lytic action of the host complement system (Puentes *et al.,* 1988).

Leishmania parasites can also select a specific mode of cell entry as a survival strategy. They can engage the host cell mannose receptor and enter cells without triggering any signaling cascades; while their interaction with Fcγ-receptors will lead to their rapid clearance by host cell defense system. It has been reported that there is low production of superoxide and cytokine during *Leishmania sp*. infection (Pham *et al.,* 2005; Olivier *et al.,* 2005). Therefore *Leishmania* parasites have evolved yet to be fully uncovered mechanisms to "silently enter host cell" (Laskay *et al.,* 2003) and evade/subvert the host immune system (Dogra *et al.,* 2007; Olivier *et al.,* 2005).

Membranes lining *Leishmania* primary and secondary PVs

Plasma membrane (PM), late endosomes and even ER seem to be the source of membranes lining the nascent phagosome (Braun *et al.,* 2004; Hackam *et al.,* 1998). PM components generally recycle back very quickly after phagocytosis. This might imply that host endocytic compartments (Desjardins *et al.,* 1997; Desjardins, 1995; Desjardins *et al.,* 1994) that interact with nascent phagosome are most likely the source of additional membranes for phagolysosomes. Such intracellular interactions usually occur within an hour or less after phagocytosis of dead particles. Live organisms such as *Leishmania* parasites, survive many days after phagocytosis, increase the size of their PVs and/or form new secondary PVs. It is likely that *Leishmania* pathogens might have developed a more selective form of interactions with the host cell endocytic pathway during their maturation.

Endoplasmic Reticulum

Endoplasmic Reticulum Structure

The endoplasmic reticulum (ER) is a continuous network of interconnected tubules, cisternae, and highly organized lamellar sheets (Figure 1-8). These elements

form ER building blocks including rough (RER), smooth (SER), transitional (tER or exit sites), sinusoidal, crystalloid, sarcoplasmic reticulum (SR), karmellae, myeloid bodies, and the nuclear envelope (NE) (Figure 1-9)(Lavoie and Paiement, 2008; Mullins and NetLibrary, 2005). Sometime, ER is simply identified morphologically by two sub-domains: rough ER (rER) with a granular texture due to the presence of bound ribosomes, and smooth ER (SER) is deprive of these organelles and is often more convoluted than RER (Prinz *et al.,* 2000).

The ER can also be grouped in three distinct membrane regions: the nuclear envelope, the peripheral reticular ER and the peripheral ER sheets (English *et al.,* 2009; Shibata *et al.,* 2006). The nuclear envelope (nER) is stabilized by the interaction of inner nuclear membrane proteins with chromatin and the nuclear lamina. The peripheral reticular ER (pER) is shaped by to three-way junctions between ER tubules (Farhan and Hauri, 2009). The peripheral ER network is a complex and highly dynamic structure. It was originally thought to be generated and maintained motor proteins and the cytoskeleton (Terasaki *et al.,* 1986) in conjunction with cytoskeleton-linking membrane proteins (CLIMPs), such as CLIMP-63 (Klopfenstein *et al.,* 1998). Recent studies suggest the peripheral ER development is complex and required reticulons and DP1/Yop1 family members, which deform membranes by providing the curvature needed to form tubules (Voeltz *et al.,* 2006; Dreier and Rapoport, 2000). Atlastin proteins mediate fusion of these tubules to create a network (Shibata *et al.,* 2009), which would be stabilized and modeled by the cytoskeleton, motor proteins (Terasaki *et al.,* 1986) and CLIMPs (Klopfenstein *et al.,* 1998).

Endoplasmic Reticulum Functions

The ER has multiple functions including the synthesis of membrane lipids, membrane and secretory proteins, drug metabolism and the regulation of intracellular calcium. These functions are distributed in distinct ER sub-regions or domains (Borgese *et al.,* 2006). Most membrane proteins are shared between RER and SER, but several proteins involved in translocation or processing of newly synthesized proteins are enriched in RER (Vogel *et al.,* 1990; Amar-Costesec *et al.,* 1989; Kreibich *et al.,* 1978).

Endoplasmic reticulum and ERAD

Secretory and membrane proteins, which constitute about one-third of all cellular proteins, are folded in the ER. ER is equipped with a rigorous quality control system (Figure 1-9), which can differentiate between the correctly folded proteins and the misfolded or unfolded proteins (Ellgaard and Helenius, 2003). Proteins that can not be folded properly in the ER are processed through ER-associated degradation (ERAD), transported from the ER into the cytosol, and subsequently ubiquitinated and degraded by the proteasome (Vembar and Brodsky, 2008). The accumulation of misfolded proteins in the ER activates the unfolded protein response (UPR), which induces the expression of molecular chaperones and ERAD components. This results in an increase ER capacity to fold and clear of accumulating misfolded polypeptides (Malhotra and Kaufman, 2007).

The ER-Golgi intermediate compartment (ERGIC)

In mammalian cells, the secretion pathway (Figure 1-11) is essential and this process starts with molecules trafficking from the endoplasmic reticulum (ER) to the Golgi complex through the tubulovesicular membrane clusters of the ER-Golgi

intermediate compartment (ERGIC) (Appenzeller-Herzog and Hauri, 2006). The exchange of proteins and membrane between ER and Golgi in eukaryotic cells is directed by COP I and COP II coat proteins. These coat proteins are essential in both selecting proper cargo proteins and deforming the lipid bilayer of appropriate donor membranes into buds and vesicles. COP II proteins are required for selective export of newly synthesized proteins from the endoplasmic reticulum (ER). COP I proteins mediate a retrograde transport pathway that selectively recycles proteins from the cis-Golgi complex to the ER. COP I coat proteins also have complex functions in intra-Golgi trafficking and in maintaining the normal structure of the mammalian interphase Golgi complex (Budnik and Stephens, 2009).

Studies involving the transport of a temperature-sensitive mutant G protein from vesicular stomatitis virus (tsO45-VSV-G) and the E1 glycoprotein of Semliki forest virus were to demonstrate that ERGIC-53-positive compartment is a distinctive intermediates in ER-to-Golgi protein transport (Saraste and Svensson, 1991; Schweizer *et al.,* 1990). The protein composition of ERGIC membranes differs from that of the neighboring ER and Golgi (Schweizer *et al.,* 1991); serial sectioning and three-dimensional ultrastructure reconstruction and other analysis of pancreatic acinar cells confirmed the ERGIC as an independent structure, which is not continuous with the ER or the cis-Golgi (Klumperman *et al.,* 1998; Bannykh *et al.,* 1996; Sesso *et al.,* 1994).

ER-Golgi transport and SNAREs

Structure of SNAREs. SNAREs (soluble NSF attachment protein receptors) (Figure 1-11) are a superfamily of proteins that function in all membrane fusion steps of the secretory pathway within eukaryotic cells (Weber *et al.,* 1998; Sollner *et al.,* 1993). Their

core structure is characterized by an evolutionary conserved stretch of 60–70 amino acids containing eight heptad repeats, also known as SNARE motif (Brunger, 2005). The number of different SNAREs varies between different organisms, ranging from 25 in yeast, 36 in mammals, to over 50 in plants. Each fusion step requires a specific set of four different SNARE motifs that is contributed by three or four different SNAREs, and each of the membranes destined to fuse contains at least one SNARE with a membrane anchor (Lang and Jahn, 2008). Membrane traffic usually consists of a sequence of steps involving the generation of a transport vesicle by budding from a precursor compartment, the transport of the vesicle to its destination, and finally the docking and fusion of the vesicle with the target compartment (Figure 1-10). SNAREs operate in the very last step of this sequence (Jahn and Scheller, 2006).

Classification of SNARES. SNAREs were originally classified functionally into t-SNAREs (target-membrane SNAREs) or v-SNAREs (vesicle-membrane SNAREs). While the v- and t-SNARE nomenclature appropriately describes SNAREs that function in the exocytic pathway, this distinction does not fit well with SNAREs involved in fusion between internal membranes, and even homotypic fusion (Wickner, 2002; Fukuda *et al.,* 2000). Therefore, another way of grouping SNAREs is suggested, and it is based on "ionic layer" found in the neuronal SNARE complex (Sutton *et al.,* 1998). This region of the canonical coiled coil sequence contains polar or charged residues in place of hydrophobic residues, which are either a glutamine (Q) for Q-SNAREs or an arginine (R) residue for R-SNARES. According to the position of the SNARE motif in the structurally conserved SNARE complex, the "Q-SNAREs" are further subdivided into Qa

(Syntaxin1a), Qb, and Qc (Kloepper *et al.,* 2007; Bock *et al.,* 2001; Fasshauer *et al.,* 1998). However, the Q-SNARE and R-SNARE designation is not also universal because SNARE complexes such as the yeast ER-Golgi v-SNARE Bet1p has neither a Q nor an R, but an S in the relevant location. Vti1b contains an aspartic acid (D) residue in this location (Zwilling *et al.,* 2007).

SNAREs involved in ER-Golgi transport. Several SNARE complexes have been defined to function in various transport events in the secretory and/or endocytic pathways of mammalian cells (Hong, 2005). The complex consisting of Syn5 (Qa), GS27 (Qb), Bet1 (Qc), and Sec22b (R) appears to function in mediating homotypic fusion of ER-derived COPII vesicles into the ERGIC (ER-Golgi intermediate compartment) also known as VTC (vesicular tubular cluster) (Zhang *et al.,* 1997). Based on systematic analysis of yeast SNAREs (Parlati *et al.,* 2000), a similar SNARE complex is found in yeast. Since yeast Bet1p is the functional v-SNARE, Bet1 is likely the v-SNARE, while Syn5, GS27, and Sec22b may form the t-SNARE, although this remains to be experimentally investigated. EGTCs are dynamic structures that undergo maturation events (including recycling of proteins back to the ER) as they move towards the Golgi apparatus (Horstmann *et al.,* 2002). The SNARE complex consisting of Syn5 (Qa), GS28 (Qb), Bet1 (Qc), and Ykt6 (R) is suggested to act in the late stage of transport from the ER to the Golgi and is likely to mediate the fusion of matured EGTCs with the cis-face of the Golgi apparatus (Horstmann *et al.,* 2002). A likely possibility is that Bet1 stays on the EGTC and acts as the v-SNARE responsible for interaction with

another t-SNARE assembled from the same heavy chain (Syn5) but two different light chains (GS28 and Ykt6) at the cis-Golgi (Hong, 2005).

COPI coat proteins mediated the retrograde transport between ER and Golgi, which help to maintain a dynamic balance of membrane traffic in the early part of the secretory pathway. The ER SNARE complex consisting of Syn18 (Qa), Sec20 (Qb-equivalent), Slt1/Use1/p31 (Qc), and Sec22b (R) may serve as target or t-SNAREs during fusion of retrograde transport vesicles with ER (Hatsuzawa, 2004; Dilcher *et al.,* 2003; Burri *et al.,* 2003; Nakajima *et al.,* 2004). The SNARE complex consisting of Syn5 (Qa), GS28 (Qb), GS15 (Qc), and Ykt6 (R) functions in intra-Golgi traffic and a similar complex is also found in yeast. Based on analysis in yeast, GS15 acts as the v-SNARE, interacting with t-SNARE assembled from Syn5, GS28, and Ykt6 (Parlati *et al.,* 2002). In addition, a recent study suggests that this SNARE complex also mediates traffic from the endosomal compartments to the Golgi apparatus (Tai *et al.,* 2004).

Ricin Trafficking in Eukaryotic Cells and the ER

Ricin is a type II ribosome inactivating protein (RIP) found in castor beans, which are the seeds of the castor plant, Ricinus communis. Due to its high toxicity and ready availability, ricin has been listed as a Category B Select Agent by the National Institutes of Health and the Centers for Disease Control and Prevention (Audi *et al.,* 2005). Ricin is a heterodimeric glycoprotein composed of a catalytically active 32 kDa A-chain (RTA) linked by a disulfide bond to a 34 kDa B-chain (RTB), a galactose- and N-acetylgalactosamine-specific lectin. The molecule enters the cells through endocytosis and undergoes retrograde translocation to the Golgi apparatus/endoplasmic reticulum. Protein disulphide-isomerase reduces ricin to its A and B chains in the endoplasmic

reticulum (Figure 1-13) (). After dissociation, a portion of the RTA reaches the cytosol where it inactivates ribosomes by depurinating a single adenine nucleotide (Spooner *et al.,* 2006; Marsden *et al.,* 2005). This depurination prevents binding of elongation factors, which leads to the inhibition of protein synthesis. However, recent evidence in yeast suggests that ribosome depurination may not by itself cause cell death (Li *et al.,* 2007). Ricin also activates stress-activated protein kinase (SAPK) signaling pathways that are induced by ribosome damage (ribotoxic stress) (Iordanov *et al.,* 1997) and induces apoptosis (Rao *et al.,* 2005; Wu *et al.,* 2004; Higuchi *et al.,* 2003)(Bhaskar *et al.,* 2005). However, the role of SAPK pathways in ricin-induced apoptosis has not been well delineated. While the RTB subunit is thought to enhance the entry of ricin into cells, several studies have shown that the RTA subunit can enter the cell on its own and induce cytotoxicity (Vago *et al.,* 2005; Svinth *et al.,* 1998; Wales *et al.,* 1993; Casellas *et al.,* 1984).

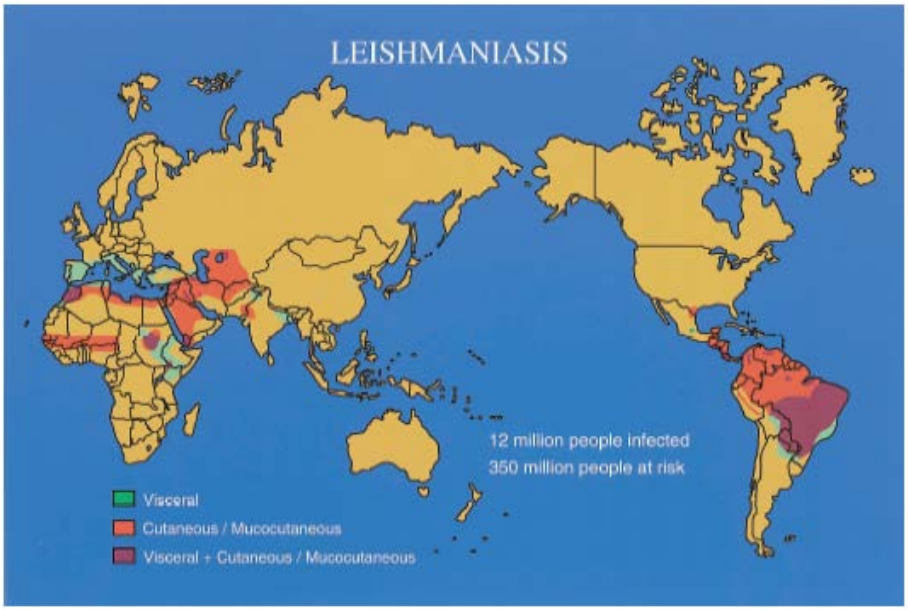


Figure 1-1. World map of leishmaniasis. This map highlights areas where cutaneous, visceral, and mucocutaneous *Leishmania*sis are endemic (Adapted from (Handman, 2001)).

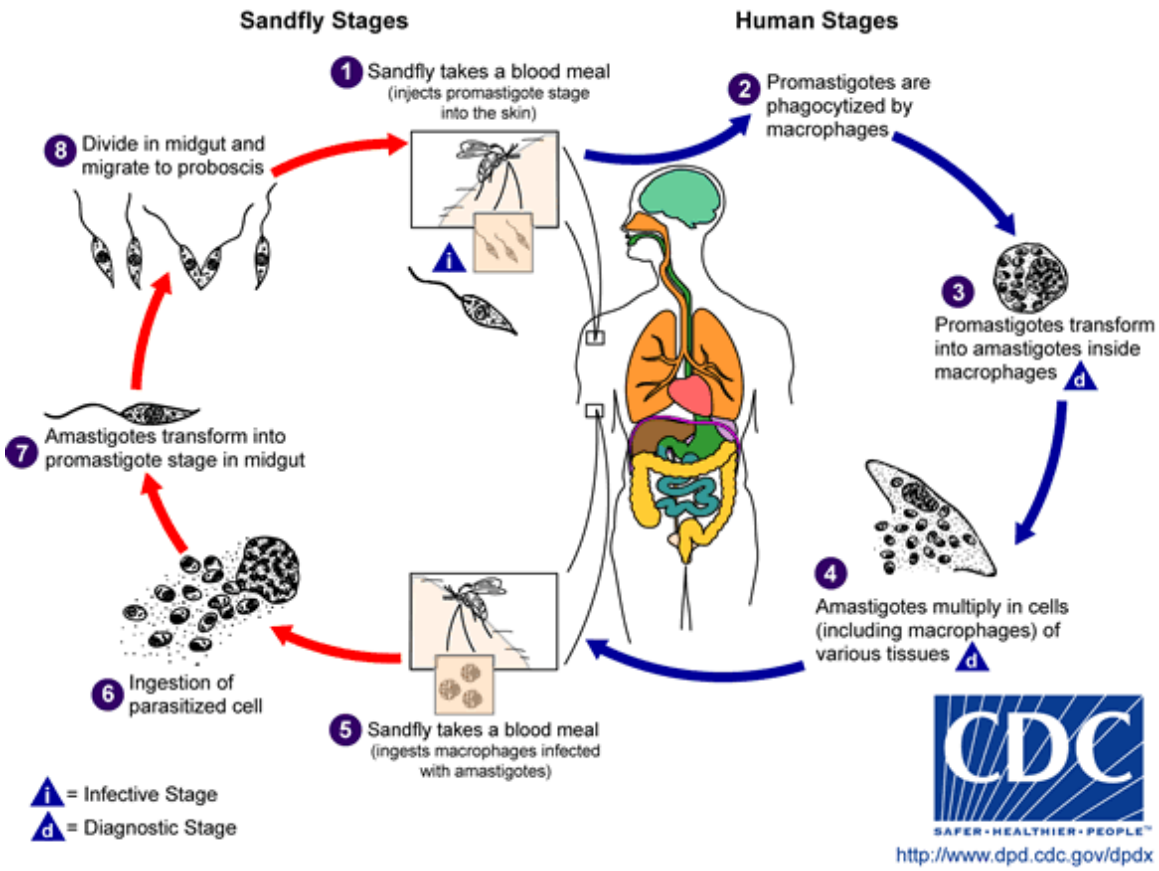


Figure 1-2. Life cycle of *Leishmania* parasites. Leishmaniasis is transmitted by the bite of infected female phlebotomine sandflies. The sandflies inject the infective stage (i.e., promastigotes) from their proboscis during blood meals ❶. Promastigotes that reach the puncture wound are phagocytized by macrophages ❷and other types of mononuclear phagocytic cells. Promastigotes transform in these cells into the tissue stage of the parasite (i.e., amastigotes) ❸, which multiply by simple division and proceed to infect other mononuclear phagocytic cells ❹. Parasite, host, and other factors affect whether the infection becomes symptomatic and whether cutaneous or visceral leishmaniasis results. Sandflies become infected by ingesting infected cells during blood meals (❺, ❻). In sandflies, amastigotes transform into promastigotes, develop in the gut ❼(in the hindgut for *Leishmania* organisms in the *Viannia* subgenus; in the midgut for organisms in the *Leishmania* subgenus), and migrate to the proboscis ❽.

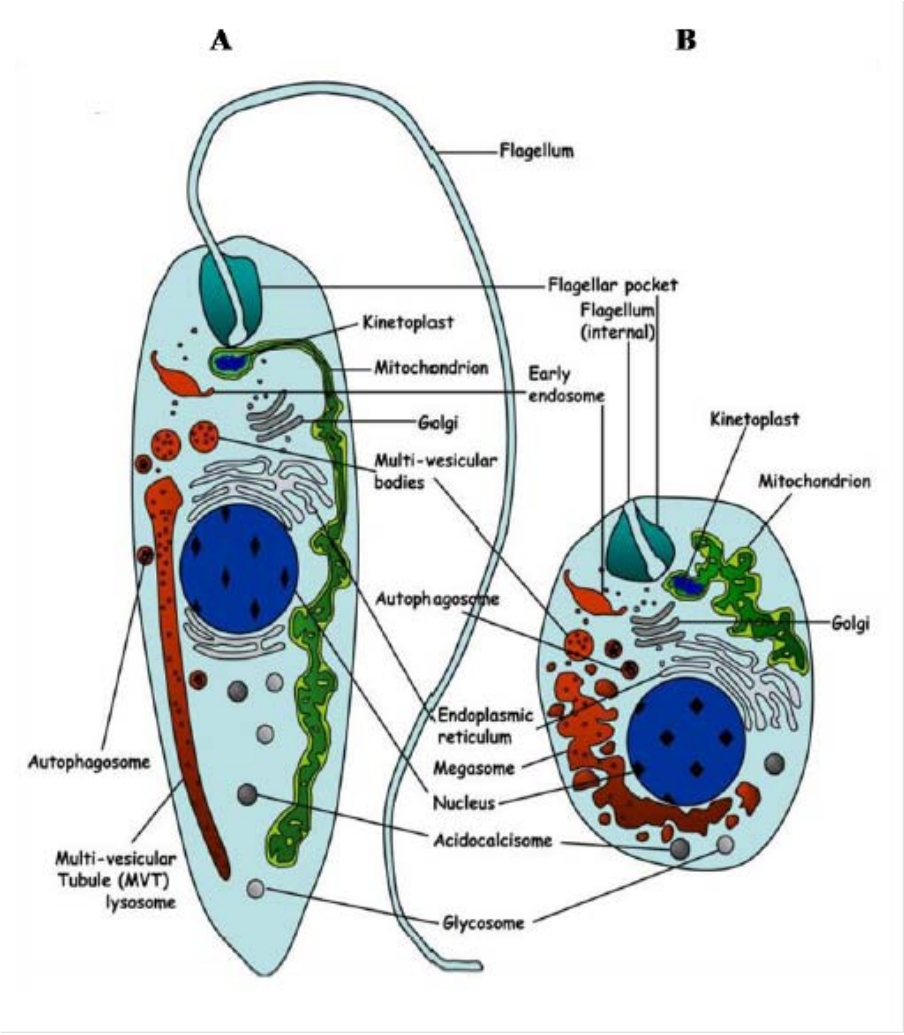


Figure 1-3. Schematic representation of the main intracellular organelles from *Leishmania* promastigote (left, A) or amastigote (right, B) forms. The flagellar pocket marks the anterior end of the cell (Adapted from (Besteiro *et al.,* 2007)).

Figure 1-4. Clinical symptoms of leishmaniasis. A- Cutaneous leishmaniasis, B-Diffuse cutaneous leishmaniasis, C-Mucocutaneous leishmaniasis, D-Visceral leishmaniasis and E-Post kala-azar (Murray *et al.,* 2005; Chappuis *et al.,* 2007; Perez *et al.,* 2006); http://bearspace.baylor.edu/Charles_Kemp/www/leishmaniasis.htm)

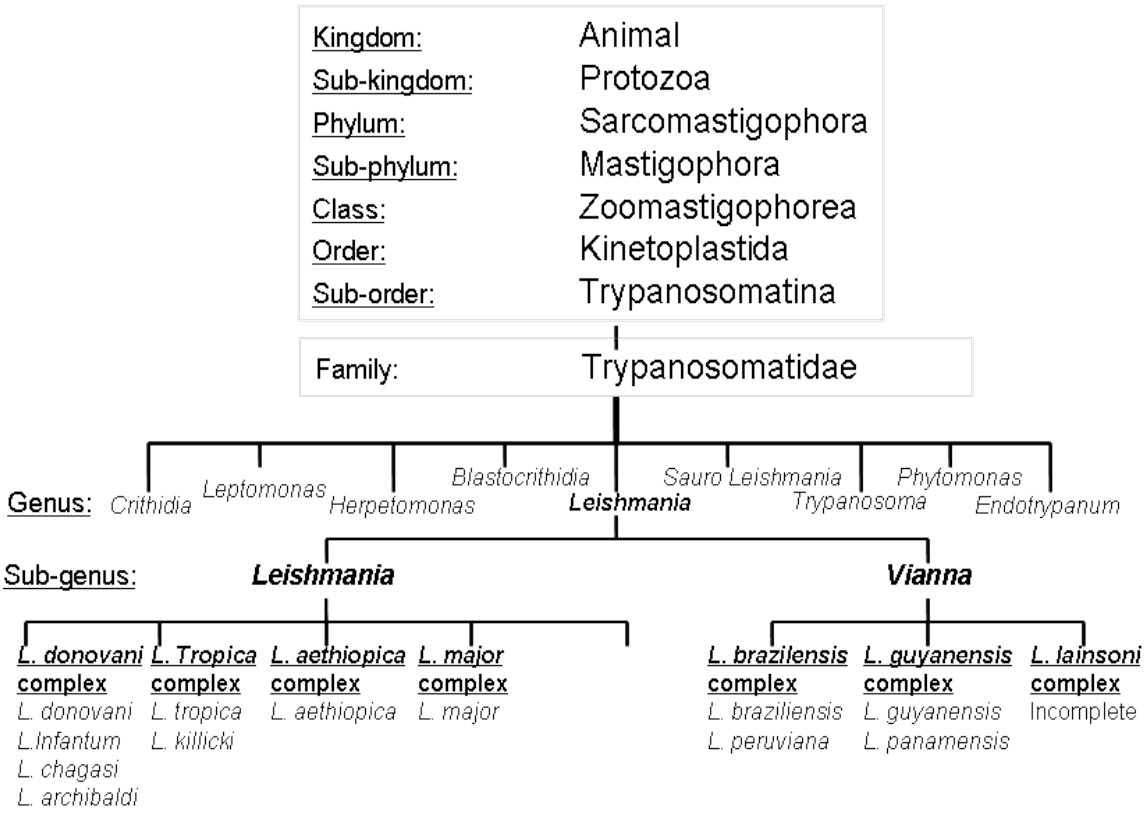
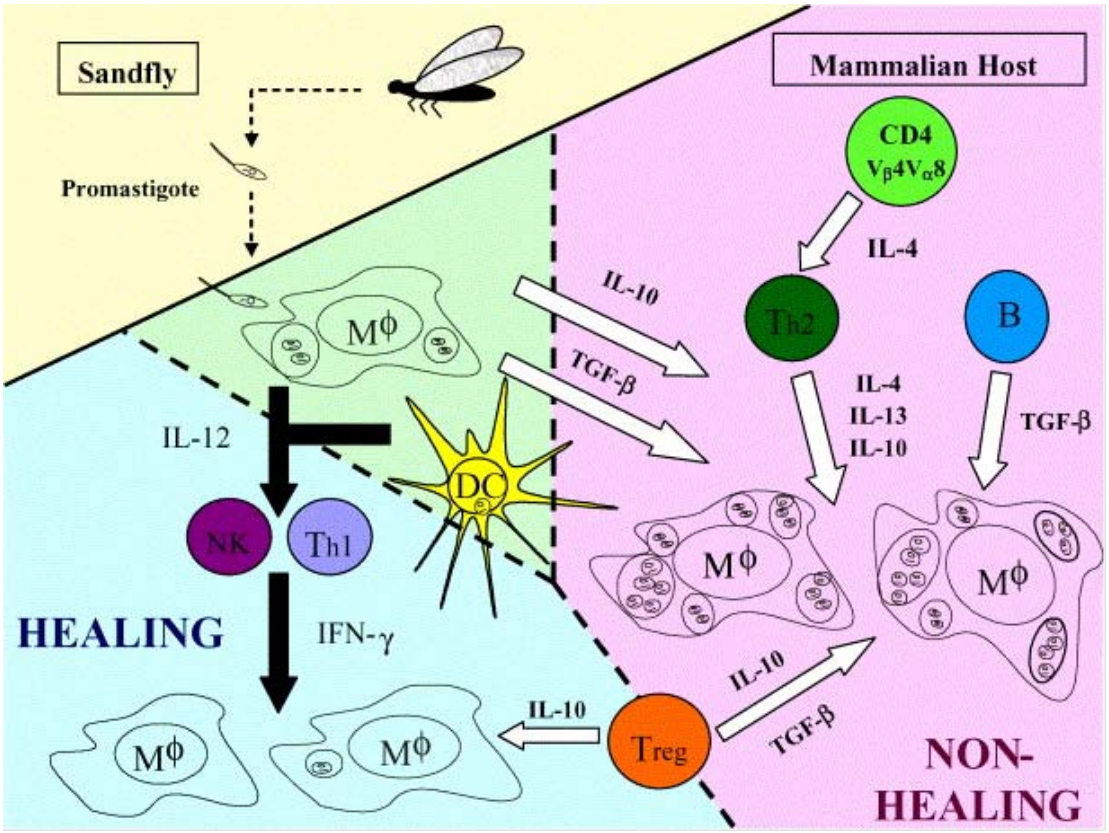


Figure 1-5. *Leishmania* taxonomy (adapted from (Ashford, 2000; Handman *et al.,* 2000))



A

Figure 1-6. *Leishmania major*: healing and non-healing immunological responses. During a blood meal an infected sandfly transmits metacyclic promastigotes to the vertebrate host, which convert to the amastigote form on entering macrophages and dendritic cells. IL-12 production from infected cells induces NK cell activation and $CD4^+$ T helper 1 differentiation and IFN-γ production. IFN-γ stimulates iNOS expression and NO production in the macrophage, which mediates parasite killing and therefore a healing response. Failure to produce IL-12 or alternatively IL-4/IL-13 production results in unregulated parasite replication within the infected cells facilitated by host cell IL-10 production. IL-10 production by $CD4^+$ $CD25^+$ T regulatory cells can both facilitate non-healing disease as well as maintaining latent infection and concomitant immunity. Ingestion of infected cells by a sandfly during a blood meal initiates the life cycle of this protozoan parasite within the insect vector(Adapted from (Alexander and Bryson, 2005)).

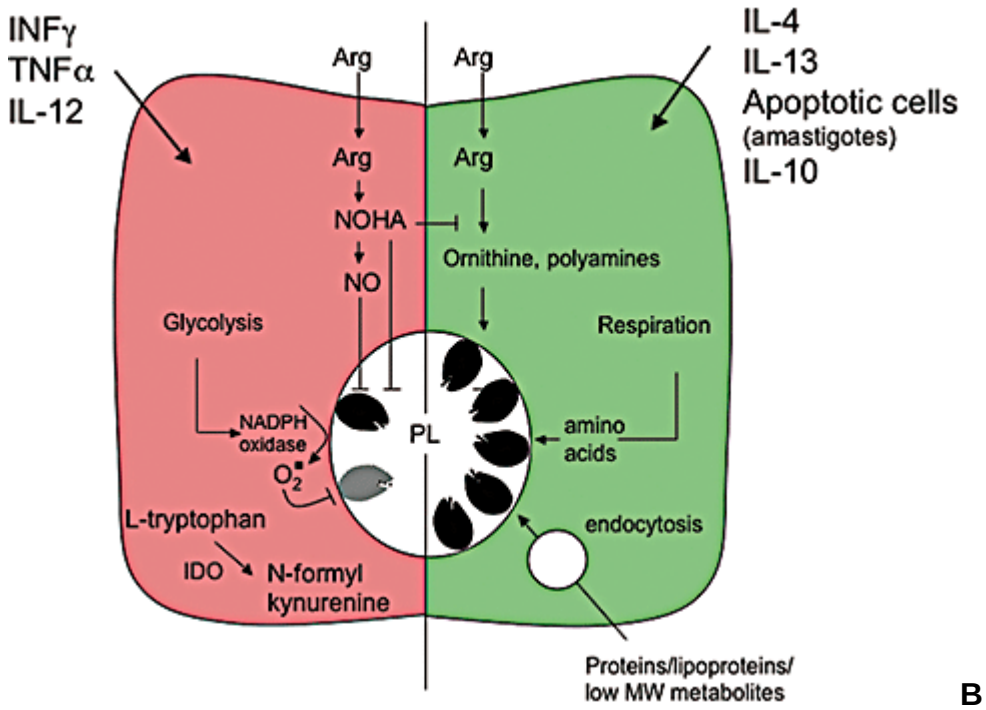


Figure 1-6 Continued. Macrophage metabolism and intracellular growth of *Leishmania* amastigotes. *Leishmania* infection of healing or non-healing mice is associated with a transient or prolonged TH2 response, respectively, and the alternative activation of macrophages (green panel). This activation state can be induced by cytokines (IL-4/IL-13 and IL-10) that predominate in non-healing mice or the presence of apoptotic host cells (macrophages, neutrophils) and the uptake of *Leishmania* amastigotes expressing surface phosphatidylserine. Alternative activation results in the upregulation of endocytic activity, mitochondrial respiration and arginase-1, that increase levels of amino acids and polyamines required for amastigote growth. Conversely, parasite growth can be controlled by an IL-12 driven TH1 response and classic activation of infected macrophages with IFN-γ and other costimulatory molecules (red panel). Classic activation results in the upregulation of glycolysis, NO synthesis, and down-regulation of arginase-1 activity. Enzymes involved in depleting other amino acids may also be up-regulated, restricting growth of amastigotes and increasing their sensitivity to oxidative stress. NOHA, N^G-hydroxy-l-arginine methyl ester; IDO, indoleamine 2,3-dioxygenase (Adapted from (Naderer *et al.,* 2008)).

