LEISHMANIA PARASITOPHOROUS VACUOLES – THE CONTRIBUTION OF THE SECRETORY PATHWAY TO PARASITOPHOROUS VACUOLE BIOGENESIS AND INTRACELLULAR PARASITE REPLICATION

By

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To my mother and father

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BCG	Bacille-Calmette Guerin
CL	Cutaneous leishmaniasis
Co-IP	Co-immunoprecipitation
D12	D12/USE-1/p31
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
DDC	Dermal dendritic cell
DMEM	Dulbecco’s Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DSP	Dead *Salmonella* containing phagosome
EDTA	Ethylene diamine tetraacetic acid
EEA1	Early Endosome Antigen 1
EGTA	Ethylene glycol tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERGIC	ER/Golgi intermediate region
fPPG	Filamentous Proteophosphoglycan
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon- γ
IL-6	Interleukin-6
LACK	*Leishmania* homologue for activated C kinase
LAMP1	Lysosomal-Associated Antigen 1
LCV	*Legionella* containing vacuole

LCV	*Listeria* containing vacuole
LeIF	*Leishmania braziliensis* elongation and initiation factor
LmSTI1	*Leishmania major* stress-inducible protein 1
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LSP	Live *Salmonella* containing phagosome
ManLAM	Mannose-capped lipoarabinomannan
MCL	Mucocutaneous leishmaniasis
MCV	*Mycobacterium*-containing vacuole
MHCII	Major histocompatibilty complex class II
MPL	Monophosphoryl lipid A
MPL-SE	Monophosphoryl lipid A in oil and water emulsion
NEM	N-ethylmaleimide
NSF	N-ethylmaleimide sensitive factor
PBS	Phosphate-buffered saline
PEC	Peritoneal Exudate Cells
PFA	Paraformaldehyde
PIM	Phosphatidyl Inositol Mannoside
PKDL	Post-kala-azar dermal leishmaniasis
PNS	Post-nuclear supernatant
PPG	Proteophosphoglycan
PSA-2	Parasite surface antigen 2
PSG	Parasite Secretory Protein
PV	parasitophorous vacuole
RCF	Relative centrifugal force

RFP	Red fluorescent protein
RIPA	Radioimmunoprecipitation Assay Buffer
SCV	*Salmonella* containing vacuole
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophresis
siRNA	Small interfering ribonucleic acid oligonucleotides
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
SPI	*Salmonella* pathogenicity islands
STX18	Syntaxin 18
STX5	Syntaxin 5
T3SS	Type III secretion system
TBST	Tris-Buffered Saline with 0.05% Tween-20
Tfr	Transferrin Receptor
TI-VAMP/VAMP7	Tetanus neurotoxin-insensitive vesicle-associated membrane protein 3
TLR3	Toll-Like Receptor 3
Tris-HCl	Tris-Hydrochlric acid
TSA	Thiol-specific antioxidant
VAMP3	Vesicle-associated membrane protein 3
VL	Visceral leishmaniasis
WHO	World Health Organization

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During the intracellular stage of their life cycle, *Leishmania amazonensis* parasites reside in a specialized, membrane-bound compartment termed a parasitophorous vacuole (PV). Well-established interactions of the PV with host cell compartments have been documented, including transient interactions with early endosomes and more sustained interactions with late endosomes and lysosomes. However, there is growing evidence for the interaction of PVs with another host cell compartment - the endoplasmic reticulum (ER). Here we extend these observations by showing, for the first time, the recruitment of several ER soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) to the PV. In addition, we show that in blocking the recruitment of host cell ER to the PV, parasite replication and PV development are compromised.

Blocking the recruitment of host cell ER to the PV was achieved by overexpressing dominant negative variants of the ER SNAREs sec22b, D12 and syntaxin 18, all of which were found to be present on the PV. Under these conditions, PVs failed to distend and parasite replication was reduced. These studies were confirmed by

knocking down the expression of the ER SNAREs sec22b, D12 and syntaxin 18, as well as, the ER Golgi SNARE syntaxin 5 by using siRNA. Once again, under these conditions, PVs failed to distend and parasite replication was significantly reduced. In both overexpression and knockdown studies, the targeting or ER/Golgi SNAREs had no measurable effect on ER morphology or activated secretion. We also extended studies on the role of syntaxin 5 by making use of a small molecule inhibitor of syntaxin 5 - retro-2. Retro-2 treatment of cells resulted in a significant reduction in parasite replication and PV distention. In a mouse model of *Leishmania amazonensis* infection, retro-2 treatment of infected mice resulted in a significant reduction in lesion size as well as parasite titer at the site of infection without any apparent effect on mouse health. Taken together, these observations suggest that the recruitment of host cell ER to the *Leishmania amazonensis* PV is important for the establishment a replicative organelle; moreover, the targeting of this interaction may represent a viable strategy for the treatment of leishmaniasis.

CHAPTER 1
INTRODUCTION

The Leishmaniases

The Leishmaniases are a group of diseases caused by the flagellated protozoan parasite of the genus *Leishmania*. The diseases are widely spread throughout tropical and sub-tropical regions on every continent with the exception of Antarctica (Figure 1-1). The leishmaniases continue to be a burden in the areas where it is endemic, indeed Current World Health Organization (WHO) statistics estimate approximately 500,000 new cases of visceral leishmaniasis and 1-1.5 million new cases of cutaneous leishmaniasis per year, an overall prevalence of 12 million reported clinical cases and an at-risk population of 350 million in 88 countries(Desjeux, 2004). Moreover, recent studies have reported the reactivation of several foci including Italy, China, Brazil and central Israel(Arias et al., 1996; Gradoni et al., 2003; Guan et al., 2003; Bañuls et al., 2007) as well as the emergence of new foci in northern and central Israel and Morocco(Jacobson et al., 2003; Al-Jawabreh et al., 2004; Guernaoui et al., 2005; Shani-Adir et al., 2005; Bañuls et al., 2007). As new risk factors continue to emerge(Desjeux, 2001), such as an increase in the cases of co-infection of the human immunodeficiency virus (HIV) and *Leishmania* , increased clearing of primary forest and increased migration from rural to urban areas, the leishmaniases continue to be a major public health concern.

The disease itself can be classified as an anthropozoonosis or a disease that is primarily a zoonosis but is transmissible to humans. There are, however, exceptions, the species *Leishmania donovani* is known to be transmissible from human to human. According to Bañuls et al., the epidemiological cycles are (i) a primitive or sylvatic cycle

in which transmission to humans is accidental, (ii) a secondary or peridomestic cycle in which the reservoir is domestic or peridomestic animals, and (iii) a tertiary, strictly anthroponotic cycle, in which there is no apparent animal reservoir and the vector is completely anthroponotic (Bañuls et al., 2007).

In a very broad sense, the distribution of the Leishmaniases can be subdivided into the “New World” and the “Old World”. Generally, all species of the subgenus *Viannia* were isolated in the “New World” and all species in the subgenus of *Leishmania* were isolated in the “Old World”; however, there are exceptions such as *Leishmania major* and *Leishmania infantum* which can be found in both the “Old” and “New World”(Bañuls et al., 2007).

Forms of Leishmaniasis

The majority of *Leishmania* species are adapted to a large range of host species and, for the most part, infections remain asymptomatic (Peters, 1987). However, it is when *Leishmania* infects the less adapted host, such as humans, that a wide range of pathologies emerges. In humans, the leishmaniases can be divided into various types of disease including visceral (VL), cutaneous (CL) and mucocutaneous (MCL) leishmaniasis. The cutaneous form of the disease can be further divided into diffuse and localized cutaneous leishmaniasis. More recently, it has been recognized that the parasite may survive for decades in asymptomatic infected humans and that these individuals are of great importance for transmission because they can transmit the visceral form of the disease through the vector.

Visceral Leishmaniasis (VL)

In the “Old World”, VL, also known as kala-azar, is caused by parasites of the *Leishmania donovani* complex, whereas, in the “New World” it is caused by *Leishmania*

chagasi. There are occasional exceptions, for example, there are reports that describe cases of VL where the causative agent is *Leishmania tropica* and *Leishmania amazonensis* both of which usually result in CL. VL is the most serious form of the disease and is almost always fatal if left untreated. This form of the disease is characterized by undulating fever, substantial weight loss, splenomegaly, hepatomegaly, lymphadenopathies and anemia. Active VL may also represent relapse (recurrence after 6-12 months after apparent successful treatment) or late reactivation (recrudescence) of subclinical or previously treated infection(Murray et al., 2005). Reactivation may be spontaneous, but often times is provoked by an insult to T (CD4) cell number or function - corticosteroid or cytotoxic therapy, anti-rejection treatment in transplant recipients, or advanced HIV disease (Pintado et al., 2001; Murray, 2004, 2004; Murray et al., 2005).

After recovering from kala-azar, patients may develop a recurring form of the disease termed post-kala-azar dermal leishmaniasis (PKDL), which requires long and expensive treatment. PKDL can appear anywhere between two to seven years post-recovery and starts out with a mottling of the skin that resembles freckles (Bañuls et al., 2007). Five to fifteen percent of VL patients in India end up developing PKDL, usually within one to two years of apparent clinical cure(Salotra and Singh, 2006).

Cutaneous Leishmaniasis (CL)

Multiple species of *Leishmania* are responsible for the cutaneous form of the disease. It is useful to make the distinction between “Old World cutaneous leishmaniasis” and “New World cutaneous leishmaniasis” not only because they are caused by different species, but also because the manifestation of the disease as well as the approach to treatment of the disease can be quite different. In the “Old World”,

CL is primarily caused by *L. major, L. tropica, L. (L) aethiopica, L. infantum,* and *L. chagasi*; whereas, in the "New World", it is primarily caused by *L. mexicana, L. (L) amazonensis, L. braziliensis, L. (V) panamensis, L. (V) peruviana,* and *L. (V) guyanensis*. An erythematous papule usually begins to form at the site of infection. It enlarges to form a painless nodule and will begin to ulcerate around one to three months post-infection. Flat plaques, hyper-keratotic or wart-like lesions may also appear(Murray et al., 2005). In some cases, the parasite may disseminate and form new papules immediately around the healed lesion. This form of *Leishmaniasis* is the most severe form of CL (leishmaniasis recidivans) and is very difficult to treat, long lasting, destructive and disfiguring. The location of the lesion on the body depends on lifestyle and clothing habits (Dowlati, 1996). For example, patients, including travelers and military personnel (Blum et al., 2004; Weina et al., 2004; Magill, 2005; Schwartz et al., 2006), often seek attention for papules or nodules that form on areas of skin exposed at night.

Diffuse Cutaneous Leishmaniasis (DCL)

DCL is more geographically restricted than CL. Indeed, DCL is restricted to Ethiopia and Kenya in the "Old World" and Venezuela and the Dominican Republic in the "New World". It usually results from infection with the same parasites responsible for CL; however, patients presenting with DCL have a specific anergy or lack of an immunological response(Ashford, 2000). The disease is characterized by multiple lesions which may be restricted, perhaps only on the ear, or may be more widespread on the body. The lesions themselves, albeit painless, are grossly disfiguring.

Mucocutaneous Leishmaniasis (ML)

Classical ML, also known as espundia, is restricted to “New World” *Leishmania (Viannia)* species infections in which, after what appears to be complete resolution of a primary lesion, metastatic secondary lesions appear on the buccal or nasal muscosa. ML is also occasionally reported in Sudan and other “Old World” foci; however, these cases seem to originate from infection on or near the mucosa as opposed to resulting from metastasis. ML causes extensive destruction of oro-nasal and pharyngeal cavities with unsightly disfiguring lesions and lifelong stress for the patient (Bañuls et al., 2007). Interestingly, a recent study reported that a metastasizing strain of *Leishmania (Viannia) guyanensis*, but not a non-metastasizing strain, has a high burden of a non-segmented, double-stranded RNA virus, *Leishmania* RNA virus (LRV). The host Toll-Like Receptor 3 (TLR3) senses the RNA virus and this results in a pro-inflammatory response, which may in turn facilitate metastasis (Ives et al., 2011; Ronet et al., 2011).

Treatment

As a result in the differences in manifestation of VL and CL, the approaches taken to the treatment and development of new treatments has been quite different and will be discussed separately in this section. The development of new forms of treatment can be complicated by several factors including the intracellular location of the target form of the parasites, amastigotes, and the varying sensitivities of strains and species compounded by their inter-relationship with the host immune system, which under some circumstances renders drugs ineffective (Croft and Olliaro, 2011).

The potentially fatal nature of VL has resulted in its inclusion as a target disease in drug research and development by product development partnerships such as the Drugs for Neglected Disease initiative, Institute of One World Health, Consortium for

Parasitic Drug Development, Bill and Melinda Gates foundation and Novartis. Pentavalent antimonials have served as the standard drug for VL for around 60 years and are available as sodium stibogluconate (Pentostam®), meglumine antimoniate (Glucantime®) (Alvar et al., 2006), or generic sodium stibogluconate. However, drug resistance in key endemic areas has rendered the use of pentavalent antimonials obsolete (Sundar, 2001). In these areas, amphotericin B has been used as a second-line treatment where resistance is evident and one liposomal formulation, AmBisome®, has become a standard treatment for VL. A recent study from India has shown that a single-course treatment with amphotericin B can resolve 95% of the cases of VL (Sundar et al., 2010). There are drawbacks to amphotericin B in that it represents a relatively expensive option, administration is intravenous and there are issues with its temperature sensitivity (Croft and Olliaro, 2011). Another drug, miltefosine, first identified in the 1980s (Croft et al., 1987), has shown 94% efficacy in adults and children in clinical trials in India (Sundar et al., 2002) and has become the first registered oral treatment for VL. This drug is also not without potential issues and has been linked to potential teratogenicity and requires 28 days of oral treatment, which results in poor compliance (Croft and Olliaro, 2011). The possibility of drug combinations in order to shorten the course of therapy, reduce toxicities through lower dosages and reduce the risk of selection for resistance mutations in infectious diseases such as malaria and tuberculosis are being pursued.

Treatment options for CL are limited and this is partially due to the issues of species variation and pharmacokinetics. Pentavalent antimonials are less efficient when it comes to CL and it has been suggested that this is in part due to the larger

range of species that result in CL. Amphotericin B has also showed a limited range of effectiveness across species causing CL (Alvar et al., 2006). Although registered for CL treatment in Columbia, oral miltefosine has variable, species dependent effectiveness against CL (Soto et al., 2004; Yardley et al., 2005; Croft and Olliaro, 2011). It is important to note that most CL lesions are self-resolving; therefore, a common strategy for the development of drugs for the treatment of CL has been to look for drugs that aid self-cure such as immunomodulators (Garnier and Croft, 2002). This sort of adjunct therapy has been trialed for years, including Bacille-Calemette Guerin and trehalose dimycolate to small molecules such as the anti-viral Toll-like receptor 7 agonist imiquimod (Croft and Olliaro, 2011). Although these studies show some improvement, it is clear that there is much more work to be done with regards to the successful treatment of leishmaniasis.

Vaccine

Currently, there is no vaccine for any of the forms of leishmaniasis. It has been known that self-healing CL confers lifelong protection against the disease and this suggests that the development of a vaccine is feasible. This observation led to the centuries old practice of scarification or leishmanization in which individuals are purposefully given the disease in an effort to develop immunity (Nadim et al., 1983). Although leishmanization had proven effective, particularly in the Middle East where it has been practiced on a large scale, the adverse effects and local lesions persisting for several months in 2 – 3% of the cases (Hosseini et al., 2005) demands improvements and alternatives (Schroeder and Aebischer, 2011). Evidence from animal models using various vaccine formulations have shown that immunization can be achieved; however, when tested in the field results have been poor (Kedzierski et al., 2006). First

generation vaccines consisting of whole killed parasites have been suggested for both therapeutic and prophylactic purposes. Whole-cell killed vaccines; however, have been poorly defined and variable in potency therefore, have rendered inconclusive results (Kedzierski, 2010).

Second-generation vaccines consisting of recombinant proteins, poly-proteins, DNA vaccines or dendritic cells loaded with peptides from *Leishmania* antigens have become the primary focus of most vaccine studies. The recombinant nature of the product means it is accessible to large-scale, reproducible and cost-effective approaches and responses elicited upon vaccination can be potentiated and refined by appropriate formulation with adjuvant (Reed et al., 2009; Duthie et al., 2011, 2012).

A variety of molecules have been looked at thus far, one of which is the major *Leishmania* coat protein gp63. Although immunization with gp63 gave promising results in the mouse model, it gave a poor T cell response when tested in humans (Burns et al., 1991; Russo et al., 1991). Other molecules such as the native parasite surface antigen 2 (PSA-2) and *Leishmania* homologue of activated C kinase (LACK) have also been tested using recombinant systems and, despite immunogenicity, failed to elicit protection from experimental leishmaniasis (Handman et al., 1995, 2000; Mougneau et al., 1995; Sjölander et al., 1998a, 1998b; Melby et al., 2001). The first defined vaccine against leishmaniasis came in the form of the recombinant fusion protein Leish-111f combined with the TLR-4 agonist monophosphoryl lipd A (MPL) in oil and water (MPL-SE). The fusion protein itself is comprised of *L. major* homologue of eukaryotic thiol-specific antioxidant (TSA), the *L. major* stress-inducible protein-1 (LmSTI1), and the *L. braziliensis* elongation and initiation factor (LeIF). This vaccine formulation protects

mice, hamsters and rhesus macaques and was the first to enter clinical trials. Thus far, Leish-111f with MPL-SE has proven safe and immunogenic in healthy subjects with and without history of previous infection with *Leishmania* and in patients with CL and ML (Campos-Neto et al., 2001; Skeiky et al., 2002; Coler et al., 2007; Vélez et al., 2009; Nascimento et al., 2010; Chakravarty et al., 2011; Duthie et al., 2012). In summary, the data suggests that there is the potential for a vaccine that can provide long-term protection and, in some instances, have therapeutic value; therefore, work continues to be done towards achieving this goal.

The Parasite

As mentioned earlier, Leishmaniasis is caused by the protozoan parasite of the genus *Leishmania*. The parasites are digenetic and thus have two basic life cycle stages, an extracellular stage in which they reside in the gut of an invertebrate host, and an intracellular stage in which they reside in a specialized intracellular compartment in a mammalian host (Figure 1-2). As a consequence, the parasites exist in two main morphologies, a flagellated, motile promastigote form and a non-flagellated amastigote form, which reside in the invertebrate and mammalian host respectively.