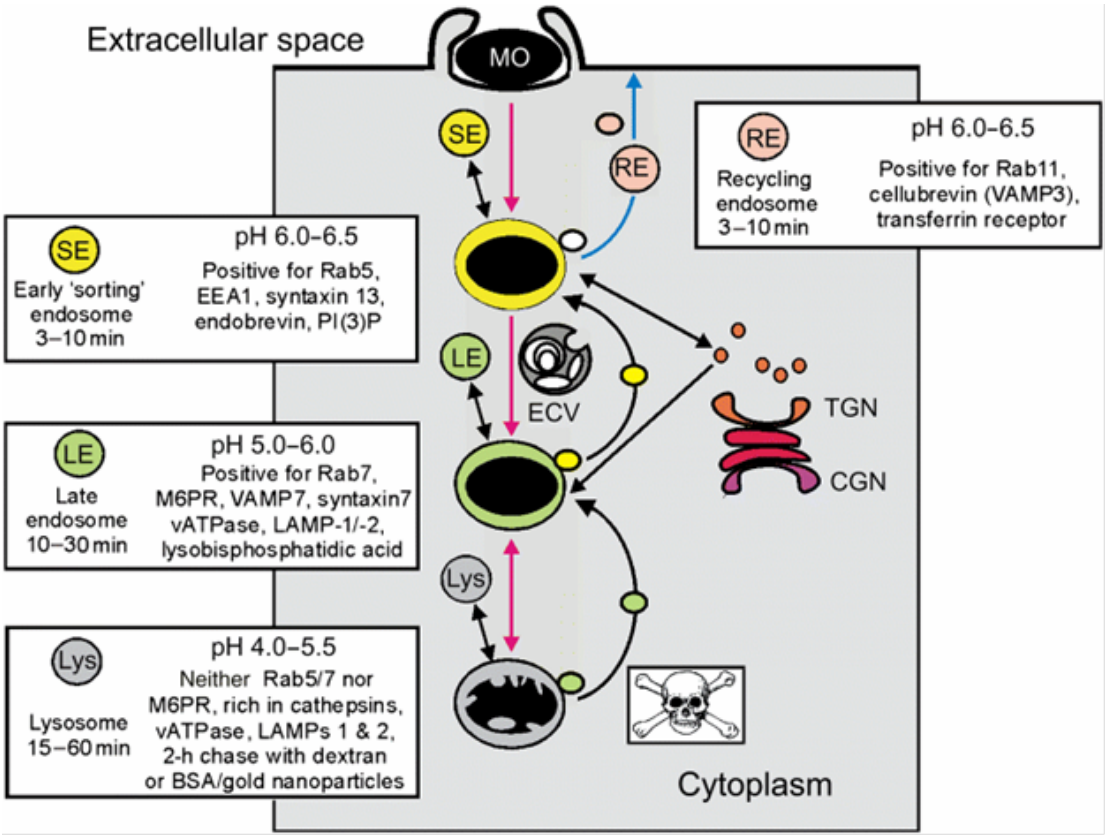


Figure 1-7. Schematic simplified view of the endocytic (phagocytic) pathway. Maturation along the degradative pathway is indicated by red and the recycling pathway by blue arrows. Endosomes mature as they consecutively undergo fusion with membranes of the next stage of maturation, followed by fission of recycling vesicles whose identity is largely unknown (small, colored vesicles with black arrows in backward direction). In macrophages, same-type endocytic organelles fuse particularly avidly with each other (double-sided black arrows). Traffic between early and late endosomes may be predominately accomplished by a vesicle (202). These are vesicles with multiple internal membranes that contain transmembrane proteins destined for degradation. Micro-organism would not fit into such comparably small vesicles. It is more likely that early phagosomes fuse directly with late endosomes to form late phagosomes. Most of the killing and digestion is accomplished in a late phagosome and in phagolysosomes. Antigen presentation through MHC class II occurs predominately from a late phagosome compartment (not included here). Vesicles with biosynthetic cargo from the TGN can fuse with early and late endosomes. Times indicate the approximate periods of time required for a particle to appear in the respective compartment. For example phagolysosomes can normally be observed starting 15 min after ingestion by macrophages, while most phagosomes have matured into phagolysosomes by 60 min of infection (times can vary between macrophage type and activation status). Other endocytic compartments may exist (20), but this simplified four-compartment view has proven valuable in the discussion of most features seen. CGN, *cis* Golgi network; EEA1, early endosome antigen 1; LE, late endosomes; M6PR, mannose 6-phosphate receptor; MO, micro-organism; MVB, multivesicular body; I(3)P, phosphatidylinositol 3-phosphate; RE, recycling endosomes; SE, sorting endosomes; TGN, *trans* Golgi network. (Adapted from (Haas, 2007)).

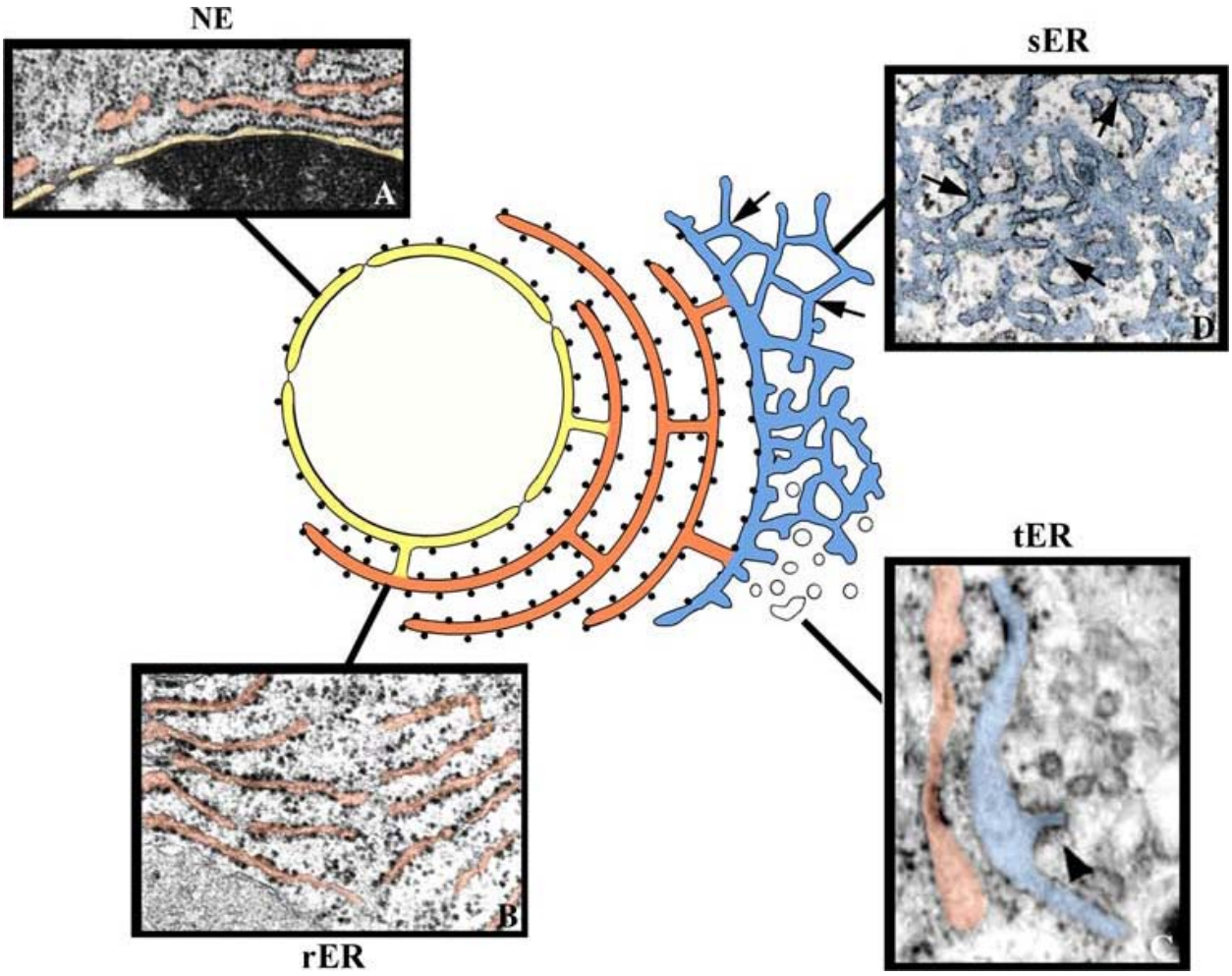


Figure 1-8. Different sub-compartments of the endoplasmic reticulum. The ER is composed of continuous but distinct subdomains. **a** The nuclear envelope (NE) is shown with nuclear pores and ribosomal particles attached to the outer membrane. **b** The rough ER (rER) is continuous with the NE and consists of stacked Xattened saccules, whose limiting membranes have numerous attached ribosomal particles. **c** Transitional ER (tER) is composed of a rER subdomain continuous with the rER and a smooth ER (sER) subdomain consisting of buds and tubules devoid of associated ribosomes (*arrowhead points to a coated bud*). **d** In some cells (e.g., steroid secreting cells and hepatocytes) the sER is composed of a large network of interconnecting tubules showing tripartite junctions (*arrows*) and fenestrations (Adapted from (Lavoie and Paiement, 2008)).

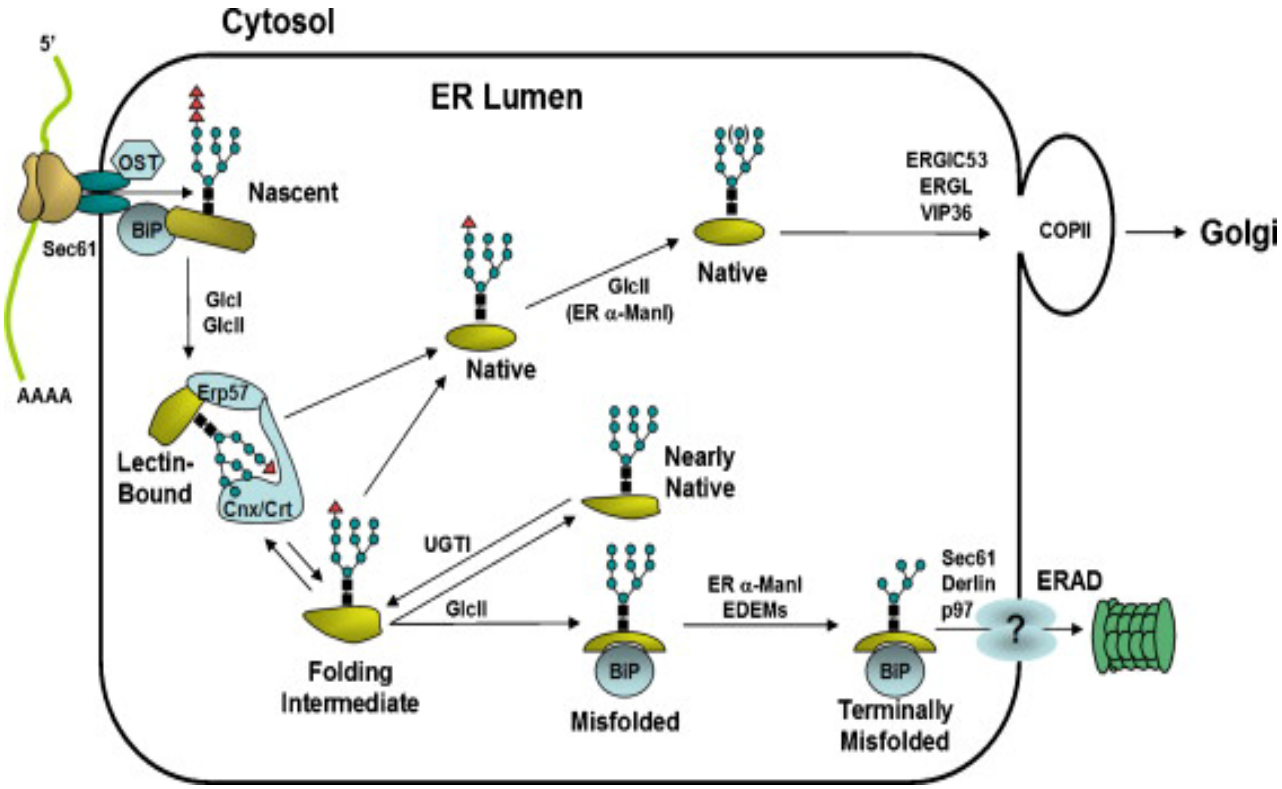


Figure 1-9. Schematic representation of the endoplasmic reticulum quality control system. Protein trafficking from the ER: upon translocation of polypeptides through the Sec61 proteinaceous channel, asparagine residues are frequently modified by covalent addition of a preassembled oligosaccharide core (*N*-acetylglucosamine$_2$-mannose$_9$-glucose$_3$). This reaction is catalyzed by the oligosaccharyltransferase (OST). To facilitate unidirectional transport through the translocon, nascent polypeptide chains in the ER lumen interact with BiP, a molecular chaperone that binds to exposed hydrophobic residues. Subsequently, rapid deglucosylation of the two outermost glucose residues on the oligosaccharide core structures, mediated by glucosidase I and II (GlcI and GlcII), prepares glycoproteins for association with the ER lectins calnexin and calreticulin. The calnexin/calreticulin-associated oxidoreductase ERp57 facilitates protein folding by catalyzing formation of intra- and inter-molecular disulfide bonds, a rate-limiting step in the protein folding process. Release from calnexin/calreticulin (Cnx/Crt) followed by glucosidase II cleavage of the innermost glucose residue prevents further interaction with calnexin and calreticulin. At this point, natively folded polypeptides transit the ER to the Golgi compartment, in a process possibly assisted by mannose-binding lectins, such as ERGIC-53, VIPL, ERGL. As an essential component of protein-folding quality control, non-native polypeptides are tagged for reassociation with Cnx/Crt by the UDP-glucose:glycoprotein glucosyltransferase (UGT1) to facilitate their ER retention and prevent anterograde transport. Polypeptides that are folding incompetent are targeted for degradation by retrotranslocation, possibly mediated by EDEM and Derlins, into the cytosol and delivery to the 26S proteosome. Triangles represent glucose residues, squares represent *N*-acetylglucosamine residues, and circles represent mannose residues (Adapted from (Malhotra and Kaufman, 2007)).

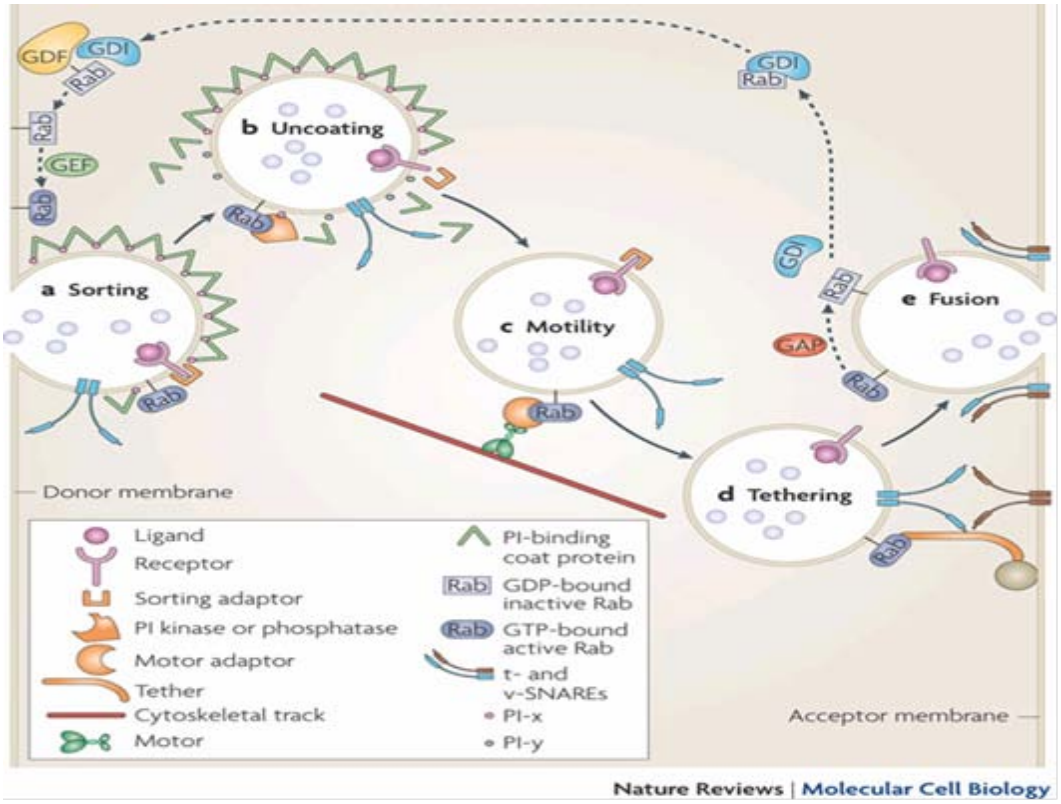


Figure 1-10. Distinct membrane trafficking steps that can be controlled by a Rab GTPase and its effectors (indicated in orange). a)-An active GTP-bound Rab can activate a sorting adaptor to sort a receptor into a budding vesicle. b)-Through recruitment of phosphoinositide (PI) kinases or phosphatases, the PI composition of a transport vesicle might be altered (the conversion of PI-x into PI-y) and thereby cause uncoating through the dissociation of PI-binding coat proteins. c)-Rab GTPases can mediate vesicle transport along actin filaments or microtubules (cytoskeletal tracts) by recruiting motor adaptors or by binding directly to motors (not shown). d | Rab GTPases can mediate vesicle tethering by recruiting rod-shaped tethering factors that interact with molecules in the acceptor membrane. Such factors might interact with SNAREs and their regulators to activate SNARE complex formation, which results in membrane fusion. e | Following membrane fusion and exocytosis, the Rab GTPase is converted to its inactive GDP-bound form through hydrolysis of GTP, which is stimulated by a GTPase-activating protein (GAP). Targeting of the Rab–GDP dissociation inhibitor (GDI) complex back to the donor membrane is mediated by interaction with a membrane-bound GDI displacement factor (GDF). Conversion of the GDP-bound Rab into the GTP-bound form is catalyzed by a guanine nucleotide exchange factor (GEF) (Adapted from Stenmark, 2009).

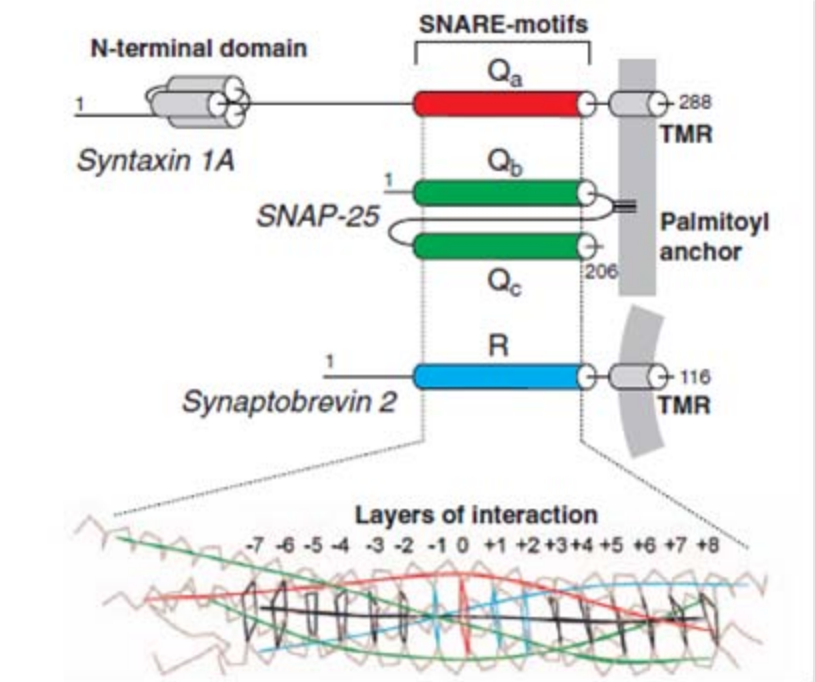


Figure 1-11. Structure of the (neuronal) SNAREs. Upper panel: domain structure of the three neuronal SNARE proteins involved in synaptic vesicle fusion. Syntaxin 1A and SNAP-25 (contains two SNARE motifs) are associated with the presynaptic membrane, whereas synaptobrevin 2 is synaptic vesicle associated. The SNARE motifs form a stable complex (core complex) whose crystal structure has been analyzed (lower panel). In the complex, each of the SNARE motifs adopts an alpha-helical structure, and the four alpha-helices are aligned in parallel forming a twisted bundle (modified from Sutton et al. 1998). Stability of the complex is mediated by layers of interaction (−7 to +8) in which amino acids from each of the four alpha-helices participate (Adapted from (Lang and Jahn, 2008)).

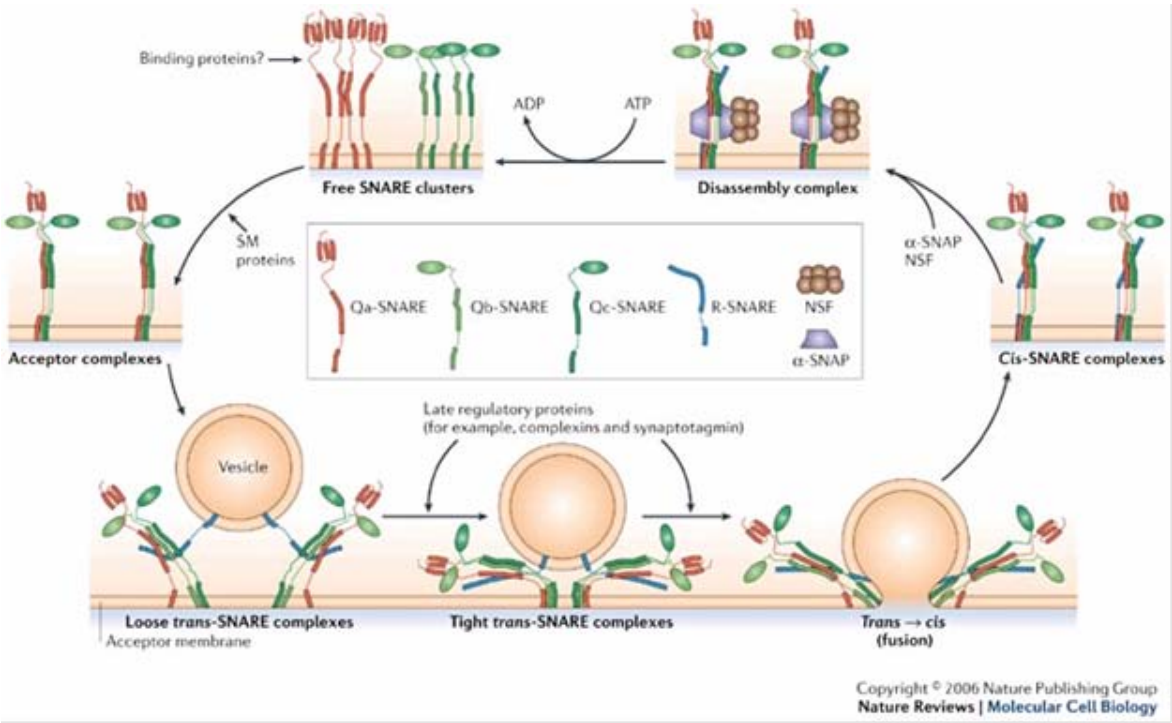
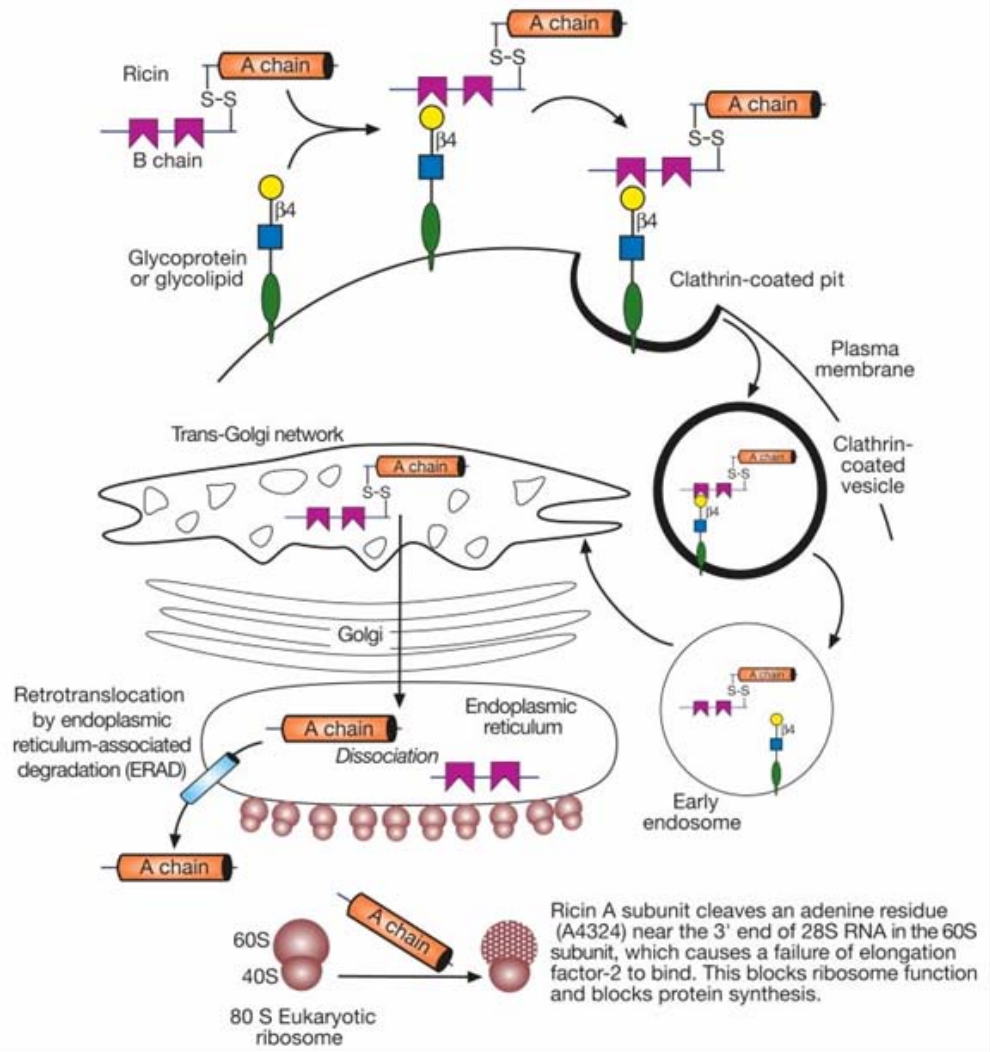


Figure 1-12. The SNARE conformational cycle during vesicle docking and fusion. As an example, we consider three Q-SNAREs (Q-soluble N-ethylmaleimide-sensitive factor attachment protein receptors) on an acceptor membrane and an R-SNARE on a vesicle. Q-SNAREs, which are organized in clusters (top left), assemble into acceptor complexes, and this assembly process might require SM (Sec1/Munc18-related) proteins. Acceptor complexes interact with the vesicular R-SNAREs through the N-terminal end of the SNARE motifs, and this nucleates the formation of a four-helical trans-complex. Trans-complexes proceed from a loose state (in which only the N-terminal portion of the SNARE motifs are 'zipped up') to a tight state (in which the zippering process is mostly completed), and this is followed by the opening of the fusion pore. In regulated exocytosis, these transition states are controlled by late regulatory proteins that include complexins (small proteins that bind to the surface of SNARE complexes) and synaptotagmin (which is activated by an influx of calcium). During fusion, the strained trans-complex relaxes into a cis-configuration. Cis-complexes are disassembled by the AAA+ (ATPases associated with various cellular activities) protein NSF (N-ethylmaleimide-sensitive factor) together with SNAPs (soluble NSF attachment proteins) that function as cofactors. The R- and Q-SNAREs are then separated by sorting (for example, by endocytosis).(Adapted from (Jahn and Scheller, 2006)).



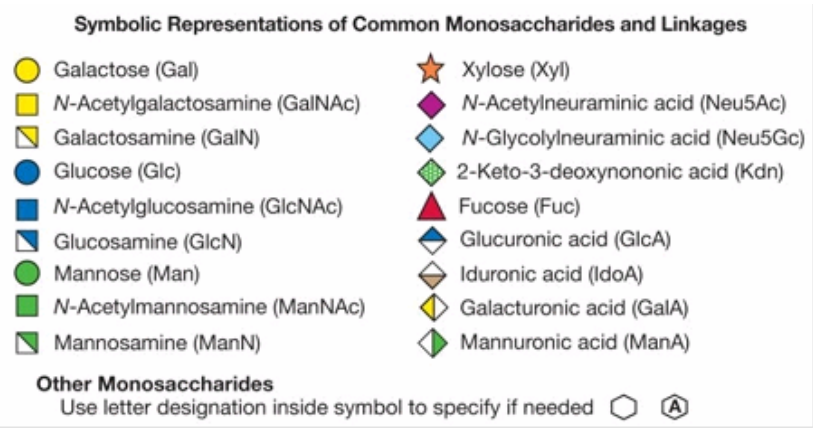


Figure 1-13. Pathway of ricin uptake by cells and the mechanism toxic activity of the A chain in the cytoplasm results in cell death. (Adapted from the Consortium of Glycobiology Editors, La Jolla,;California http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=glyco2&part=ch)

Project Rational and Design

Within the past decade an increasing number of studies have proposed that the endoplasmic reticulum (ER) is also a key participant in phagocytosis of large particles and in the uptake of microorganisms by phagocytes. In 2001 Muller-Taubenberger et al. (Muller-Taubenberger *et al.,* 2001) showed that the knockout of genes that encode two ER proteins (calnexin and calreticulin) severely inhibits inert particles phagocytosis in Dictyostelium sp. During the same year, proteomic analyses revealed that purified latex bead phagosomes contained ER resident molecules such as calnexin, calreticulin and GRp78 (Garin *et al.,* 2001).

Proteomic analysis of phagosomes combined with confocal microscopy and electron microscopy (EM) studies later showed a direct association between ER and PM during early phagocytosis (Gagnon *et al.,* 2002)(Garin *et al.,* 2001). These authors then proposed that ER-mediated phagocytosis is an alternative mechanism employed by phagocytes to internalize large particles and microorganisms without significant depletion of their surface area (Gagnon *et al.,* 2002). Further confirmation of the participation of ER in phagocytosis has been obtained in gene silencing and overexpression studies involving the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) syntaxin 18 (Hatsuzawa *et al.,* 2006) and Sec22b (Becker *et al.,* 2005). Both of these ER-associated SNAREs were shown to regulate fusion between ER and PM during phagocytosis.

However, a study on quantitative and dynamic assessment of ER contributions to latex bead phagosome biogenesis (Touret *et al.,* 2005b) confirmed the presence of PM molecules at the phagosomal membrane, but could not confirm ER-mediated

phagocytosis of inert particles as well as live organisms such as *Leishmania* in macrophages.

This notwithstanding, there have been several observations that have suggested that *Leishmania* parasitophorous vacuoles (PVs), which are formed after phagocytosis of *Leishmania* parasites, have interactions with the host ER. This evidence includes the fact that PVs that harbor *Leishmania* parasites display ER molecules (Garin *et al.,* 2001; Gueirard *et al.,* 2008; Kima and Dunn, 2005); in addition, it has been shown that *Leishmania*-derived molecules can access the MHC class I pathway of presentation through a transporter associated with antigen processing (TAP)-independent mechanism (Bertholet *et al.,* 2006), which implies that parasite-derived peptides in PVs have direct access to MHC class I molecules.

Leishmania parasites reside in PVs with different morphologies: parasites of the *Leishmania mexicana* complex (*L. mexicana*, *Leishmania pifanoi* and *Leishmania amazonensis*) reside in communal PVs that continuously enlarge as the parasites replicate. In contrast, parasites of the *Leishmania donovani* complex (*L. donovani*, *Leishmania infantum*) reside for the most part in individual compartments from which daughter parasites segregate into new compartments or secondary PVs after parasite replication. Although the involvement of endocytic compartments including lysosomes in the biogenesis of *Leishmania* PVs is well established (Courret *et al.,* 2002) the extent of the interactions of the host cell's ER with nascent and secondary PV has not been assessed.

In this study the interactions between the ER and nascent and maturing PVs in macrophages was assessed. Murine Raw246.7 macrophages either transiently or

stably transfected with representative green fluorescence protein (GFP)- or YFP-tagged ER markers (calnexin and Sec22b, D12 and Syntaxin18) were infected; the resultant PVs were monitored for their recruitment of these molecules over the infection course both by fluorescence and Immuno-Electron microscopy. We also exploited the trafficking of ricin, a toxin that accesses the cytosol through a retrograde pathway that traverses the ER (Audi *et al.,* 2005), to further characterize host ER and PVs association. Finally, we employed dominant negative constructs from ER-membrane associated SNAREs (Sec22b, D12, and Syntaxin18) that lack the transmembrane domain, to assess the consequences of host ER interaction on *Leishmania* replication and PVs development in macrophages.

CHAPTER 2
MATERIAL AND METHODS

Material

Chemicals and Reagents

Most of the chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburg, PA). BrefeldinA, Prolong antifade mounting medium were purchased from Invitrogen Inc (Carlsbad, CA). DNA restriction endonuclease and 1kb DNA ladder were purchased from New England Biolabs, Inc (Ipswich, MA). Desalted oligonucleotides were from Integrated DNA Technologies (Coralville, IA). The fluorescein labeled *Ricinus communis* agglutinin II (RCA II) was purchased from Vector Laboratories Inc (Burlingame, CA)

Commonly use buffers and media:

DNA Loading Dye (10X)- 0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 0.1M
LB Broth- 10g Bacto Tryptone, 5g Yeast Extract, 10g NaCl.
TAE- 40mM Tris-Acetate, 1mM EDTA (pH 8.0).
TE- 10mM Tris, 39mM Glycine, 15% Methanol.

Biochemical Kits

Plasmid extraction, DNA gel-extraction, and RNeasy kits were purchased from Qiagen Inc (Valencia, CA). The Fast-Link DNA Ligation Kit was purchased from Epicentre Biotechnologies (Madison, WI). The nucleofection kit was purchased from Amaxa Inc. (Gaithersburg, MD).

Plasmids

pShooter vector pCMV/ER/GFP/c-myc vector (Invitrogen Inc; Carlsbad, CA). The pmVenus, pmVenus/Sec22b, pmVenus/syntaxin 18, pmVenus/D12, ∆-tm-Sec22b/RFP, ∆-tm-Synatxin/RFP and ∆-tm-D12/RFP were generously provided to us by Dr. Kiyotaka Hatsuzawa at the Fukushima Medical University, Japan)

Antibodies

Early endosome antigen 1 (EEA1), calnexin, GM130 (Santa Cruz Biotechnology, San Jose CA), BiP, (BD Biosciences, San José CA); the rat anti-late endosome and lysosomal associated membrane proteins, 1D4B, was obtained from the Developmental Studies Hybridoma bank, Iowa City, IA.

Cell Lines and Maintenance

Parasites

The *Leishmania pifanoi* promastigotes (MHOM/VE/57/LL1) line was obtained from the American Type Culture Collection (ATCC)(Manassas, VA). It was grown in Schneiders medium supplemented with 10% fetal calf serum and 10 μg/ml gentamicin at 23°C. *L. donovani* strain 1S-CL2D from Sudan, World Health Organization (WHO) designation: (MHOM/SD/62/1S-CL2D) was obtained from Dr. Debrabant (USDA, MD). Promastigotes of this parasite strain were grown in Medium-199 (with Hank's salts, Gibco Invitrogen Corp.) supplemented to a final concentration of 2 mM L-glutamine, 100 μM adenosine, 23 μM folic acid, 100 IU and 100 μg/ml each of penicillin G and streptomycin, respectively, 1×BME vitamin mix, 25 mM Hepes, and 10% (v/v) heat-inactivated (45 min at 56 °C) fetal bovine serum, adjusted with 1 N HCl to pH 6.8 at 26 °C. Generation of amastigotes forms was carried out as described (Debrabant *et al.,* 2004). *L. donovani* axenic amastigotes parasites were maintained in RPMI-640/MES/pH 5.5 medium at 37 °C in a humidified atmosphere containing 5.5% CO_2 in air. Similarly, *L. pifanoi* amastigotes were maintained in the amastigote medium above at 34 °C.

Macrophages

The RAW 264.7 murine macrophage cell line (obtained from ATCC) was cultured in RPMI supplemented with 10 % fetal calf serum and 100 units Penicillin/Streptomycin

at 37°C under a 5% CO_2 atmosphere. G418 sulfate antibiotics at a final concentration of 1mg/ml were added to the complete growth medium of Raw 264.7 cells transfected with either pCMV/ER/GFP/c-myc or pCMV/ER/GFP-calnexin vector. Culture medium of Raw 264.7 or J774 cells expressing pmVenus/YFP or RFP based DNA constructs (Sec22b, syntaxin, and D12) was supplemented with puromycin antibiotics at a final concentration of 2ug/ml, as previously recommended by (Hatsuzawa *et al.,* 2006; Hatsuzawa *et al.,* 2009). These cells were routinely maintained every 2 to 3 days as follow, cells were dislodged from the bottom surface of a 75 cm2 culture flask or other culture vessels with a sterile cell scraper. An appropriate volume of cell suspension was transferred into a new culture vessel containing a fresh culture medium at a sub-cultivation ratio of 1:1 to 1:10. For long-term storage, these cells were frozen in complete growth medium supplemented with 5% (v/v) DMSO and stored at -80 °C, as recommended by the provider.

Bacteria

DH5α and TOP10 Escherichia coli strains (Life Technologies) were used for routine cloning. Bacteria were grown on LB agar plates or LB broth. Media of transformed bacteria were supplemented with antibiotics at a final concentration of 50ug/ml (for kanamycin) and 100ug/ml (for ampicillin) depending of the resistant or selective gene on the plasmid of interest. Bacterial cultures were stored at 4ºC for up to a week in LB broth or a month on LB/agar plates. For a long-term storage, 850µl of the bacterial fresh overnight culture was added to 150µl of sterile glycerol. This 10% bacterial culture was rapidly frozen in liquid nitrogen for 30 seconds, and stored at -80ºC.

Molecular Cloning

RNA Extraction and Purification

Total RNA was extracted from murine Raw246.7 macrophages using the RNeasy Mini kit for animal cells (Qiagen). A volume of overnight fresh culture containing approximately 1 x 10^7 Raw246.7 cells was centrifuged at 300 g for 5 min. To lyse the plasma membrane, resuspend the cells in 600 µl of pre-cooled (4ºC) Buffer RLN and incubate on ice for 5 min. Centrifuge the lysate at 4ºC for 2 min at 300 *g*. Transfer the supernatant to a new centrifuge tube, and discard the pellet. Add 600 µl Buffer RLT to the supernatant, and mix well by vigorously vortexing. Add 430 µl of 100% ethanol to the homogenized lysate, and mix well by pipetting. Transfer the sample to the RNeasy spin column placed in a 2 ml collection tube, and centrifuge at 13,000 rpm for 15 s.

To eliminate genomic DNA contamination, add 80 µl of RNase-Free DNase I solution to the column membrane and incubate at RT for 15 min. Wash the column by adding 350 µl of RW1 buffer and after centrifugation at maximum speed for 15s, discard the flow-through. Repeat this step respectively with 700 µl of RW1 buffer, and 500 µl Buffer RPE (centrifuge for 2 min). Centrifuge again the RNeasy spin column in a new 2 ml collection tube for 1 min, to eliminate any possible carryover of Buffer RPE. To elute the RNA, place the RNeasy spin column in a new 1.5 ml collection tube and add 50 µl RNase-free water directly to the spin column membrane, and centrifuge for 1 min at 13000 rpm. The concentration of the total RNA was determined from A260nm. Quality of RNA was determined by 0.8 % (w/v) agarose gel electrophoresis in 1 × TAE buffer after heating the RNA samples to 50°C for 15 minutes. The purified RNA was stored at -70°C until further use.

DNA Extraction and Purification

One-step RT-PCR and DNA amplification

A DNA fragment containing the calnexin gene was directly amplified from purified total RNA using specific primer sequences (Forward: 5' *AGCG GAT CCG GGA GGC TCG AGA TAG ATC ATG GA* 3'and Reverse: 5' *AGCG GAT CCG GGA GGC TCG AGA TAG ATC ATG GA* 3') and following a one-step RT-PCR protocol from Qiagen Inc. PCR and calnexin gene amplification

The Calnexin gene was amplified from the DNA template obtained from the total RNA sample. Specific primer sequences containing a NotI restriction enzyme site (Forward: 5' *AAG GAA AAA A GCG GCC GC C ATG ATG GAC ATG ATG ATG ACG* 3' and Reverse: 5' *AAG GAA AAA AA GCG GCC GCT CAC TCT CTT CGT GGC TTT CTG* 3')

Agarose gel electrophoresis

Sizes of PCR products and plasmids were analyzed by electrophoresis using 0.7 – 1.2 % (w/v) agarose gels in TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.5) with 1kb DNA ladder (from New England Biolabs Inc) as the DNA molecular weight standard. The gels were stained with ethidium bromide at 0.5 µg/ml. The image of the gels was captured with the Kodak Gel Logic 200 Imaging System, and the process with Carestream Molecular imaging software purchased from Carestream Health, Inc (Rochester, NY).

Mini-prep plasmid DNA isolation

Plasmids were isolated by Qiagen Miniprep kit according to Manufacturer's protocols (Qiagen Inc., Valencia, CA). When applicable, plasmids were purified from agarose slices by QIAquick gel extraction kit (Qiagen). The alkali lysis method of

plasmid isolation was performed as previously described (Sambrook et al. 1989). Briefly, bacteria containing the desired plasmid were inoculated into 2ml of LB broth with the appropriate selectable drug and grown overnight with shaking at 37ºC. The following day, 1.5ml of the culture was pelleted by centrifugation at 10,000 rpm for 30 seconds. The pellet was resuspended in 100µl of STET buffer followed by the addition of 200µl of freshly prepared lysis buffer (0.2N NaOH, 1% SDS) and incubated at 24ºC for 5 mins.

Following the incubation, 150µl of 3 M potassium acetate (pH 5.5) was added to the lysed mixture, which was then mixed gently and incubated on wet ice for 5 mins. The mixture was then centrifuged at 4ºC for 15 mins. The supernatant was transferred to an empty microfuge tube to which 0.7 volumes of isopropanol was added, mixed and centrifuged for a further 15 mins at 4ºC. The pellet was air dried and resuspended in 150µl of TE buffer and incubated with 20µg/ml RNaseA at 37ºC for 30 mins. The plasmid preparation was then extracted sequentially with Tris-saturated Phenol (pH 8.0) and Chloroform. The aqueous phase was precipitated with equal volumes of 7.5M ammonium acetate and 2.5 volumes of 95% ethanol. The pellet was washed with 70% ethanol, air-dried and resuspended in 20µl of ddH2O.

Endotoxin- free maxi-prep DNA isolation

To generate a large-scale DNA production, 250 ml of fresh overnight culture of DH-5α cells transformed with a plasmid of interest was processed using the Endo-Free Plasmid Maxi Kit (Qiagen Inc. Chatsworth, CA) was used to generate a large amount of endotoxin-free and high purity DNA constructs for mammalian cell transfection. Kit ale plasmid isolations were performed using the Qiagen Maxi-Prep Kit Isolation of plasmid DNA from 250ml cultures was carried out according to the manufacturer's instructions.

Plasmid DNA was purified using an anion-exchange column. The purified DNA was stored at -20°C until further use.

DNA Cloning

Restriction enzyme digestion of DNA (vector and insert)

The pCMV/ER/GFP/c-myc vector and the calnexin gene were digested with NotI restriction enzyme purchased from New England Biolabs. digestion of plasmid DNA or DNA insert was performed using 2 units of restriction enzyme per µg of DNA at 37°C for 2 hours in the provided buffers from the manufacturer.

Dephosphorylation of digested DNA

Dephosphorylation of digested plasmids was carried out to prevent subsequent religation of the plasmid and to facilitate sub-cloning of restriction digested fragments. Dephosphorylation of plasmid DNA performed by digesting 1pico-molar termini of DNA with 1 unit Shrimp Alkaline Phosphatase (Fermentas Inc; Glen Burnie, MD) at 37ºC for 30 min. To stop the reaction, the sample was incubated at 70ºC for 15 min, and chilled on ice for at 5min before usage or storage at -20ºC.

DNA ligation

Both insert and plasmid DNA digested with endonucleases was purified using a Gel extraction and purification Kit (Qiagen Inc.). Their concentration and purity were assessed using a NanoDrop spectrophotometer. The ligation reaction at the ratio of 1:3 (vector:insert) was performed using the Fast-Link DNA ligation kit purchased from Epicentre Biotechnologies Inc (Madison, WI). 1.5 ml 10X Fast-Link Ligation Buffer, 1.5 ml 10 mM ATP, x ml vector DNA (1); x ml insert DNA (2); x ml sterile water to a volume of 14 ml 1 ml Fast-Link DNA Ligase. 2. Incubate the reaction at least 30 min. The reaction was transferred to 70oC for 15 minutes to inactivate the Fast-Link DNA ligase.

After inactivation, the sample was chill on ice for at least 5min, and centrifuge at 13000 for 15 seconds. DH-5α electro-competent cells were transformed with 1/10 of the ligation reaction, keeping the volume of the ligation to no more than 5% of the volume of competent cells,4 or follow the manufacturer's recommendations. If electroporating the ligated molecules, use no more than 2 ml of the ligation reaction with 50 ml of electrocompetent cells. To determine the extent of ligation, inactivate the ligase and run 5 ml of the ligation reaction on an agarose gel and visualize.

DNA and RNA Analysis

DNA and RNA quantity and quality measurement

The quantity and quality of DNA and RNA were measured using a NanoDrop spectrometer equipped with the ND-1000 V3.3.0 software from NanoDrop Technologies Inc (Wilmington, DE).

DNA sequencing

To confirm the nature and origin of DNA inserts amplified by PCR or RT-PCR, DNA constructs or clones were sent and sequenced at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR), DNA Sequencing Facilities.

DNA agarose Gels

DNA was separated on 0.7%-.8% agarose gels depending on the size of the DNA fragments to be separated according to Sambrook *et al.*(1989). Gels were cast using pure Agarose (Invitrogen) in 1x TAE buffer by boiling. Ethidium bromide was added (0.5µg/ml) to the agarose after it had cooled (to <50oC) and cast in the gel-casting tray. DNA samples were mixed with DNA loading dye to a final concentration of 1x before separation in agarose gels. Electrophoresis was carried out at 100V constant in 1x TAE buffer.

Preparation of *E.coli* DH5-α competent cells

Competent cells were created as previously described (Mandel and Higa 1970). Briefly, 500µl of an overnight bacterial culture was used to inoculate 30ml of LB media and grown with shaking at 37ºC until the OD600 = 1.0 (approx. 2 hours). Cells were pelleted at 6000rpm using a Sorval SS34 rotor for 10 mins at 4ºC. The pellet was resuspended in 10ml of sterile ice-cold CaCl2 solution (0.1M CaCl2, 15% glycerol) and incubated for 30 mins on wet ice. Cells were pelleted once again and the pellet was resuspended in 1ml of ice-cold CaCl2 solution and incubated on ice for one hour. The cells were aliquoted into 100µl volumes, snap-frozen in liquid nitrogen and stored at -80ºC until required. For transformation of DH5-α competent cells Plasmid DNA was added directly to 100µl of competent cells and gently mixed and placed on ice for 10 mins. The cells were then placed at 45ºC for 2 minutes, after which 1ml of LB media was added to the tubes, mixed and then placed at 37ºC for one hour. Cells were centrifuged at 10,000rpm for 30 seconds at 24ºC and the pellet was resuspended in 200µl of LB media. Cells were spread on pre-warmed LB plates containing the appropriate selectable antibiotic. The plates were placed at 37ºC for 12 hours. Several bacterial colonies were then analyzed for the appropriate plasmid. Cells containing the appropriate plasmid were grown in LB media containing the selectable antibiotic for 12 hours with shaking at 37ºC. 850µl of this saturated culture was added to 150µl of sterile glycerol and stored at -80ºC.

Transformation of DH-5α cells

This process was performed following a modified protocol from Epicentre Biotechnologies Inc. Frozen bacteria sample was thawed on ice, and 20 ul of this sample was added into an ice pre-chilled 1.5 ml microcentrifuge test tube containing 1

ul of inactivated ligation reaction. The bottom of the test tube was gently flicked to mix DNA and cells. This mixture was transferred into a 0.2 cm cuvette (Bio-Rad Inc); the electroporation was performed using the Gene Pulse electroporator (Bio-Rad, Inc) with 25uF capacitance, 200 Ω resistance and 2.5 kV settings. 979 ul of SOC medium was immediately added to the cuvette; the electroporated cells in suspension were transferred into a sterile round bottom 15 ml culture tube, which was then incubated at 37˚C shaking (225 rpm). After an hour, 100 ul of the transformation culture was spread on an LB/agar plate containing 100ug/ml ampicilin antibiotics. The culture plate was incubated at 37C to allow transformed cells to grow overnight. The next day, the plate was screened for colony growth.

Screening of bacterial colonies

Colonies were picked and screened first using a PCR based approach. PCR positive either plasmid extraction/endonuclease or PCR based approach for potential positive clones, which were sequenced at University of Florida ICBR sequencing facilities. The true-positive DNA constructs were purified at large scale using the endotoxin-free DNA Maxi-prep kit (Qiagen Inc.) to transfect Raw264.7 macrophages. PCR based screening was only used when screening 10 or more distinct bacterial colonies. Each single colony was picked with a sterile micro-pipet tip, transferred respectively on a master LB/agar/antibiotics plate and into a 0.2ml PCR tube containing the pre-mix PCR reaction with specific primers to the gene or DNA segment of interest. The amplification was proceeded as described by the PCR kit provider. 0.8% agarose gel electrophoresis was performed and the image of the gel was analyzed to identify DNA fragment with the right expected size.

Endonclease-based screening was performed when analyzing either less than 10 distinct bacterial colonies, or PCR-positive colonies. Plasmid mini-prep reaction was prepares for each single colony, and the plasmid was digested with one or multiples specific restriction enzymes. After digestion, an agarose gel electrophoresis was performed and the gel image was screened for the DNA fragments with the appropriate or expected size.

Vectors Construction and Expression

To prepare the calnexin construct, total RNA was extracted from murine Raw264.7 macrophages using the RNeasy Kit from Qiagen Inc (Valencia, CA). The calnexin gene was directly amplified from total RNA employing specific primer sequences (Forward: 5' *AAG GAA AAA A GCG GCC GC C ATG ATG GAC ATG ATG ATG ACG* 3'and Reverse: 5' *AAG GAA AAA AA GCG GCC GCT CAC TCT CTT CGT GGC TTT CTG* 3') in a one-step RT-PCR protocol (Qiagen Inc). The amplified gene was cloned in frame at the Not I site of the pShooter vector pCMV/ER/GFP/c-myc vector (Invitrogen Inc; Carlsbad, CA), such that GFP is expressed at the N-terminus of the protein. The signal peptide sequence (from the vector) and retention signal (from gene) were selected to direct and localize the expressed protein in host ER compartments. The selected clone was sent to University of Florida Genetics and Cancer Institute, Sequencing Core facility (Gainesville, FL) for sequencing and to confirm the RT-PCR product. After sequencing, endotoxin- free plasmids containing the calnexin/GFP tagged gene were obtained and used to transfect murine Raw 264.7 macrophages with a nucleofection kit from Amaxa Inc. (Gaithersburg, MD). Sec22b constructs in pmVenus plasmid as well as J774 cells expressing these Sec22b/YFP proteins were described previously (Hatsuzawa *et al.,*

2006; Hatsuzawa *et al.,* 2009). The pmVenus/Sec22b/YFP plasmid and the pmVenus/YFP plasmids were purified and used to transfect Raw 264.7 cells.

Transfection (Nucleofection) of Raw264.7 Macrophages

Approximately 1.7x10 ^{7} cells at the exponential growth phase were put into 50 ml centrifuge tubes and centrifuged at 90xg (RCF) for 10 min. The supernatant was carefully discarded and 100 uL of the nucleofection solution was added to the cell pellet. Approximately 15 ug of DNA was transferred into cells, and the mixture was gently transferred into a 0.4 cm cuvette (Amaxa). Specific conditions for nucleofection of Raw 264.7 supplied by the manufacturer (Amaxa Inc.) was selected to electroporate the cells. After electroporation, 500 uL of DMEM complete medium pre-warmed at 37C was immediately added to the cuvette containing electroporated cells. They were transferred into a sterile cell culture dish holding 12 mm glass cover-slides, and incubated for 24h at 37C under 5% CO2 atmosphere, bef ore subsequent use.

Infection of Raw 264.7 Macrophages with *Leishmania* Parasites

Cell counting (macrophages and parasites)

Macrophages- The number of Raw264.7 macrophages was estimated using the Trypan blue assay. Under a safety hood, Raw 264.7 adherent cells in the culture flask or dish were gently detached with a sterile rubber policeman. The medium containing cells in suspension was diluted at a ratio of 1:1 with a Trypan-blue solution (0.4% Trypan bleu, 0.81% NaCl, 0.06% $K2HPO_4$, 0.02%, NaN_3) and incubated at RT for 5min. 10 ul the mixture was transferred on a hemocytometer, which was then placed under a 20x objective of a Leica Microsystems DM/LS microscope. The number of cells was counted using a cell counter device purchased from Fisher Scientific Inc.

Parasites- To estimate the number of *Leishmania*, 5ul of the parasite culture was diluted in 95 ul of 2% PFA (ratio of 1:20) and incubated at RT for at least 20 min to fix the parasites. 10ul of the diluted culture was transferred in a hemocytometer, and the parasites were counted under a 40x objective of a Leica Microsystems DM/LS microscope.

Plating Raw 264.7 macrophages on glass cover slips

The number of Raw264.7 macrophages with the tissue culture flask was estimated using a hemocytometer. The appropriate volume of cells in suspension was transferred on glass cover-slides inside a sterile cell culture dish. This dish was incubated at 37˚C with 5% CO2 for over -night, to allow cells to adhere on cover-slides.

Incubation of Raw264.7 macrophages with *Leishmania* parasites

Infections were carried out following standard protocols previously described (Kima, 2007; Ruhland and Kima, 2009). Raw 264.7 macrophages were seeded on 12mm round glass cover slips inside cell culture Petri dishes and incubated at 37˚C under 5% CO2 atmosphere. The next day, Raw 264.7 macrophages on cover slips were co-incubated with *Leishmania* parasites in RMPI complete medium at a 1:10 cell-to-parasites ratio. The infections were performed at either 37˚C (for *L. donovani*) or 34˚C (for *L. pifanoi* and *L. amazonensis*) under 5% CO2 atmosphere. In experiments in which the time course of ER recruitment to PVs was assessed, infections were initiated with 1:20 cell-to parasites ratio and then the cultures were washed at the first sampling time to remove free parasites. To stop the infection, cover-slips of infected macrophages were washed three times in 1x phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde (PFA)/1x PBS solution for at least 20min at room temperature.

Immunofluorescence Labeling and Imaging

Transfected or non-transfected cells infected on cover slips were fixed with 2% para-formaldehyde (PFA) for at least 20 minutes at room temperature and processed as previously described (Pham *et al.,* 2005). These cover slips were washed twice in 1xPBS and incubated in 50mM NH4Cl/1xPBS solution for 5 min. After two washes in 1x PBS, the preparations were blocked in 2% fat-free milk/1x PBS binding buffer (BB) supplemented with 0.05% saponin, as a cell membrane permeabilizing agent. Some cover slips were then incubated with one of the following primary antibodies to early endosome antigen 1 (EEA1), calnexin, GM130 (Santa Cruz Biotechnology, San Jose CA), BiP, (BD Biosciences, San José CA) and/or 1D4B reactive with late endosome and lysosomal associated membrane proteins (LAMP-1), (obtained from the Developmental Studies Hybridoma bank, Iowa City, IA). Cover slips were then incubated with the appropriate Alexa Fluor secondary antibodies (Molecular Probes Carlsbad CA) into which the nucleic acid dye 4',6-diamidino-2 phenylindole dihydrochloride (DAPI) had been added. Cover slips were mounted on glass slides with Prolong antifade (Molecular Probes). They were examined on a Zeiss Axiovert 200M microscope with a plan neofluar 100x/1.3 oil immersion objective. Images were captured with an AxioCam MRm camera controlled by AxioVision software. Images series over a defined z-focus range were acquired and processed with 3D deconvolution software supplied with AxioVision. An extended focus function was used to merge optical sections to generate images presented in the figures.

Immuno-Electron Microscopy

Infected macrophage cultures were spun down and resuspended in the growth medium supplemented with 0.15 M sucrose. The macrophages were gently spun down

and the cell pellet was rapidly frozen with a HPM 100 high-pressure freezer (Leica, Bannockburn, IL). The whole process from cell harvesting to freezing was completed within several minutes. The frozen cell samples were freeze-substituted in 0.1% uranyl acetate and 0.25% glutaraldehyde in acetone at -80^{o} C for 2 days. After freeze-substitution, the samples were warmed up to -50 ^{o}C over 30 hrs, washed 4 times with dry acetone at -50^{o} C, then, embedded in HM20 acrylic resin (Ted Pella, Inc., Redding, CA) at -50^{o}C. The resin was polymerized under ultraviolet light at -50^{o}C for 36 hrs. All the freeze-substitution, temperature transition, resin embedding, and UV-polymerization were carried out in the AFS2 automatic freeze substitution system (Leica, Bannockburn, IL). The HM20 embedded samples were sliced into 100 nm thin sections that were placed on nickel grids, which were then immunogold labeled with an anti-GFP antibody (1/50 dilution v/v) as described by Kang and Staehelin (Kang and Staehelin, 2008). The immunogold labeled sections were post-stained with an aqueous uranylacetate solution (2% w/v) and a lead citrate solution (26g L^{-1} lead nitrate and 35g L^{-1} sodium citrate) and examined with a Hitachi TEM H-7000 (Pleasanton, CA) operated at 80 kV

Ricin Experiment

Ricin pulse-chase experiment

Ice-cold fluorescein labeled *Ricinus communis* agglutinin II (RCA II) (called ricin here) purchased from Vector Laboratories Inc (Burlingame, CA) was added to cell cultures at a final concentration of 10ug/ml. The cultures were incubated on ice for 10 min to ensure the adherence of ricin molecules to the surface of cells. They were then incubated at 37ºC under 5% C02 atmosphere to initiate the internalization of ricin-infected macrophages. After a five min pulse excess ricin was removed by rinsing the culture twice with RPMI complete medium. Ricin was chased into cells at 37ºC under

5% C02 atmosphere; samples were collected at 30 min, 60 min, 3 h and 6 h respectively. In some experiments, cells were treated with 5ug/mL BFA (from Invitrogen) for 2 h prior to adding ricin. BFA was maintained in those cells for the duration of the experiment. In ricin experiments of infected cells, Raw 264.7 macrophages were incubated with either *L. donovani* or *L. pifanoi* parasites for at least 4 h at 34 or 37ºC with 5% C02, to generate mature PVs that would no longer interact with early endosomes containing ricin. The samples were washed three times in 1xPBS solution to remove non-internalized parasites. Growth medium was added to the infected cell culture and incubated for an additional 2hrs to ensure complete internalization of parasites attached to host cell membranes.

The infected cell cultures were then placed on ice for 10 min before incubation with ricin. Following the initiation of the ricin chase, infected macrophages on cover slips were collected at the same intervals listed above. Some samples of infected macrophages were incubated with BFA as described above. Samples from different time-points were processed in immunofluorescence assays as described above and cover slips were mounted on glass slides with Prolong antifade (Molecular Probes). They were examined on a Zeiss Axiovert 200M microscope with a plan neofluar 100x/1.3 oil immersion objective. Images were captured as described above.

Targeting strategy of ricin into *Leishmania* parasitophorous vacuoles

Green fluorescent Ricin molecules were targeted into LPVs following schematic design below.

Statistical Analysis

Data analysis and generation of graphs was performed on sigma-plot software. Each data point is the mean ± standard deviation from at least three observations. T-test was performed to assess differences; they were considered significant at a *P* value of ≤0.05.