Life in a Sand Fly

The invertebrate host for *Leishmania* is the phlebotamine sandfly which belongs to the order Diptera, belonging to the subfamily Phlebotiminae. All known vectors of importance for *Leishmania* transmission fall under two genera, *Phlebotomus* and *Lutzomyia* in the “Old” and “New World” respectively. For several reasons, the majority of sand fly species have no part to play in the transmission of leishmaniasis: they may never bite man; their distribution may not overlap with that of a reservoir host for *Leishmania*; or they may be unable to support the development of parasites (Killick-

Kendrick, 1999). Indeed, among the five hundred species of phlebotamine sandflies, only thirty-one have been positively identified as vectors of pathogenic species of *Leishmania* and 43 as probable vectors (Killick-Kendrick, 1990, 1999; Bañuls et al., 2007).

The female sand flies obtain *Leishmania* parasites by a taking a blood meal from an infected mammalian host. It is the amastigote form of the parasite that is taken up during the blood meal (Figure 1-2). The parasites are present in the skin of the host itself and cannot be found in the peripheral circulation. The cutting action of the sand fly's mouth part results in the creation of a "pool" of blood into which infected macrophages are released and subsequently taken up by the "pool feeding" sand fly (Bates, 2007).

As the amastigote moves from the mammalian host to the sand fly vector, it experiences a change in conditions: decrease in temperature and increase in pH. It is this change in conditions that triggers development of the parasite in the invertebrate host (Bates and Rogers, 2004; Kamhawi, 2006; Bates, 2007). The non-motile amastigotes transform into motile promastigotes with a flagellum at the anterior end of the parasite. This form of the parasite is termed the procyclic promastigote and it undergoes replication in the blood meal within the sand fly gut. After a few days, replication is slowed and the procyclic promastigote differentiates into a more vigorously motile nectomonad promastigote. Nectomonad promastigotes migrate toward the midgut and attach to the epithelia to avoid removal via defecation. One of the major parasite surface glycoconjugates involved in attachment is lipophosphoglycan (LPG) (Pimenta et al., 1992; Kamhawi et al., 2004). The parasites resume replication as

leptomonad promastigotes. The final form in the sand fly is the metacyclic promastigote (Rogers et al., 2002) which is capable of infecting mammalian hosts.

The Transfer of Promastigotes to a Mammalian Host

The development of parasites in the midgut and foregut of the sand fly led to two early hypotheses about the mechanism by which *Leishmania* parasites are deposited in the mammalian host. One idea was that metacyclic promastigotes present in the foregut were directly deposited during proboscis probing and the other was that regurgitation of promastigotes from the midgut resulted in depositing of parasites (Peters, 1987; Bates, 2007). The finding that a previously unidentified gel-like substance present in the sandfly gut was in fact a parasite product added some credence to the regurgitation hypothesis. The gel-like substance was termed promastigote secretory gel (PSG) (Stierhof et al., 1999; Rogers et al., 2002) and the primary component was found to be filamentous proteophosphoglycan (fPPG) (Ilg et al., 1996). The PSG forms a plug in the anterior midgut and contains metacyclic promastigotes at the poles of the plug. Following up on these observations, it was shown that the primary mechanism of metacyclic promastigote depositing was indeed regurgitation of the PSG plug (Rogers et al., 2004).

Neutrophils and *Leishmania*

Leishmania had been widely regarded as fastidious, obligate intracellular pathogens of macrophages, but recent studies have confirmed that these parasites have a far greater degree of promiscuity in host cell range (Kaye and Scott, 2011). After being deposited by the sand fly, the parasite must invade and establish it's intracellular niche while at the same time avoid innate defense mechanisms of the host. It was suggested that neutrophils may serve as "Trojan Horses" for the promastigotes to

help evade innate defenses (Laskay et al., 2003). In this *in vitro* study, *Leishmania* infected neutrophils in culture and survived in neutrophil phagosomes. After inducing the neutrophils for apoptosis, macrophages were added to the culture of infected neutrophils. Mechanisms exist for the "silent" uptake of neutrophils undergoing apoptosis by macrophages in order to clear neutrophils without triggering macrophage defense mechanisms (Ravichandran and Lorenz, 2007); therefore, promastigotes were efficiently and safely shuttled to the macrophage phagosome (Laskay et al., 2003; Kaye and Scott, 2011). This model is supported by *in vivo* studies using two-photon intravital imaging which demonstrated that neutrophils are indeed recruited to the site of sand fly bite or needle inoculation of *Leishmania major* and that neutrophils were infected by parasites. Moreover, depletion of neutrophils reduced, rather than enhanced, the ability of parasites to establish a productive infection (Peters et al., 2008). However, there are studies that are contrary to the "Trojan Horse" hypothesis. *In situ* imaging studies have shown that the neutrophils that engulf promastigotes are relatively short-lived and release promastigotes before being phagocytosed by macrophages (Peters et al., 2008) and go on to show that depletion of neutrophils has no effect on the number of parasites in macrophages of mice. Taken together, these studies do suggest that neutrophils participate in the early response to *Leishmania* challenge; but the exact role that neutrophils are playing in establishing infection has yet to be clearly defined.

Mononuclear Phagocytes and *Leishmania*

Although parasites are taken up by neutrophils, it is well established that it is within mononuclear phagocytes that replication and long-term survival occurs. *In vivo* studies using two-photon intravital imaging have revealed that dermal dendritic cells (DDCs) take up *Leishmania major* promastigotes within hours of inoculation (Ng et al., 2008). In

addition to DDCs, it has been demonstrated that dermal macrophages also take up *Leishmania major* promastigotes at the site of inoculation, and that it is this population that becomes the primary infected population (Peters et al., 2008). The number of resident macrophages and DDCs are not sufficient to support the multiplication of parasites, as a consequence, the recruitment of monocytes is required for survival (Kaye and Scott, 2011). Many of the recruited monocytes differentiate into monocyte-derived dendritic cells which can support the multiplication of parasites at the lesion (León et al., 2007).

Phagocytosis and *Leishmania*

Various studies utilizing state-of-the-art microscopy and *in vivo* imaging techniques have demonstrated that after *Leishmania* has attached to the host cell membrane there appears to be no further requirement for active invasion by the parasite; instead, they can rely on the phagocytic mechanisms of the host cell for uptake (Antoine et al., 1998a; Courret et al., 2002; Forestier et al., 2011). Early studies indicated that complement receptors were involved in the uptake of *Leishmania* promastigotes by primary macrophages and the complement receptors CR1 and Mac-1(CR3) have indeed emerged as two of the main receptors for promastigote attachment and subsequent uptake (Mosser and Edelson, 1984; Blackwell et al., 1985; Wozencraft et al., 1986; Russell, 1987; Brittingham and Mosser, 1996). Cell membrane attachment triggers actin-dependent uptake by macrophages (Alexander, 1975), as a result of transient F-actin accumulation around the nascent phagosome (Holm A et al., 2001; Beattie and Kaye, 2011). Phagocytosis occurs rapidly and parasites have been shown to be internalized as soon as 10-20 minutes post-attachment. A recent study has suggested that during uptake and nascent phagosome formation *Leishmania*

promastigotes are in fact highly active and the persistent beating of flagella during phagosome formation results in wounding of the plasma membrane and subsequent recruitment of lysosomes to the site of wounding (Forestier et al., 2011).

Immediately after phagocytosis, the *Leishmania* promastigotes reside in a newly formed parasitophorous vacuole (PV) with much of its membrane derived from the host cell plasma membrane (Figure 1-3). Typically, phagosomes mature with a series of interactions with the endocytic pathway allowing cargo to be shuttled along various pathways, including recycling pathways, retrograde pathways and targeting to lysosomes for degradation (Huotari and Helenius, 2011). Initially, it was difficult to determine the extent to which the *Leishmania* phagosome or PV maturation was similar to normal phagosome maturation. Attempts to characterize the transition from a compartment containing early endosomal/recycling markers proved difficult because as early as 10-20 minutes post-phagocytosis nascent PVs were displaying late-endosomal/lysosomal markers (Courret et al., 2002). A more recent study using real-time imaging showed just how transient the interaction of nascent PVs with early endosomal compartments can be. The study utilized macrophages from transgenic mice expressing a RAB5-eGFP construct. RAB5, an early endosomal marker, was shown to be massively recruited to the early PV for only 1-2 minutes post-phagocytosis compared to latex bead phagosomes which retained RAB5 for greater than 30 minutes post-phagocytosis (Lippuner et al., 2009). A separate study showed that after 10-30 minutes the large majority of PVs do not display the early endosomal markers Early Endosome Antigen 1 (EEA1) and Transferrin receptor (Tfr); rather, at this time-point the overwhelming majority of PVs (about 95%) display late endosomal/lysosomal markers

such as macrosialin and Lysosome Associated Membrane Protein 1 (LAMP1) (Courret et al., 2002). By about 1 hour post-phagocytosis virtually all PVs will be positive for macrosialin and LAMP1 and other late endosmal/lysosomal markers such as cathepsins, major histocompatibility complex class II (MHCII) and RAB7p also increase with time (Figure 1-3).

The Selective Fusogenicity of *Leishmania* Parasitophorous Vacuoles

The observation that *Leishmania* PVs are fusogenic with other host cell compartments started with the early observations that in *Leishmania* infected cells, the lysosomal compartment was extensively depleted and that lysosomal markers, both luminal and membrane-bound, could be found on PVs (Alexander and Vickerman, 1975; Barbieri et al., 1985; Barbiéri et al., 1990). It is believed that the extensive fusion of lysosomes with PVs is, at least in part, responsible for the impressive aggrandizement of PVs during long-term infections. However, whether the interactions of the PV with the endocytic pathway were passive or if *Leishmania* PVs were more selective in which organelles they were fusing with was yet to be explored. One study, in the early 90s describing the transfer of Zymosan from Zymosan-containing phagosomes (ZCPs) to PVs reported selectivity in the fusion of PVs with ZCPs (Veras et al., 1992). Transfer of material to the PVs was selective in the sense that, Zymosan, beta glucan or heat-killed yeast particles were transferred, but not latex beads, aldehyde-fixed or immunoglobulin G-coated erythrocytes. It was suggested by the authors of the study that the selectivity of the fusion may be related to the high density of carbohydrate ligands displayed on the surface of yeast-derived particles, to ligand resistance to lysosomal degradation or to signals encoded in the cytosolic tails of the receptors engaged during uptake of the individual particles. These observations were

confirmed and extended by a later study showing that the *Listeria* containing vacuole (LCV) with live *Listeria*, but not heat-killed *Listeria*, was fusogenic with *Leishmania* PVs (Collins et al., 1997). Importantly, these reports hinted at, but did not show, that parasites can alter the fusogenic selectivity of the compartment in which they reside. A later study described the alteration of *Leishmania* PV interaction with the endocytic pathway. It made use of *Leishmania donovani* mutants deficient for a major promastigote surface molecule, lipophosphglycan (LPG). The authors describe an inhibition of the fusogenic capabilities of *Leishmania* PVs with endocytic organelles as compared to latex-bead phagosomes. Moreover, the inhibition could be reduced by infecting macrophages with LPG-deficient mutants of *L. donovani* (Desjardins and Descoteaux, 1997). It was suggested that LPG interferes with the molecular structure of lipid bilayers and would disrupt the fine-tuned events of membrane fusion (Miao et al., 1995). Therefore, the restriction of fusion events allows the promastigotes time to develop into amastigotes, which are more capable of survival in late endosomal/lysosomal compartments. Another study supported this work with evidence that *Leishmania major* promastigotes lacking LPG survived poorly in peritoneal exudate macrophages (Späth et al., 2000). However, the generality of this model began to be questioned when studies using LPG-deficient *Leishmania mexicana* promastigotes showed that they survived just as well as wild-type promastigotes in peritoneal exudate macrophages (Ilg, 2000). Moreover, recent studies have shown that interactions of the PV with early endosomes occurs on an extremely rapid timescale (<2 minutes post-infection) (Lippuner et al., 2009); therefore, the times probed in the Desjardins and Descoteaux study meant for early endosome fusion events (15 minutes post-infection)

may have been far beyond the appropriate time window. Another study suggested that, at least for *Leishmania chagasi* promastigotes, it was the mode of entry and not LPG content that determined the delay in fusion of PVs with late endocytic/lysosomal organelles. It showed that *L. chagasi* promastigotes enter through caveolae and that by disrupting caveolae, the delay in fusion could be reduced. Indeed, the observation held in both wild-type and LPG-deficient promastigotes (Rodríguez et al., 2006).

Although the interaction of *Leishmania* PVs with endocytic organelles, including lysosomes, is well established, the interaction of PVs with other host cell compartments is less understood. Despite the lack of knowledge, there is growing evidence for an important role for the interaction between the PV and the host cell endoplasmic reticulum (ER). Following up on reports that the ER played an important role in the phagocytosis of large particles, as well as some intracellular pathogens, one study reported the efficient isolation of *Leishmania* PVs from macrophages using the ER molecule calnexin as an identifier (Kima and Dunn, 2005). Later, it was reported that *Leishmania donovani* promastigotes resided in compartments within neutrophils that displayed the ER molecules calnexin and glucose-6-phosphate. The report went on to suggest that the LPG content of the *Leishmania donovani* promastigotes determined the ER content of the PV; in that, LPG-deficient promastigotes lost ER markers and acquired lysosomal markers more rapidly than wild-type promastigotes (Gueirard et al., 2008). Ndjamen et al. went on to show that, in macrophages, the ER molecules calnexin and sec22b were continuously recruited to the PV membrane during the course of infection. In addition, ricin, which uses retrograde trafficking to reach the ER, was also trafficked to the PV indicating that ER luminal contents were also reaching the

PV during the course of infection (Ndjamen et al., 2010). These observations notwithstanding, the exact role that ER is playing in the development of the PV is yet to be determined.

Parasitophorous Vacuole Size

Most species of *Leishmania* reside within small "tight" PVs that only house one or two parasites. However, the parasites of the *L. mexicana* complex reside in large, communal PVs housing many parasites (Figure 1-3). These large compartments have been the primary focus of studies on PV biogenesis, maturation and fusogenicity. Although not yet clear, there have been some suggestions as to what may be providing the material, such as membrane, for the impressive aggrandizement of PVs of the *L. mexicana* complex parasites. As mentioned previously, early studies reported extensive fusion of the host cell lysosomal compartment with PVs (Alexander and Vickerman, 1975; Barbieri et al., 1985; Barbiéri et al., 1990). These observations imply that the fusion of lysosomes with PVs is partially responsible for the aggrandizement of PVs. In support of this, a recent study has shown that the LYST/beige molecule, which regulates lysosome size, also regulates PV size in infected macrophages. Overexpression of the LYST/beige molecule resulted in significantly smaller PVs and reduced parasite replication (Wilson et al., 2008). Importantly, this study suggested that by indirectly limiting PV expansion the intracellular survival of *L. amazonensis* is compromised. Also in support of a lysosomal contribution to PV size, it was shown that *L. donovani* can inhibit the acquisition of flotillin-1 by the PV (Dermine et al., 2001). Flotillin-1 is involved in the formation of lipid raft domains on phagosomes and the reduced acquisition was shown to limit interactions with late endosomes/lysosomes. It

is conceivable that the reduced interactions of *L. donovani* PVs with these late compartments could, in part, result in the small PVs characteristic of this species.

In addition to the lysosomal/phagolysosomal contribution to PV expansion, other studies have reported that homotypic fusion of PVs can also result in PV expansion. One study showed that *L. amazonensis* PVs fuse with one another by demonstrating a reduction in PV numbers over time, as well as, super-infecting with fluorescently labeled parasites and enumerating PVs housing both fluorescently labeled and non-labeled parasites (Real et al., 2008).

The endocytic compartment also appears to have a role in PV expansion. As described earlier, the PV acquires markers of various endocytic organelles during its maturation. However, the exact nature of these interactions is unclear and many seem to be very short-lived. Despite the short-lived nature of some of these interactions, such as Rab5, they appear to have bearing on PV size. In one study using macrophages expressing a constitutively active form of Rab5, it was shown that *L. dononvani* PVs, which are normally tight, became “giant” phagosomes and rendered the macrophage less adept at controlling infection (Duclos et al., 2000).

The recent observation that the ER chaperone calnexin and the ER molecule sec22b are continuously recruited to the PV during the course of infection (Ndjamen et al., 2010) also implicates the ER as a potential source of material for PV aggrandizement; however, more work needs to be done to explore whether this membrane-rich compartment contributes to PV size.

Despite the observations that fusion of *Leishmania* PVs with host cell compartments occurs and may result in PV expansion, there is very little knowledge of

how exactly *Leishmania* parasites are capable of modulating PV size. One interesting study suggested that an amastigote molecule, proteophosphglycan (PPG), shown to be secreted into the PV (Ilg et al., 1996) may be responsible for the formation of large vacuoles. The study described the isolation of PPG and treatment macrophages with the purified molecule. They observed significant "vacuolization" of macrophages and went on to suggest that the vacuoles induced by PPG treatment were similar to *Leishmania* PVs, although this was not explored experimentally. They also suggested that since *L. major* parasites do not secrete PPG as do *L. mexicana* parasites, this molecule may be responsible for the discrepancy in PV size between species (Peters et al., 1997). An in depth understanding, of why *L. mexicana* complex parasites form large PVs, while all other species do not, is lacking and further investigation is certainly required. That said, parasites of *L. mexicana* seem to be more adept at intracellular survival (Gomes et al., 2003; McMahon-Pratt and Alexander, 2004; Qi et al., 2004) and whether this is a direct result of PV size is also of interest.

Survival in the Parasitophorous Vacuole

The PV presents an acidic, strongly hydrolytic environment in which *Leishmania* parasites must persist. Interestingly, the parasites do not seem to attenuate this relatively harsh environment, although some have suggested that the formation of large vacuoles by the *L. mexicana* complex parasites serves to dilute some potentially leishmanicidal factors (Wilson et al., 2008). Instead, amastigotes seem to benefit from the low molecular weight nutrients generated by the digestive processes in the PV. A more complete understanding of how parasites are able to thrive in their intracellular niche, which has been shown to have a pH of approximately 5 (Antoine et al., 1990) and a slew of hydrolases and proteases (Antoine et al., 1998a), is beginning to emerge.