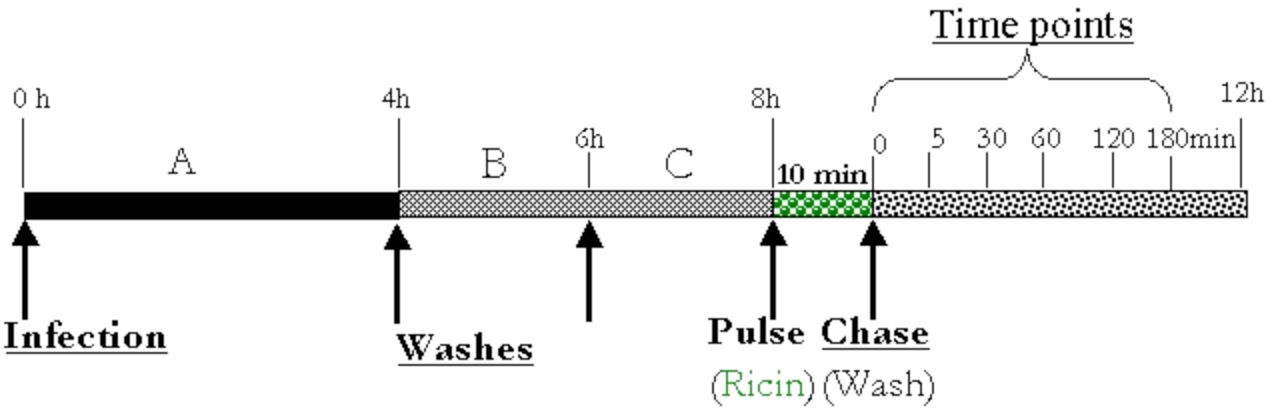


Figure 2-1. Design of ricin targeting experiment

Table 2-1. List of primers

Names	Sequences	Usage
NB02R NB02F		DNA external fragment
NB.Fi11:	*AAG GAA AAA A GCG GCC GC C ATG ATG GAC ATG ATG ATG ACG*	PCR - calnexin
NB.Ri11:	*AAG GAA AAA AA GCG GCC GCT CAC TCT CTT CGT GGC TTT CTG*	
Pcmv Forward3:	*CGC AAA TGG GCG GTA GGC GTG*	Sequencing
BGH Reverse3:	*TAG AAG GTT CAC AGT AGG*	

CHAPTER 3
HOST ENDOPLASMIC RETICULUM CONTRIBUTIONS TO THE LEISHMANIA PARASITOPHOROUS VACUOLE MEMBRANE

Introduction

The endoplasmic reticulum (ER) is a single continuous membrane-enclosed organelle made up of functionally and structurally distinct sub-domains: the nuclear envelope (NE) and the peripheral ER (pER) (Lavoie and Paiement, 2008). The pER is a network of tubules and sheets spread throughout the cytoplasm. ER functions include the translocation of secretory proteins, and integration of proteins into its membrane (English *et al.,* 2009). The ER is also the site of proteins folding and modification, synthesis of phospholipids and steroids, and storage and release of calcium ions (Ma and Hendershot, 2001; Meldolesi and Pozzan, 1998; Matlack *et al.,* 1998). Proteins that transit the secretory pathway in eukaryotic cells first enter the endoplasmic reticulum (ER) where they are folded, and in some cases assembled into multi-subunit complexes and glycosylated prior to transit to the Golgi compartment (Kaufman *et al.,* 2002). The ER is equipped with a 'quality control system', which is a surveillance mechanism that scans and targets misfolded proteins for recycling, permitting properly folded proteins to exit the ER *en route* to other intracellular organelles and the cell surface. Misfolded proteins are either retained within the ER lumen in complex with molecular chaperones or are directed toward degradation through the 26S proteasome in a process called ER-associated degradation (ERAD) or through autophagy.

Calnexin is a type I trans-membrane protein that primarily resides in the ER (Leach *et al.,* 2002; Schrag *et al.,* 2001). To investigate the recruitment of host ER components to the membrane of PVs that harbor *Leishmania* parasites, we engineered a DNA construct in which the calnexin gene sequence was fused to the c- terminus of a

green fluorescence protein (GFP) gene. The ER signal sequence was supplied in the vector but the ER retention signals of calnexin were included in the construct. This construct as well as the empty plasmid, pCMV/myc/ER/GFP, was used to transiently transfect Raw 264.7 macrophages. The distribution pattern of calnexin/GFP was assessed in transfected cells that were infected or not with *Leishmania* parasites.

Membrane fusion in macrophages and other eukaryotic cells is thought to be mediated by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Coppolino *et al.,* 2001). Although SNARE complexes are the critical machinery of membrane fusion, their role in determining the specific sites of fusion within the endomembrane system remains to be fully established (Okumura *et al.,* 2006). SNAREs are a group of membrane proteins that localize and function in the diverse endomembrane system, where docking and fusion between membranes take place. All SNAREs are characterized by homologous stretches of 60-70 amino acids referred to as SNARE motifs, which are adjacent to the membrane anchor domains. SNAREs can be classified into subgroups: the syntaxin and SNAP-25 families contain a conserved glutamine at a central position called the “0” layer of the SNARE motif and are therefore called Q-SNAREs, and the VAMP (also called synaptobrevin) family contains a conserved arginine at this position and are therefore called R-SNAREs (Jahn and Scheller, 2006).

In this study, Raw246.7 macrophages were transfected with DNA constructs containing ER-SNAREs molecules, Sec22b and D12 (R-SNAREs), or Syntaxin-18(Q-SNARE), fused to yellow fluorescence protein. These transfected macrophages expressing each SNARE molecule listed above were infected with either parasites or

ZymosanA. Samples were processed in immuno-fluorescence assays and analyzed by fluorescence microscopy to assess the recruitment of host ER molecules to *Leishmania* PVs during the course of infection. The recruitment of ER components to *Leishmania* PV's membranes was confirmed by immuno-EM analysis of J774 macrophages stably expressing the Sec22b/YFP fusion gene.

Results

Recruitment of Calnexin and LAMP-1 to the *Leishmania* PV Membrane

Calnexin/GFP expression in Raw 264.7

Prior to analyzing the recruitment of calnexin to *Leishmania* PVs, a series of experiments were performed in order to determine whether the GFP-fusion to calnexin had achieved the natural distribution pattern of calnexin in cells. In cells transfected with the empty vector, pCMV/ER/GFP (appendix A, Figure A), the GFP signal was diffuse and non specific (Figure 3-1A). However, in Raw264.7 macrophages expressing the calnexin/GFP chimeric protein, the GFP distribution was very specific; intense and high labeling of GFP around the nucleus (on the nuclear membrane), and a mesh-like pattern in the cytosol (Figure 3-1B). This GFP distribution in cells transfected with the calnexin/GFP vector, did not display any co-localization with the fluorescent signal of LAMP-1, a marker of late endosome and lysosome compartments (Figure 3-1B, merged chanels). However, the staining of these calnexin/GFP expressing cells with antibodies against the endogenous calnexin, showed a complete co-localization between the chimeric and the endogenous calnexin (Figure 3-1C). The calnexin/GFP distribution (Figure 3-1B) appeared identical to the calnexin sub-cellular distribution in wild type cells stained with anti-calnexin antibodies (Figure 3-1D).

Recruitment of calnexin/GFP and LAMP-1 to *L. pifanoi* PV membranes

After validating that calnexin/GFP localized in ER compartments within Raw264.7 macrophages, calnexin/GFP expressing cells were infected with *L. pifanoi* parasites. Calnexin/GFP expressing Raw264.7 macrophages displayed a high level of GFP and LAMP-1 signals on the membrane of PVs containing *L. pifanoi*; these two markers only co-localized on *Leishmania* PV membranes within these transfected cells (Figure 3-2). PVs that harbor *L. pifanoi* parasites became progressively distended over the 24h time course (Figure 3-2) as the parasites within them replicated. Similar observations were made in cells infected with *L. donovani* parasites. *L. donovani* infected cells had many PVs over time, and each PV had a tight lumen that generally contained only a single parasite (Figure 3-3). *L. donovani* PVs exhibited a high level of GFP and LAMP-1 signals on their membrane, where both markers only co-localized in host cells (Figure 3-3).

Enumeration of calnexin/GFP and LAMP-1 recruitment to *L. pifanoi* PVs

The proportion of PVs that had their membrane positively stained with the calnexin/GFP signal was estimated. The data showed that at 15 min post infection, over 90% of all LPV harboring live *L. pifanoi* parasites had their membrane positively stained with the calnexin/GFP marker (Figure 3-4). This calnexin/GFP rate was maintained through out the course of a 24h infection. In the same samples, the proportion of LPVs that were positively stained with LAMP-1, was approximately 60% at 15 min, and over 90% after about 2h and through out the rest of the 24h infection course.

The pattern of calnexin recruitment to *Leishmania* PVs was compared to its recruitment to phagosomes that harbor *Zymosan* particles or dead parasites. When the transfected cells were incubated with heat killed parasites, the proportion of phagosome

membranes positively stained with the calnexin/GFP signal started at about 65% at 15min, decreased to 30% at 1h, and returned to the initial level at 65% at 4h. After the 6h post infection, the heat-killed parasites were completely destroyed and their PVs cleared out within infected cells (Figure 3-4). Moreover, in calnexin/GFP expressing Raw264.7 macrophages that were fed with *Zymosan* particles, the proportion of phagosome membranes positively stained with calnexin/GFP marker was approximately 23% at 15min, and 20% after an hour and through out the rest of the infection (Figure 3-4). In the same sample, the proportion of *Zymosan*-containing phagosome membranes intensely labeled with the LAMP-1 signal was about 55% at 15min, and more than 90% after an hour and during the rest of the infection (not shown on the graph).

Recruitment of calnexin/GFP and LAMP-1 to *L. donovani* PV membranes

L. donovani parasites cause the visceral leishmaniasis in their rather than the skin related form of the disease generated by members of the *L. mexicana* complex (*L. pifanoi*, *L. amazonensis*, and *L. mexicana*). To have a broad understanding of the scheme of *Leishmania* parasites interaction with the host ER, we also investigated the recruitment of calnexin/GFP to PVs harboring *L. donovani* parasites. Our data showed that at 15 min post infection, over 90% of all LPVs harboring live *L. donovani* parasites had their membrane positively stained with the calnexin/GFP marker (Figure 3-5). This recruitment rate of the calnexin/GFP marker was relatively retained through out the course of 24h infection. The proportion of LPV that was positively stained with LAMP-1 was approximately 60% at 15 min, and over 90% after about 2h and through out the rest of the 24h infection course (Figure 3-5).

When the transfected cells were incubated with heat killed *L. donovani* parasites, the proportion of phagosome membranes positively stained with the calnexin/GFP

signal started at about 65% at 15min, decreased to 40% at 1h, and returned to the initial level at 65% at 2h. This rate was maintained for 4h, but after 6h all the heat-killed parasites were completely destroyed and their PVs cleared out within infected cells (Figure 3-5).

Recruitment of Host ER Membrane-Associated SNAREs the LPV Membrane

To obtain additional evidence on the interactions between PVs and the host cell's ER, we examined the recruitment of ER membrane associated SNAREs; Sec22b, D12, and Syntaxin18 to *Leishmania* PVs. These SNARE proteins have been reported to mediate ER vesicles transport and fusion in eukaryotic cells (Aoki *et al.,* 2008; Verrier *et al.,* 2008). Therefore, their recruitment to PVs would provide some insight into the mechanism by which ER molecules are recruited to PVs. These genes were inserted into the pmVenus vectors. The construction of the pmVenus vectors that encode YFP fused to the N-terminus of Sec22b, D12, or Syntaxin18 was described by Hatsuzawa and collaborators (Hatsuzawa *et al.,* 2006). Stable lines of J774 cells expressing these molecules and transiently transfected Raw 264.7 were first assessed for appropriate expression before they were infected with *Leishmania* parasites.

ER-SNARE (Sec22b, D12, and Syntaxin18)/YFP expression in Raw264.7

Even though the expression pattern of Sec22b/YFP, D12/YFP, and Syntaxin18/YFP was previously described (Hatsuzawa *et al.,* 2006), in this study, a series of experiments were performed in order to determine what was the distribution of the YFP-SNARE fusion molecules in our system. The data showed that in cells transfected with the empty vector, pmVenus, the expression pattern of YFP was diffuse and non specific (Figure 3-6A). Moreover, there was also no YFP signals on *Leishmania* PV membranes in these transfected control cells (Figure 3-6B).

However, in Raw264.7 macrophages transfected with Sec/YFP, D12 or Syntaxin18, an intensely high level of YFP was observed around the cell's nucleus or the nuclear membrane, and a mesh-like YFP pattern in the cytosol (Figure 3-6C). The distribution pattern of Sec22b/YFP, D12/YFP, or Syntaxin18/YFP observed in this study was consistent with their endogenous distribution pattern, which is representative of the ER distribution in mammalian cells. This was confirmed by the Sec22b/YFP distribution, which overlapped with the pattern of endogenous BiP (Figure 3-6D) and calnexin (Figure 3-6E), but not with the endogenous late endosomes and lysosomes marker, LAMP-1 (Figure 3-6C).

Raw 264.7 macrophages expressing Sec22b/YFP, D12/YFP, or Syntaxin18/YFP were then infected with *Leishmania* parasites and sampled over a 48h infection period. These infected cells were also stained with anti-LAMP-1 antibodies. The LAMP-1 distribution co-localized with the ER SNARE (Sec22b, D12, or Syntaxin18)/YFP signal only on the PV membrane containing at least a parasite (Figures 3-7 to 3-10). Like with cells expressing calnexin/GFP, distinctive morphological features of the PVs form by parasites of both *L.donovani* and *L. mexicana* complexes were observed in cells expressing ER SNARE (Sec22b, D12, or Syntaxin18). Parasites of the *L. mexicana* complex (*L. pifanoi* and *L. amazonensis*) form a primary PV with a large lumen (Figure 3-7A), which size significantly increased over time to accommodate multiple parasites (Figure 3-7B; Figure 3-9; Figure 3-10) generated after replication.

During our study, *L. donovani* parasites were only available when we had access to purified pmVenus and pmVenus/Sec22b/YFP vectors. For this reason, only the recruitment of Sec22b/YFP, but not D12/YFP or Syntaxin18/YFP, to PV membranes

containing *L. donovani* parasites was investigated. In Sec22b/YFP expressing cells that were infected with *L. donovani* parasites, the majority of the primary PVs had a tight lumen with a single parasite (Figure 3-8A); over time these host cells were fill with many secondary individual PVs (Figure 3-8B).

Enumeration of SNARE/YFP recruitment to *L. pifanoi and L. amazonensis* PV membranes

The proportion of *Leishmania* PVs that had their membrane positively stained with ER SNARE (Sec22b, D12, or Syntaxin18)/YFP, as well as with the LAMP-1 marker was estimated through out a period of 48h infection. Our data showed that, in transfected cells infected with live *L. pifanoi* parasites, the proportion of PVs positive for Sec22b/YFP signals was approximately 90% at 15 min and throughout the course of the 48h infection (Figure 3-11). Similar proportion was observed with all the transfected cell types (Sec22b, D12, and Syntaxin18)/YFP infected with *L. amazonensis* (Figure 3-12).

Recruitment of Sec22b/YFP to *L. donovani* PV membranes

The recruitment of Sec22b/YFP to *L. donovani* PVs was also determined. At 15 min post infection, over 90% of all LPVs harboring live *L. donovani* parasites had their membrane positively stained with the Sec22b/YFP marker (Figure 3-11). This high level of Sec22b/YFP signal was maintained on the membrane of approximately 90% of PVs, throughout the course of infection. The profile of LAMP-1 recruitment on *Leishmania* PVs in ER-SNARE transfected cells over time was identical of that in cell transfected with calnexin/GFP described above.

Electron Microscopy (EM) Analysis

To further confirm that ER membrane molecules are recruited to and displayed on the membrane of *Leishmania* PVs, we performed immuno-EM analyses on cells

expressing Sec22b/YFP. The stable J774 cell line expressing Sec22b/YFP was used since all cells expressed the chimeric molecule, as oppose to the transiently expressed cell samples, in which only a portion of the cells contained expressed proteins. YFP distribution in these cells as well as in cells transfected with the pmVenus/YFP was evaluated with an antibody to GFP (see Materials and Methods section), which was followed with a secondary antibody conjugated to gold particles.

Distribution of GFP/gold particles in control samples

In Raw264.7 transiently transfected with the pmVenus plasmid, gold particles had a random distribution including in the cell nucleus, cytosol, and various organelles or vesicles (Figure 3-13A). This random distribution of gold particles was also observed in pmVenus transfected J774 cells (controls) that were infected with *Leishmania* parasites (Figure 3-13B).

Distribution of Sec22b/GFP/gold particles in macrophages

In transiently transfected Raw264.7 macrophages expressing Sec22b/YFP, the distribution of gold particles were restricted to the cell nuclear membrane, and to ER compartments within the cell cytosol (Figure 3-14A). This Sec22b/YFP distribution seems therefore to target the two sub-domains of the ER in eukaryotic cells; the nuclear ER, and the peripheral ER. When Sec22b/YFP expressing cells were infected with *Leishmania* parasites (*L. donovani*), the specificity of the gold particle distribution was extended to membrane delimiting both primary (Figure 3-14B) and secondary PVs or multiples PVs (Figure 3-14C).

These results complement the evidence obtained from fluorescence assays that had shown that the host cell ER membrane associated molecules are on the PV membrane.

Discussion and Conclusion

Phagocytosis enables professional phagocytes to internalize large particles, while their total cell surface remains relatively constant. Numerous studies have considered the biogenesis and maturation of the new membrane-bound structures that harbor large particles; nascent phagosomes sequentially interact with early endosomes, late endosomes and lysosomes to mature into phagolysosomes (Harrison *et al.,* 2003; Vieira *et al.,* 2003). The evidence prevailing to date suggests that *Leishmania* parasites during the process of phagocytic internalization are directed into PVs that follow a maturation scheme similar to that of phagosomes harboring inert particles.

The focus of our studies was to assess the evolving composition of the PV membrane; specifically, their acquisition of ER components overtime. We generated a DNA construct in which calnexin gene was fused to GFP, and demonstrated that the distribution of the chimeric proteins (calnexin/GFP, Sec22b/YFP, D12/YFP and Syntaxin18/YFP) was specific and identical to that of their wild type forms. First, these fusion proteins were validated as appropriate markers to trace ER distribution in macrophages fluorescence and immuno-EM approaches. This result confirmed previous studies in which fusion of Sec22b, D12 and Syntaxin18 tagged with an YFP molecule, did not affect their distribution (Hatsuzawa et al., 2006).

A high proportion (more than 90%) of PVs containing *Leishmania* parasites had their membrane intensely labeled with ER markers at a very early time point, 15min. It

strongly suggests that ER components such as calnexin (Muller-Taubenberger et al., 2001) may be recruited to the site of phagocytosis; their fusion to the phagocytic-cup membrane to facilitate parasites entry in macrophage may be mediated by ER-SNAREs such as Sec22b, D12, and Syntaxin18. Our results corroborated previous studies that suggested ER- SNAREs such as Sec22b (Becker et al., 2005), syntaxin18 and D12 (Hatsuzawa 2006, 2009) may play a significant role during phagocytosis. Our data showed a gradual recruitment of the LAMP-1 marker on *Leishmania* PV membranes before an hour, which confirmed the fact that PVs maturation is similar to phagosomes maturation that undergoes a sequential and orderly process. It respectively involved the fusion of nascent phagosomes with early endosomes at about 5min; late endosomes at about 15min and LAMP-1 after an hour.

Although we did not provide EM data illustrating or confirming the co-localization LAMP1 and Sec22b or other ER marker, our results strongly demonstrated recruitment of ER molecules. Both our fluorescence and immuno-EM data indicate that in addition to interactions with late endocytic pathway vesicles, *Leishmania* PVs interact continuously with the host ER and acquire both membrane and membrane associated molecules. Thus, the biogenesis and maturation scheme of *Leishmania* PVs differs from the maturation of phagosomes that harbor inert particles; PVs are hybrid compartments.

Both membranous and luminal contents of endocytic compartments are believed to be acquired by mechanisms including local or focal exocytosis, which involve secretion and fusion of endosomes and lysosomes to the site of phagosome formation (Bajno *et al.,* 2000; Tapper *et al.,* 2002). The presence of endogenous ER resident proteins such as calnexin and calreticulin on phagosome membranes has been

reported (Gagnon *et al.,* 2002; Lee *et al.,* 2010; Houde *et al.,* 2003). However, the mechanisms by which these molecules access phagosomal compartments are still being debated (Gagnon *et al.,* 2002; Huynh and Grinstein, 2007). Suppression of genes responsible for both calnexin and calreticulin expression significantly hindered the process of phagocytosis in Dictyostelium sp (Muller-Taubenberger *et al.,* 2001). Other studies have also shown that Sec22b and syntaxin 18 (ER related SNAREs) form complexes with Sso1-Sec9C (PM associated SNAREs) to regulate the fusion between ER and PM under the phagocytic cup (Becker *et al.,* 2005; Hatsuzawa *et al.,* 2006). Phagosome proteomic analysis revealed the abundance of ERS-24/Sec22b molecules at the early stage of phagosomes biogenesis (Gagnon *et al.,* 2002). In the studies presented above we observed that at the earliest sampling time (15 min) the majority of nascent PVs displayed ER molecules, which suggested that these molecules were delivered / recruited at the earliest point of PV formation.

As we noted, the situation with phagosomes containing dead parasites was intermediate between the observations of Zymosan particles and live *Leishmania* parasites. Differences between the Zymosan phagosomes that recruited ER molecules and those that were devoid of these molecules were not readily obvious. Since a higher proportion of PVs recruited ER marker molecules as compared to Zymosan phagosomes and phagosomes that harbor dead particles, this suggested that there is a unique interaction between *Leishmania* and host cells that promotes their PVs association with the ER. The observations of ER-PV interactions are in part in agreement with a recent report that found that a sub-population of *L. donovani* promastigotes in neutrophils selectively develop in ER-like compartments (Glucose 6-

phosphatase and calnexin positive) that are non-lytic and devoid of lysosomal properties (Gueirard *et al.,* 2008). Unlike the findings in that study that parasites in compartments with lysosomal properties were dead, our studies show that in macrophages, live *Leishmania* parasites reside in compartments that contain both ER-like and lysosomal properties. The differences in PV characteristics observed in both of these studies might suggest that PV development is determined in part by the characteristics of the host cell.

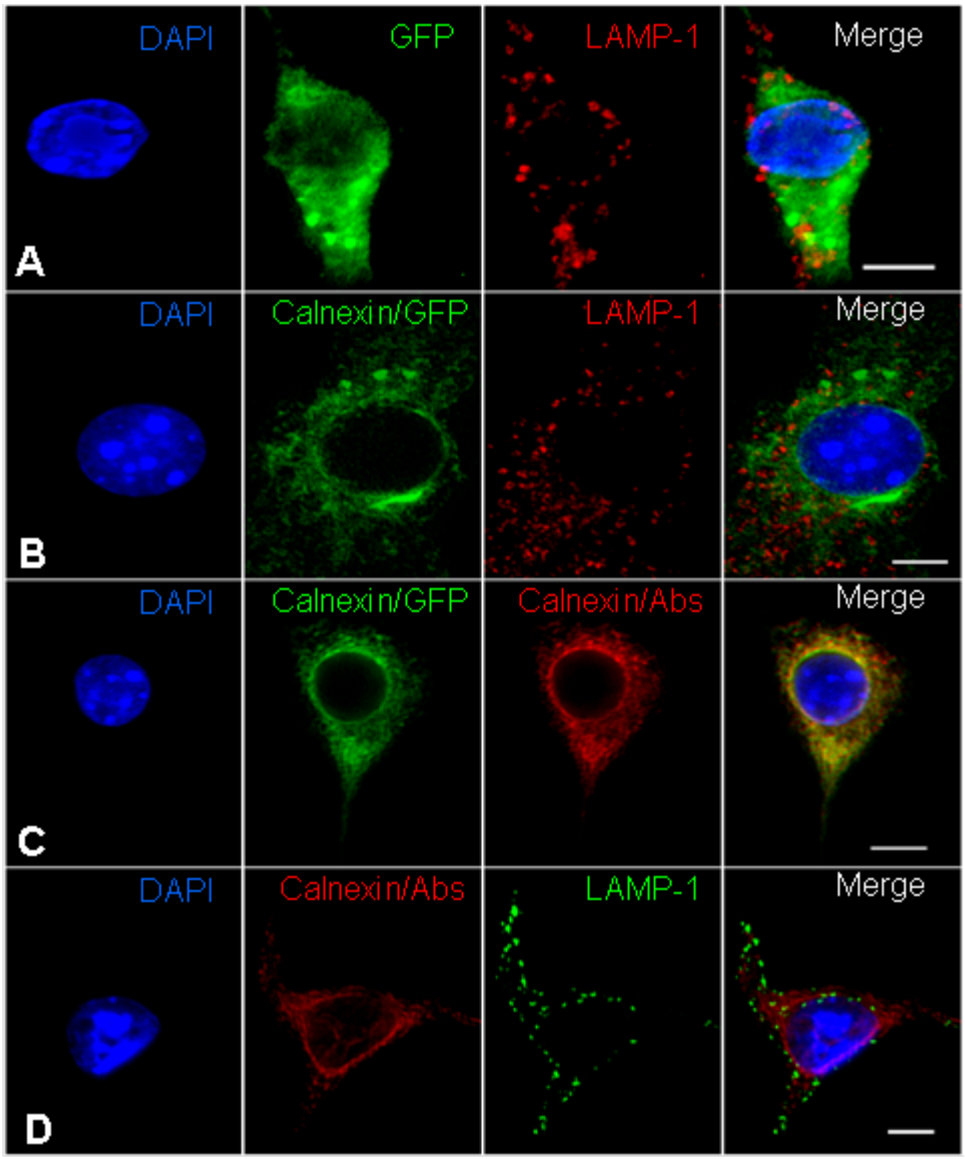


Figure 3-1. Distribution of GFP and calnexin/GFP in Raw264.7 macrophages. Murine raw264.7 macrophages were transfected either with the empty plasmid containing the GFP alone (pCMV/ER/GFP) (A) or the DNA vector in which calnexin was tag to a GFP marker (pCMV/calnexin/GFP) (B). These transfected cells were then processed in immuno-fluorescence assays (IFA) with 4',6-diamidino-2-phenylindole (DAPI) to reveal nuclei (blue) and anti-LAMP 1 antibody to label late endosomes and lysosomes (red). In addition, some cells transfected with pCMV/Calnexin/GFP were probed with DAPI and an antibody against an endogenous ER protein, BiP (C). Wild-type Raw264.7 cells were stained DAPI and an antibody against the endogenous calnexin (D), an important ER marker. Images were captured with a Zeiss Axiovert 200M fluorescence microscope controlled with Axiovision software. Optical sections from a Z-series were combined to create the images shown. Scale bar = 5 micrometers (5µm).

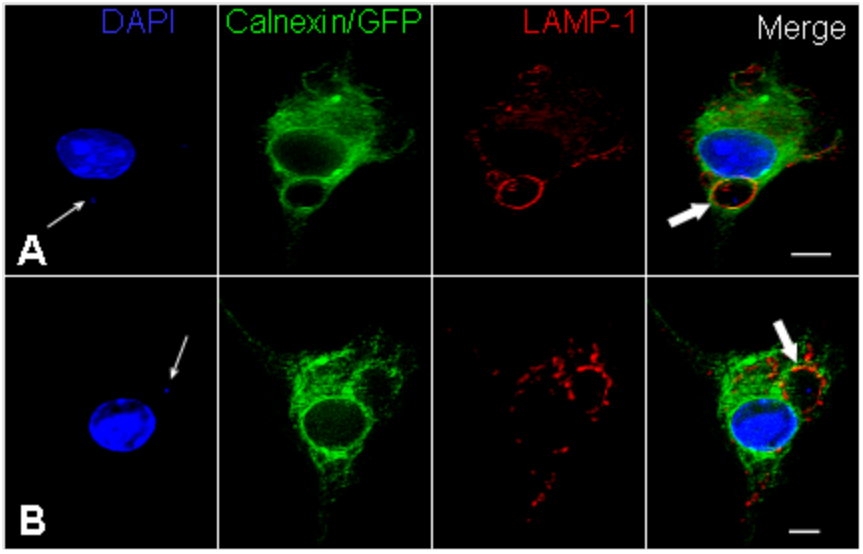


Figure 3-2. The recruitment of calnexin/GFP to *L. pifanoi* PVs in Raw264.7 macrophages. Murine Raw264.7 macrophages transfected with the pCMV/calnexin/GFP plasmid were infected with *L. pifanoi* amastigotes for 2 h (A) or 12 h (B), then processed in IFA with DAPI (blue) and anti-LAMP 1 antibody (red). Thin white arrows point to parasites while thick white arrows show the PV membrane. Images were captured with a Zeiss Axiovert 200M fluorescence microscope controlled with Axiovision software. Optical sections from a Z-series were combined to create the images shown. Scale bar = 5 micrometers (5µm).

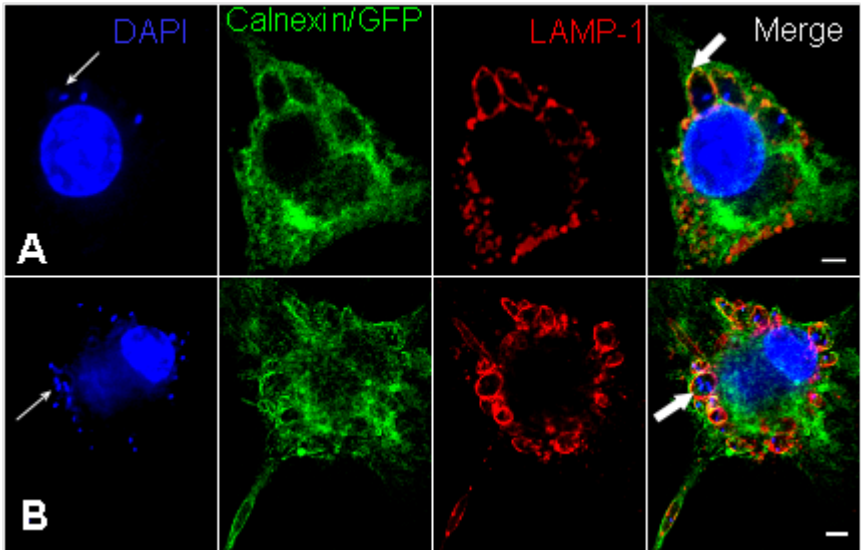


Figure 3-3. The recruitment of calnexin/GFP to *L. donovani* PVs in Raw264.7 macrophages. Murine Raw264.7 macrophages were transfected with the pCMV/calnexin/GFP construct, and infected with *L. donovani* amastigotes for 2 h (A) or 24 h (B). The samples were processed in IFA with DAPI to reveal nuclei (blue) and anti-LAMP 1 antibody to label late endosomes and lysosomes (red). Thin white arrows point to representative parasites while thick white arrows show the PV membrane. Images were captured with a Zeiss Axiovert 200M fluorescence microscope controlled with Axiovision software. Optical sections from a Z-series were combined to create the images shown. Scale bar = 5 micrometers (5µm).

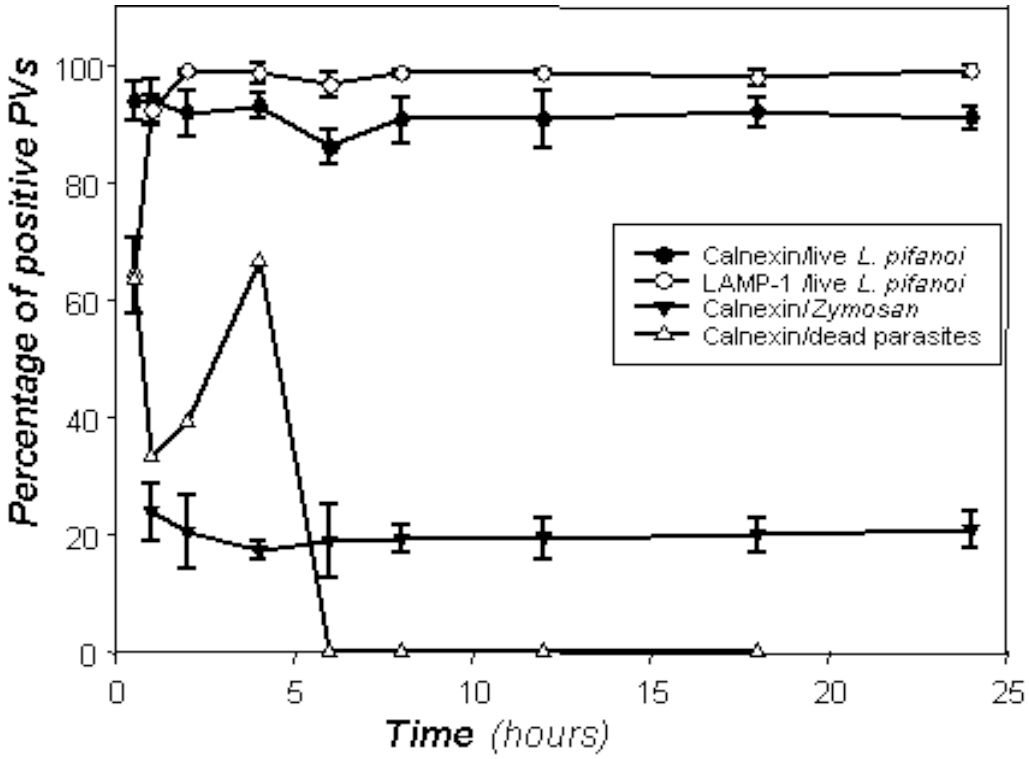


Figure 3-4. Proportion of calnexin and LAMP-1 recruitment to *L. pifanoi* PVs. The proportion of PVs harboring live *L. pifanoi* parasites that are positively displaying calnexin/GFP (black circles) or LAMP-1 (open circles) during a 24 h course of infection were enumerated and plotted. Also included is the proportion of Zymosan phagosomes displaying calnexin/GFP (black triangles), the proportion of dead *L. pifanoi* parasites (Open triangles). Each data point is the mean value from at least three experiments in which at least 150 vacuoles were counted per experiment. Graphs were made using the Sigma plot software. The difference between the proportions of PVs that recruited calnexin as compared to Zymosan phagosomes was tested in a paired t-test at all time points. The p-value in each case was <0.001.