Studies aimed at the development of a culture media for axenic amastigotes has taught us about the PV luminal environment in that the media must, to some degree, mimic that environment. Indeed, it has been found that conditions that presumably mimic the pH and temperature encountered by the parasites in a PV allow for the continuous culture of amastigote-like forms (Pan, 1984; Rainey et al., 1991; Bates et al., 1992). Despite the acidic environment, *Leishmania* amastigotes require a neutral pH for intracellular metabolism and it has been shown that amastigotes are capable of maintaining an intracellular pH of 7 when exposed to environmental pHs as low as 4 (Glaser et al., 1988). Although it has been shown that the maintenance of intracellular pH homeostasis by amastigotes is sensitive to ATPase inhibitors and the glucose-content of the media, suggesting it to be an energy dependent process, the exact mechanisms by which amastigotes maintain their pH is still unclear. However, some studies suggest a role for P-type ATPases upregulated by amastigotes in the maintenance of the steep pH gradient (Meade et al., 1989). The surface of amastigotes is covered with densely packed glycolipids which may also serve as protection from the harshly acidic envrionment in the PV (McConville and Blackwell, 1991). Indeed, enzymes involved in the synthesis of this surface coat have been shown to be important virulence factors for *Leishmania* (Ilgoutz et al., 1999; Garami and Ilg, 2001).

As detailed earlier, multiple vacuole trafficking pathways intersect with the PV. These interactions may be a source of nutrients for parasites residing in the PV. For example, phagocytosis by the host cell encloses large, potentially nutrient-rich structures within phagosomes, which traffic through the endocytic pathway. Indeed, phagosomes containing various cargoes, such as heat-killed yeast and beta-glucan,

have been shown to fuse with *Leishmania* PVs (Veras et al., 1992; Collins et al., 1997) and may serve as a nutrient source. In addition, autophagosomes have been shown to fuse with PVs (Schaible et al., 1999). The majority of cellular RNA degradation occurs by sequestration in autophagosomes, and subsequent catabolism in endosomes (Lardeux and Mortimore, 1987; Burchmore and Barrett, 2001). The presence of enzymes such as cathepsins and glucoronidase in the PV (Prina et al., 1990) may provide a mechanism for the degradation of macromolecules into structures easily incorporated by amastigotes. These processes provide a source of sugars, lipids, amino-acids, phosphate and sulphate (Burchmore and Barrett, 2001). In light of the mounting evidence that recruitment of host ER to the PV does occur, it will be interesting to learn if there are components of this host cell compartment that are contributing to the survival of amastigotes in their intracellular niche.

Phagocytosis

Phagocytosis is a process employed by eukaryotic cells for the internalization of large particles (typically 0.5 micrometers or more) that can be as diverse as inert beads, dying cells and other organisms. The actual process employed by cells to phagocytose large particles has proven to be an extremely complex and varied phenomenon. Indeed, various forms of phagocytosis, such as “zippering” and “coiling” have been shown to occur in response to different stimuli. It is the recognition of specific ligands on the particle surface that initiates the process of phagocytosis. Mammalian professional phagocytes, such as macrophages and dendritic cells, display a substantial array of phagocytic receptors, coupled to distinct signal transuction pathways (Jutras and Desjardins, 2005). Various sets of receptors can be engaged by any given particle and this “crosstalk” can result in a complex cascade of intracellular signaling pathways

(Underhill and Ozinsky, 2002). The number of molecules involved in these signaling pathways is growing rapidly and although it is difficult to define how these pathways are organized, calcium, phospholipases, kinases and GTPases have all been implicated in early events that occur during phagosome formation (Desjardins, 2003). Engagement of signaling pathways results in cytoskeletal rearrangements, which in turn leads to the formation of a "phagocytic cup" at the base of the particle about to be internalized. The particle is internalized and the resulting membrane-bound intracellular compartment is termed the phagosome. The nascent phagosome undergoes a series of interactions with the host cell endocytic pathway. Sequential interactions with early, late and lysosomal compartments result in the acquisition of hydrolytic enzymes and a lowering of the pH. The resulting compartment is termed the phagolysosome and is capable of degrading its luminal contents. It is the degradative or "digestive" properties of the phagolysosome that has allowed these organelles to play a central role in both the innate and adaptive immune processes.

Source of Membrane for Phagosome Biogenesis

It is generally accepted that the main source of membrane for the phagosome is the plasma membrane. Early studies showed that immediately post-phagocytosis there is a decrease in plasma membrane content; moreover, they showed that the membrane of the early phagosome resembled that of the plasma membrane (Werb and Cohn, 1972; Muller et al., 1980). However, another early study described the differential uptake of labeled markers by the plasma membrane and phagosome membrane suggesting that there must be membrane synthesis or some other source of membrane that is incorporated into the phagosome (Vicker, 1977). Interesting studies, using membrane capacitance techniques have also shown that phagocytosis is

accompanied by a decrease in membrane capacitance at the plasma membrane (Holevinsky and Nelson, 1998). A decrease in membrane capacitance is associated with exocytosis of internal compartments possibly of endocytic origin. Similarly, it was observed that the phagocytosis of opsonized zymosan by J774 macrophages was accompanied by the exocytosis of endosomes (Hackam et al., 1998). Moreover, the effect was shown to be sensitive to inhibition of vesicle-soluble N-ethylmaleimide sensitive factor attachment protein recepors (v-SNAREs), which are required for membrane fusion events. Following up on these observations, it was shown that the exocytosis of VAMP-3 vesicles occurs in the vicinity of and preceding phagosome formation suggesting that this may contribute to membrane extension during phagocytosis (Bajno et al., 2000).

Intracellular Interactions of Phagosomes

Shortly after their formation at the cell surface, phagosomes interact with early endosomes, late endosomes and lysosomes in a sequential manner (Desjardins et al., 1994a, 1994b; Desjardins, 2003). Interestingly, one study showed that the nature of the interactions between phagosomes and endosomes was not one of complete fusion of the two compartments but rather a transient interaction in which a pore is formed through which material can be transferred (Desjardins et al., 1994b). This transient association was termed the “kiss-and-run” hypothesis (Desjardins, 1995). Interactions of phagosomes with early endosomes results in the acquisition of early endosomal markers such as Tfr, EEA1, and Rab5 but not of late endosome and lysosome markers (Pitt et al., 1992; Scianimanico et al., 1999; Duclos et al., 2000). Integral and peripheral proteins such as Tfr and EEA1 are removed from the phagosome membrane during maturation (Vieira et al., 2002).

As the maturation process proceeds and phagosomes continue to lose early endosomal markers, they begin to acquire late endosomal markers. The acquisition of late endocytic markers typically begins 10-30 minutes post-uptake and is characterized by the accumulation of markers such as Rab7, mannose-6-phosphate receptor and lysobisphosphatidic acid (Pitt et al., 1992; Via et al., 1997; Fratti et al., 2001). The presence of late endocytic markers on the phagosome is also transitory and the phagosomes will ultimately transition to phagolysosomes displaying features of the lysosomal compartment. This stage is characterized by the acquisition of lysosomal properties such as the presence of hydrolytic proteases, and the lowering of the intraluminal pH to extremely acidic conditions, reported to get as low as pH 4.5 (Vieira et al., 2002).

Phagocytosis and the ER

The finding that latex-bead-containing phagosomes can be isolated from other cellular organelles using a sucrose gradient, due to the low density of latex beads, greatly facilitated proteomics analyses of phagosomes (Desjardins et al., 1994a; Desjardins, 2003). Early observations using this technique yielded the somewhat unexpected finding that ER molecules, including calnexin and calreticulin, appeared to be present on the phagosome (Garin et al., 2001). It was suggested that the presence of ER in the preparations in this study, which was the first global characterization of a complex intracellular organelle using a proteomics approach, must have been a result of contamination. However, the authors also suggested that the presence of ER molecules may not have been the result of contamination and may suggest that the ER plays some role in the biogenesis of the phagosome. In an elegant set of experiments using cellular biology techniques, the recruitment of ER components to the nascent

phagosome were confirmed (Gagnon et al., 2002). In these studies it was shown that the ER molecules calnexin and calreticulin were enriched in phagosomes and that they were present in their native form as early as phagocytic cup formation; in addition, newly synthesized, unfolded proteins were also delivered to early phagosomes. This study also showed that ER molecules are delivered to the early *Leishmania donovani* PV and in ultra-structural studies showed the direct association of ER cisternae with the newly formed PV suggesting that ER-mediated phagocytosis may be important for the uptake of pathogens as well. These observations led to the proposal of a model of ER-mediated phagocytosis suggested by Michel Desjardins and colleagues (Desjardins, 2003). In this model, particles in contact with the cell surface are rapidly trapped in short pseudopodia that are present on resting macrophages. During this process the ER is recruited to the cell surface, where it fuses with the cell surface and opens up at the plasma membrane (Figure 1-4). This fusion may provide membrane required for the extension of pseudopodia around the particle to be internalized. Indeed, during phagocytosis by the amoeba *Dictyostelium*, calnexin and calreticulin double-null mutants display arrested outgrowth of the phagocytic cup (Müller-Taubenberger et al., 2001). The particle then slides into the opened ER and the membrane is resealed at the plasma membrane.

The ER-mediated phagocytosis model did not arrive without controversy. A follow up study to test this new model using a combination of biochemical, fluorescence imaging and electron microscopy techniques to quantitatively and dynamically assess the contribution of the plasmalemma and of the ER to phagocytosis could not verify the observations made by the previous study. This new study reported that the only

interactions between intracellular compartments and the phagosome that could be confirmed were with the endocytic pathway (Touret et al., 2005b). Furthermore, Touret et al. goes on to suggest that several aspects of phagosomal physiology are not reconciled easily with the ER-mediated model, such as the acidification of phagosomes during maturation in which the rapid acquisition of v-ATPases results in acidification yet ER membranes are functionally devoid of v-ATPases (Touret et al., 2005a). They suggest that the experimental observations resulting in the ER-mediated model may have arisen from techniques prone to artifacts, leading the proponents of the ER-mediated model to contest the findings (Gagnon et al., 2005). Although ER-mediated phagocytosis remains somewhat controversial support for this model has come in recent years in the form of studies involving ER SNARE molecules.

Phagocytosis and Endocytic SNAREs

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins were first characterized in the late 1980s and since then have been identified as key elements in intracellular membrane fusion. Growing evidence suggests that SNARE molecules are involved in membrane fusion events at all trafficking steps of the secretory pathway. In a generally accepted model of SNARE-mediated membrane fusion, the energy liberated from the coupling of four SNARE molecules on opposing membranes provides the energy to drive membrane fusion. The dissociation of the quaternary SNARE complex is mediated by the action of the AAA+ protein N-ethylmaleimide sensitive factor (NSF) leaving the SNAREs ready for a second round of complex formation (Jahn and Scheller, 2006).

A growing body of evidence suggests that SNAREs are important, if not necessary, players in the interaction between various endocytic compartment

organelles and the nascent/maturing phagosome. One of the first studies that suggested that SNARE molecules have a role in phagocytosis came in the late 1990s. In this study, it was reported that during phagocytosis there is a net increase in membrane surface area. It was thus suggested that an internal compartment must be contributing to the increase in surface area. The introduction of tetanus or botulinum toxins, which degrade v-SNAREs, resulted in an inhibition of this effect suggesting that these SNAREs were involved in exocytic event that accompanies phagocytosis (Hackam et al., 1998). It was later shown that vesicles containing the SNARE VAMP3 accumulate at the base of a forming phagosome (Bajno et al., 2000). Furthermore, it was shown that the overexpression of dominant-negative NSF, which is an essential regulator of SNARE complex formation, inhibited the phagocytosis of the bacteria *Salmonella typhimurium* (Coppolino et al., 2001). However, NSF is known to function globally on SNARE function and the introduction of a dominant-negative form of this molecule will, theoretically, affect all membrane trafficking events in the cell and the physiology of the cell would conceivably be compromised; therefore, it is difficult to conclude whether the inhibition of phagocytosis in this study is a direct result of inhibited exocytosis or a more global effect. Another SNARE, tetanus neurotoxin-insensitive vesicle-associated membrane protein (TI-VAMP/VAMP7), has also been shown to have some bearing on phagocytosis. In experiments where TI-VAMP function was compromised, through overexpression of dominant-negative amino terminal TI-VAMP or introduction of TI-VAMP siRNA, phagocytosis was once again significantly inhibited and a blockade of phagocytic cup extension was demonstrated (Braun et al., 2004). Moreover, the recruitment of TI-VAMP to the phagocytic cup was also demonstrated.

Other SNAREs that have been shown to interact with phagosomes include syntaxin 13 and syntaxin 7, which are localized to recycling endosomes and late endosomes/lysosomes respectively. Overexpression of dominant negative syntaxin 13 and syntaxin 7 were shown to have no effect on phagocytosis but did inhibit phagosome maturation (Collins et al., 2002). Taken together, these studies demonstrate the importance of SNAREs in the sequential interaction of phagosomes with the endocytic pathway.

Phagocytosis and ER SNAREs

As mentioned earlier, there is evidence that the ER interacts with phagosomes from very early on and may provide at least some of the membrane required for phagosome formation and maturation. More recently groups have been exploring the role that ER-resident SNAREs might be playing in the phagocytic process. One of the initial studies to explore the role of ER SNAREs in phagocytosis described the presence of the ER SNAREs Syntaxin 18, D12 and Sec22b on the membrane of isolated phagosomes. Moreover, the same study showed that overexpression of the dominant negative form of syntaxin 18, or knockdown using siRNA, resulted in an inhibition of phagocytosis. The study went on to show that direct interactions between syntaxin 18 and plasma membrane SNAREs were also possible in *in vitro* manipulations (Hatsuzawa et al., 2006). In marked contrast to the observations with syntaxin 18, the same group went on to show that overexpression of functional ER SNARE sec22b resulted in the near abolition of phagocytosis; moreover, knockdown of endogenous sec22b using siRNA increased phagocytosis (Hatsuzawa et al., 2009). The authors suggest that sec22b functions as a negative regulator of phagocytosis in macrophages by affecting the availability of free syntaxin 18 and/or D12 at the site of phagocytosis.

These results differ from an earlier study that showed that the introduction of a dominant-negative form of sec22b to macrophages results in an inhibition of phagocytosis (Becker et al., 2005). In a more recent study in dendritic cells, it was shown that sec22b is recruited to phagosomes in dendritic cells as well and is required for the efficient cross-presentation of antigens (Cebrian et al., 2011). Taken together, the reports exploring the role of ER SNAREs supports the model of ER-mediated phagocytosis and suggests that ER SNAREs may be involved in phagosome maturation and the acquisition of the machinery required for cross-presentation.

SNAREs and Intracellular Organisms

Cells of the innate immune system, such as macrophages and neutrophils, play an important role in the detection and elimination of invading pathogens. Phagocytosis is the primary mechanism of eliminating pathogens by this group of cells. As discussed earlier, as the phagosome matures into a phagolysosome, acquiring microbicidal and degradative properties along the way, it becomes more adept at eliminating the contained organism. On the other hand, phagocytosis can serve as a mechanisim of entry for some pathogens that rely on intracellular sequestration for their survival. Indeed, pathogens such as *Leishmania* are phagocytosed and employ mechanisms, once internalized, to modify phagolysosome maturation and avoid destruction by the host cell. In light of the recent evidence that SNAREs are vital players in phagocytosis and phagosome maturation, several studies have emerged showing that various intracellular pathogens manipulate the host cell SNARE machinery to facilitate their intracellular survival.

In this section, I will describe the interactions of pathogen-containing vacuoles with the host cell SNARE machinery. Indeed, the number of reports describing such

interactions is growing and seems to represent a new trend in the field of cellular microbiology. Unfortunately, knowledge on this sort of interaction for many intracellular organisms is still lacking; therefore, I will focus on the pathogens for which there is evidence of host cell SNARE manipulation such as *Mycobacterium, Chlamydia, Legionella, Salmonella* and *Leishmania*.

SNAREs and *Mycobacterium*

Mycobacteria lack the ability to actively invade host cells; instead, they rely on the phagocytic capacity of host cells for entry. Once inside, mycobacteria are capable of altering normal phagosomal maturation of the *Mycobacterium*-containing vacuole (MCV). The resulting arrest of phagosomal maturation prevents the degradation of the mycobacteria and also shelters it from the immune system (Scott et al., 2003). The observation that the MCV rapidly acquires Rab5 (early endosomal marker) but does not acquire Rab7 or LAMP1 (late endosomal/lysosomal marker) (Via et al., 1997) led to the hypothesis that MCVs maintain the capacity to fuse with early endosomes but do not fuse with late endosomes and lysosomes. Rab5 is known to function upstream of the recycling endosomal/plasma membrane SNARE syntaxin 4. Indeed, it has been shown that syntaxin 4 is recruited to the MCV membrane with similar, if not identical, kinetics to Rab5 (Perskvist et al., 2002). It was suggested that the maintainenance of the Rab5/syntaxin 4 complex at the MCV membrane plays a role in phagosome arrest as knockdown of Rab5 reduced recruitment of syntaxin 4 and altered the fusogenic capacity of the MCV. In a separate study aimed at understanding the mycobacterial effector phosphatidyl innositol mannoside (PIM), it was found that syntaxin 4 accumulated to a higher extent on PIM-coated latex bead phagosomes than on control bead phagosomes. The accumulation of syntaxin 4 coincided with the reduced fusion

of phagosomes with lysosomes (Vergne et al., 2004). In addition to syntaxin 4, the dynamics of acquisition of syntaxin 3, a plasma membrane SNARE, syntaxin 13, associated with endosomal SNARE recycling, and syntaxin 8, a target SNARE that overlaps with Rab5 on endosomes, by MCVs were all similar, albeit with small exceptions, compared to model latex bead phagosomes (Fratti et al., 2003).

In addition to the aberrant acquisition or accumulation of SNARE, which can result in a preferential fusion with one host cell compartment, pathogens may exclude certain SNAREs from the pathogen-containing vacuole membrane in order to limit the fusion with other host cell compartments. Indeed, syntaxin 6, a SNARE involved in vesicular trafficking between the trans Golgi network and the endocytic pathway is excluded from the MCV; whereas, model latex bead phagosomes acquire syntaxin 6 (Fratti et al., 2003). The exclusion of syntaxin 6 has been shown to be mediated by the mycobacterial phosphatidylinosotil (mannose-capped lipoarabinomannan) ManLAM (Fratti et al., 2003). The exclusion of syntaxin 6 results in a block in communication between the trans Golgi network and the MCV. This results in a block in the delivery of lysosomal enzymes and proton pump subunits to the MCV and prevents the assembly of a functional H+ ATPase complex. Another interesting observation was that the endosomal SNARE VAMP3 is also acquired by MCVs; however, in contrast to latex-bead phagosomes, VAMP3 is degraded on the MCV by an unknown mechanism (Fratti et al., 2002). VAMP3 has also been implicated in traffic from the trans Golgi network to the endocytic pathway and its degradation may also contribute to the observed block in communication between the trans Golgi network and MCVs.

SNAREs and *Chlamydia*

Chlamydia is an obligate intracellular pathogen that resides within a host cell in a membrane-bound compartment termed an inclusion. The membrane of the inclusion is initially formed from the invagination and subsequent pinching off of the plasma membrane. Interestingly, the newly formed inclusion does not appear to interact with the endocytic pathway as fluid-phase endocytic tracers as well as membrane markers of endosomes are not observed in/on the inclusion (Heinzen et al., 1996; Scidmore et al., 1996a; Taraska et al., 1996). On the other hand, the inclusion appears to get sphingolipids from exocytic vesicles in transit from the trans Golgi network to the plasma membrane (Hackstadt et al., 1995, 1996). It was suggested that the sequestration of *Chlamydia* in such a vesicle allows the inclusion to be perceived by the host cell as a vesicle not destined for fusion with lysosomes (Hackstadt et al., 1997). Based on observations that the acquisition of sphingolipids by chlamydiae in an inclusion is dependent on early protein synthesis by the bacteria (Scidmore et al., 1996b), it is believed that chlamydiae actively modify the inclusion to intersect exocytice vesicles. Indeed, in the absence of early protein synthesis, chlamydiae are rapidly degraded in phagolysosomes (Scidmore et al., 1996b).

The first implication that chlamydiae may subvert the host cell SNARE machinery came from studies of the effector protein IncA. Working from observations that strains lacking IncA were not capable of homotypic inclusion fusion (Hackstadt et al., 1999; Fields et al., 2002), it was found that heterologous expression of IncA, which localized to the ER in host cells, completely disrupted inclusion development (Delevoye et al., 2004). Moreover, it was shown that the disruption in inclusion development was a result of interactions of IncA on the inclusion with IncA on the ER, most likely resulting in the

aberrant fusion of these two compartments. The apparent role in membrane fusion prompted the group to model IncA tetramers in parallel four helix bundles based on the structure of the SNARE complex. These structures were highly stable in the model and it was suggested that IncA proteins may have co-evolved with SNARE proteins for a common function in membrane fusion (Delevoye et al., 2004). In a follow up study, the same group employed bioinformatics techniques to search for SNARE-like proteins belonging to the Inc family of proteins. A number of proteins contained SNARE-like motifs. In addition, the recruitment of the host SNAREs VAMP3, VAMP7 and VAMP8, but not Sec22b and VAMP4, to the inclusion was demonstrated. Interestingly, the deletion of the SNARE motif from VAMP7 blocked its recruitment to the inclusion, indicating that a functional SNARE motif was required for recruitment. Moreover, IncA was found to co-immunoprecipitate with host SNAREs VAMP3, VAMP7 and VAMP8 (Delevoye et al., 2008). In addition to IncA, it was found that another *Chlamydia* protein CT813 is also campable of interacting with host SNAREs (Delevoye et al., 2008). However, the recruitment of these SNAREs, some of which are characteristic of early endosomes, seemed to contrast previous reports that chlamydiae avoid interactions with the endocytic pathway. Another group extended theses studies using an *in vitro* liposome fusion assay and a cellular assay, they showed that IncA was capable of blocking membrane fusion in eukaryotic cells by directly inhibiting SNARE-mediated membrane fusion (Paumet et al., 2009). They were also able to demonstrate that the inhibitory function was encoded in the SNARE-like motif of IncA. Importantly, the role of direct inhibition of membrane fusion allows for the recruitment of endocytic SNAREs to the inclusion without fusion, perhaps explaining the recruitment of endocytic organelles

in previous studies. At the same time, the formation of nonfunctional SNARE complexes allows *Chlamydia* to exclude certain host-cell compartments from the inclusion. In a more recent study, it was shown that the trans Golgi network SNARE syntaxin 6 is recruited the inclusion membrane (Moore et al., 2011). It was suggested that this may, in part, account for the interception of exocytic vesicles.

SNAREs and *Salmonella*

Once inside of a host cell, *Salmonella* resides in a specialized compartment termed a *Salmonella*-containing vacuole (SCV). To invade host cells as well as control the fate of the SCV the *Salmonella* employ protein effectors that are injected into the host cell using two type III secretion systems (T3SSs). The two T3SSs are encoded on two separate *Salmonella* pathogenicity islands, SPI-1 and SPI-2. In general, the SCV matures with similar endocytic interactions to a model phagosome. As the SCV matures, it acquires early endosomal markers such as EEA1 and Rab5 (Steele-Mortimer et al., 1999), followed by the acquisition of the late endosomal marker Rab7 as well as lysosomal glycoproteins, such as LAMP1 (Garcia-del Portillo et al., 1993). However, it differs from normal phagosome maturation in that it excludes mannose-6-phosphate receptor, which is known to deliver lysosomal hydrolases to the endosomal system (Garcia-del Portillo and Finlay, 1995).

The first indication that SNAREs play a role in SCV maturation came from a study of live *Salmonella* containing phagosomes (LSPs) and dead *Salmonella* containing phagosomes (DSPs) in J774 macrophages. It was observed that NSF, required for the disassembly of SNARE complexes and recycling of SNAREs, is enriched on the LSP as compared to the DSP (Mukherjee et al., 2000). The selective enrichment of NSF on LSPs suggests that *Salmonella* actively recruits NSF, a known SNARE regulator. In a

later study, it was shown that the overexpression of non-functional NSF in host cells rendered the SCV less capable of acquiring LAMP1, a lysosomal marker, suggesting that the maturation of SCVs is NSF-dependent (Coppolino et al., 2001). The same study showed that cell invasion by *Salmonella* was unaffected by non-functional NSF, implying that cell invasion is NSF-independent; therefore, there is a differential requirement for NSF at different stages of infection.

VAMP3 is another endosomal SNARE recruited to the nascent SCV. Interestingly, it was shown that degradation of VAMP3 with tetanus toxin and inhibition of recruitment of VAMP3 to the nascent SCV by overexpression of dominant-negative NSF had no bearing on *Salmonella* uptake. However, the inhibition of the recruitment of another ealry endosomal SNARE, VAMP8, resulted in a significant reduction in cell invasion capacity (Dai et al., 2007).

The maturation of the SCV and the ultimate acquisition of LAMP1 appear to be crucial in the establishment of a replicative niche for *Salmonella* (Madan et al., 2012). The acquisition of two host cell SNAREs, early endosomal SNARE syntaxin 13 and trans Golgi network SNARE syntaxin 6, appear to have some role in the acquisition of the lysosomal marker LAMP1. Syntaxin 13 was shown to be massively recruited to the SCV (Smith et al., 2005) and the inhibition of syntaxin 13 function resulted in impaired SCV maturation as interpreted by the delayed acquisition of LAMP1 (Smith et al., 2005). In a more recent study, it was shown that syntaxin 6 is recruited to the SCV via the *Salmonella* effector SipC (Madan et al., 2012). The study goes on to show that the SCV acquires LAMP1 via syntaxin 6 mediated fusion with Golgi derived vesicles. Indeed, depletion of syntaxin 6 significantly reduced the recruitment of LAMP1 to the SCV

membrane. Interestingly, SipC(-):*Salmonella* mutants survivial in mice is significantly inhibited. Also of interest to note is that *Mycobacterium*, as mentioned earlier, excludes syntaxin 6 from its vacuole and it is suggested that the block in trans Golgi network to phagosome communication is in part responsible for the lack of late endosomal/lysosomal markers of the MCV.

SNAREs and *Legionella*

After uptake by a eukaryotic cell, *Legionella* resides inside a vacuole, primarily composed of membrane from the plasma membrane, termed the *Legionella* containing vacuole (LCV). Unlike model phagosomes, the LCV avoids sequential interactions with the endocytic pathway and intercepts early secretory pathway vesicles exiting the ER (Horwitz, 1983b; Horwitz and Maxfield, 1984; Roy et al., 1998; Wiater et al., 1998; Kagan and Roy, 2002). Modulation of the vacuole trafficking requires a specialized secretion system termed the Dot/Icm system (Kagan and Roy, 2002). It is within the ER-derived organelle that *Leigionella* begins to replicate (Horwitz, 1983a).

In an attempt to better understand the machinery that mediates fusion between ER-derived vesicles and the LCV, Kagan et al. elected to look for the presence of sec22b, an ER SNARE that functions in ER to pre-Golgi traffic, on the LCV (Kagan et al., 2004). Indeed, sec22b was found to be present on the LCV of wild-type *Legionella;* whereas, a non-functional Dot/Icm mutant was not capable of recruiting sec22b. Interestingly, membrin, a described SNARE partner of sec22b, was not present on the LCV. This, at first, is surprising in that four SNAREs are required to form a quaternary complex and all members of the complex can be expected to be present on the target membrane (Jahn and Scheller, 2006). The implication is that sec22b may be interacting with noncognate SNARE partners at the LCV membrane. It was also observed that the

titrating of sec22b by overexpression of membrin resulted in suppression of *Legionella* replication suggesting that sec22b function is important for establishment of the replicative niche (Kagan et al., 2004). Some clarification of what sec22b is partnering with on the LCV membrane came from a study by Arasaki and Roy in which plasma membrane SNAREs syntaxin 2, 3 and 4 were found to be present on the LCV. In addition, these plasma membrane SNAREs formed functional SNARE complexes with sec22b (Arasaki and Roy, 2010). This noncogante SNARE partnering was found to be dependent on the presence of a functional Dot/Icm system. In a follow up study by the same group, it was demonstrated that the *Legionella* effector DrrA is sufficient to stimulate the noncanonical SNARE partnering and promote membrane fusion. It was suggested that DrrA activation of the Rab1 GTPase on the newly-formed plasma membrane derived LCV stimulates the tethering of ER derived vesicles to allow for vesicle fusion (Arasaki et al., 2012).

It is also of interest that, similar to *Chlamydia*, *Legionella* also expresses SNARE-like proteins using the Dot/Icm system. One of the SNARE-like molecules, IcmG/DotF, was shown to inhibit SNARE mediated membrane fusion *in vitro* (Paumet et al., 2009). The role, if any, that these SNARE mimics are playing *in vivo* may help to better understand LCV biogenesis.

SNAREs and *Leishmania*

As discussed in detail in earlier sections, the biogenesis of the *Leishmania* PV involves sequential interactions with the host cell endocytic pathway (Antoine et al., 1998b; Courret et al., 2002). Despite, the apparent complete depletion of host cell lysosomal compartment by PVs (Alexander and Vickerman, 1975; Barbieri et al., 1985; Barbiéri et al., 1990) as well as growing evidence for the acquisition of ER components

(Kima and Dunn, 2005; Ndjamen et al., 2010), there is a poor understanding of the molecular players mediating fusion events with the PV. Membrane fusion events seem particularly key to the intracellular survival of *Leishmania*. Indeed, it was demonstrated that by indirectly affecting PV size, the survival and replication of amastigotes is adversely affected (Wilson et al., 2008).

In a recent study describing the gradual acquisition of ER components by the PV, the ER SNARE sec22b was confirmed to be present on the PV (Ndjamen et al., 2010). This observation suggested that ER SNAREs may play a role in the acquisition of ER components by the PV. Indeed, in another intracellular organism, *Legionella*, it was shown that sec22b is an important player in the delivery of ER molecules to the LCV and that by inhibiting sec22b function, *Legionella* replication can be reduced (Kagan et al., 2004; Arasaki and Roy, 2010). Whether or not sec22b plays an important role in the intracellular survival and replication of *Leishmania* is yet to be determined. Moreover, in order for sec22b to be present on a target membrane, the PV membrane in this case, it must partner with three additional partner SNAREs. In the *Legionella* system, it was shown that sec22b undergoes noncognate SNARE pairing, in that an ER SNARE, sec22b, partners with plasma membrane SNAREs (Arasaki and Roy, 2010). Whether or not *Leishmania* infection results in such noncognate interactions is also yet to be determined. A better understanding of how *Leishmania* is capable of subverting the host cell SNARE machinery may provide insight into how it is capable of establishing its intracellular niche.

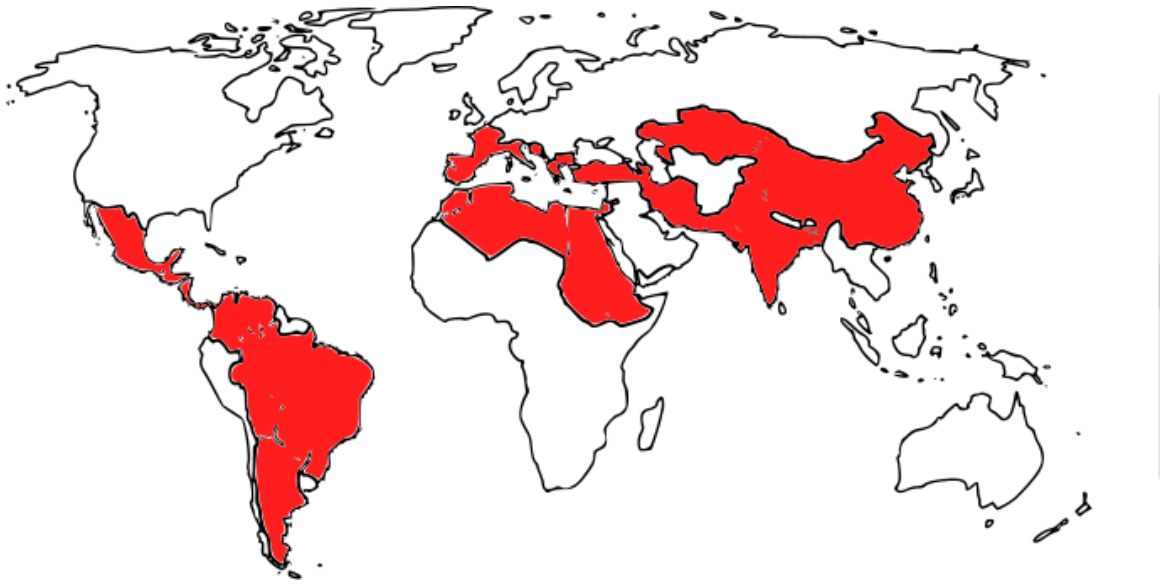

Figure 1-1. Map showing countries at risk for leishmaniasis. This figure does not distinguish between visceral and cutaneous leishmaniasis.

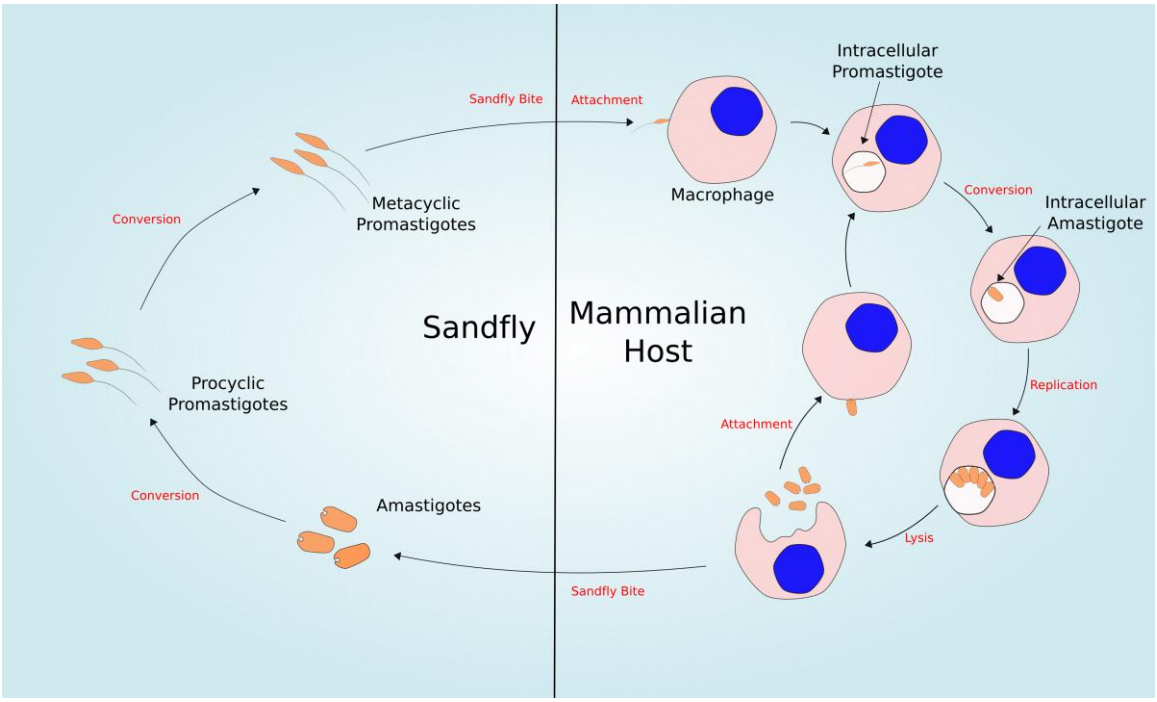

Figure 1-2. The Life cycle of *Leishmania*. The parasites are digenetic and thus have two basic life cycle stages, an extracellular stage in which they reside in the gut of an invertebrate host, and an intracellular stage in which they reside in a specialized intracellular compartment in a mammalian host. Amastigotes are taken up as the invertebrate host takes a blood meal from a mammalian host. The amastigotes transform into procyclic promastigotes in the midgut of the invertebrate host. Promastigotes replicate in the midgut and migrate to the foregut where they ransform into infective metacyclic promastigotes. When the invertebrate host takes a blood meal, metacylcic promastigotes are deposited in the mammalian host. Promastigotes are then phagocytosed by host cells. In host cells, promastigotes transform into amastigotes and replicate inside a specialized compartment termed the parasitophorous vacuole (PV). When host cells lyse, amastigotes can be phagocytosed by host cells and form a new PV.