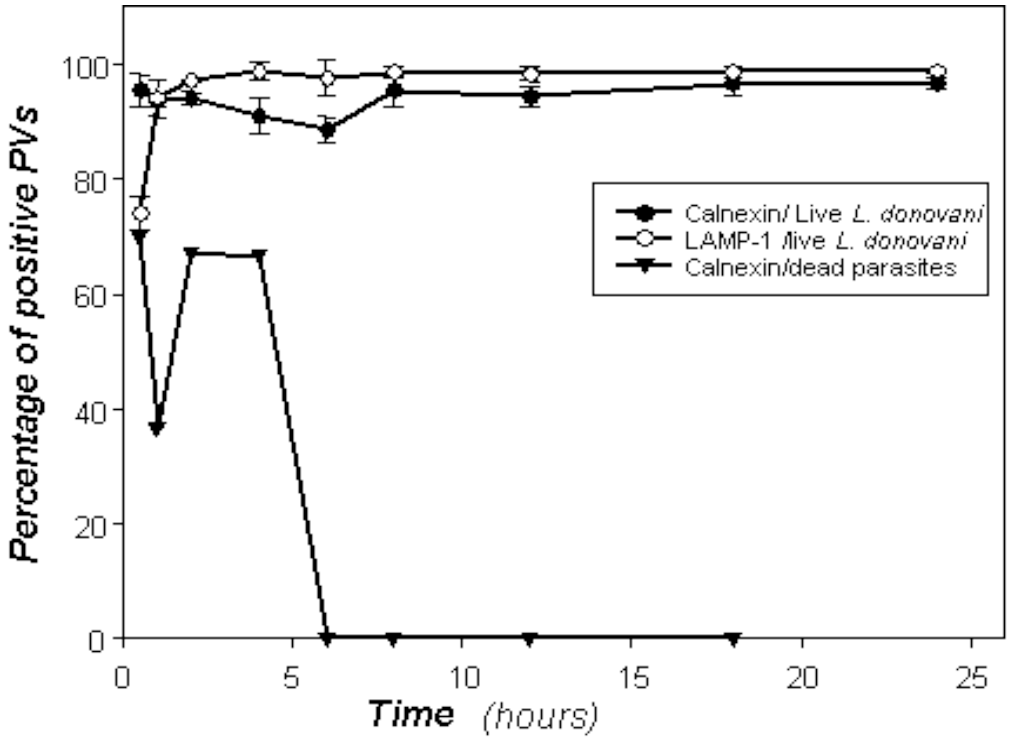


Figure 3-5. Proportion of calnexin and LAMP-1 recruitment to *L. donovani* PVs. The proportion of PVs harboring live L.donovani parasites that are positively displaying calnexin/GFP (black circles) or LAMP-1 (open circles) during a 24 h course of infection were enumerated and plotted. Also included is the proportion of Zymosan phagosomes displaying calnexin/GFP (black triangles), the proportion of dead *L. donovani* parasites (Open triangles). Each data point is the mean value from at least three experiments in which at least 150 vacuoles were counted per experiment. Graphs were made using the Sigma plot software. The difference between the proportions of PVs that recruited calnexin as compared to Zymosan phagosomes was tested in a paired t-test at all time points. The p-value in each case was <0.001.

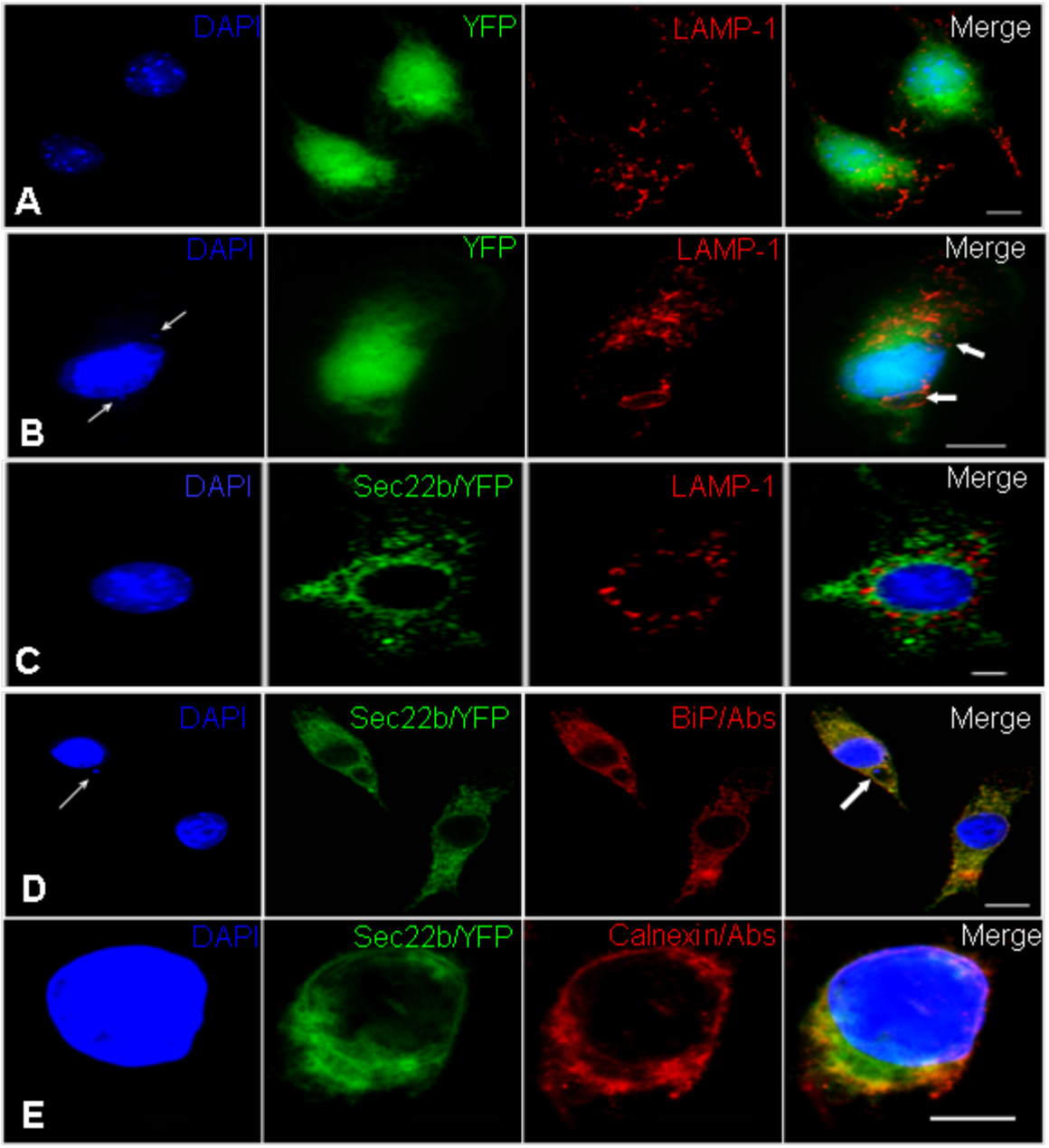


Figure 3-6. Distribution of YFP and Sec22b/YFP in Raw264.7 macrophages. Murine Raw264.7 macrophages were transfected either with the empty plasmid containing the YFP alone (pmVenus) (A -B) or the DNA vector in which calnexin was tagged to a YFP marker (pmVenus/Sec22b/YFP) (C-E). Some cells transfected with the pmVenus plasmid were infected with *Leishmania* parasites (B). These transfected cells were processed in IFA with DAPI to reveal nuclei (blue) and anti-LAMP 1 antibody to label late endosomes and lysosomes (red)(A-C). In addition, portion of cells transfected with the Sec22b/YFP vector probed with DAPI and antibodies against an endogenous ER protein, BiP (D) or calnexin (E). Images were captured with a Zeiss Axiovert 200M fluorescence microscope controlled with Axiovision software. Optical sections from a Z-series were combined to create the images shown. Scale bar = 5 micrometers (5µm).

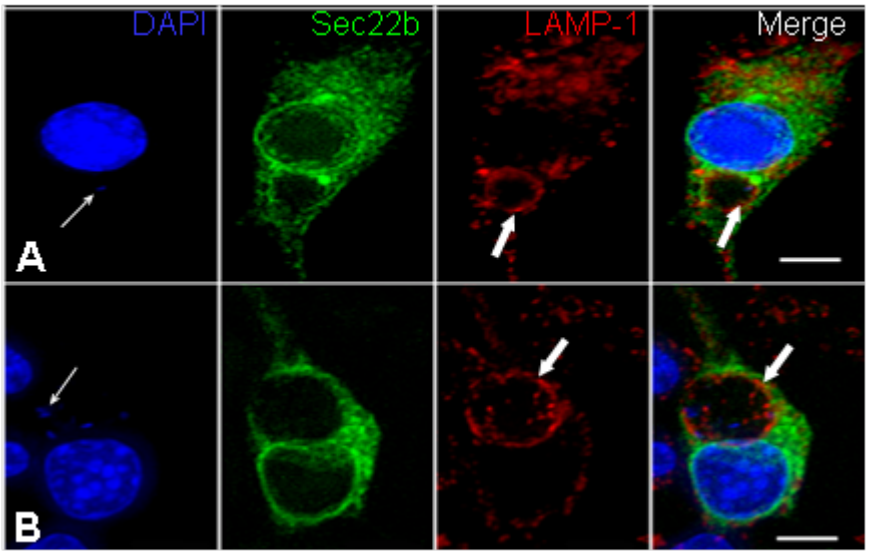


Figure 3-7. The recruitment of Sec22b/YFP to *L. pifanoi* PVs in Raw264.7 macrophages. Murine Raw264.7 macrophages transfected with the Sec22b/YFP plasmid were infected with *L. pifanoi* amastigotes for 2 h (A) or 48h (B), then processed in IFA with DAPI (blue) and anti-LAMP 1 antibody (red). Thin white arrows point to parasites while thick white arrows show the PV membrane. Images were captured with a Zeiss Axiovert 200M fluorescence microscope controlled with Axiovision software. Optical sections from a Z-series were combined to create the images shown. Scale bar = 5 micrometers (5µm).

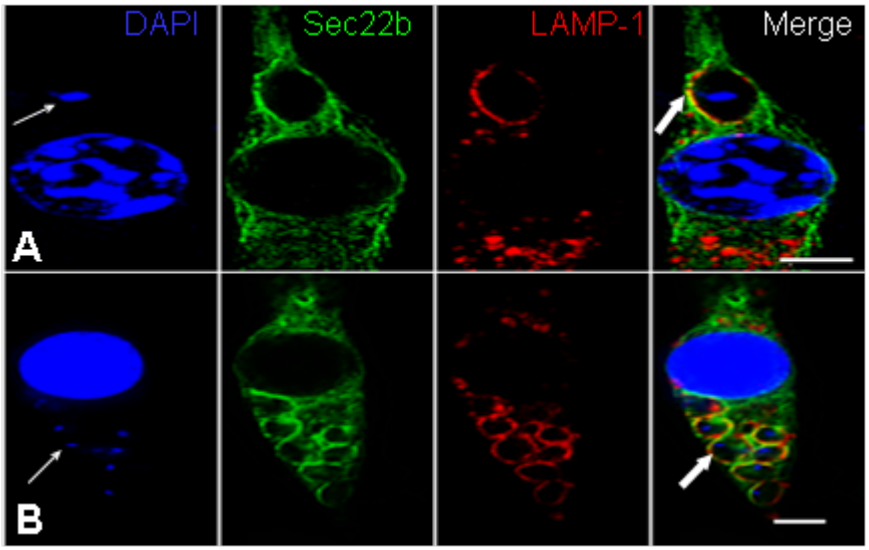


Figure 3-8. The recruitment of Sec22b/YFP to *L. donovani* PVs in Raw264.7 macrophages. Murine Raw264.7 macrophages transfected with the Sec22b/YFP plasmid were infected with *L. donovani* amastigotes for 2 h (A) or 48h (B), then processed in IFA with DAPI (blue) and anti-LAMP 1 antibody (red). Thin white arrows point to parasites while thick white arrows show the PV membrane. Images were captured with a Zeiss Axiovert 200M fluorescence microscope controlled with Axiovision software. Optical sections from a Z-series were combined to create the images shown. Scale bar = 5 micrometers (5µm).

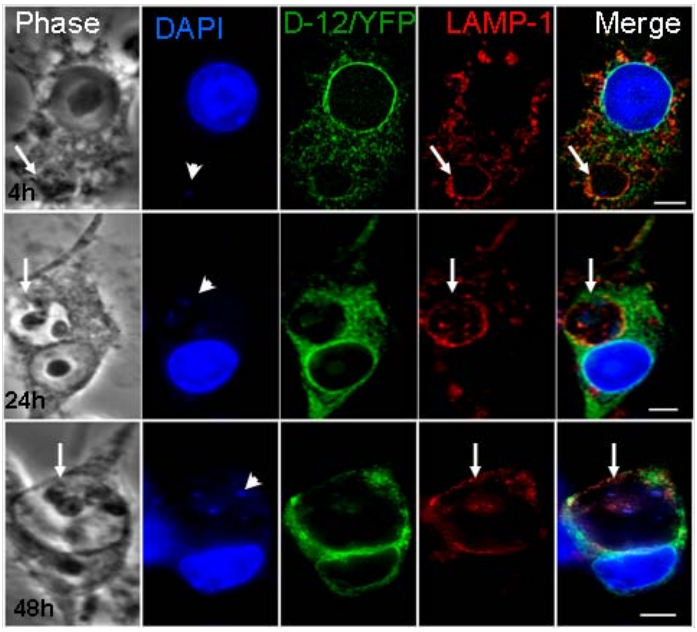


Figure 3-9. D12/YFP expressing Raw264.7 macrophages infected with *L. amazonensis*. Murine Raw264.7 macrophages transfected with the D12/YFP plasmid were infected with *L. amazonensis* promastigotes for 4h (A), 24h (B) or 48h (C), then processed in IFA with DAPI (blue) and anti-LAMP 1 antibody (red). The arrow-head points to parasites while the full arrows show the PV membrane. Images were captured with a Zeiss Axiovert 200M fluorescence microscope controlled with Axiovision software. Optical sections from a Z-series were combined to create the images shown. Scale bar = 5 micrometers (5µm).

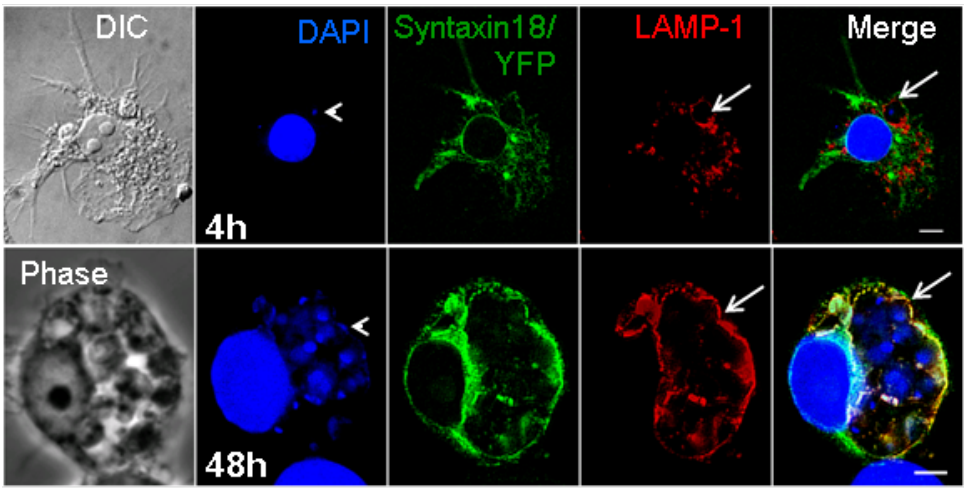


Figure 3-10. Syntaxin18/YFP expressing Raw264.7 macrophages infected with *L. amazonensis*. Murine Raw264.7 macrophages transfected with the Syntaxin18/YFP plasmid were infected with *L. amazonensis* promastigotes for 4h (A) or 48h (B), then processed in IFA with DAPI (blue) and anti-LAMP 1 antibody (red). The arrow-head points to parasites while the full arrows show the PV membrane. Images were captured with a Zeiss Axiovert 200M fluorescence microscope controlled with Axiovision software. Optical sections from a Z-series were combined to create the images shown. Scale bar = 5 micrometers (5µm).

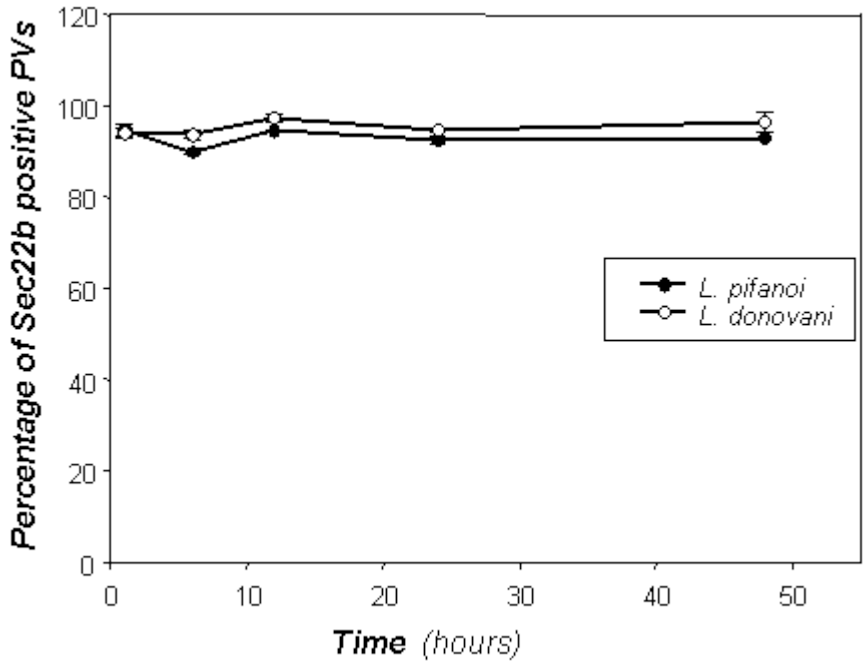


Figure 3-11. Recruitment of Sec22b to *Leishmania* PVs. Murine Raw264.7 macrophages transfected with Sec22b/YFP were co-incubated with *L. pifanoi* (close circle) and *L. donovani* (open circle) amastigotes at a ratio of 1:10 (one macrophage to five parasites). The cultures were washed twice with sterile 1xPBS 2h after the infection. Samples were removed from the culture and fixed in 2% PFA at several time-points (2, 4, 12, 24, and 48h). Fixed samples were processed by IFA, and analyzed with a Zeiss fluorescence microscope. The proportion of *L. pifanoi* (close circle) and *L. donovani* (open circle) PVs that positively recruited Sec22b/YFP during a 48 h course of infection was estimated and plotted. More than 150 PVs containing live *Leishmania* parasites were screened. The experiments were repeated at least three times, graphs of the mean values were made using the Sigma plot software.

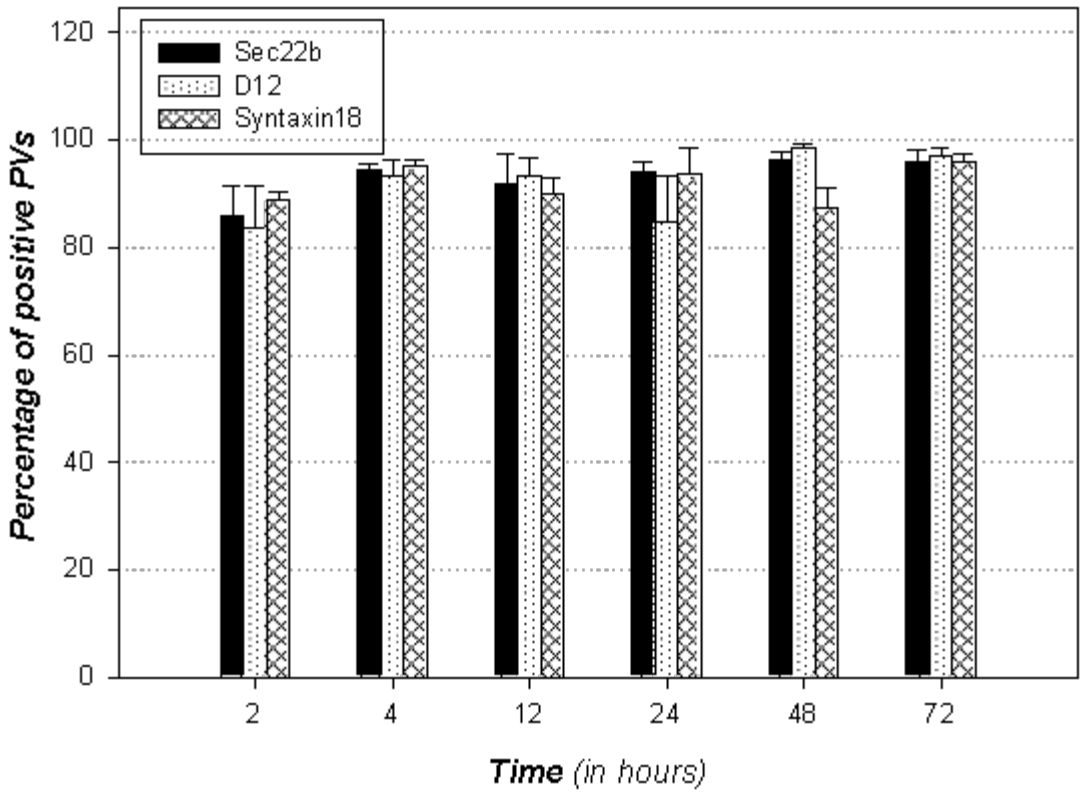


Figure 3-12. Endoplasmic reticulum membrane associated SNAREs recruitment to *L. amazonensis* parasitophorous vacuoles during infection. Murine Raw264.7 macrophages transfected with Sec22b/YFP, D12/YFP or Syntaxin18/YFP were co-incubated with *L. amazonensis* promastigotes at a ratio of 1:10 (one macrophage to five parasites). The cultures were washed twice with sterile 1xPBS 2h after the infection to remove free and unattached parasites in the culture. After washing, fresh medium was added to the cultures, which were returned in the incubator. Samples were removed from the culture and fixed in 2% PFA at several time-points (2, 4, 12, 24, 48, and 72h). Fixed samples were processed through immunofluorescence assays, and analyzed with a Zeiss fluorescence microscope. The proportion of *Leishmania* PVs that were positively stained with the YFP was estimated through out the course of 72h infection.

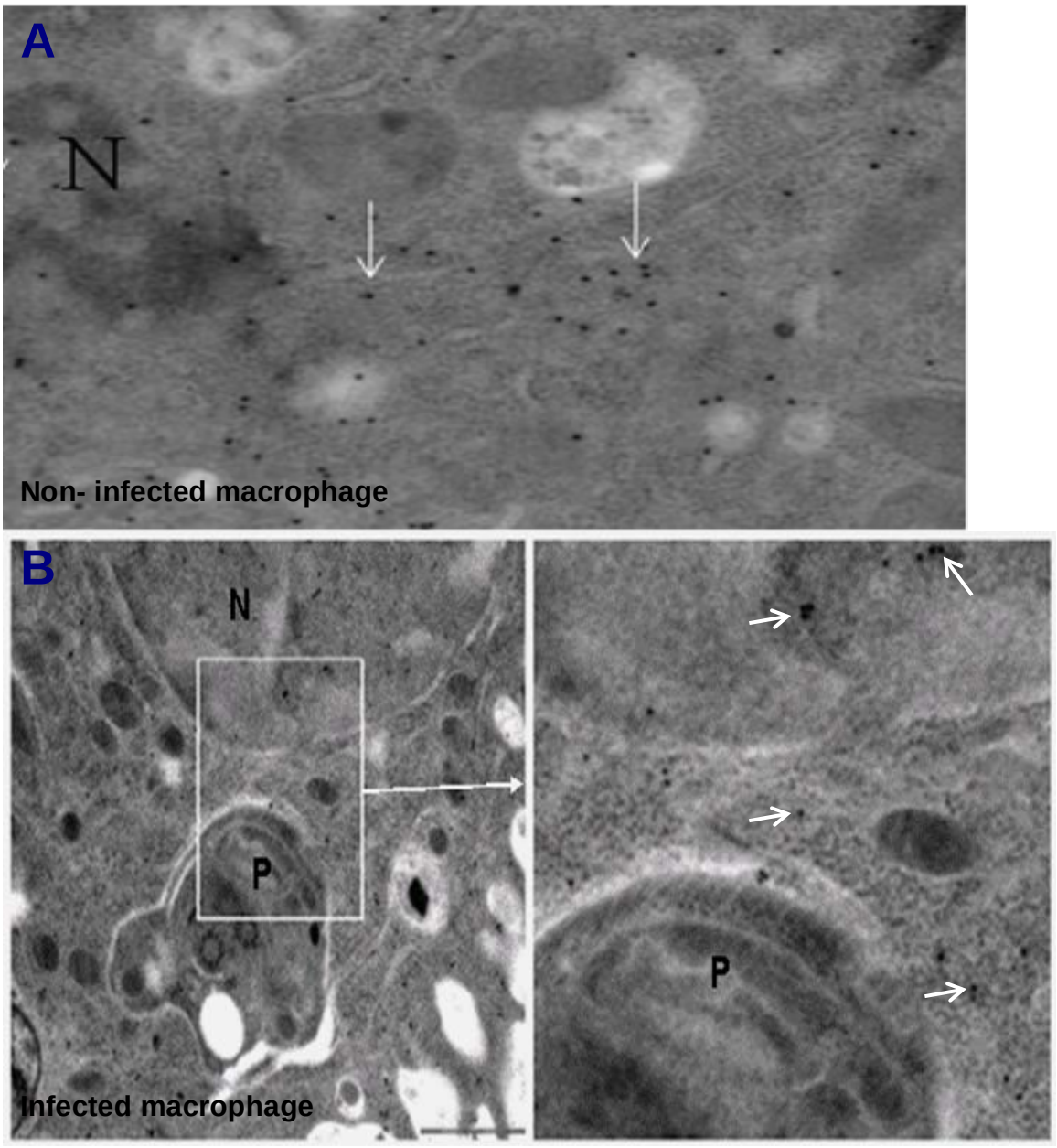


Figure 3-13. Anti-GFP labeling of macrophages expressing pmVenus (YFP). A Raw264.7 expressing pmVenus (YFP) (A); J774 macrophages stably transfected with pmVenus vector (B) were infected with *L. donovani* parasites. These cells were processed for immuno-EM analysis. Sections on Nickel grids were incubated with a rabbit anti-GFP antibody followed by a secondary antibody conjugated to 18nM gold particles. The grids were post-stained with 0.5% uranyl acetate and lead citrate and were examined with a Zeiss EM-10CA transmission electron microscope. Arrows point to 18nM gold particles randomly distributed in both infected (B) and non-infected (A) cells. These images are representative of images obtained from at least 2 experiments. N= macrophage nucleus; P= *Leishmania* parasite

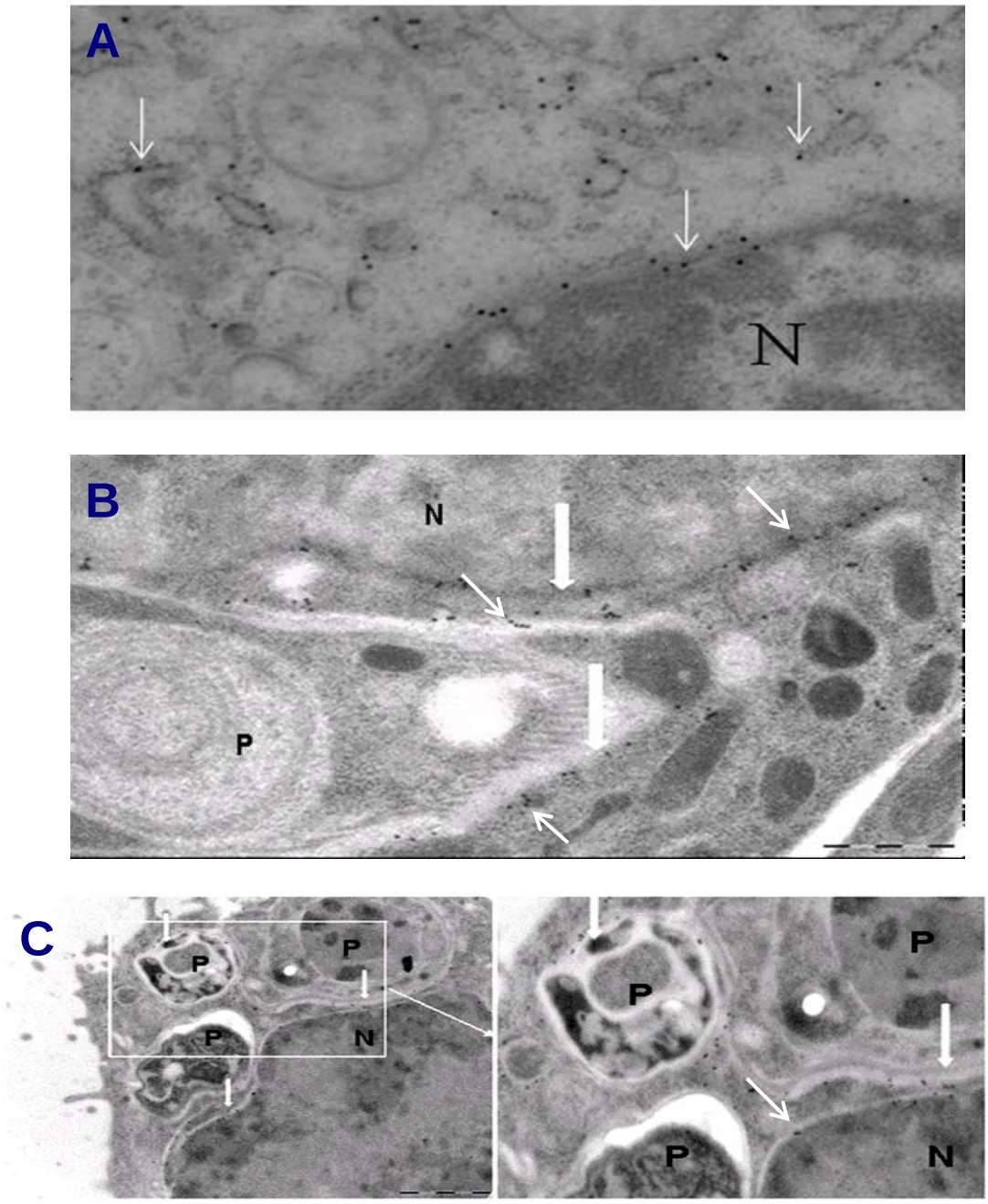


Figure 3-14. Immuno-EM analysis of ER components recruitment to *Leishmania* PVs. J774 cells stably transfected with pmVenus/Sec22b/YFP (A) were infected with *L. donovani* parasites for 12h (B) and 48h (C), and then processed for immuno-EM analysis. Sections on Nickel grids were incubated with a rabbit anti-GFP antibody followed by a secondary antibody conjugated to 15nm gold particles. The grids were post-stained with 0.5% uranyl acetate and lead citrate and were examined with a Hitachi TEM H-7000 (Pleasanton, CA) operated at 80 kV. A) A non-infected J774 cell stably expressing Sec22b/YFP. B)- Cell with a PV with single parasite; c) A cell with multiple PVs; and Zoomed out area. Bold white arrows point to perinuclear ER and PV membrane lined with gold particles.