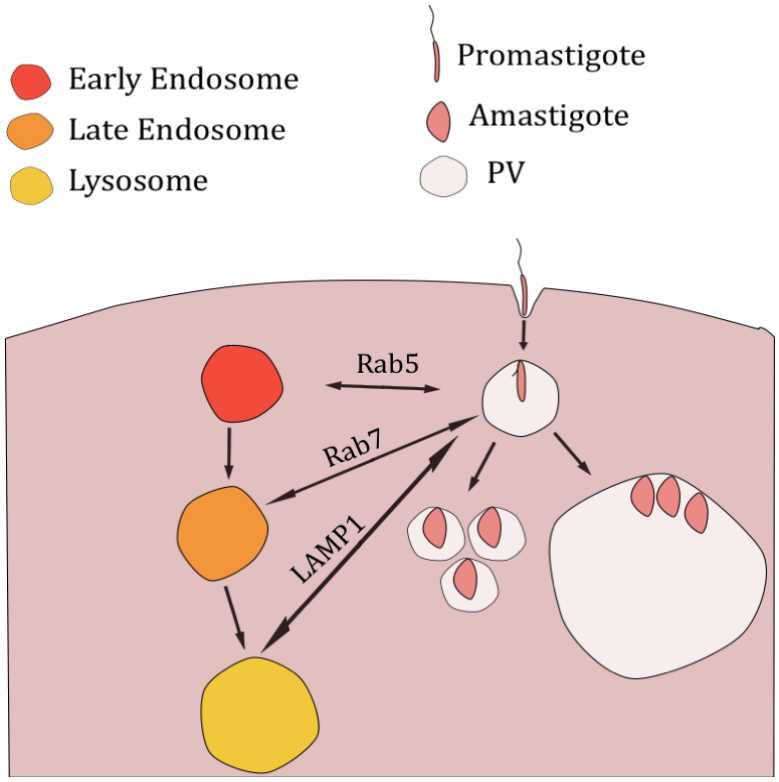

Figure 1-3. The interaction of *Leishmania* with the endocytic pathway. After phagocytosis, *Leishmania* resides in a membrane bound compartment termed a parasitophorus vacuole. Similar to phagosomes containing inert particles, such as latex beads, the PV undergoes sequential interactions with endosomes. Very early interactions occur with early endosomes and are maintained for only 1-2 minutes. Subsequently, the PV begins to acquire markers of late endosomes and lysosomes. These interactions are maintained for the course of the infection. Depending on the species of *Leishmania*, PVs can either develop into large, communal compartments (e.g. *Leishmania amazonensis)* or tight, individual compartments (e.g. *Leishmania donovani*).

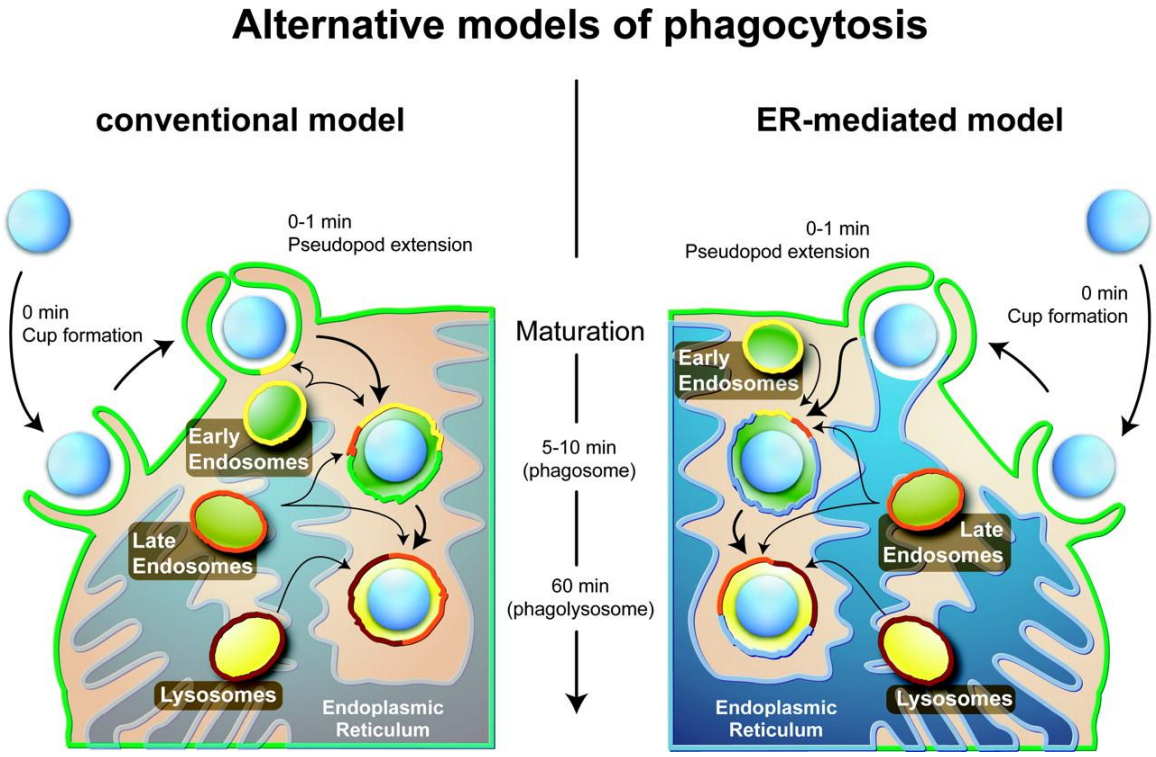

Figure 1-4. Representation of the two models of phagocytosis. Upon engagement of phagocytic receptors [e.g., Fcγ receptors (FcγRs)], the plasmalemma surrounds a target particle by extension of pseudopods. In the conventional model (left panel), the phagocytic vacuole is formed by the fusion of pseudopods at their tips and is composed largely of plasmalemmal constituents with a varying contribution of endosomes, perhaps depending on the particle size. The sealed phagosome proceeds to mature by sequential fusion of additional early and late endosomes and ultimately, lysosomes. The ER-mediated model proposes the recruitment to the nascent phagosome as early as phagocytic cup formation. The nascent phagosome consists largely of ER, which remains present in the phagosome for hours (adapted from Touret et al. 2005).

CHAPTER 2
MATERIALS AND METHODS

Parasites, Cell Lines, Animals and Infections

Leishmania amazonensis promastigotes (MHOM/BR/77/LTB0016) were obtained from American Type Culture Collection (ATCC™). Promastigotes were cultured in Schneider's Drosophila Medium (Gibco ®) supplemented with 20% Heat-Inactivated Fetal Bovine Serum (Atlanta Biologicals ®) and $10\ \mu g\ ml^{-1}$ gentamicin (Gibco ®) and grown at 23˚C. The pathogenicity of the parasites was maintained by regular passage through mice.

The RAW264.7 murine macrophage-like cell line was obtained from ATCC™ and cultured in RPMI medium (Cellgro®) with L-glutamine supplemented with 10% heat-inactivated Fetal Bovine Serum (Atlanta Biologicals ®) and 100 units mL-1 of penicillin/streptomycin (Cellgro®) at 37 ˚C and under a 5% carbon dioxide atmosphere. Primary mouse macrophages were obtained from the peritoneal exudate of Balb/c mice stimulated with thioglycolate 4 days prior to macrophage recovery and cultured under the same conditions as RAW264.7 macrophages.

Balb/c mice at 6-8 weeks old (The Jackson Laboratory, Bar Harbor, ME) were maintained in specific pathogen-free conditions at the Association for Assessment and Accreditation for Laboratory Animal Care–accredited University of Florida under the supervision of the Institutional Animal Care and Use Committee in strict accordance to approved protocols.

For infections, macrophages were seeded on coverslips and grown as described above overnight. Stationary phase *Leishmania amazonensis* promastigotes were added to the macrophages and incubated at 34˚C under a 5% carbon dioxide

atmosphere. After 1 hour of incubation, non-internalized parasites were washed using fresh medium. The cultures were then returned to the incubators for the times indicated in each experiment. To assess the effect of expression of dominant-negative SNAREs on parasite internalization infections were only run for 2 hours before washing fixing using 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). The number of parasites per macrophage was determined by inspection under the microscope.

Parasite Intoxication Assay

Retro-2, 2-{[(5-methyl-2-thienyl)methylene]amino}-N-phenylbenzamide (5374762) and control molecules (5322770 and 5322213) were obtained from the ChemBridge corporation (San Diego, CA). 1 X 10^7 parasites were seeded in each well of a 24 well plate containing 1 mL of Schneider's Drosophila medium supplemented as described above. Wells were treated with vehicle (DMSO) or 25, 50, 75, 100 μM Retro-2. Each group was performed in triplicate (3 wells). Parasites were left to grow at 23°C. Parasite counts for each well were counted every 24 hours for 14 days using a hemacytometer.

In Vivo Infection and Retro-2 Treatment of Mice

Balb/c mice were infected in their hind feet with 2 x 10^6 stationary stage cultured *L. amazonensis* promastigotes. The course of infection in at least 10 mice was monitored by measurement of foot size using a dial gauge caliper. At the indicated time, mice were sacrificed to determine parasite burdens at the site of infection by limiting dilution analysis. For drug-treated groups, Retro-2 dosage was dissolved in DMSO and made up to the respective doses (100mg/Kg or 20 mg/Kg) in sterile PBS. Mice were weighed and the average weight of mice used was approximately 18g. Retro-2 (made up to 150

μL) was administered intra-peritonealy at 24 hours post-infection or 3 weeks post infection. Each drug-treated group consisted of 8-12 mice.

Vectors, Constructs and Oligonucleotides

The sec22b, syntaxin 18 and D12/USE1/p31 (D12) in pmVENUS-C1 constructs and also the SNAREΔTMDs of sec22b, syntaxin 18 and D12 expressed as red fluorescent protein (RFP) chimeras were gifts from Dr. Kiyotaka Hatsuzawa at the Fukushima Medical University School of Medicine in Fukushima, Japan. Further details on the design and construction of the above-mentioned constructs can be found in previous reports (Hatsuzawa et al., 2006, 2009). The plasmids were propagated in laboratory strains of *Escherichia coli* and were purified and used in the nucleofection protocol described below. The expression of the recombinant proteins was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting described below.

The sec22b, syntaxin 18, D12, syntaxin 5 and scrambled siRNA oligonucleotides that usually consist of pools of three to five target-specific siRNA oligonucleotides were obtained from Santa Cruz Biotechnology®. Inc. The siRNAs were used at a concentration of 50 nM in the nucleofection protocol described below.

Nucleofection of RAW264.7 Macrophages

Approximately 1.7 X 10^7 macrophages in exponential growth phase were harvested and placed into a 50 mL conical tube and centrifuged at 90 XG (RCF) for 10 minutes. The supernatant was aspirated and 100 μL of Ingenio™ electroporation solution (Mirus®) was carefully placed on the pellet. Approximately 15 μg of DNA was transferred to the cell suspension and the resulting mixture was gently transferred into a

0.4 cm electroporation cuvette (Amaxa). Electroporation was performed using the Amaxa Nucleofector™ II and the appropriate settings were selected in the options menu. After electroporation, 0.5 mL of Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich®) supplemented with 10% heat-inactivated fetal bovine serum and 100 units mL^{-1} of penicillin/streptomycin was added to the electroporated cells and the solution was gently transferred to 100 mM dishes for overnight incubation at 37˚C under a 5% carbon dioxide atmosphere. Efficiency was assessed by visualization under the microscope before proceeding with further protocols.

Antibodies, Immunofluorescence Labeling and Imaging

The sec22b, D12, syntaxin 18, syntaxin 5 and HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology®. Inc. The 1D4B (anti-LAMP1) and JLA20 (anti-actin) antibodies were obtained from the Developmental Studies Hybridoma Bank, Iowa City, Iowa. The anti-GFP antibody was obtained from Novus Biologicals®, Littleton, CO. Hybridoma clone 5C6 (anti-CR3) and 2.4G2 (anti-FcRII) were obtained from ATCC™ and hybridoma supernatants were prepared in the laboratory. Alexa Fluor® secondary antibodies were obtained from Molecular Probes®, Carlsbad, Ca.

For immunofluorescence labeling, cells grown on coverslips were fixed using 4% PFA in PBS for a minimum of 20 minutes at room temperature. Following fixation, coverslips were washed three times in PBS and reactive agents were quenched using 50 mM ammonium chloride in PBS for 7 minutes. Cells were then permeabilized using 0.1% saponin (Sigma-Aldrich®) in PBS for 10 minutes. Coverslips were next blocked using blocking buffer (0.1% saponin, 2% nonfat dried milk in PBS) for 30 minutes.

Coverslips were then incubated with primary antibodies diluted in blocking buffer for 1 hour at room temperature. After primary antibody incubation, coverslips were washed three times using blocking buffer and then incubated with secondary antibody diluted in blocking buffer containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich®) for 1 hour. Following secondary antibody incubation, coverslips were washed three times with blocking buffer, three times with PBS and three times with de-ionized water. Coverslips were then mounted onto slides using FluoroGel with Tris Buffer from Electron Microscopy Sciences. Labeled coverslips were examined on a Zeiss Axiovert 200 M microscope with a plain neofluar 100X/1.3 oil immersion objective. Images were captured with an AxioCam MRm camera controlled by AxioVision software. Image series over a define z-focus range were acquired and processed with 3D deconvolution software provided by AxioVision. The extended focus function in the AxioVision software was used to merge the optical sections to generate the images presented.

PV Measurement and Parasite Counts

Z-stack images of infected cells were acquired as described above. Z stack heights were selected to cover the entire host cell nucleus and any PVs being measured so as to be sure that the widest point of the PV as well as the host cell nucleus was captured. Images were processed as described above and all Z images were collapsed using the AxioVision software. Using the "Outline Spline" tool available in the AxioVision software, the circumference of the PV as well as the host cell nucleus was measured in micrometers. Normalization of PV size was carried out by dividing the circumference of the PV by the circumference of the nucleus for each cell analyzed. The ratio is presented in the results section as "Relative PV Size".

Infected cells were processed for immunofluorescence and the DAPI stain was used to visualize the host and parasite nuclei. Parasite counting was performed on the immunofluorescence microscope described above. Parasite nuclei and host cell nuclei were enumerated at time points indicated in the results section.

Western Blot Analysis

RAW264.7 cells to be lysed were washed twice with ice-cold PBS and were lysed using Radioimmunoprecipitation Assay Buffer (RIPA) with Roche® Complete Mini protease inhibitors. Lysates were then cleared of cellular debris by centrifugation at 10,000 XG and 4°C. Protein concentrations were read using Pierce® 660 nm protein assay reagent (Thermo Scientific®). SDS-PAGE was run on 50 μG of each sample on a 12% polyacrylamide gel. Protein was transferred to an Immobilon®-P membrane (Millipore), the membranes were blocked in 5% nonfat milk in Tris-buffered Saline with 0.05% Tween-20 (TBST) followed by incubation with primary antibodies. After removal of primary antibodies and washing, blots were incubated with HRP-conjugated secondary antibodies. After removal of secondary antibodies and washing, blots were incubated with Western Lightning® chemiluminescence substrate (PerkinElmer™). Antibody reactivity was assessed by exposure of blots to x-ray film. Some blots were stripped by incubation 62.5 mM Tris-Hydrochloric acid (Tris-HCl) pH 6.8 supplemented with 20 mM 2-mercaptoethanol and 2% Sodium dodecyl sulfate (SDS) for 30 minutes at 56˚C. The blots were then available for re-probing.

Co-immunoprecipitation

Plates to be processed for co-immunoprecipitation (co-IP) were lysed in 50mM Tris-HCL pH7.4, 15mM ethylenediaminetetraacetic acid (EDTA), 100mM sodium

chloride (NaCl), 1mM N-ethylmaleimide (NEM) and 1% Triton X-100 with Roche® Complete Mini protease inhibitors. Lysis was carried out for 30 minutes at 4°C with gentle rocking. Lysate was collected and spun down at 10,000g for 10 minutes. 1 mG of the cleared lysate was adjusted to 500 uL with co-IP buffer (50mM Tris-HCL pH7.4, 15mM EDTA, 100mM NaCl, 1mM NEM and 0.1% Triton X-100). 25 μL of a 50% slurry of pre-washed protein G beads (Amersham, Protein G 4-fast flow) was added to the lysate and placed at 4°C for 25 minutes. Protein G beads were removed and 2 micrograms of the appropriate antibody was added to the cleared lysate. Tubes were placed at 4°C with gentle rocking for 2 hours. 75 μL of the 50% slurry of Protein G beads was added and the tubes were placed back at 4°C for an additional hour. Beads were spun down and washed 5 times with 1 mL of co-IP buffer. Sample buffer (Laemmli) was added to the bead pellet after the final wash. Beads were boiled in sample buffer for 5 minutes. Beads were spun down and the supernatant was saved for SDS-PAGE.

Isolation of *Leishmania* PVs

Using 15 confluent 100 mm tissue culture plates of RAW264.7 cells, a 12 hour infection with *Leishmania amazonensis* was performed as described above. Cells were then washed with ice cold PBS and scraped into lysis buffer (20 mM Hepes, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 0.25 M sucrose and 0.1% gelatin) containing Roche® Complete Mini protease inhibitors. The cell suspension was passed through a 23 gauge needle 12 times. Lysed cells were brought up to 8 ml with lysis buffer and centrifuged at 200 XG (RCF) for 10 minutes. The post-nuclear supernatant (PNS) was recovered and loaded onto a step gradient containing 4 ml per step of 20%, 40% and

60% sucrose in gradient buffer [30 mM Hepes, 100 mM NaCl, 0.5 mM calcium chloride ($CaCl_2$), 0.5 mM magnesium chloride ($MgCl_2$) pH 7.0]. The gradient was centriguged at 700 XG (RCF) for 25 minutes at 4°C. The enriched PV fraction was recovered from the 40-60% interface of the sucrose gradient. The protein content of the fraction was determined as described above and the sucrose concentration of the fraction was brought to 0.25 M using gradient buffer and centrifuged at 12,000 XG (RCF). The pellet was re-suspended on lysis buffer and loaded onto SDS-PAGE for western blotting.

Lipopolysaccharide (LPS) and Interferon-γ (IFN-γ) Activation

RAW264.7 cells were plated at 1.8 X 10^6 cells mL^{-1} in 60 mm cell culture dishes in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 100 units mL^{-1} of penicillin/streptomycin (DMEM complete) and incubated as described above. After overnight incubation (approximately 16 hours), the medium was aspirated and replaced with IFN-γ at 100 units mL^{-1} and LPS at 10 μG mL^{-1} in DMEM complete and placed back in the incubator for a 24 hour incubation. The supernatant was removed and cellular debris was removed by centrifugation. An enzyme-linked immunosorbent assay (ELISA) for Interleukin-6 (IL-6) (Becton Dickinson) was performed. To account for cells lost as a result of transfection, ELISA results were normalized using the average number of cells per field from 20 fields counted from the plates before the removal of supernatant.

Statistics

Data analysis and generation of graphs was performed using Sigma-Plot and Microsoft Excel software. Each data point is presented as the mean with standard error indicated by y-error bars. Box-plots for PV size graphs were generated using Sigma-

Plot. Boxes represent the range of PV sizes for the given condition and points falling outside the boxes represent each individual outlier. Significance is indicated by an (*) and assessed by the student's *t*-test. Two arrays were considered significantly different if the P-value is ≤ 0.05.

CHAPTER 3
RESULTS

The Role of ER SNAREs in the Acquisition of ER-Derived Vesicles by the *Leishmania* Parasitophorous Vacuole

In eukaryotes, communication between membrane bound organelles, such as the ER and the Golgi apparatus, occurs through vesicle trafficking. Vesicles are usually generated at the precursor membrane and trafficked to the target membrane where fusion of the vesicle with its target can occur. Currently, SNAREs are recognized as key components of the protein complexes that drive membrane fusion. SNAREs present on vesicles interact with SNAREs on the target membrane resulting in the formation of SNARE complexes which is a requirement for the fusion of the two apposing membranes (Jahn and Scheller, 2006). A functional SNARE complex is formed by the hetero-oligomeric association of four SNARE motifs; a Qa-, a Qb-, a Qc- and an R-SNARE.

In a recent study, it was shown that 90% of *Leishmania* PVs display ER molecules on their PV during the course of infection(Ndjamen et al., 2010). In addition, it was shown that ricin, which traffics through the retrograde pathway, accumulated in the ER in a Brefeldin A sensitive manner. These observations suggest that, in addition to established interactions with the endocytic pathway, *Leishmania* PVs also interact with the host cell ER. Of particular interest was the observation that the ER SNARE sec22b is displayed on the *Leishmania* PV. Sec22b, an R-SNARE, functions in the trafficking of ER-derived vesicles in ER to Golgi directed traffic and has also been implicated in the delivery of ER-derived vesicles to another pathogen-containing compartment, the *Legionella*-containing vacuole (Kagan et al., 2004). The presence of sec22b on the

Leishmania PV suggests that ER SNAREs such as sec22b may play an important role in the delivery of ER-derived vesicles to the PV.

Leishmania Parasitophorous Vacuoles (PVs) Display Host Cell ER SNAREs

As a first step, we sought to identify potential SNARE partners for sec22b on the PV and to assess their role in the acquisition of ER molecules during the course of infection. In addition to sec22b, the SNAREs syntaxin 18 (STX-18), D12/USE-1/p31 (D12) and syntaxin 5 (STX-5) have been shown to function in the ER and ER/Golgi intermediate region (ERGIC) (Hay et al., 1997; Nichols and Pelham, 1998; Xu et al., 2000; Hong, 2005; Okumura et al., 2006). We proceeded to determine whether these molecules could also be found on the PV. For D12 and STX-18, we took advantage of the availability of YFP-tagged chimeras of these molecules described in previous studies (Hatsuzawa et al., 2006, 2009; Okumura et al., 2006). Following transfection of RAW264.7 macrophages with STX18-YFP and D12-YFP, the distribution of the molecules in uninfected cells was assessed by immunofluorescence microscopy. In order to show that the distribution of the YFP-tagged molecules overlapped with endogenous molecules we co-labeled transfected cells with antibodies to D12 and STX-18 (Figure 2-1a). Appropriate expression of the YFP-tagged molecules was also assessed by western blotting analysis, which confirmed that molecules of the appropriate size were being expressed in transfected cells (Figure 2-1b). The YFP-molecules showed the typical perinuclear distribution characteristic of ER labeling which did not overlap with LAMP1 labeling, a marker of the lysosomal compartment (Figure 2a). In contrast, cells transfected with the YFP molecule alone showed a diffuse pattern that had some overlap with the lysosomal compartment (Figure 2-2a). Next, the distribution of the YFP-molecules was assessed in RAW264.7 cells infected with

Leishmania amazonensis. At 48 hours post-infection, LAMP1, a lysosomal marker, shows a characterisitic vesicular pattern of labeling and is present on the limiting membrane of the PV. The D12-YFP and STX18-YFP transfected cells show perinuclear labeling, characteristic of ER labeling, and are also present on the limiting membrane of the PV (Figure 2-2b). An overlap of the LAMP1 and the D12-YFP and STX18-YFP labeling is evident on the PV membrane. The cells transfected with vector alone show no labeling around the PV, instead the YFP signal is diffuse around the cell (Figure 2-2b). These observations indicate that, in addition to sec22b-YFP, the ER SNARE chimeras D12-YFP and STX18-YFP localize to the PV membrane.

In addition to the SNAREs described above, the localization of STX5, a SNARE that functions in vesicle traffic in the ER-Golgi intermediate region, was assessed using a monoclonal antibody in immunofluorescence and immuno-electron microscopy techniques. In infected cells, STX5, which is normally localized to the Golgi and ER-Golgi intermediate compartment, is recruited to the PV membrane (Figure 2-3d). Although the label appears different to the label displayed by the aforementioned ER SNAREs, the punctate distribution may be more representative as it represents endogenous STX5. For immuno-electron microscopy analysis, infected cells were processed for electron microscopy by high pressure freezing and thin sections were subsequently labeled using monoclonal STX5 antibody, followed by 10 nm gold-conjugated secondary antibody. STX5 was present on the PV membrane (Figure 2-3b) as well as the Golgi and Golgi intermediate compartment (IC) (Figure 2-3c) as has been described in previous studies (Hay et al., 1998).

Parasitophorous Vacuole Growth and Parasite Replication are Mediated by ER and ER-Golgi Intermediate SNAREs

As mentioned in previous sections, the PVs housing *L. amazonensis* parasites gradually grow into large communal vacuoles. PVs can take up much of the cytoplasmic space and can achieve sizes that rival the host cell nucleus. In a previous study, it was shown that by limiting lysosome size, which is considered to be a source of membrane for PV aggrandizement, the size of the PV can also be limited (Wilson et al., 2008). In addition to the lysosomal contribution to PV size, it is believed that the homotypic fusion of PVs in infected cells as well as fusion with other host cell vesicles can result in PV growth (Real et al., 2008). In light of the observation that the PV also displays various ER and ER/Golgi SNARE molecules, which are involved in the trafficking of early secretory vesicles from the ER, we explored whether the recruitment of early secretory vesicles also played a role in PV growth. The approach we used to assess the role of ER-derived vesicle fusion at the PV membrane was to overexpress dominant-negative constructs of the ER SNAREs found to be present on the PV – sec22b, D12 and STX18. Indeed, in a somewhat related study, it was shown that by inhibiting sec22b function the recruitment of ER to phagosomes containing latex beads could be reduced (Cebrian et al., 2011). The dominant negative constructs used in our study lacked the transmembrane domain which is required for SNARE function. These constructs have been described and partially characterized in previous studies (Hatsuzawa et al., 2006, 2009; Okumura et al., 2006). As a first step, we assessed whether the overexpression of the dominant negative constructs had an effect on normal host cell function. The localization of two macrophage surface markers, complement receptor 3 (CR3) and Fc receptor (FcR), were assessed in cells

overexpressing dominant negative constructs. The expression and localization of both CR3 and FcR were unaffected (Figure 2-4a). Moreover, the secretion of Interleukin 6 (IL-6) after activation with LPS/IFN-γ was not affected by overexpression of the dominant negative SNARE constructs (Figure 2-4b). These observations suggest that normal cell functions such as trafficking of surface markers to the plasma membrane and the activated secretion of a cytokine are unaffected by the overexpression of dominant negative SNARE constructs.

In order to assess PV size and parasite replication, RAW264.7 macrophages were transfected with wild-type SNAREs (sec22b-YFP, D12-YFP or STX18-YFP) or with dominant negative SNAREs (sec22bΔTMD-RFP, D12ΔTMD-RFP or STX18ΔTMD-RFP) and PV size and the number of parasites per infected macrophage were monitored at 4 and 48 hours post-infection. In cells transfected with vector alone, the PVs grow from approximately half the size of the host cell nucleus at early time points (4 hours post-infection) to become approximately the same size as the host cell nucleus at late time points (48 hours post-infection) (Figure 2-5a). The transition from several small PVs at 4 hours to a single large PV at 48 hours is the result of homotypic fusion of PVs and is normal for *L. amazonensis* PVs. The overexpression of wild-type ER SNAREs resulted in an increase in PV size at 48 hours compared to the vector alone. On the other hand, the overexpression of dominant negative sec22b and D12, but not STX18, resulted in a decrease in PV size at 48 hours. Figure 2-5b and 2-5c show representative images with a sketch to accentuate PV size. In addition to smaller PV size at 48 hours post infection, the number of PVs per infected cell in cells transfected with dominant negative constructs was greater suggesting impaired homotypic fusion of primary PVs. Taken

together, these results suggest that overexpression of dominant negative sec22b and D12, but not STX18, result in decreased PV distention and fusion without having any apparent effect of host cell function.

Next, we assessed the effect of overexpressing wild-type and dominant negative ER SNAREs on parasite replication. Dominant negative sec22b and D12, but not STX18, overexpression resulted in a significant decrease in the number of parasites per infected cell at 72 hours post infection compared to cells transfected with vector alone (Figure 2-6). The overexpression of wild-type sec22b, D12 and STX18 resulted in a small, although not significant, increase in the number of parasites per infected cell.

As mentioned earlier, a somewhat related study showed that in dendritic cells the recruitment of ER to a latex bead-containing phagosome can be inhibited by blocking sec22b function (Cebrian et al., 2011). To assess whether the overexpression of the dominant negative ER SNARE constructs had any effect on the recruitment of ER to the PV, we monitored the recruitment of the ER molecule calnexin to the PV. Figure 2-7 shows that, whereas the display of calnexin is evident on the PV in an untransfected cell, PVs in cells transfected with dominant negative sec22b, D12 and STX18 are devoid of calnexin. These observations suggest that the function of the ER SNAREs sec22b, D12 and STX18 are essential for the fusion and acquisition of host ER by the PV.

It has been demonstrated that the ER participates in the phagocytosis of large particles (>0.5 μM) and that ER SNAREs are involved in the regulation of ER-mediated phagocytosis (Becker et al., 2005; Hatsuzawa et al., 2006, 2009). Therefore, the observed decrease in the number of parasites per infected cell may be a result of

inhibition of parasite uptake in the presence of dominant negative ER SNAREs . To address this issue, we assessed parasite uptake in cells expressing wild-type or dominant negative ER SNAREs. Only the overexpression of wild-type D12 and STX18 resulted in a small, but significant, increase in parasite uptake (Figure 2-8). There was no significant change for all other constructs used including wild-type sec22b and dominant negative sec22b, D12 and STX18 (Figure 2-8). Therefore, although ER SNAREs have been shown to regulate the phagocytosis of large inert particles, they appear to have a minimal role in the uptake of live *L. amazonensis* parasites.

ER SNARE Knockdown Results in Reduced Parasitophorous Vacuole Size and Parasite Replication

In order to confirm the role of ER-PV interactions in PV growth and parasite replication, we chose to knockdown the ER SNAREs discussed using siRNA to limiting levels. In addition to the ER SNAREs sec22b, D12 and STX18, we chose to include STX5 in these studies as it was also found to be present on the PV (Figure 2-3d) and is known to be involved in vesicular transport in the ER Golgi intermediate compartment.

Knockdown of sec22b and STX5 resulted consistently in an 80–90% reduction in expression level compared to control, scrambled siRNA as determined by western blotting and densitometry measurements (Figure 2-9). Knockdown of STX18 resulted in a 50-60% reduction in the expression level. The expression level of D12 was determined by immuno-fluorescence intensity measurements and was consistently reduced by 80-90% as compared to the control, scrambled siRNA (Figure 2-9). As was done for the experiments using ER SNARE constructs, the expression of surface markers CR3 and FcR, as well as the activated secretion of IL-6, were assessed to determine whether or not siRNA knockdown resulted in a disruption of normal cell

function. No significant change in CR3 and FcR localalization or activated secretion of IL-6 was detected (Figure 2-10).

Cells treated with control or specific siRNA, were infected 12 hours after the introduction of the siRNA and, as described earlier, the PV size and the number of parasites per infected cell were assessed at 4 and 48 hours post-infection. An impressive and statistically significant reduction in average PV size, from approximately 0.9 times the size of the host cell nucleus to approximately 0.6 times the size of the host cell nucleus, was observed in cells in which sec22b, D12 and STX5 had been knocked down compared to control, scrambled siRNA (Figure 2-11). Although the range in PV sizes appeared reduced for STX18 knockdown, the average PV size only reduced from approximately 0.9 the size of the host cell nucleus to approximately 0.8 times the size of the host cell nucleus. In addition, at 48 hours post-infection, in cells in which sec22b, D12 and STX5 had been knocked down, a significant reduction in the number of parasites per infected cell as compared to control siRNA treated cells was observed (Figure 2-11). In cells in which STX18 had been knocked down, there was no significant change in the number of parasites per infected cell at 48 hours post-infection (Figure 2-11). Taken together, these observations suggest that by blocking the interaction of the *Leishmania* PV with the host cell ER by blocking ER SNARE function, the development of the PV as well as parasite replication can be adversely affected.

A Small Molecule Inhibitor of STX5, Retro-2, Limits PV Distention and Parasite Replication

In a recent study, it was shown that the retrograde trafficking of ricin could be blocked by a small molecule inhibitor of STX5, retro-2 (Stechmann et al., 2010). Retro-2, named for its ability to block retrograde traffic, was shown to function by resulting in

the dramatic mislocalization of STX5. In the presence of retro-2, STX5, which is normally located in the Golgi and ER Golgi intermediate compartment, is dispersed throughout the cell in a punctate pattern (Stechmann et al., 2010). The mechanism by which retro-2 results in the aberrant localization of STX5 is unknown, but the redistribution is sufficient to inhibit STX5 function in Golgi to ER directed traffic. Our work, as shown in the previous section, has shown that STX5 regulates PV development as well as parasite replication during *L. amazonensis* infection. These observations led us to explore the effect of retro-2 on PV development and parasite replication.

As a first step, we chose to evaluate the effect of retro-2 on RAW264.7 cell function. The distributions of the surface markers CR3 and FcR were determined at increasing concentrations of retro-2 (Figure 2-12a and b). Retro-2 had no effect on the localization of CR3 at all concentrations tested, but at higher concentrations of retro-2 (50-100 μM), FcR began to be retained in an internal compartment (Figure 2-12b). Next, the distribution of STX5 relative to the Golgi marker GM130 was assessed at increasing concentrations of retro-2. STX5 is normally localized to the Golgi and when cells were treated with vehicle alone (DMSO), the two molecules colocalized (Figure 2-13). In the presence of retro-2, STX5 is dispersed; meanwhile, GM130 labeling is unaffected (Figure 2-13). Taken together, these observations suggest that retro-2 has no effect on CR3 and GM130 localization and a limited effect of FcR localization; however, it results in the mislocalization of STX5. These results are in agreement with those of Stechmann et. al (Stechmann et al., 2010).

As with previous experiments using dominant negative constructs and siRNA, the effect of retro-2 on the secretion of IL-6 from RAW264.7 cells activated with LPS and IFNγ was also assessed. There was no significant difference in the activated secretion of IL-6 by cells treated with retro-2 at increasing concentrations compared to cells treated with vehicle alone (Figure 2-14). These results suggest that the trafficking of various molecules along the secretory pathway are not affected by treatment of cells with retro-2 once again confirming the results of Stechmann et al. (Stechmann et al., 2010).

The observation that STX5 knockdown by siRNA results in reduced PV size and parasite replication prompted us to study the effect of retro-2 on PV size and parasite replication. RAW264.7 cells were infected with *L. amazonensis* for 2 hours and then washed and treated with retro-2 at increasing concentrations for the course of the infection. The addition of retro-2 after parasite internalization allowed us to study the effect of retro-2 on parasites already in PVs and eliminated an effect on parasite entry as a variable. In RAW264.7 cells, treatment with retro-2 resulted in a dose dependent decrease in PV size at 48 hours post infection (Figure 2-15a). In addition, the number of parasites per infected cells did not increase from 4 hours to 48 hours post infection in cells treated with 100 μM retro-2; whereas, the number of parasites per infected cell nearly doubled in cells treated with vehicle alone (Figure 2-15b).

As our next step, we chose to study the effect of retro-2 on macrophages from mouse peritoneal exudate (PECs). PECs offer a convenient system for studying PV distention and parasite replication; in that, in PECs, PVs distend to more impressive sizes that exceed the size of the host cell nucleus and parasite numbers after 48 to 72

hours of infection tend to be greater than in RAW264.7 cells. Figure 2-16a shows representative images of the effect of retro-2 on PV size in PECs at 48 hours post infection. The trace is used to outline PVs. There is a dose-dependent decrease in PV size with increasing concentrations of retro-2 (Figure 2-16a). Interestingly, at early timepoints (4 hours) there is a decrease in PV size at 75 μM and 100 μM retro-2, suggesting that ER-PV interactions play a role in PV aggrandizement from very early timepoints (Figure 2-16b). More impressive, though, is the effect of retro-2 on PV size at later timepoints (48 hours) where PV size goes from an average of 1.1 times the size of the host cell nucleus in cells treated with vehicle alone to about 0.5 times the size of the host cell nucleus in cells treated with 100 μM retro-2 (Figure 2-16b).

The number of parasites per cell was assessed at 4, 48 and 72 hours post infection (Figure 2-17). In cells treated with vehicle alone there is an increase in the number of parasites per macrophage from an average of 3 at 4 hours to approximately 5.5 parasites per infected cell at 72 hours. At 75 μM retro-2 there is no change in the number of parasites per infected cell at any of the timepoints and at 100 μM retro-2 there is a decrease in the number of parasites per infected cell (Figure 2-17). These results indicate that targeting STX5 function using retro-2 results in a significant reduction in PV size as well as parasite replication, without any apparent effect of host cell function.

In Retro-2 Treated Cells, STX5 is Not Recruited to the PV

Next we sought to determine whether the mislocalizationn of STX5 caused by treatment of host cells with retro-2 results in a limited association with its cognate SNARE partner, sec22b. To achieve this goal, co-immunoprecipitation experiments

were performed in which RAW264.7 cells, treated with vehicle alone or retro-2, were infected for 12 hours followed by lysis. When sec22b was immunoprecipitated from lysates treated with either vehicle alone or retro-2, similar amounts of STX5 co-immunoprecipitated in both samples (Figure 2-18a). In addition to STX5, other cognate SNARE partners of sec22b-D12 and STX18-also co-immunoprecipitated at comparable levels. However, in samples in which the PV fraction was enriched on a sucrose density gradient (as described in Kima and Dunn, 2005), STX5 did not co-immunoprecipitate with sec22b in retro-2 treated samples (Figure 2-18b). Moreover, the partnering of sec22b and STX18 is not affected in the PV fraction in retro-2 treated samples (Figure 2-18b). These observations demonstrate that the interaction of STX5 and sec22b are affected by retro-2 at the PV membrane; however, their interaction in the whole cell is unaffected. This differential effect of retro-2 may explain why it can have such a dramatic effect on PV size and replication while having no apparent effect on host cell function.