CHAPTER 4
CONTRIBUTIONS OF HOST ER TO LEISHMANIA PV LUMEN AND VESICULAR TRANSPORT

Introduction

In chapter 3, we made the observations that an integral ER membrane protein and an ER membrane associated molecules are recruited to PVs. In this chapter, we assessed the accumulation of molecules in the ER lumen into the lumen of *Leishmania* PVs.

Results

Ricin trafficking in Raw264.7 macrophages

To determine whether molecules in the ER lumen also gain access to the lumen of *Leishmania* PVs, we elected to monitor the trafficking of ricin toxin in macrophages infected with either *L. donovani* or *L. pifanoi* strains. Ricin enters cells by endocytosis then traffics by a retrograde pathway through early endosomes, trans-Golgi network (TGN) and then the ER before reaching the cytosol (Audi *et al.,* 2005; Skanland *et al.,* 2007; Slominska-Wojewodzka *et al.,* 2006). Some of the evidence in support of this pathway included the observation that ricin trafficking to the cytosol is independent of Rab7. We reasoned that if ricin is recruited to PVs, it would imply that the contents of the compartments in the pathway of ricin including the ER lumen could be delivered to PVs.

Initial experiments were performed to confirm the trafficking scheme of ricin in uninfected RAW 264.7 macrophages (Figure 4-1). After a brief pulse with ricin, the plasma membrane (PM) of cells became coated with fluorescent ricin molecules (green) (Figure 4-1A); within 5 min, ricin was internalized and enclosed in endosomes. This was verified by demonstrating that ricin is found in vesicles that display the early endosome

antigen (EEA1) (Figure 4-1B – [arrows point to endosomes labeling with both ricin and EEA1]). From early endosomes, ricin was transferred successively into the TGN (Figure 4-1C) and ER (Figure 4-1D) before reaching the cytosol (Figure 4-1E). At no point is ricin observed in LAMP-1 positive compartments. Further confirmation that ricin traverses the TGN and ER was obtained in experiments with brefeldin A (BFA), which blocks retrograde transport from the TGN to the ER (Plaut and Carbonetti, 2008; Mardones *et al.,* 2006). Figure 4-1F&G show that in the presence of BFA, ricin accumulates in a compartment that is no longer reactive with anti-EEA1 antibodies (Fig. 4-1F). It however accumulates in a compartment that is partially reactive with anti-GM130, which suggests that it is most likely retained in the TGN (Figure 4-1G). GM130 is a matrix protein localized in the cis-Golgi, which explains its partial co-localization with ricin (Nakamura *et al.,* 1995).

Targeting Ricin into *Leishmania* PVs

Due to potential interactions of ricin in early endosomes (Sandvig *et al.,* 2002) with nascent PVs, infections were established for 4-6h before pulsing cells with ricin for five minutes. By 4h post-infection, PVs are LAMP-1 positive and therefore would not be expected to interact with early endosomes. After the ricin pulse, ricin was chased into infected cells, which were evaluated after an additional 5 min, 30 min, 1 h, 3 h and 6 h. Figure 4-2 and Figure 4-3 show representative images of ricin in infected cells at 5 min, 1 h and 3 h. After 5min, no ricin was found associated with PVs of both *L. pifanoi* (Figure 4-2A) and *L. donovani* (Figure 4-3A). At 1h, ricin had reached the *Leishmania* PV membrane and lumen (Figure 4-2B). By the 3h point ricin was within PVs that are clearly delimited by a membrane that contains LAMP-1 (Figure 4-2C&D; Figure 4-3B).

Enumeration of ricin accumulation into *Leishmania* PVs

The proportion of *Leishmania* PVs that had accumulated fluoresceinated ricin molecules was estimated over a 6h post-chase period (Figure 4-4). At 30min, less than 5% of both *L. pifanoi* and *L. donovani* PVs contained ricin molecules on their membrane or lumen. At 1h post-chase, approximately 40% of both *L. pifanoi* and *L. donovani* PVs was positively labeled with ricin signals. When the samples were chased for a longer period, the proportion of both *L. pifanoi* and *L. donovani* PVs that had accumulated ricin was about 75% at 3h, and 80% at 6h. This enumeration of PVs with ricin showed that there is a gradual increase in the number of PVs in both *L. donovani* and *L. pifanoi* infected cells that accumulated ricin (Figure 4-5). When infected Raw264.7 cells were pretreated with BFA before the ricin pulse-chase experiment, the proportion of both *L. pifanoi* and *L. donovani* PVs that had accumulated the ricin marker was less than 5% throughout the 6h chase time. The gradual accumulation of ricin in PVs suggested that recruitment of ER contents into the PV might be the result of vesicular delivery.

Discussion and Conclusion

To further assess the extent of host ER association with *Leishmania* PVs, we exploited the pathway that ricin toxin traverses to access the cytosol of cells. Ricin has been shown to enter cells by endocytosis and to then migrate from endosomes through a retrograde pathway that traverses the trans-Golgi network (TGN) and ER before reaching the cytoplasm (Sandvig *et al.,* 2002). Access to this pathway is sensitive to BFA treatment (Audi *et al.,* 2005; Skanland *et al.,* 2007). Our data indicated that ricin can access the *Leishmania* PV lumen via the TGN and ER. The transport of ricin from Golgi to ER, and subsequently from ER to *Leishmania* PVs was effectively blocked by

BFA treatment. A delay was observed between the accumulation of ricin in the ER and its accumulation in *Leishmania* PVs. This suggests that these two compartments are not continuous. ER contents are most likely delivered to PVs through vesicular fusion; the presence of ER-SNAREs, which mediates ER vesicle fusion, on PVs supports this view. Our data are not consistent with an alternative delivery scenario in which ER and PV membranes fuse and become continuous, which would then permit the exchange of ER contents with PVs. Such a mechanism was suggested in a recent study on the interactions of the *Toxoplasma* containing vacuole and the host cell's ER in which the existence of a pore between these compartments through which ER molecules could be delivered was proposed (Goldszmid *et al.,* 2009).

The observation that *Leishmania* PVs recruit both endocytic pathway and endoplasmic reticulum components suggests that PVs are atypical compartments, which differ from other pathogen containing vacuoles that also interact extensively with the host cell's ER. *Legionella* containing vacuoles (LCV) for example, avoid interactions with the endocytic pathway by elaborating molecules that are delivered to the cell cytosol via the type IV secretion apparatus (Paumet *et al.,* 2009; Roy *et al.,* 1998). LCVs recruit ER components through a mechanism that is sensitive to BFA was shown to be ARF1 dependent (Shin and Roy, 2008).

Experiments aimed at assessing the accumulation of ricin molecules in phagosomes containing dead particles or Zymosan was not feasible in this study. Dead particles within the phagosomes could not survive more 4h or 6h (see Chapter #3, Figure 3-4 and Figure 5), which was the required incubation time before the ricin pulse/chase experiment. There were additional technical difficulties with using

Zymosan/Texas Red in these experiments; there was the limitation of having to localize phagosome compartments with a fourth marker, giving that ricin fluoresced green, and the host nucleus was stained in blue by the DAPI dye.

The observations presented here documenting continuous interactions of PVs with the ER has implications for our understanding of how *Leishmania* molecules might access the MHC class I pathway of antigen presentation. Although a few reports have addressed this issue, it is still not known where *Leishmania* molecules are processed and loaded onto MHC class I molecules for presentation to CD8+ T cells by infected macrophages. The data presented here suggest that MHC class I molecules in the ER might be accessible to parasite molecules within PVs. Future studies on that topic and on how the presence of ER components in PVs benefits the *Leishmania* parasite should prove to be quite informative.

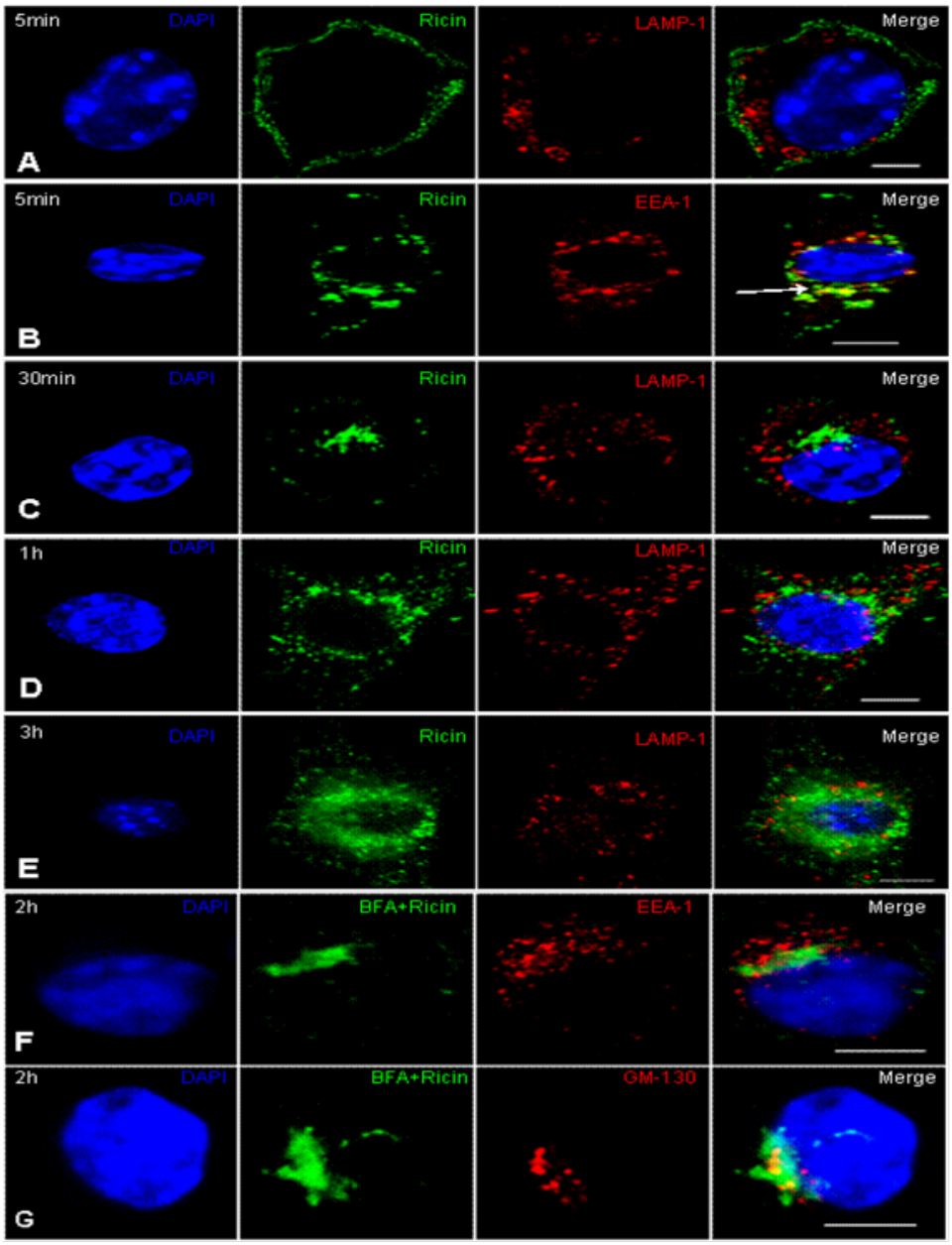


Figure 4-1. Trafficking of ricin in non-infected Raw 264.7 macrophages. Raw 264.7 macrophages on cover slips were pulsed with fluoresceinated ricin at a concentration of 10ug/ml in complete RPMI medium. The cultures were rinsed and incubated with complete medium at 37 ˚C and under 5% CO2. Cover slips were removed after the pulse (0 min) (A) then after chase for 5 min (B); some cover slips were stained with anti-EEA1 to visualize early endosomes. Bold arrow in the merged image shows vesicles that are both EEA1 and ricin positive. Representative images after 30 min (C), and 1 h (D) and 3 h (E) show labeling of cell nucleus with DAPI dye (blue) and LAMP-1 using an anti-LAMP-1 antibody (red). Some samples were pre-treated with BFA at a concentration of 5ug/ml for 2 h before ricin treatment. Cover slips obtained after 2 h chase were labeled with anti EEA1 (F) or anti-GM130 (G) to localize compartment in which ricin was arrested after BFA treatment. Samples were captured with a Zeiss Axiovert 200M fluorescence microscope controlled with Axiovision software. These images are representative of images from 3 experiments. Scale bar = 5µm.

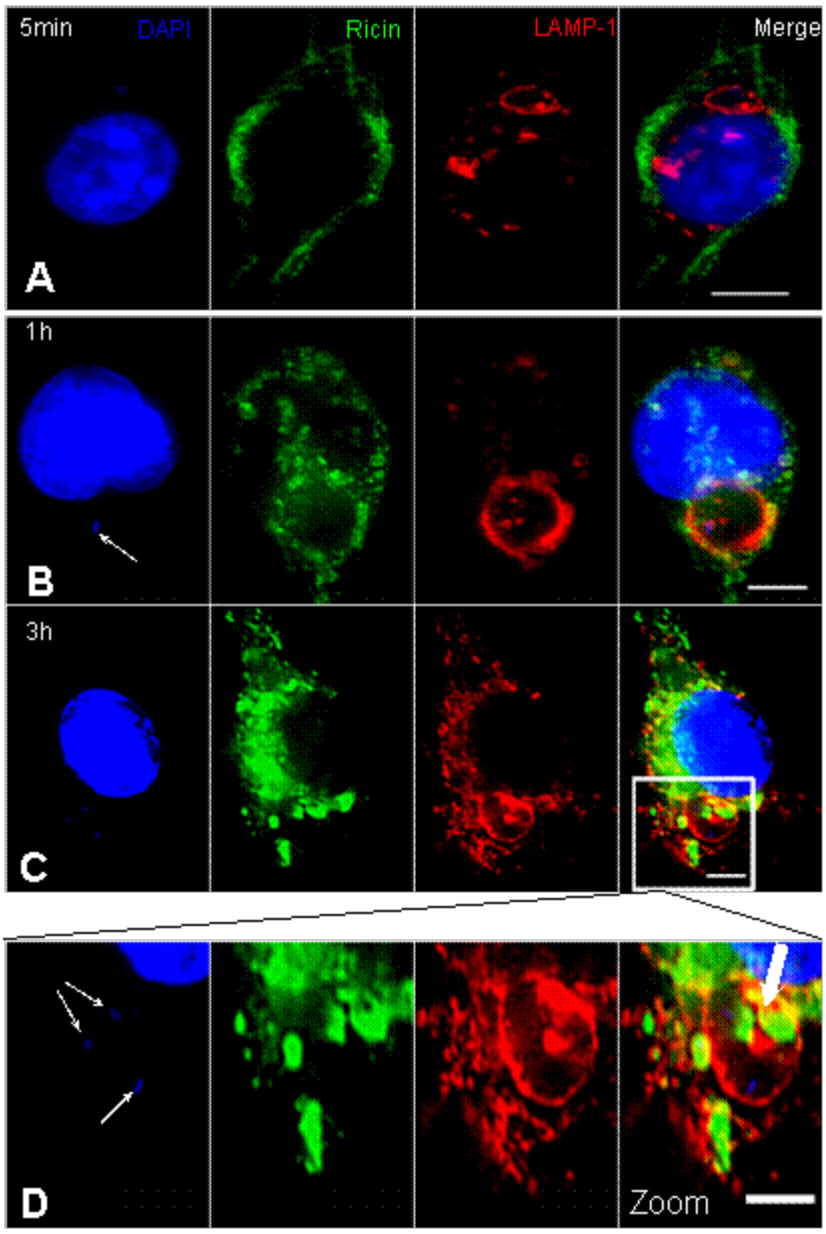


Figure 4-2. Ricin accumulates in *Leishmania* PVs during infection in Raw 264.7 macrophages. Raw 264.7 macrophages were infected for at least 4h before the cultures were incubated with ricin at a concentration of 10ug/ml, and the reaction was chased at 37˚C and under 5% CO2 atmosphere. At 0 min (A), 1 h (B) and 3 h (C) the cover slips were recovered and processed to reveal nuclei (blue) and LAMP-1 (red). Thin white arrows point to parasites in the infected cells. Thick arrow points to ricin within a PV. Scale bar = 5µm. PVs harboring either *L. pifanoi* or *L. donovani* that were ricin positive were enumerated after 30 min, 1 h, 3 h and 6 h. The percentage of PVs that were ricin positive was plotted (E). Incubations were done in the presence of BFA and enumerated as well. The difference in the percentage of PVs that were positively displaying ricin was significantly different from the percentage of PV with ricin in cells incubated with BFA. * denotes a P value < 0.01. Each data point was compiled from at least 3 experiments.

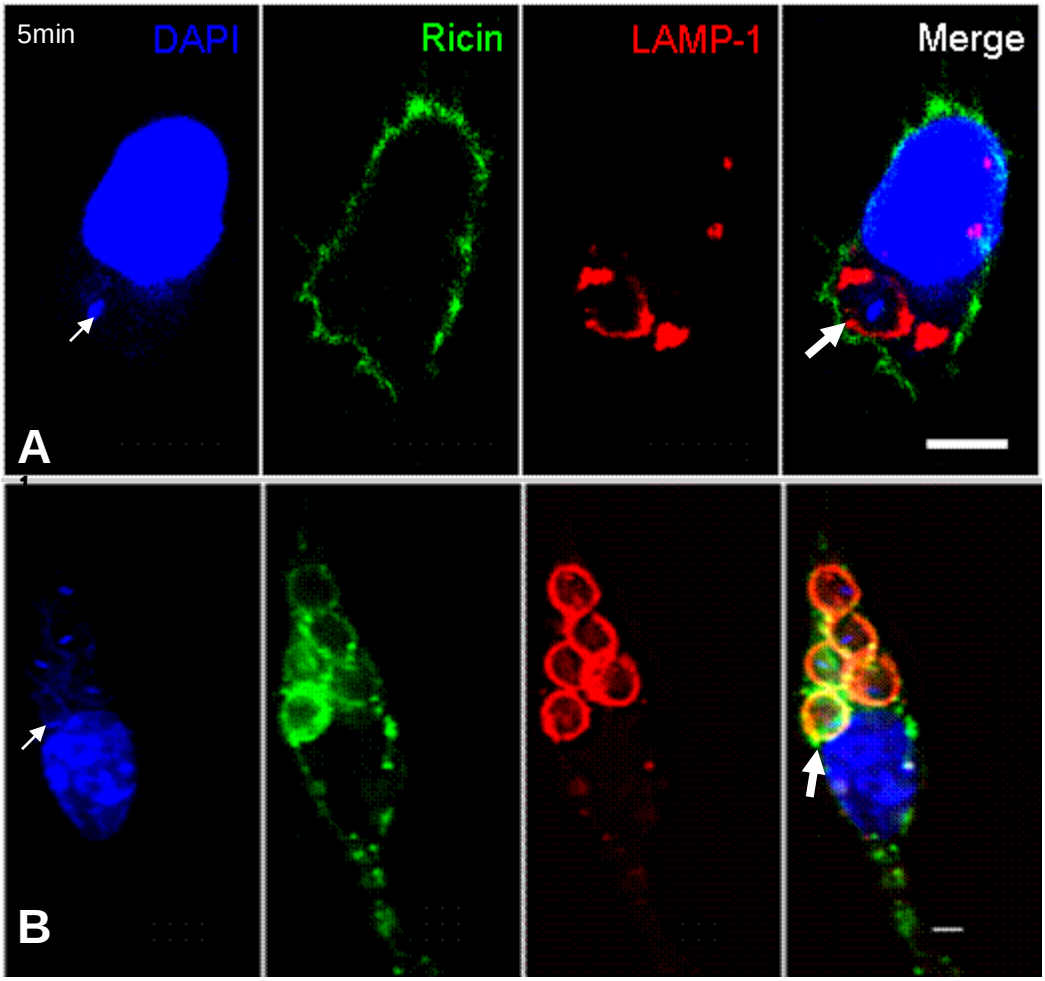


Figure 4-3. Ricin accumulates in *Leishmania donovani* PVs during infection in Raw 264.7 macrophages. Raw 264.7 macrophages were infected for at least 4h before the cultures were incubated with ricin at a concentration of 10ug/ml, and the reaction was chased at 37℃ a nd under 5% CO2 atmosphere. At 5min (A) and 3h (B) the cover slips were recovered and processed to reveal nuclei (blue) and LAMP-1 (red). Thin white arrows point to parasites in the infected cells. Thick arrow points to ricin within a PV. Scale bar = 5µm.

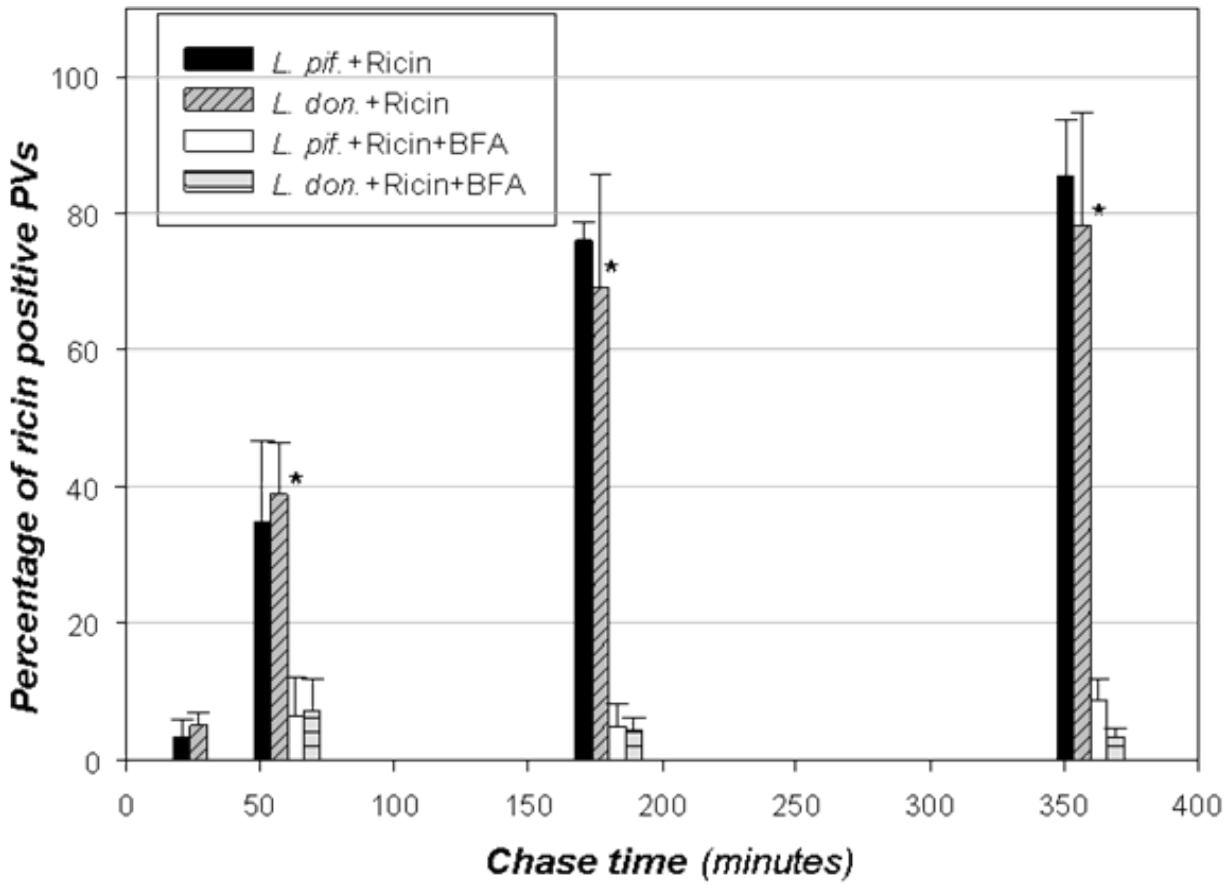


Figure 4-4. The proportion of ricin positive *Leishmania* PVs. Incubations were done in the presence of BFA and enumerated as well. The difference in the percentage of PVs that were positively displaying ricin was significantly different from the percentage of PV with ricin in cells incubated with BFA. * denotes a P value < 0.01. Each data point was compiled from at least 3 experiments.

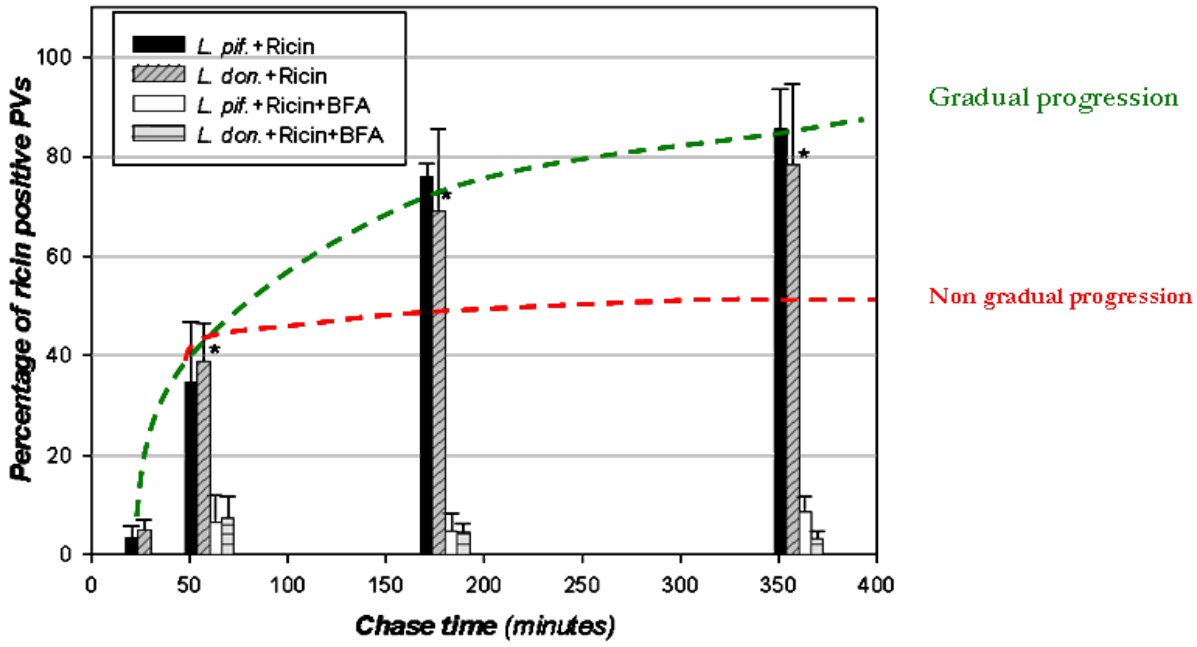


Figure 4-5. The proportion of ricin positive *Leishmania* PVs. Incubations were done in the presence of BFA and enumerated as well. The difference in the percentage of PVs that were positively displaying ricin was significantly different from the percentage of PV with ricin in cells incubated with BFA. * denotes a P value < 0.01. Each data point was compiled from at least 3 experiments. The green line demonstrates a gradual accumulation of ricin in the PV; the red line represents a non-gradual accumulation of ricin in PVs.

CHAPTER 5
EFFECT OF HOST ENDOPLASMIC RETICULUM ON LEISHMANIA DEVELOPMENT IN MAMMALIAN CELLS

Introduction

In the yeast Saccharomyces cerevisiae, the ER-localized SNARE proteins Ufe1p, Sec22p, Sec20p, and Use1p/Slt1p form a SNARE complex required for vesicular transport between the ER and the Golgi (Dilcher *et al.,* 2003; Burri *et al.,* 2003). In addition to the previously known mammalian orthologues syntaxin 18 and Sec22b, and BNIP1 and D12 (also called p31) were recently identified as mammalian orthologues of Sec20p and Use1p/Slt1p, respectively (Nakajima *et al.,* 2004; Okumura *et al.,* 2006; Hirose *et al.,* 2004). BNIP1, unlike other ER-localized SNARE proteins, has been shown to be mainly required for apoptotic cell death and homotypic ER membrane fusion rather than for vesicle trafficking (Nakajima *et al.,* 2004). The data presented in chapter 4 suggest that ER derived vesicles containing ricin molecules are transported in vesicles that fuse with both the nascent and maturing *Leishmania* parasitophorous vacuoles in Raw264.7 macrophages. In this chapter, the effect of ER membrane-associated SNAREs (Sec22b, D12 and Syntaxin18) on *L. amazonensis* promastigotes entry, parasite replication and PV development in murine Raw246.7 macrophages is investigated.

Results

Effect of Host ER SNAREs on *L. amazonensis* Entry in Raw264.7

Many pathogens such as *Leishmania* enter professional phagocytes through the process of phagocytosis. Recent studies have revealed the ER-mediated phagocytosis in which ER compartments fuse to the plasma membrane at the phagocytic cup to

facilitate the internalization of large inert or live particles by macrophages (Gagnon *et al.,* 2002; Hatsuzawa *et al.,* 2006). Knowing that membrane fusion is mediated by SNAREs; we assessed the effect of ER SNAREs during *L. amazonensis* entry in macrophages. In this study, murine Raw264.7 macrophages were employed and transiently transfected with vectors from which SNAREs (Sec22b, D12 or Syntaxin18) tagged with YFP were over-expressed, or vectors encoding dominant negative forms of these ER SNAREs; the dominant negative SNAREs are devoid of their transmembrane domain (^tm-Sec22b/RFP; ^tm-D12/RFP or ^tm-Syntaxin-18/RFP) (Hatsuzawa *et al.,* 2006).

Transfected cells were incubated with *L. amazonensis* promastigotes at 34°C and 5% CO_2 at a ratio of 1:10 (one macrophage for ten parasites) in RPMI complete medium. The samples were washed twice after 1h infection to remove all free parasites in the preparations. Fresh culture medium was added to the preparations, which were returned to the incubator. One hour after washing, samples were removed and fixed in 2% PFA. They were processed through immunofluorescence assays, and analyzed with a Zeiss fluorescence microscope.