Retro-2 Treatment Results in Reduced Lesion Size and Parasite Titer in Experimental *L. amazonensis* Infection

Encouraged by the effect of retro-2 on PV size and parasite replication *in vitro*, we sought to determine the effect of retro-2 on *L. amazonensis* in a mouse model of infection. In previous studies, it has been shown that retro-2 has no apparent effect of mouse health when used at dosages as high as 400 mg/Kg (Stechmann et al., 2010). We chose to treat mice with a single dose of retro-2 at 20 mg/Kg or 100 mg/Kg either 1 day post infection or 3 weeks post infection. In both cases, mice were infected with stationary phase *L. amazonensis* promastigotes in the footpad. Infection can be monitored by regular measurements of footpad swelling which indicates lesion growth

and by measuring parasite titer at the site of infection as assessed by limiting dilution assay. The 20 mg/Kg dose had no effect on the course of lesion development in that lesion growth was comparable to mice treated with vehicle alone (DMSO) (Figure 2-19a). However, a dose of 100 mg/Kg of retro-2 resulted in a significant reduction in lesion size compared to vehicle alone (Figure 2-19a). Similarly, a 20 mg/Kg dose of retro-2 had no effect on parasite titer at the site of infection; however, a dose of 100 mg/Kg administered either 1 day or 3 weeks post-infection resulted in a significant decrease in parasite titer (Figure 2-19b). The reduction in parasite titer in mice treated 3 weeks post-infection suggests that retro-2 can adversely affect an *L. amazonensis* infection that is already established. These observations show that, in addition to the *in vitro* effects of retro-2 on *L. amazonensis* infection, retro-2 can be used to control *L. amazonensis* infection *in vivo* without any apparent effects on mouse health.

Retro-2 Affected *Leishmania* Replication in Axenic Culture

Leishmania parasites have been shown to have a number of SNARE homologs (Besteiro et al., 2006), including a STX5 homolog; therefore, we chose to assess the effect of retro-2 on the axenic growth of *L. amazonensis* parasites. Retro-2 at 50, 75 and 100 μM concentrations has an inhibitory effect on the growth of parasites (Figure 2-20). Although parasites remained viable, they were unable to replicate unlike RAW264.7 cells, which were able to replicate in the presence of retro-2. These observations show that in addition to its effect on STX5 of the host cell, retro-2 directly inhibits the growth of *Leishmania* parasites.

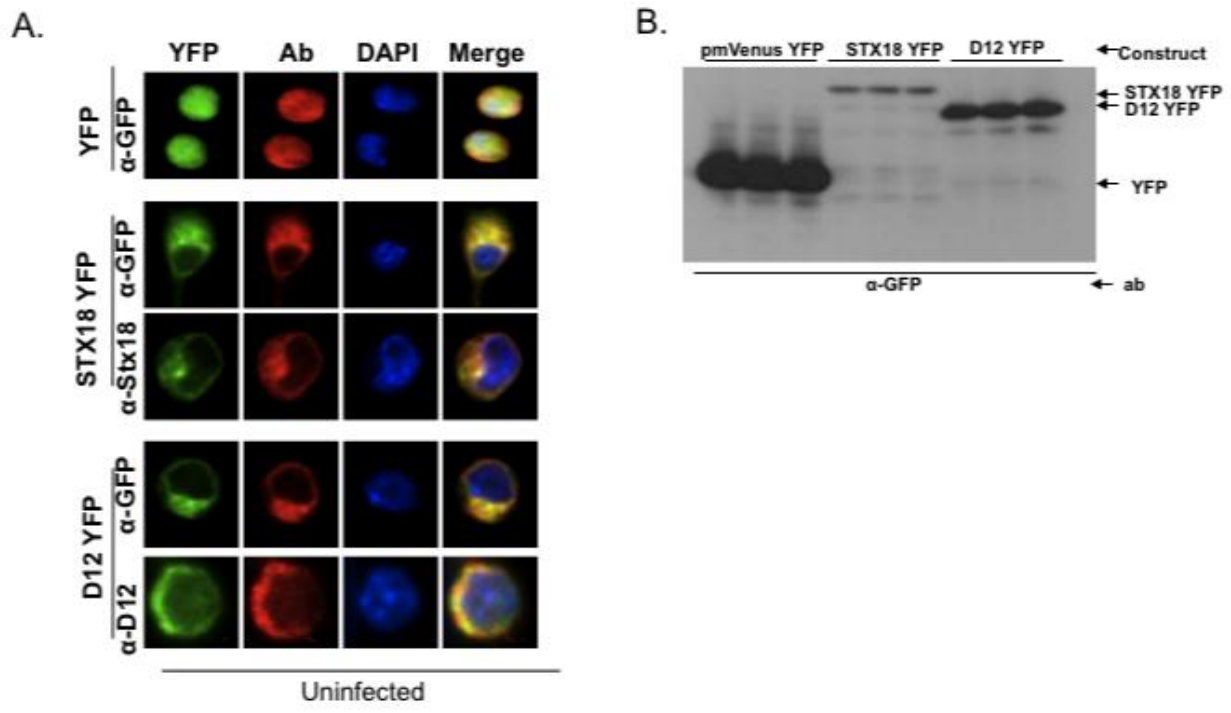

Figure 2-1. Assessment of Expression of SNARE-YFP Constructs in RAW264.7 cells. RAW264.7 cells were transfected with the pmVenus vector alone, or with STX18-YFP or D12-YFP constructs. (A) Transfected cells were labeled with antibodies to STX18 or D12 to show that the localization of the construct and the endogenous SNARE overlapped. Transfected cells were also labeled with anti-GFP antibody to show that the localization of the YFP-tagged molecules was controlled by the SNARE localization. Cells were also incubated with DAPI to show the nucleus. (B) Transfected cells were lysed and probed with anti-GFP antibody to show expression level of the constructs. Transfected cells were routinely lysed to assess the level of expression.

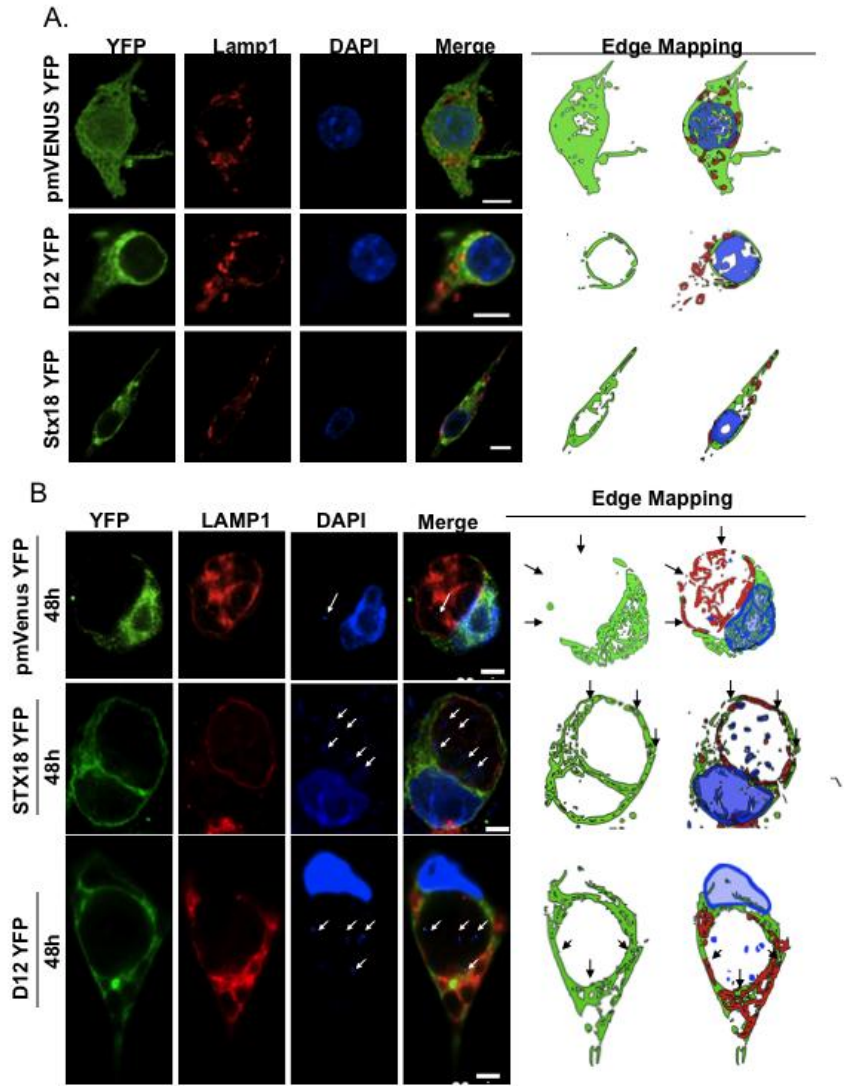

Figure 2-2. Distribution of YFP-tagged ER SNAREs in Uninfected and Infected RAW264.7 Cells. (A) RAW264.7 cells transfected with pmVenus vector alone or D12-YFP or STX18-YFP were labeled with antibodies to the lysosomal marker LAMP1 and incubated with DAPI to show both host cell and parasite nuclei. Edge mapping drawings, created with the "Trace Bitmap" tool in the InkScape software aid in the visualization of the LAMP1 and GFP localization. (B) Transfected cells were infected with *Leishmania amazonensis* for 48 hours and labeled with LAMP1 and DAPI. Black arrows indicate the PV limiting membrane. White arrows indicate parasite nuclei.

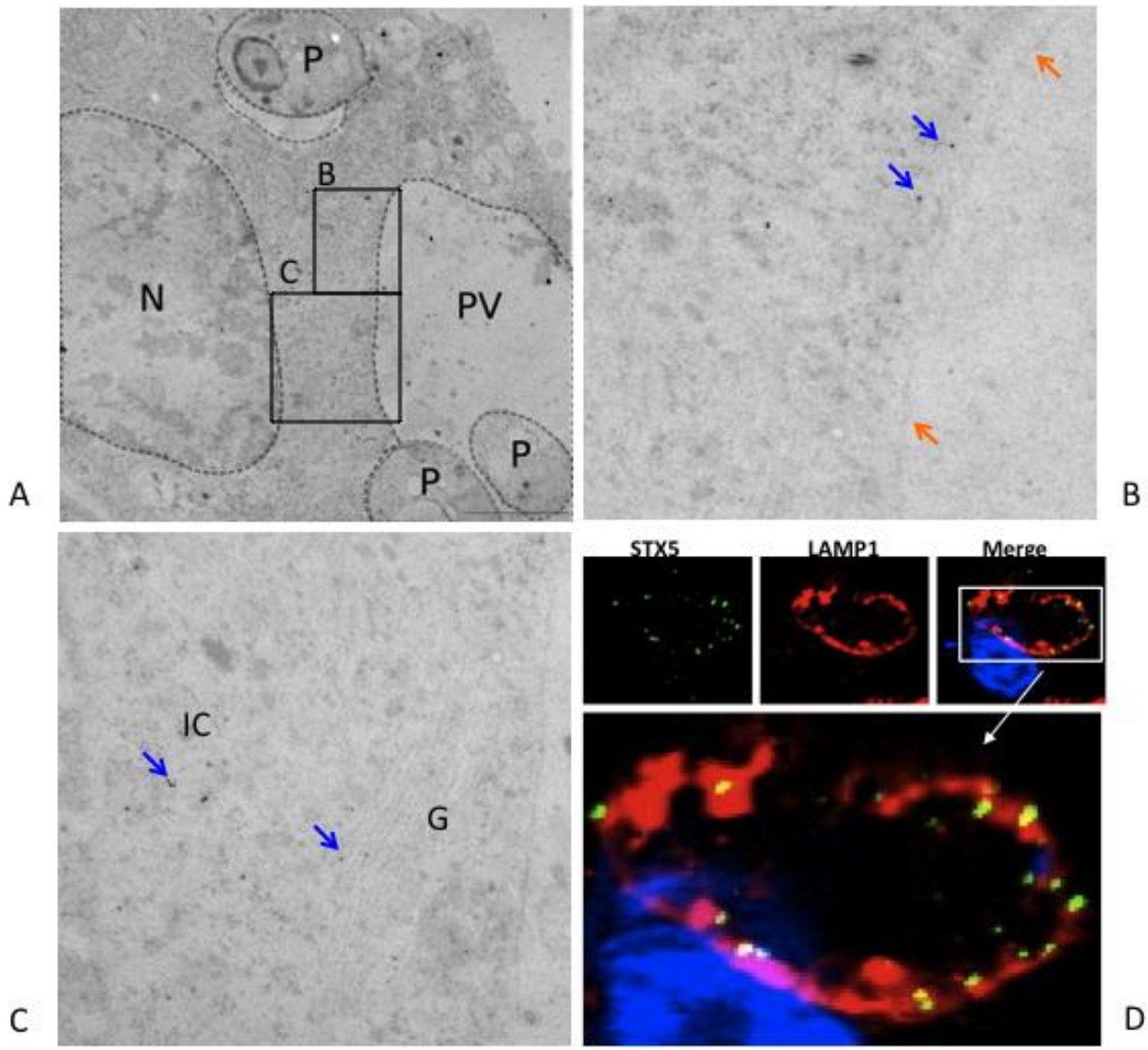

Figure 2-3. Distribution of STX5 in RAW264.7 cells infected with *Leishmania amazonensis*. Infected RAW264.7 cells were prepared for immuno-electron microscopy by high-pressure freezing. Sections were labeled with anti-STX5 antibody followed by secondary antibody conjugated to 10 nm gold particles. Post-staining with unranyl acetate and lead citrate were performed for 1 minute each, followed by analysis on a Hitachi TEM H-7000 operated at 100 kV. (A) Shown is an infected cell in which the nucleus (N), a parasitophorous vacuole (PV) and intracellular parasites (P) are clearly visible. The boxed off areas B and C are amplified in panel (B) and (C). Panel (B) shows gold particles (blue arrows) present on the PV membrane (orange arrows). Panel (C) shows the host cell Golgi (G) as well as the intermediate compartment (IC). The normal localization of STX5 is evident by the presence of STX5 on both the Golgi (G) and the intermediate compartment (IC). Panel (D) shows an infected cell processed for immunofluorescence. The red label shows the lysosomal marker LAMP1 that outlines the nucleus. In green is STX5 that colocalizes with LAMP1 at the PV membrane.

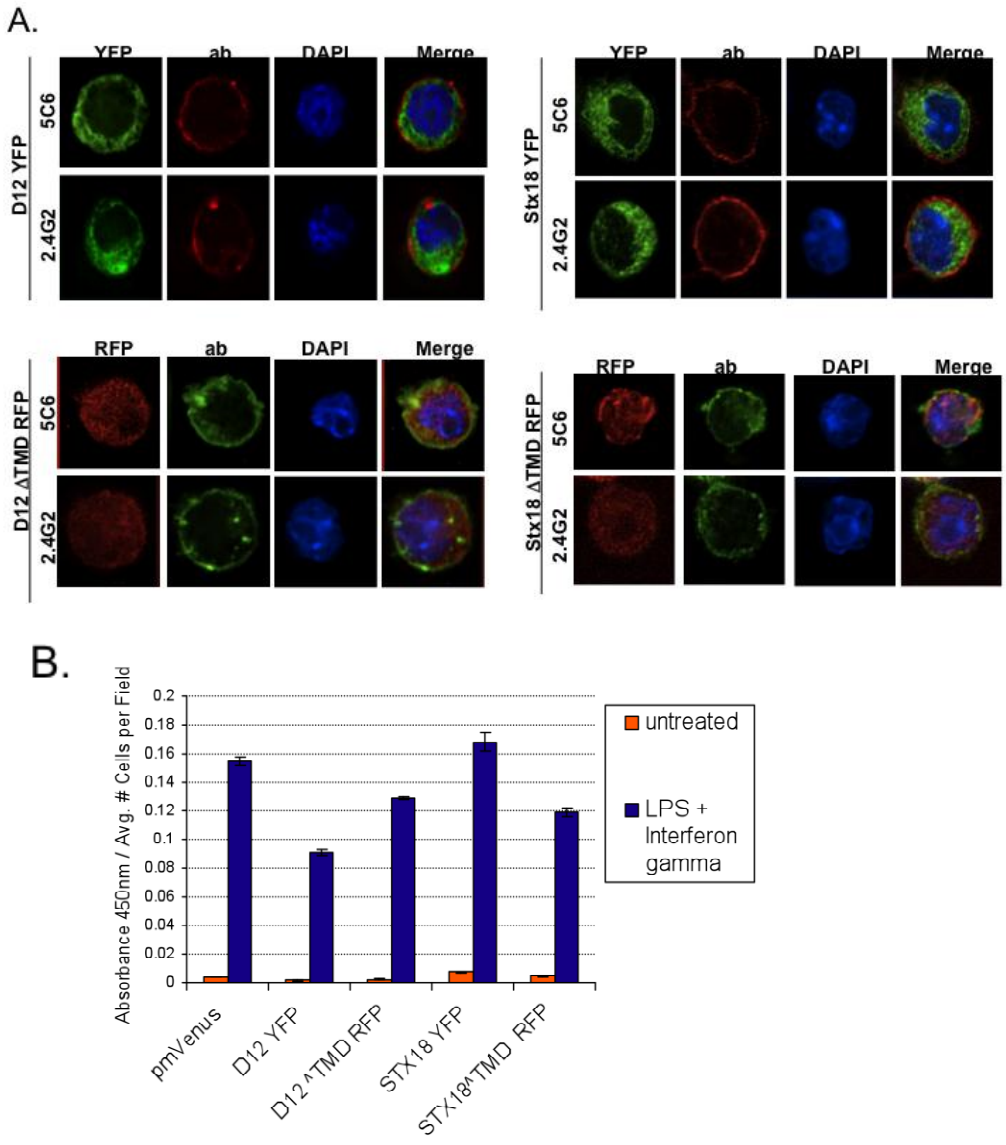

Figure 2-4. Effect of dominant negative SNARE constructs on surface marker distribution and secretion of IL-6. RAW264.7 cells were transfected with either wild-type D12, dominant-negative D12, wild-type STX18, or dominant negative STX18. In Panel (A) cells were labeled with antibodies to the surface markers CR3 (5C6) or FcR (2.4G2) and processed for immunofluorescence. Dominant negative constructs had no effect on distribution of these surface markers. In Panel (B) transfected cells were incubated with LPS/IFNg and IL-6 secretion was measured by ELISA. There was no significant difference in activated secretion of IL-6 in cells transfected with either of the constructs compared to the pmVenus vector alone.