After a 2h infection period, the proportion of cells that had internalized at least one parasite was estimated. The number of parasites in each cell was also counted in order to assess the infection load. Non transfected cells (wild type) and cells transfected with the empty vector, pmVenus/YFP, were used as controls in these experiments. We observed that approximately 20% of the cells in the control samples (non transfected Raw264.7 and pmVenus transfected cells) had internalized at least one *L. amazonensis* promastigote during the course of the two hour infection (Figure 5-1). A mean infection

rate of approximately 30% was scored for Raw264.7 macrophages transfected with the R-SNAREs, pmVenus/Sec22b/YFP or pmVenus/D12/YFP, constructs; and 23% for those transfected with the Q-SNARE, pmVenus/Syntaxin-18. Cells transfected with dominant negative constructs were infected at 17% (^tm-D12), 19% (^tm-Sec22b) and 23% (^tm-Syntaxin18) (Figure 5-1).

A paired t-test statistical analysis of the data suggests the increase in the number of *L. amazonensis*-infected cells in the groups of macrophages transfected with either pmVenus/Sec22b/YFP or pmVenus/D12/YFP was statistically significant (P value≤ 0.05). That analysis also revealed that there were no significant differences between the control samples and the rest of the cells expressing Syntaxin18, and the dominant negatives (Δtm -Sec22b, Δtm-D12, or Δtm-Syntaxin18)/RFP. We also observed that the number of parasites within each infected cell was the same across all the cell types, except in those transfected with pmVenus/Sec22b/YFP construct, which have a significantly higher parasites load compare to the controls (Figure 5-2). Taken together, the over-expression of ER membrane-associates R-SNAREs (Sec22b and D12), but not Q-SNARE (Syntaxin18), may significantly stimulate parasite entry into macrophages. Down-regulation of any single ER membrane-associated SNARE molecules (Sec22b, D12, or Syntaxin18) does not affect significantly the efficiency of *L. amazonensis* promastigotes to enter Raw246.7 macrophages.

Effect of Host ER SNAREs on *L. amazonensis* Replication

During chronic infection, amastigotes of the *Leishmania mexicana* complex (*L. mexicana*, *Leishmania amazonensis* and *Leishmania pifanoi*) generally form a PV characterized by a large lumen; daughter parasites remain in a communal PV that

increases in size after parasite replication (Kima, 2007). In this study, we assessed the effect of host ER SNAREs on the parasite load in *L. amazonensis* PVs throughout the course of 72h infection. Murine Raw264.7 macrophages transiently transfected with the SNAREs (Sec22b, D12 or Syntaxin18)/YFP, or their dominant negative forms (^tm-Sec22b/RFP; ^tm-D12/RFP or ^tm-Syntaxin-18/RFP), were infected with *L. amazonensis* promastigotes. The infection ratio was set at 1:5 ratio, which reduces the initial number of parasites entering the host. Infections were sampled and number of parasites inside the PV was scored at 2, 4, 12, 24, 48, and 72h time points.

As illustrated in Figures (5-5; 5-6; 5-7), we observed that the mean number of parasites within the PV was one, which remained relatively constant for all cell types investigated (both transfected and non transfected cells) up to 24h post infection. After 24h post infection, the mean number of parasites per PV increases over time. At 72h post infection, the mean number of parasites inside the PV was six parasites per PV; this was significantly higher in samples of macrophages over-expressing Sec22b and D12 molecules. In comparison, there were four parasites per PV in the control samples (pmVenus, and non-transfected cells). A similar number of parasites per PV was obtained in samples over-expressing Syntaxin18. However, after 72h infection, the mean number of parasites per PV was significant reduced by about a third in cells expressing dominant negative∆tm -Sec22b, and by half compare to controls expressing ∆tm-D12. Dominant negative Syntaxin18 did not affect the parasite per PV count. Taken together, our data suggest that Sec22b and D12, but not Syntaxin 18, play a significant role in the biogenesis of PVs, which ultimately affects the successful replication of *Leishmania* parasites in macrophages.

Effect of Host ER SNAREs on *L. amazonensis* PV size

For the studies in this section, our objective was to investigate whether host ER SNAREs contribute significantly to PV size during *L. amazonensis* chronic infection. To achieve this objective, we monitored the *L. amazonensis* PV size, in relation with the size of the host nucleus within infected Raw264.7 macrophages transiently transfected with the SNAREs (Sec22b, D12 or Syntaxin18)/YFP, or their dominant negative forms (^tm-Sec22b/RFP; ^tm-D12/RFP or ^tm-Syntaxin-18/RFP). Representative images of some cell types infected with *Leishmania* parasites before and after 24h infections are illustrated in Figure 5-3 (controls cells) and Figure 5-4 (cells transfected with D12/YFP (A-C) and ^tm-D12/RFP (D-E)).

The ratio of the *Leishmania* PV size over the host nucleus size (pv/n) was estimated for all the infected groups at 4h and 48h time points. The contour or perimeter of the *Leishmania* PV membrane and the host cell nucleus was measured using the ImageJ software from NIH. The pv/n ratio was estimated at about 0.6 for all groups at 4h; which means that the size of the host cell nucleus at this early stage is approximately twice the size of the *Leishmania* PVs.

After the 24h infection time, the *Leishmania* PV size was at least one and an half times bigger that the host cell nucleus. The pv/n ratio was significantly higher in more than 90% of infected cells in the control infected cells, and in cells over-expressing ER SNAREs (Sec22b, D12 and Syntaxin18). This increase in *Leishmania* PV size at the later time point during the infection is most likely to accommodate the increase in parasites after their replication. However, in cells that were transfected with dominant negative constructs, the pv/n ratio was significant decreased (pv/n< 0.3) in more than

80% of the cells investigated in this category. This indicated that the inhibition of ER vesicle fusion by targeting ER SNAREs hindered the size increase of *Leishmania* PVs (Figure 5-4 D & C).

Discussion and Conclusion

In this chapter, we investigated the effects of modulating the levels of the host ER membrane-associated molecules, Sec22b, D12, and Syntaxin 18, with the goal of limiting the interaction of the ER and *Leishmania* PVs. The effect of this on *Leishmania* entry, *Leishmania* replication, and the development of their PV in Raw264.7 macrophages was determined. Our data suggest that knocking down these SNAREs individually does not significantly disrupt *Leishmania* promastigotes entry in macrophages. However, overexpression of the R-SNAREs (Sec22b and Syntaxin18) resulted in a modest but significant increase in the entry of *Leishmania* promastigotes.

The fact that down-regulating the ER SNAREs (sec22b, D12, and Syntaxin 18) did not significantly reduce *L. amazonensis* entry in Raw264.7 macrophages, can not exclude the possibility that these SNAREs play an essential role during phagocytosis (Becker *et al.,* 2005; Hatsuzawa *et al.,* 2006). Based on the knowledge of how SNAREs act within fusion complex machinery, at least two scenarios can explain how the fusion machinery reacts so robustly to the down-regulation or loss of some of its main players. First, the functional redundancy of these molecules may enable SNAREs of the same subfamily to substitute for each other. For instance, some SNAREs such as syntaxin 1, synaptobrevin, Snc and Sso have several isoforms with identical functions, with their redundancy occasionally arising from recent gene duplication events (Aalto *et al.,* 1993); Snc1/2 (Protopopov *et al.,* 1993). Moreover, SNAREs of the same subfamily,

even if not so clearly related, can functionally replace each other to varying degrees, probably because SNAREs can and do associate in complexes rather promiscuously. For instance, in synaptobrevin knockout mice, secretion of chromaffin cells remains normal because of full compensation by endogenous cellubrevin (Borisovska *et al.,* 2005), in contrast to the severe synaptic dysfunction in these mice (with cellubrevin being absent in synapses). Examples of incomplete compensation by related SNAREs include SNAP-25 that can be partially substituted by exogenously expressed SNAP-23 in chromaffin cells (Delgado-Martinez *et al.,* 2007). The yeast SNARE, Sec22 is partially rescue by Ykt6 (Liu *et al.,* 2002), and Pep12p is also partially rescue by Vam3p (Darsow *et al.,* 1997).

Second, the SNAREs are constitutively expressed at high levels, which may drastically surpass the cellular needs. In a study in which there was 90% efficient knockdown of the early endosomal SNAREs syntaxin 13, 6 and vti1a, and the exocytic SNARE synaptobrevi in PC12 cell, the residual proteins were found to efficiently associate in SNARE complexes, at levels that were surprisingly close to the wild-type situation (Bethani *et al.,* 2009). This led the authors to conclude that the fusion machinery is expressed in higher levels than the cell's demand, because less than 11% of the total amount of SNAREs produced is still sufficient to enable fusion within the cell. Fluorescence imaging revealed that the SNAREs are organized in multi-molecular clusters in the wild-type cells, which constitutes a possible mechanism to restrict the SNARE activity (Bethani *et al.,* 2009). For example, neuronal exocytic SNAREs are expressed at hugely abundant levels (Holt *et al.,* 2006; Walch-Solimena *et al.,* 1995),

with fusion persisting even when the free SNARE pool is substantially depleted (Kawasaki *et al.,* 1998).

The dominant-negative expression of soluble mutants of syntaxin 18 and D12 lacking the transmembrane domain, but not the Sec22b mutant, was believed to significantly suppress the rate of phagocytosis in J774 macrophages by an amount similar to Arf6 T27N (Hatsuzawa *et al.,* 2006); which causes inhibition of Fc receptor–mediated phagocytosis (Uchida *et al.,* 2001; Niedergang et al., 2003). Moreover, other independent studies confirm previous findings that functions of Sec22b (also called ERS24) functions selectively in phagocytosis triggered by IgG-opsonized large particles (3.0 µm in diameter) in J774 macrophages (Becker *et al.,* 2005).

Taken together, I have shown in this chapter that *Leishmania* entry into macrophages may be positively affected by host ER membrane-associated R-SNAREs. Inside PVs, *Leishmania* promastigotes required at least 24h to transform into amastigotes, which are the only *Leishmania* form that replicate in mammalian host cells. More importantly, the functions of R-SNAREs (Sec22b and D12), but not Q-SNARE (Syntaxin18) seem to be required by *Leishmania* parasites to successful replicate and develop within their hosts, macrophages.

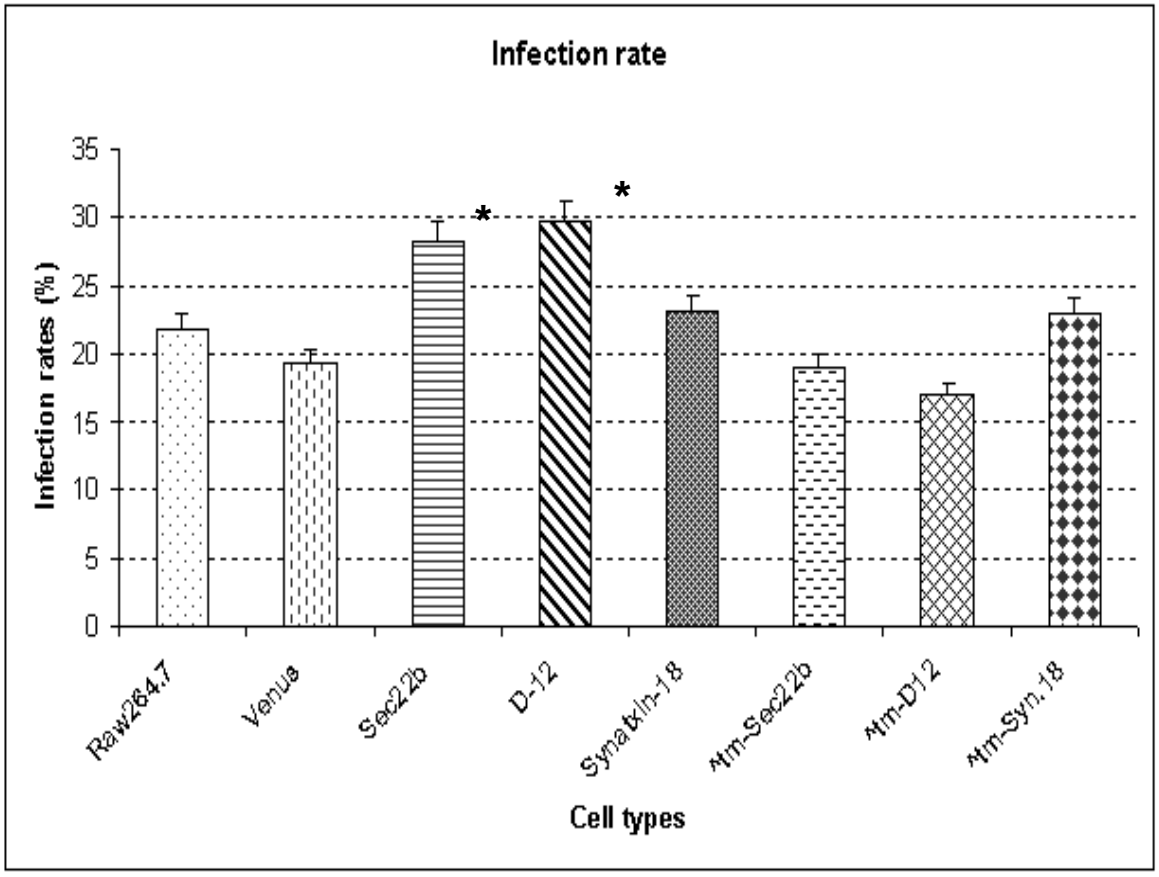


Figure 5-1. Effect of ER-membrane associated SNAREs on *Leishmania* parasites entry into macrophages. Murine Raw264.7 macrophages transfected either with over-expressing constructs (Sec22b/YFP; D12/YFP or Syntaxin18/YFP) or their dominant negative constructs (^tm-Sec22b/RFP; ^tm-D12/RFP or ^tm-Syntaxin-18/RFP) were co-incubated at 34°C and 5% CO2 with *L. amazonensis* promastigotes at a ratio of 1:10. The samples were washed twice with the culture medium after 1h infection to remove all free parasites in the preparations. After adding a fresh culture medium, they were returned to the incubator. One hour later, samples were removed and fixed in 2% PFA. They were processed in immunofluorescence assays, and analyzed with a Zeiss fluorescence microscope. The proportion of cells that had internalized at least one parasite was estimated. The experiment was repeated at least three times. The graphs and t-test analysis were done using the Microsoft Excel software. *Data are significantly different from the control (T-test value $P \leq 0.05$).

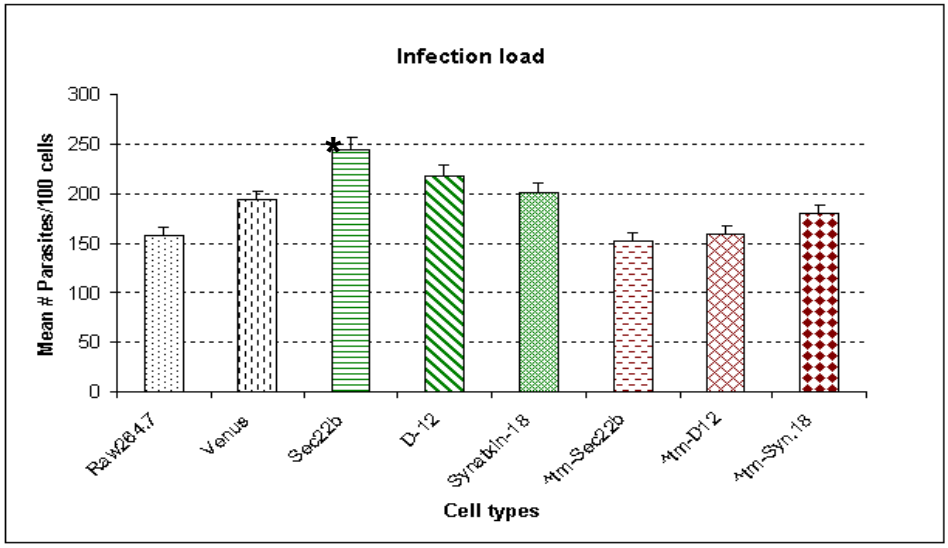


Figure 5-2. Effect of ER-membrane associated SNAREs on *Leishmania* parasites load in newly infected macrophages. Murine Raw264.7 macrophages transfected either with over-expressing constructs (Sec22b/YFP; D12/YFP or Syntaxin18/YFP) or their dominant negative constructs (^tm-Sec22b/RFP; ^tm-D12/RFP or ^tm-Syntaxin-18/RFP) were co-incubated at 34°C and 5% CO2 with *L. amazonensis* promastigotes at a ratio of 1:10. The samples were washed twice with the culture medium after 1h infection to remove all free parasites in the preparations. After adding a fresh culture medium, they were returned to the incubator. One hour later, samples were removed and fixed in 2% PFA. They were processed in immunofluorescence assays, and analyzed with a Zeiss fluorescence microscope. The number of parasites internalized by each infected cell was estimated. The experiment was repeated at least three times. The graphs and t-test analysis were done using the Microsoft Excel software. *Data are significantly different from the control (T-test value $P \leq 0.05$).

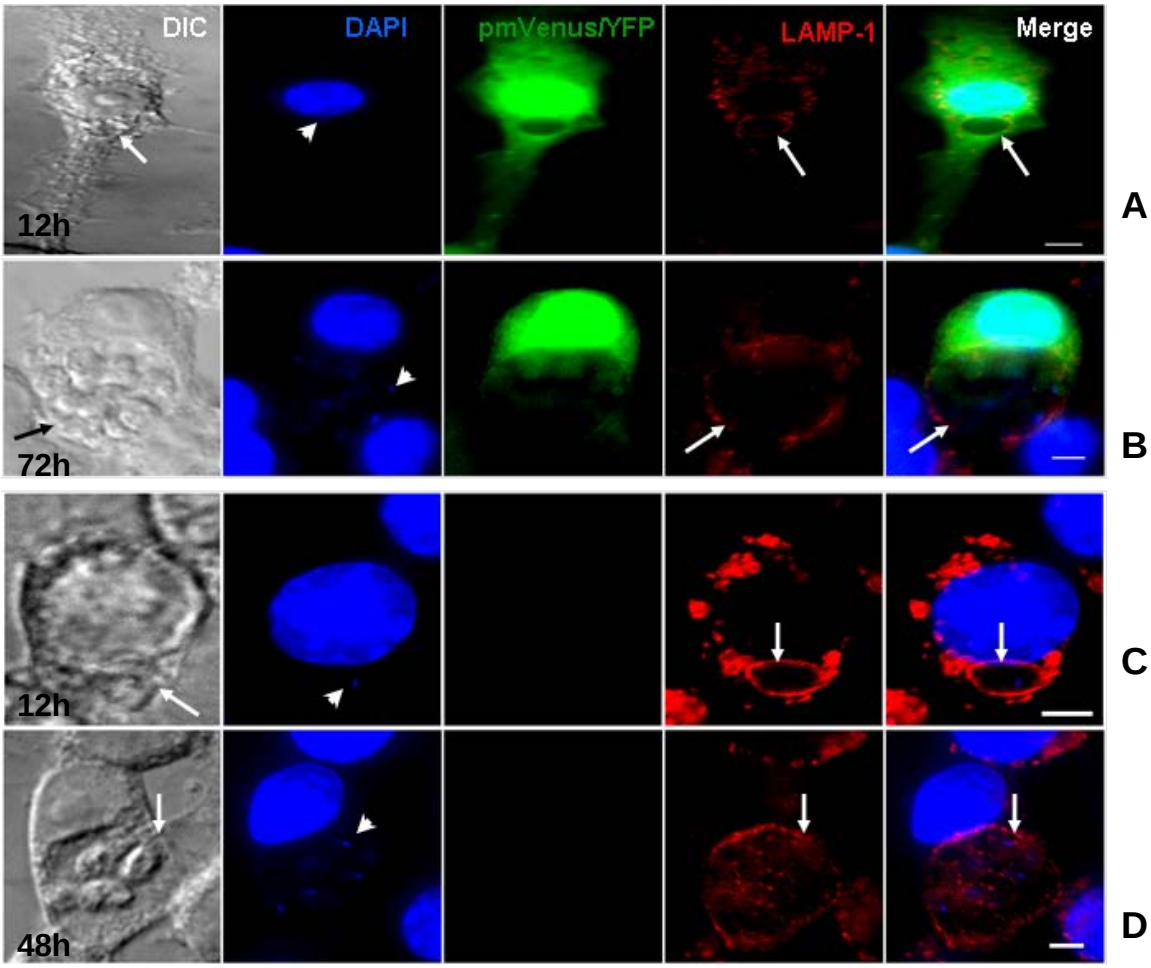


Figure 5-3. Representative images of the controls used. Non-transfected Raw264.7 macrophages and Raw264.7 in the study to the effect of host ER membrane associated SNAREs on *L. amazonensis* replication and PV development. Murine Raw264.7 macrophages non-transfected (C and D) or transfected with pmVenus/YFP (A and B). Fixed samples were processed through immunofluorescence assays, and images taken and analyzed with a Zeiss fluorescence microscope.

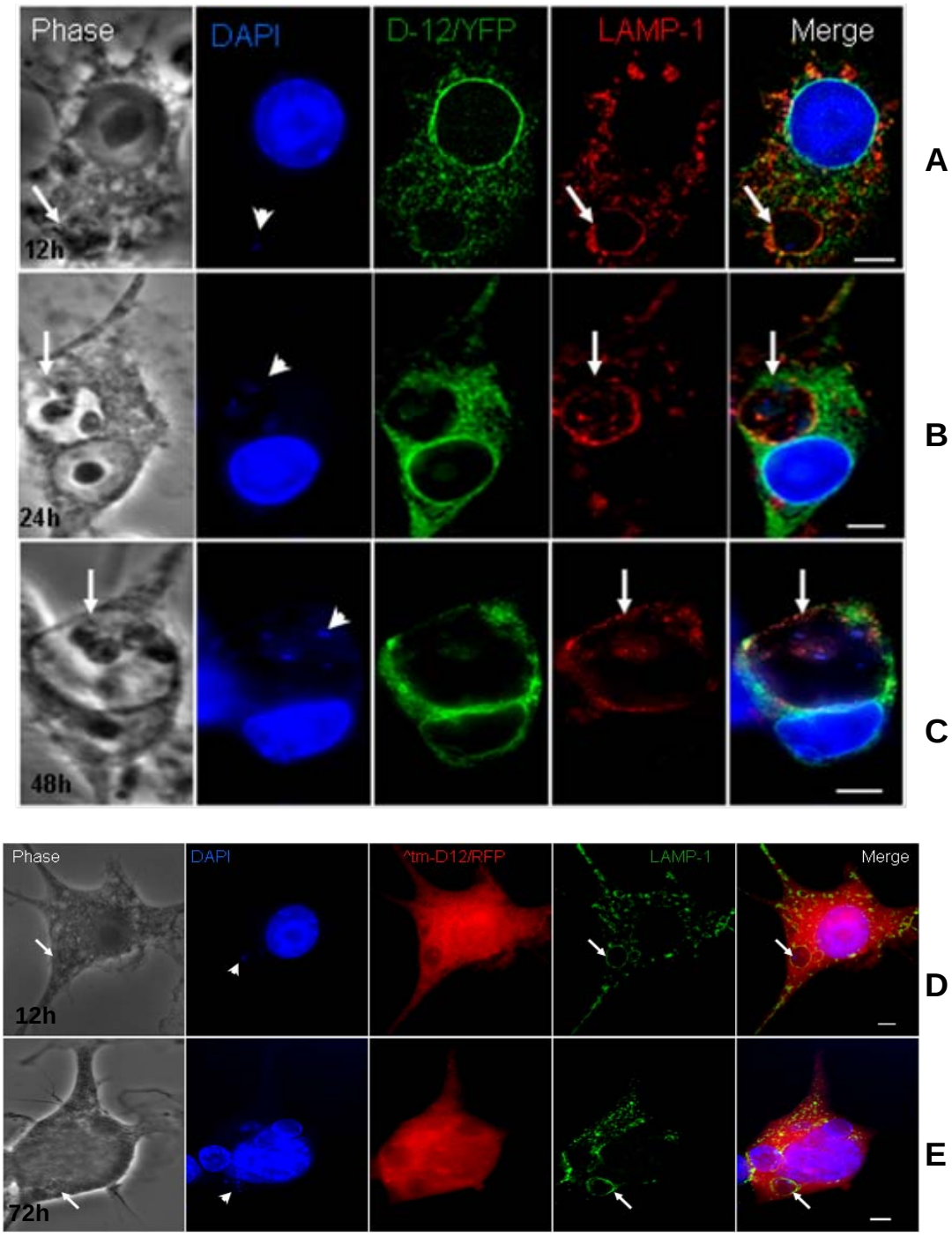


Figure 5-4. Representative images of the effect of host ER SNAREs on *L. amazonensis* replication and PV development. Murine Raw264.7 macrophages transfected either with D12/YFP (A-C) or ^tm-D12/RFP (dominant negative)(were co-incubated with *L. amazonensis* promastigotes at a ratio of 1:5 (one macrophage to five parasites)(D-E). Fixed samples were processed in immunofluorescence assays, and images taken and analyzed with a Zeiss fluorescence microscope.

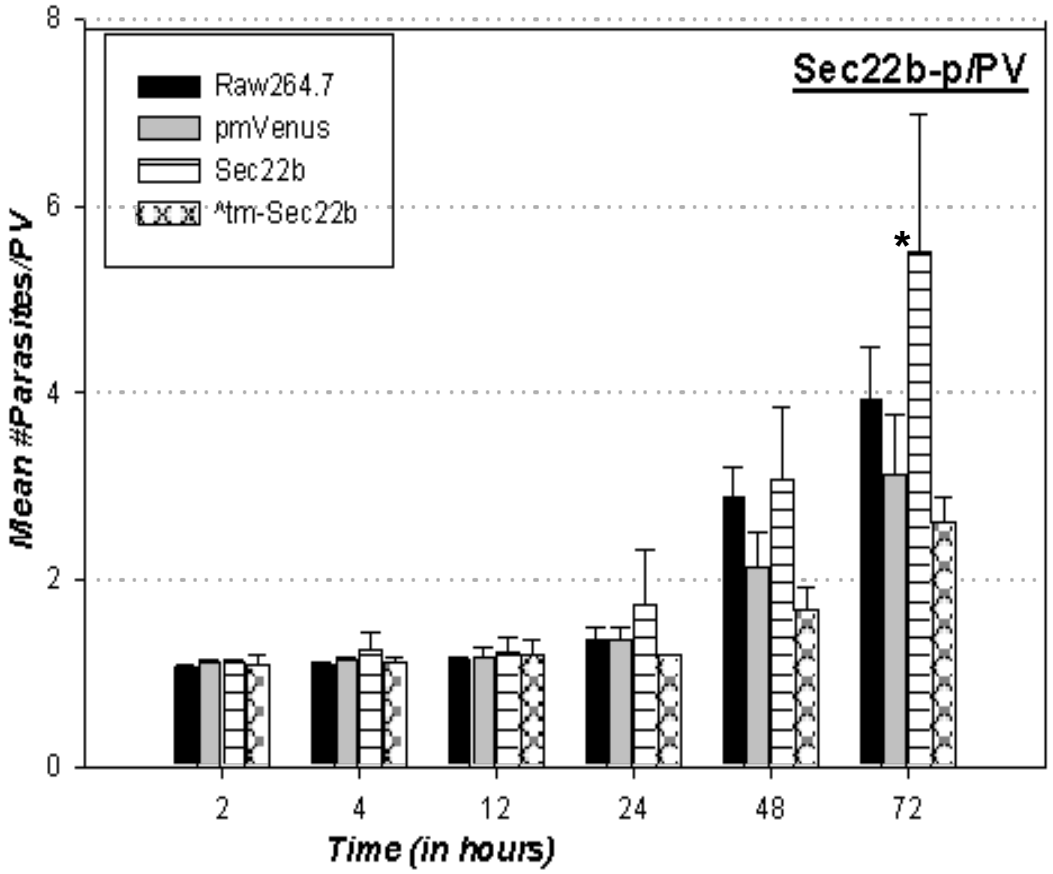


Figure 5-5. Effect of Sec22b on parasite load in *L. amazonensis* PV within Raw264.7 macrophages. Murine Raw264.7 macrophages transfected with Sec22b/YFP or dominant negative Sec22b (^tm-Sec22b), were co-incubated at 34°C and 5% CO2 with *L. amazonensis* promastigotes at a ratio of 1:5 (one macrophage to five parasites) in RPMI complete medium. The cultures were washed twice with sterile 1xPBS after 2h infection to remove all free parasites in the preparations. Samples were removed from the culture and fixed in 2% PFA at several time-points (2, 4, 12, 24, 48, and 72h). Fixed samples were processed in immunofluorescence assays, and analyzed with a Zeiss fluorescence microscope. The number of parasites within the PVs was estimated using a cell counter device purchased from Fisher Scientific Inc. The experiment was repeated at least three times. *Data are significantly different from the control (T-test value P≤ 0.05) .

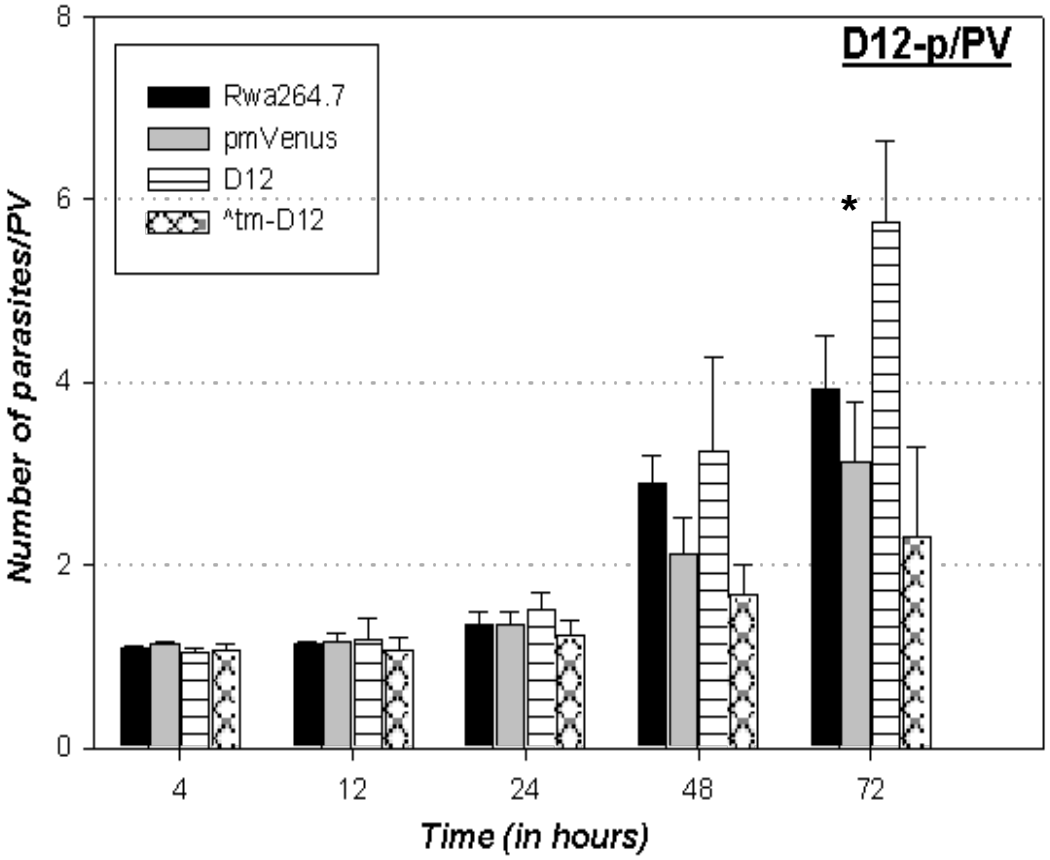


Figure 5-6. Effect of D12 on parasite load in *L. amazonensis* PV with Raw264.7 macrophages. Murine Raw264.7 macrophages transfected with D12/YFP or dominant negative D12 (^tm-D12), were co-incubated at 34°C and 5% CO2 with *L. amazonensis* promastigotes at a ratio of 1:5 (one macrophage to five parasites) in RPMI complete medium. The cultures were washed twice with sterile 1xPBS after 2h infection to remove all free parasites in the preparations. Samples were removed from the culture and fixed in 2% PFA at several time-points (2, 4, 12, 24, 48, and 72h). Fixed samples were processed in immunofluorescence assays, and analyzed with a Zeiss fluorescence microscope. The number of parasites within the PVs was estimated using a cell counter device purchased from Fisher Scientific Inc. The experiment was repeated at least three times. *Data are significantly different from the control (T-test value $P \leq 0.05$).

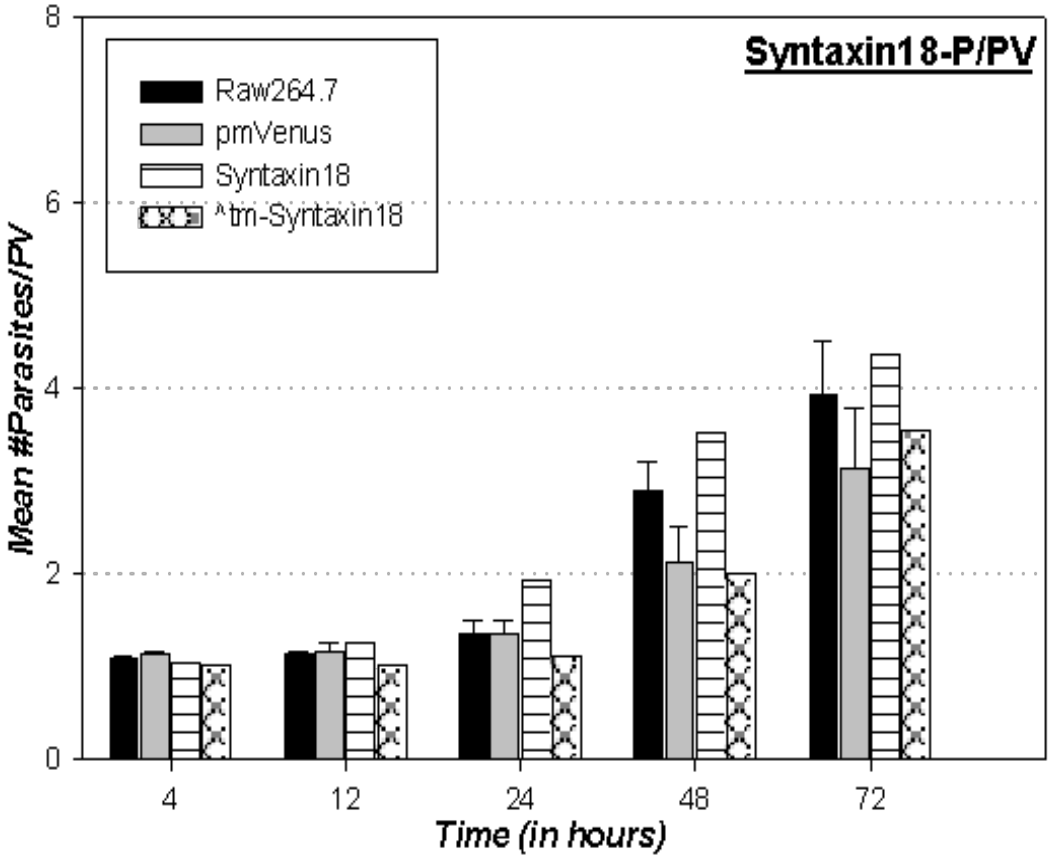


Figure 5-7. Effect of Syntaxin18 on parasite load in *L. amazonensis* PV within Raw264.7 macrophages. Murine Raw264.7 macrophages transfected with Syntaxin18/YFP or dominant negative Syntaxin18 (^tm- Syntaxin18), were co-incubated at 34°C and 5% CO2 with *L. amazonensis* promastigotes at a ratio of 1:5 (one macrophage to five parasites) in RPMI complete medium. The cultures were washed twice with sterile 1xPBS after 2h infection to remove all free parasites in the preparations. Samples were removed from the culture and fixed in 2% PFA at several time-points (2, 4, 12, 24, 48, and 72h). Fixed samples were processed in immunofluorescence assays, and analyzed with a Zeiss fluorescence microscope. The number of parasites within the PVs was estimated using a cell counter device purchased from Fisher Scientific Inc. The experiment was repeated at least three times. *Data are significantly different from the control (T-test value $P \leq 0.05$).

CHAPTER 6
OVERALL CONCLUSION AND PERSPECTIVES

Overall Conclusion

The goals of this study were to advance our understanding of the contributions of host ER on *Leishmania* parasitophorous vacuoles biogenesis and maturation, as well as its effect on parasites replication in macrophages. Recent studies have provided new insight on phagocytosis, which is the process deployed by phagocytes to capture and internalize large inert particles and pathogens such as *Leishmania* parasites. Proteomic analyses demonstrated enrichment of many ER markers such as calnexin and calreticulin to latex bead phagosomes (Garin *et al.,* 2001). The deletion of these ER markers in *Dictyostelium sp*, significantly impaired the capacities of these cells to internalize latex bead particles by phagocytosis (Muller-Taubenberger *et al.,* 2001). A combination of several approaches including proteomics, confocal microscopy and electron microscopy (EM) to investigate latex bead phagosomes properties demonstrated a direct association between ER and PM during phagocytosis (Gagnon *et al.,* 2002).

Thus, the ER-mediated phagocytosis was suggested as an alternative mechanism employed by phagocytes to internalize large particles and microorganisms without significant depletion of their surface area (Gagnon *et al.,* 2002). Other studies involving both down regulation and up regulation of fusogenic molecules associated with the ER (Syntaxin18, D12, and Sec22b), indicated that these ER-SNAREs regulate fusion between the ER and PM during phagocytosis (Hatsuzawa *et al.,* 2006). It has been also demonstrated that molecules in phagosomes can access MHC class I via cross-presentation in phagosomes (Houde *et al.,* 2003; Ackerman *et al.,* 2003; Guermonprez

et al., 2003), as well as the export of antigens out of the phagosome require ER components (Ackerman *et al.,* 2006). However, the involvement of the ER in the process of latex bead phagocytosis in J774 cells, RAW264.7 cells, and dendritic cells was not confirmed by some independent studies employing biochemical and immunogold EM techniques (Groothuis and Neefjes, 2005; Touret *et al.,* 2005a). Therefore the role of the ER in the process of phagocytosis and phagosome formation still remains controversial.

Leishmania parasites enter macrophages by the process of phagocytosis and reside in PVs with different morphologies (see description above). Several studies have shown that like inert particles phagosomes, nascent *Leishmania* PVs interact with endocytic compartments; early endosomes, late endosomes and lysosomes, to acquire phagolysosomal properties (Courret *et al.,* 2002). However, the extent of the interactions of the host cell's ER with nascent and secondary PV has not been assessed. The present Dissertation project assessed the contributions of the host ER to nascent and maturing *Leishmania* PVs in macrophages. Macrophages either transiently or stably expressing ER marker tagged fluorescent protein were employed to monitor the recruitment of host ER components to *Leishmania* PVs by fluorescence and Immuno-Electron microscopy. We also exploited the retrograde pathway of ricin in macrophages to demonstrate the accumulation of host ER contents into *Leishmania* PVs lumen. Finally, dominant negative constructs generated from ER-SNAREs (Sec22b, D12, and Syntaxin18) were used to determine how the interaction of the ER and PVs affects *Leishmania* parasites entry and replication in macrophages, as well as the size and development of their PVs in macrophages.

Our data demonstrated that *Leishmania* parasites within macrophages, reside inside a membranes-bound structure called PVs, which is a hybrid compartment composed of components of both the host endocytic pathways (late endosomes and lysosomes) and the ER. This study also provided evidence that host ER contents are continuously delivered to *Leishmania* PVs , which may occur through the process of vesicular transport and membrane fusion involving ER membrane-associated R-SNAREs (Sec22b and D12), but not the Q-SNARE (syntaxin18). The R-SNAREs had a significant effect on PVs development at the later time point during infection, and on *Leishmania* parasite replication. Therefore, *Leishmania* amastigotes positively select host ER components to survive, replicate and established a chronic infection in their mammalian hosts.

These findings are significant because they can lead to the development of new therapeutic strategies to combat *Leishmania*sis, which affect more that 12 millions people around the globe. The evidence presented in this study and other previous ones had led us to propose a model summarizing (Figure 6) of our understanding of the interactions of the host ER with *Leishmania* PVs during their biogenesis and maturation within macrophages. In this model, *Leishmania* parasite ligands (reviewed in (Lodge and Descoteaux, 2005)) make contact with the host cell surface receptors (reviewed in (Haas, 2007)); this interaction should initiate a sequence of signal transductions within the macrophages (Haas, 2007) that will lead to the formation of a phagocytic cup by the plasma membrane (PM). ER- derived vesicles will be transported along the microtubules to the phagocytic cup (PC), where there will fuse with the PM underneath the PC through a process mediated by ER-SNAREs such as Sec22b (Becker *et al.,*

2005) and D12, and even Syntaxin18 (Hatsuzawa *et al.,* 2006), and other fusion molecules (Jahn and Scheller, 2006; Hong, 2005). The fusion of host ER-derived vesicles to the PC provide molecules and membranes (Gagnon *et al.,* 2002; Muller-Taubenberger *et al.,* 2001) that will facilitate the phagocytosis process while keeping the cell surface area relative constant. The end of the phagocytosis process or parasite entry inside the host cell is marked by the internalization of the *Leishmania* parasite, which is enclosed in a newly formed PV. In order to mature, the nascent PV will continuously fuse with host ER-derived vesicles through out the course of the infection. The nascent PV will also in a sequential and orderly manner fused with early and recycling endosomes, late endosomes, and lysosomes, which are also transported by microtubules and associated motor proteins. A mature PV, which generally contains the amastigote form of the parasites, has a hybrid membrane that is mainly composed of host late endosomes, lysosomes and ER.

Perspectives

Future investigation as a continuation of this work will focus on four main points. First, the concept of vesicular transport of ER-derived vesicles proposed for the first time in this study, rather than that there is a continuity between *Leishmania* PVs and the host ER compartment. This can be further characterized by employing the a 3-dimensional immuno-EM tomography approach. This approach has been previously used to successful define the 3-dimensional ultrastructural organization of the complex cellular sub-compartments and functions such as ERGIC and ER-to Golgi transport in plant cells (Kang and Staehelin, 2008). Moreover, the possibility of the involvement of cell cytoskeleton components in ER-derived vesicles transport remains experimentally untested. From the present study, the functions or role of ER-SNAREs in *Leishmania*

parasites entry in macrophages or phagocytosis is not fully characterized. Therefore, other approaches adapting tools such as siRNA, drug assays, gene knock-down, to simultaneously inhibit the function of multiple ER-SNAREs could be applied.

Although the present study clearly demonstrated that *Leishmania* PVs are hybrid compartments containing components of lysosomes and the host ER, the effect of host ER components on the properties of lysosomal molecules within the PVs, or on the PV's composition in general remains unknown. Equally, the effect of the endocytic components on ER functions within the PV still need to be defined. Answers to these questions can significantly help uncover the mechanisms by which *Leishmania* parasites survive the microbiocidal factors generated by normal phagosomes.

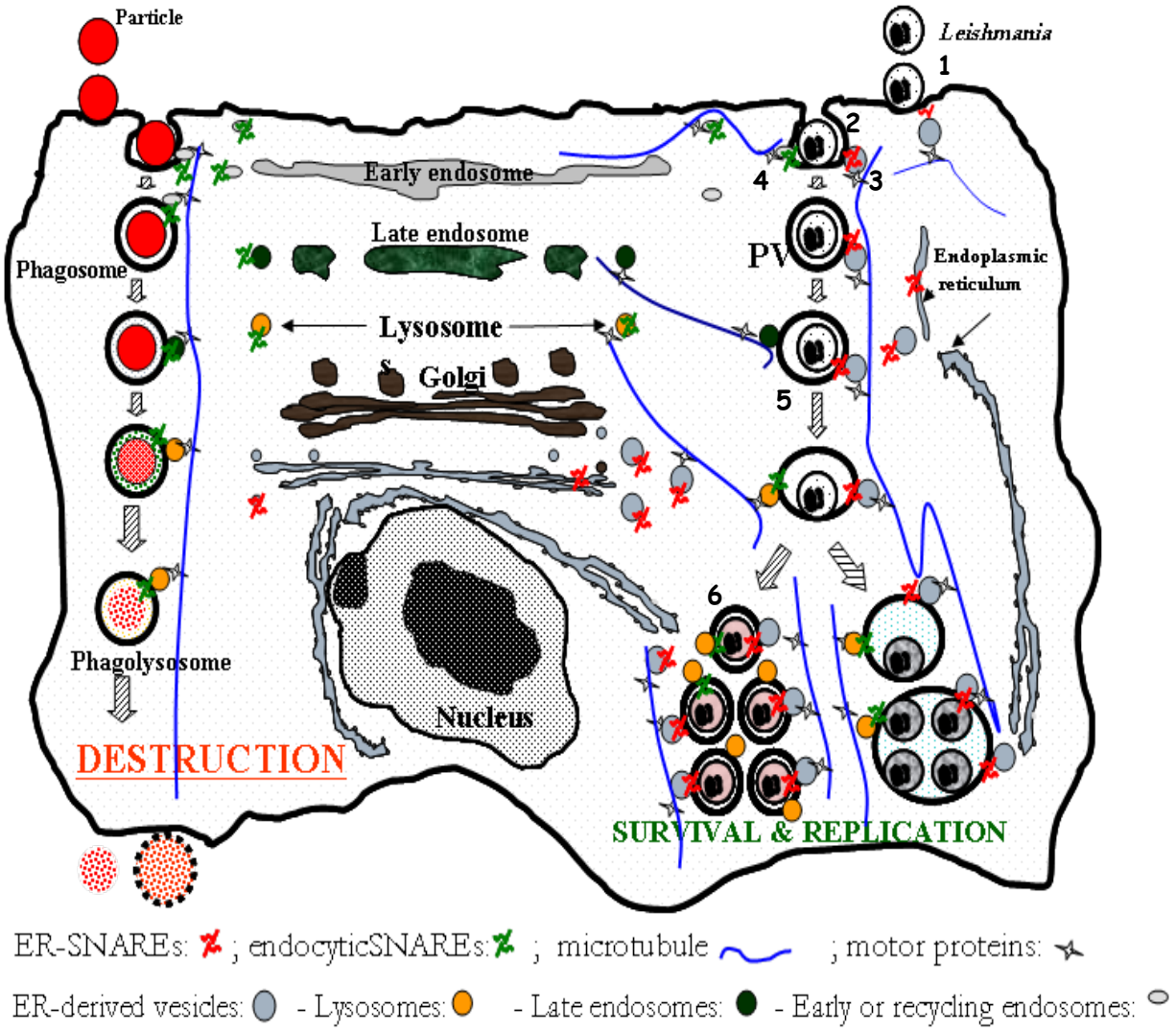
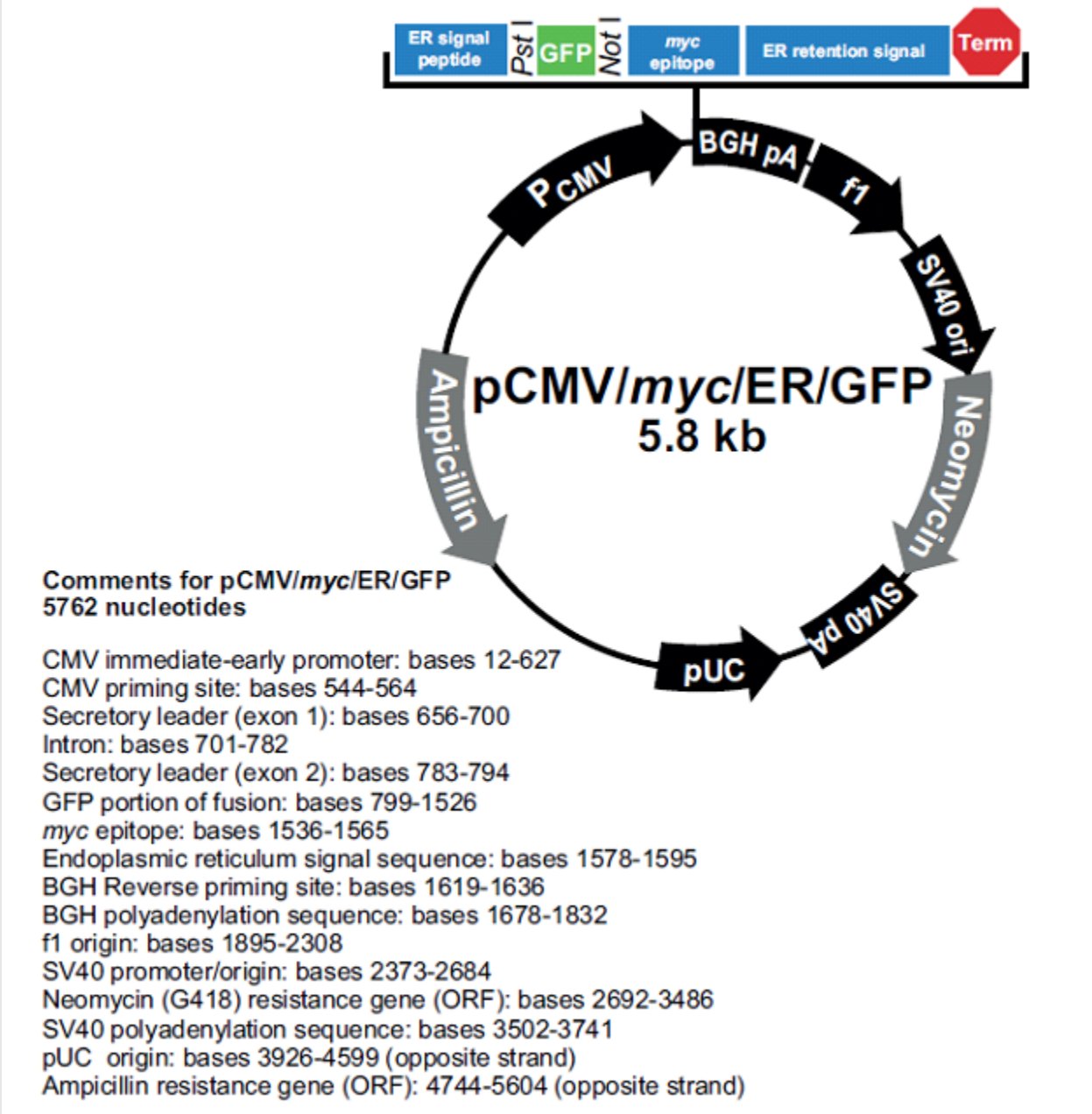


Figure 6-1. Model of the macrophage endoplasmic reticulum contributions to *Leishmania* PV biogenesis and maturation. 1- contact *Leishmania* parasite and host cell surface. 2- Formation of the phagocytic cup (PC). 3- Transport and fusion of ER-derived vesicles to the PC, mediated by ER-SNAREs (Sec22b, D12, and Syntaxin18) and other fusion molecules. Fusion of host ER-derived vesicles to the PC provides molecules and membranes and facilitates the phagocytosis. 4- The newly formed PV interacts with host ER-derived vesicles, and early and recycling endosomes. 5- Fusion of the PV with late endosomes and ER vesicles, and later lysosomes. Microtubules and associated motor proteins enable the vesicular transport. 6- A mature PV has a hybrid membrane made of host late endosomes, lysosomes and ER. The formation and maturation of the inert particle phagosome (Left) do not require fusion or interaction with host ER components.

APPENDIX A
ANALYSIS OF THE CALNEXIN GENE AND THE PCMV/ER/GFP VECTOR



Comments for pCMV/*myc*/ER/GFP
5762 nucleotides

CMV immediate-early promoter: bases 12-627
CMV priming site: bases 544-564
Secretory leader (exon 1): bases 656-700
Intron: bases 701-782
Secretory leader (exon 2): bases 783-794
GFP portion of fusion: bases 799-1526
myc epitope: bases 1536-1565
Endoplasmic reticulum signal sequence: bases 1578-1595
BGH Reverse priming site: bases 1619-1636
BGH polyadenylation sequence: bases 1678-1832
f1 origin: bases 1895-2308
SV40 promoter/origin: bases 2373-2684
Neomycin (G418) resistance gene (ORF): bases 2692-3486
SV40 polyadenylation sequence: bases 3502-3741
pUC origin: bases 3926-4599 (opposite strand)
Ampicillin resistance gene (ORF): 4744-5604 (opposite strand)

Figure A-1. pCMV/myc/ER/GFP Map. Adapted from Invitrogen Inc. (www.invitrogen.com)

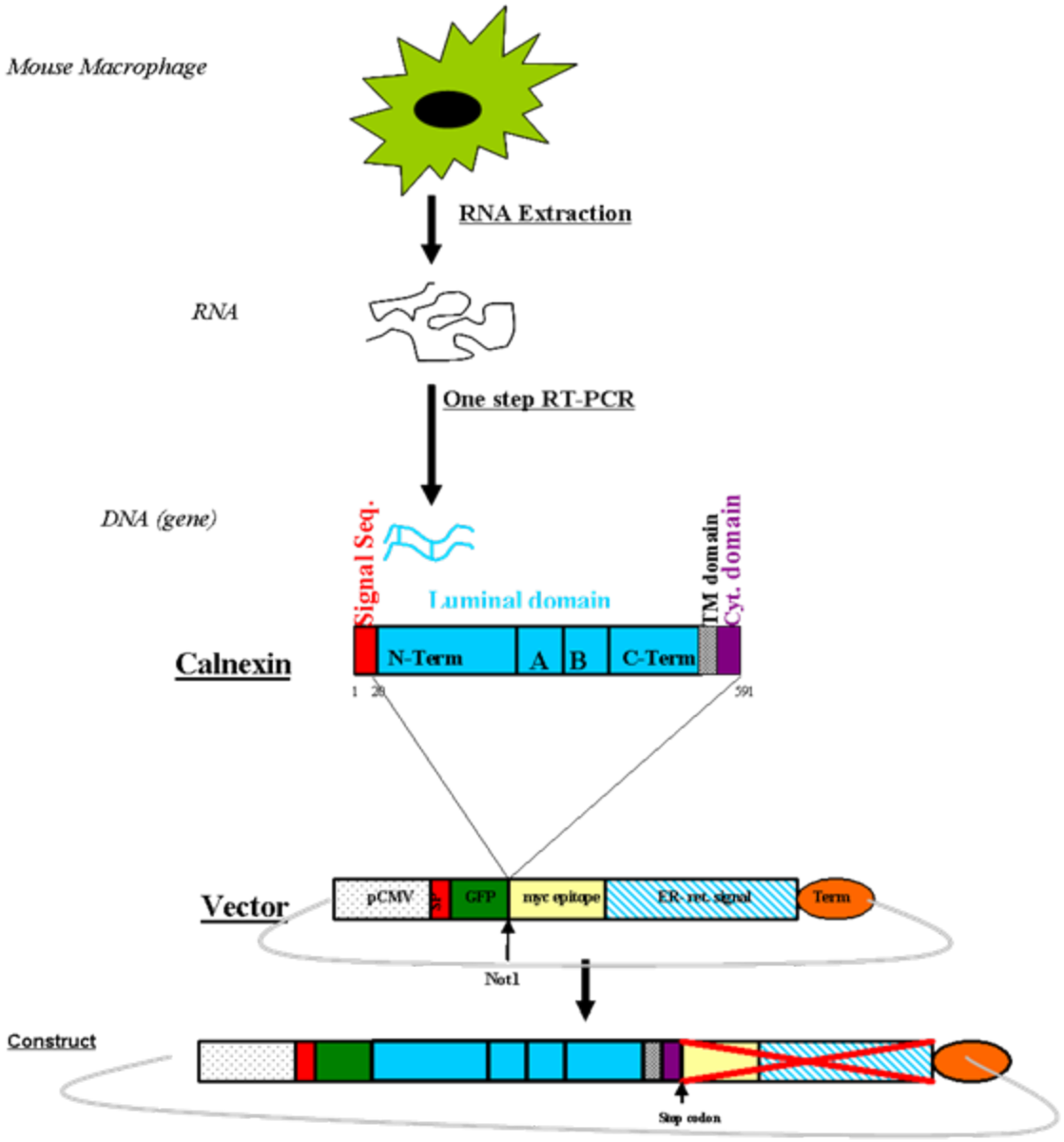


Figure A-2. Schematic design of the pCMV/GFP/calnexin vector construction

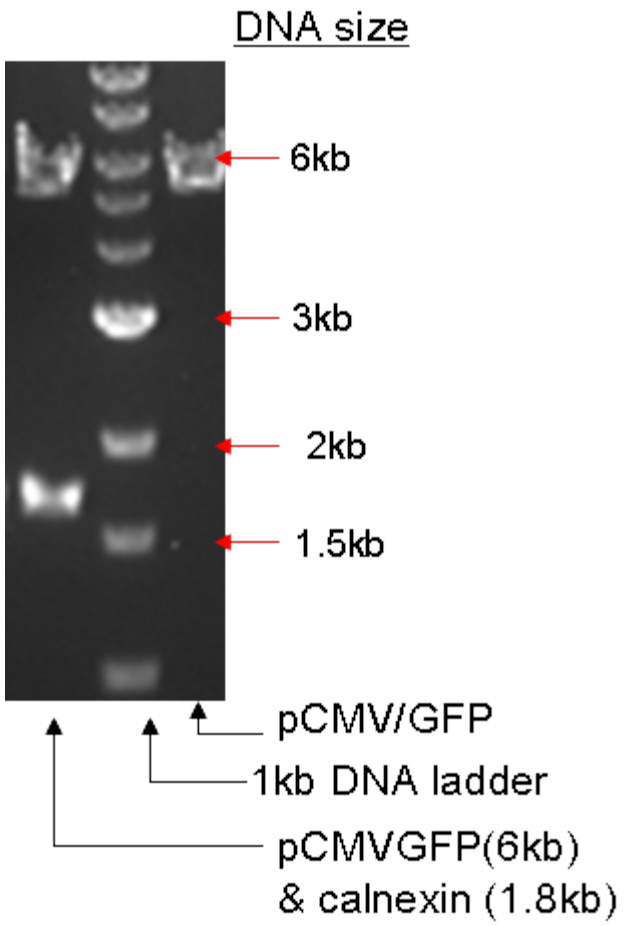


Figure A-3. 0.8% agarose gel electrophoresis. pCMV/GFP and pCMV/GFP/Calnexin plasmids were digested with NotI restriction enzyme for 4h at 37°C.

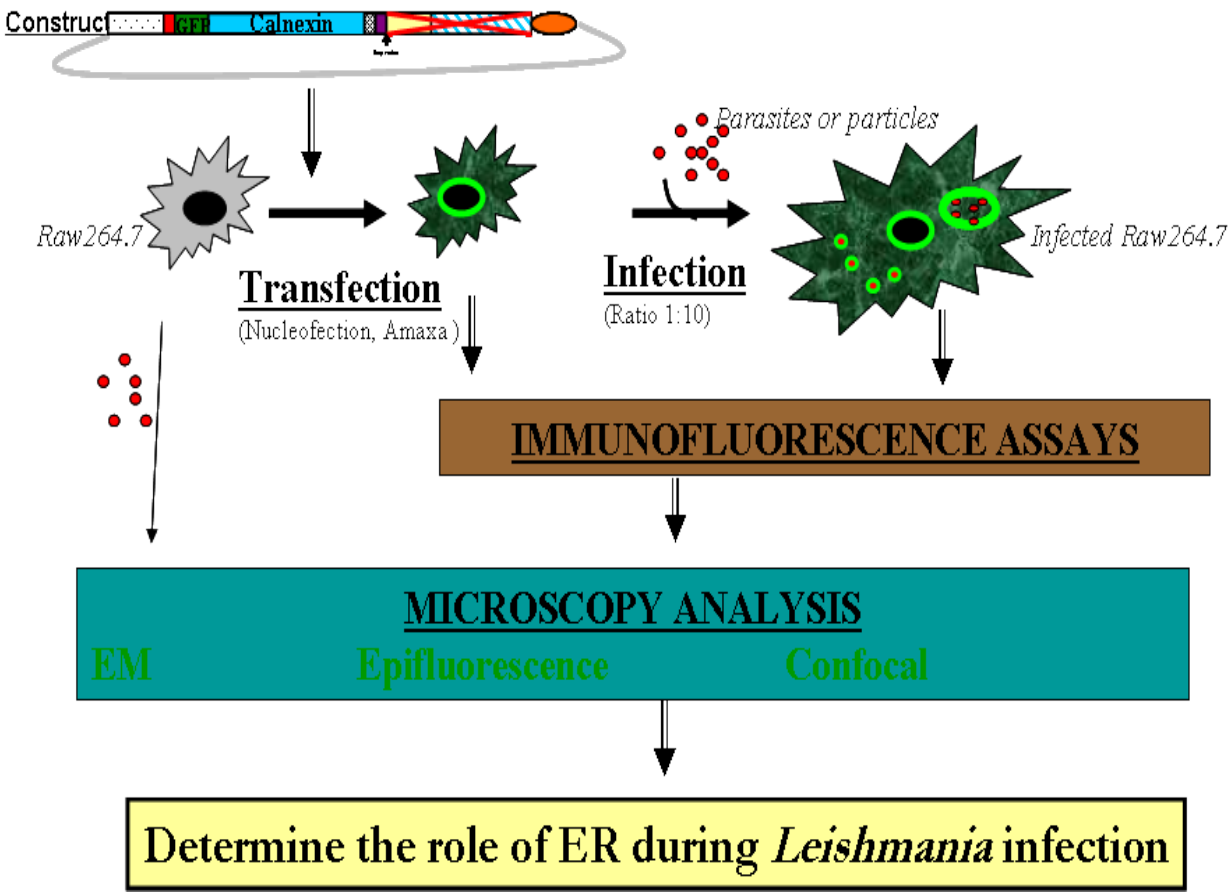


Figure A-4. Experimental design to assess host ER recruitment to *Leishmania* PVs. EM: Electron microscope.

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BIOGRAPHICAL SKETCH

Blaise Ndjamen was born in February of 1973 in Ndoungue, Littoral Province, Cameroon. He received a Bachelor of Science in biology from University of Yaoundé I in August of 1996 in Yaoundé, Cameroon. He also received a master's degree in parasitology in August 1998, and an Advanced Professional Degree in parasitology in 2000, both at University of Yaoundé. He won the prize of Best Student in the Department of Animal Biology in 1999, and an Award of Academic Excellence from the Cameroon Ministry of High Education in 2000. He worked as Research Assistant at the Centre for Schistosomiasis, and a Research Associate at the Medicinal Research Center of the Ministry of Scientific Research and Innovation in 2002; where he did both laboratory and field work in project aiming at controlling the schistosomiasis disease, vectors, and parasites. His research work in public health had lead to the identification and characterization of a new focus of Schistosomiasis in the village of Yoro, in the Central Province of Cameroon. He received a Fulbright Fellowship from the United States of America's Government in 2003, to travel to the United States of America to complete a master's degree in public health at Tulane University. He joined the graduate program at the Department of Microbiology and Cell Science at the University of Florida in August of 2005. He began working with Dr. Peter Kima on host endoplasmic reticulum interaction with *Leishmania* parasitophorous vacuoles. He married Ann Lee Grimstad on December 23, 2009 in Gainesville, Florida. He has attended and presented papers at several national and international scientific meetings. Blaise Ndjamen plans on pursuing a career in cancer biology and drug discovery.