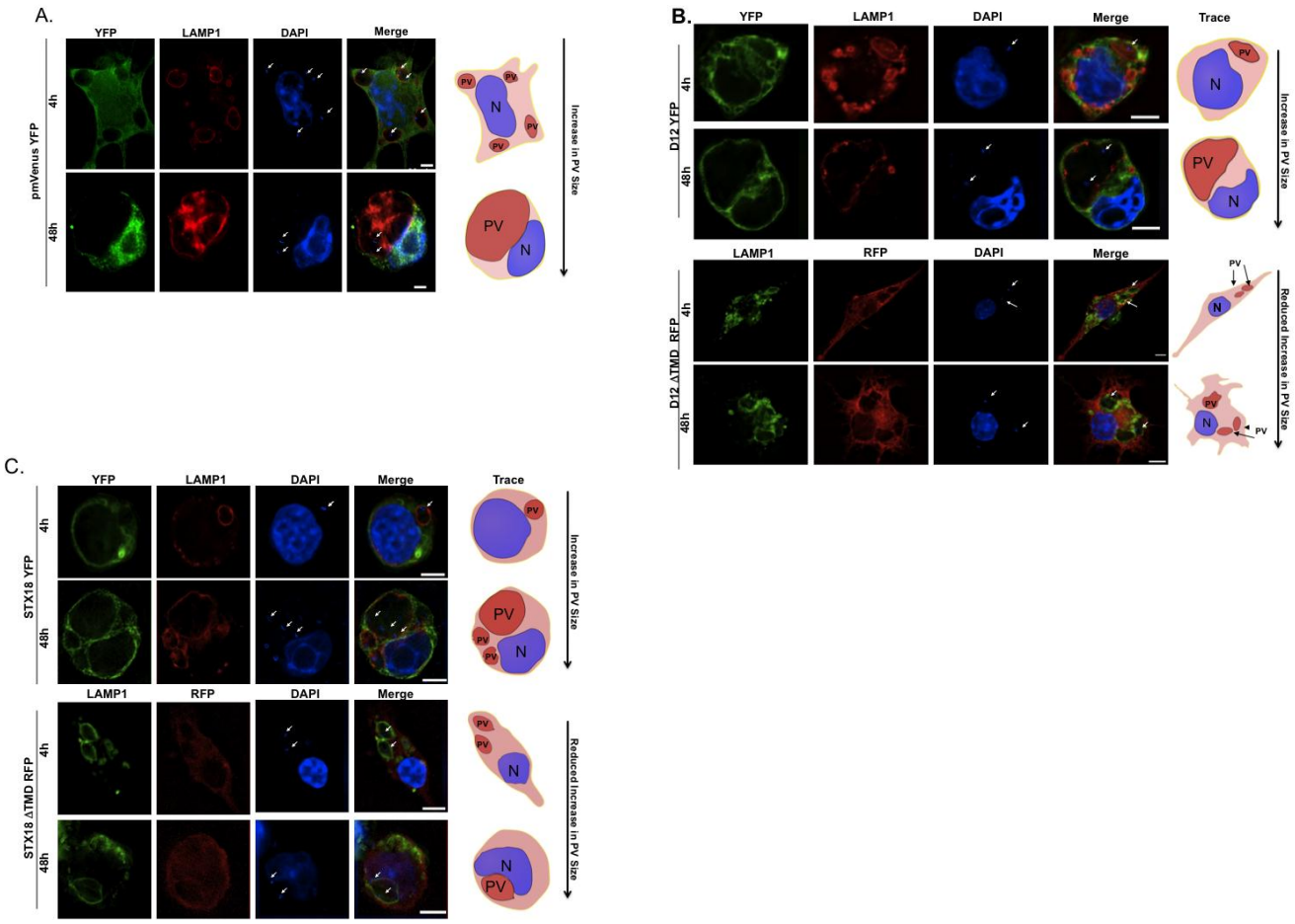

Figure 2-5. Overexpression of wild-type or ER dominant negative SNAREs modulates PV development. Panel (A) shows RAW264.7 cells transfected with the pmVenus vector alone and infected with *Leishmania amazonensis*. Cells were labeled with LAMP1 to visualize PVs and PVs were traced using the "Trace Bitmap" tool in Inkscape. Cells were also labeled with DAPI to visualize nuclei. In panels (B) and (C) cells were transfceted with either wild-type or dominant negative D12 or STX18. PV size was visualized at both 4 and 48 hours post infection to assess PV growth.

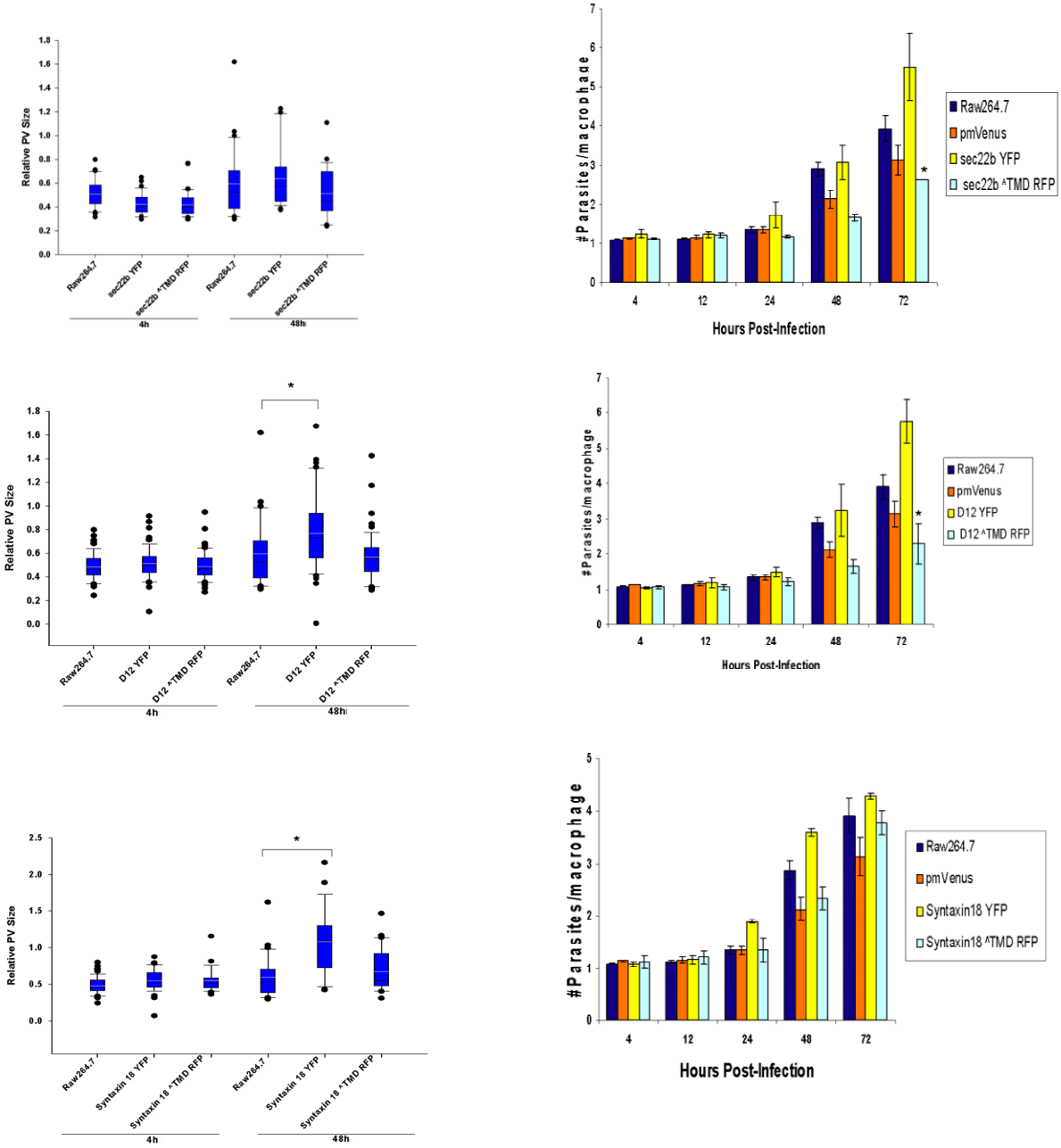

Figure 2-6. Overexpression of wild-type and dominant negative SNAREs affects PV size and parasite replication. RAW264.7 cells were transfected with wild-type or dominant negative sec22b, D12 or STX18 constructs. Transfected cells were infected with *Leishmania amazonensis* for 4, 48 or 72 hours. At each timepoint, cells were processed for immunofluorescence microscopy and PV sizes were measured (box plots) using the "outline spline" tool on the AxioVision software and the number of parasites per infected cell was counted (bar charts). PV sizes (box plots) are presented relative to the size of the host cell nucleus and white lines represent the mean, black lines represent the median. For each condition, a minimum of 50 cells was considered. The (*) indicates significance at a p value less than 0.05.

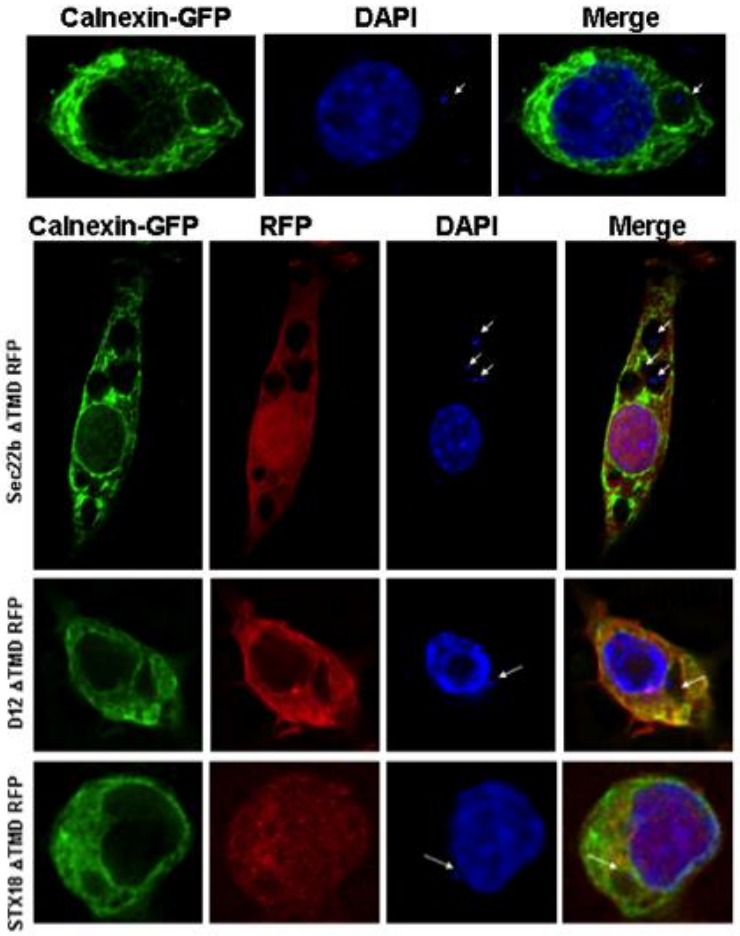

Figure 2-7. Overexpression of dominant negative constructs blocks the recruitment of the ER molecule Calnexin to the PV. RAW264.7 cells were co-transfected with dominant negative sec22b, D12 or STX18 (red) along with calnexin-GFP (green). Transfected cells were infected with *Leishmania amazonensis* for 24 hours and processed for immunofluorescence microscopy. White arrows indicate parasite nuclei.

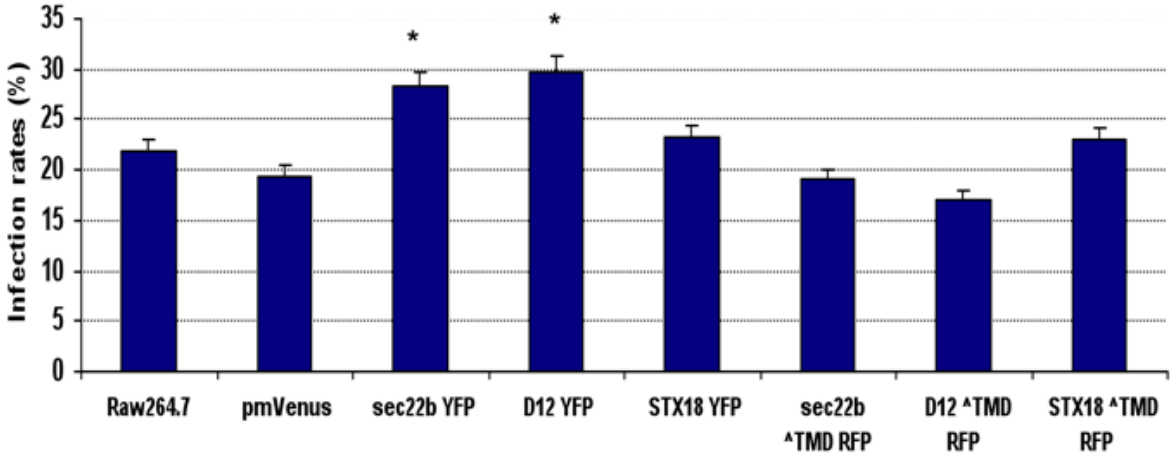

Figure 2-8. Effect of expressing wild type or dominant negative ER SNAREs on parasite internalization. RAW264.7 cells expressing either wildtype SNAREs or dominant negative variants were incubated with *Leishmania amazonensis* promastigotes for 2 hours. Cells were then washed to remove uninternalized parasites and were processed for immunofluorescence microscopy labeling with LAMP1 and DAPI. The percentage of transfected cells that were infected was plotted. Data above is compiled from at least three separate experiments. The (*) represents statistical difference as compared to the cells transfected with pmVenus vector alone. Statistical significance is indicated where p values are less than 0.05.

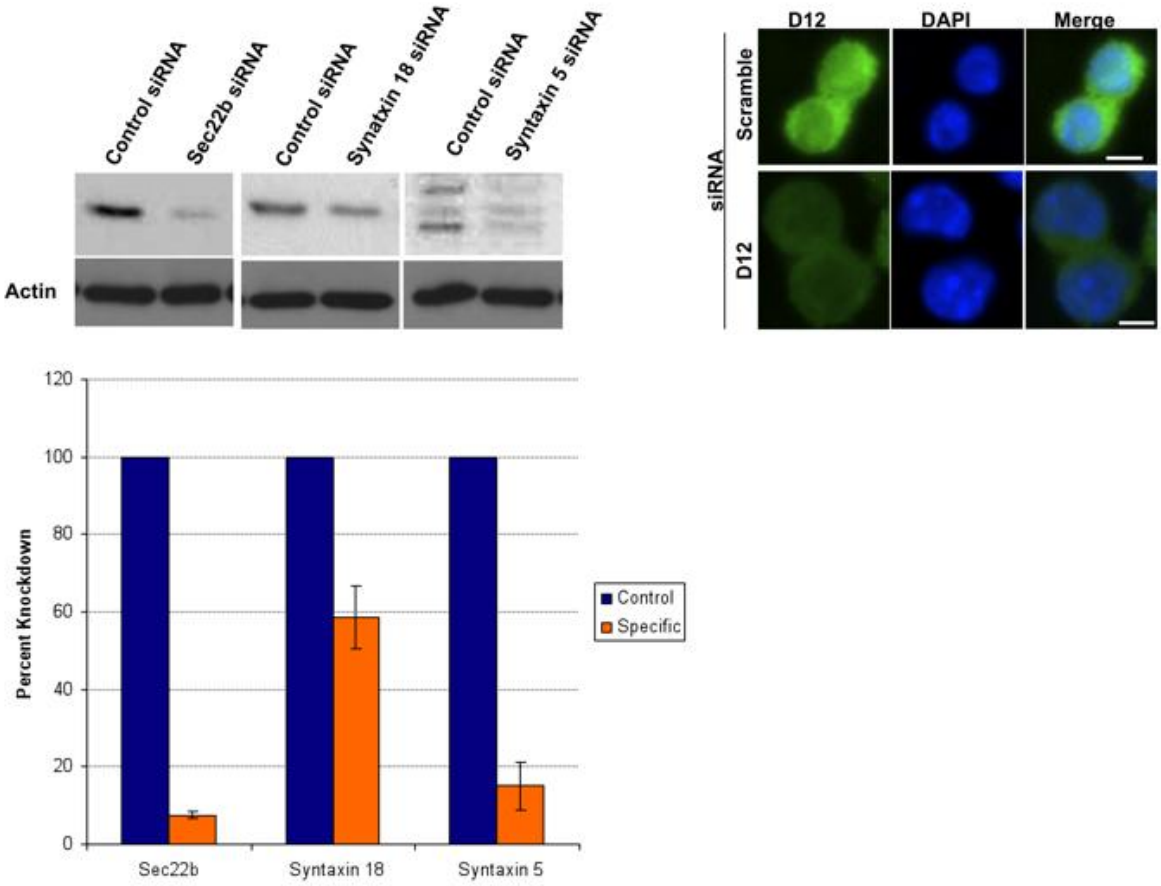

Figure 2-9. Assessment of ER/Golgi SNARE knockdowns. RAW264.7 cells were transfected with siRNA targeted to sec22b, STX18, D12 or STX5. A control scrambled siRNA was also used for comparison. 24 hours after transfection cells were lysed and analysed by Western Blot for the SNARE of interest. Representative blots are shown in the top left image. Knockdowns were also quantified by densitometry. The graph at the bottom left shows the level of knockdown relative to samples transfected with control siRNA. Data is compiled from at least three separate experiments. Knockdown was also assessed by immunofluorescence microscopy. The top right image shows cells transfected with either D12 siRNA or control siRNA which were processed for immunofluorescence and labeled with anti-D12 antibody and DAPI. The relative difference in fluorescence intensity was used to estimate the D12 knockdown.

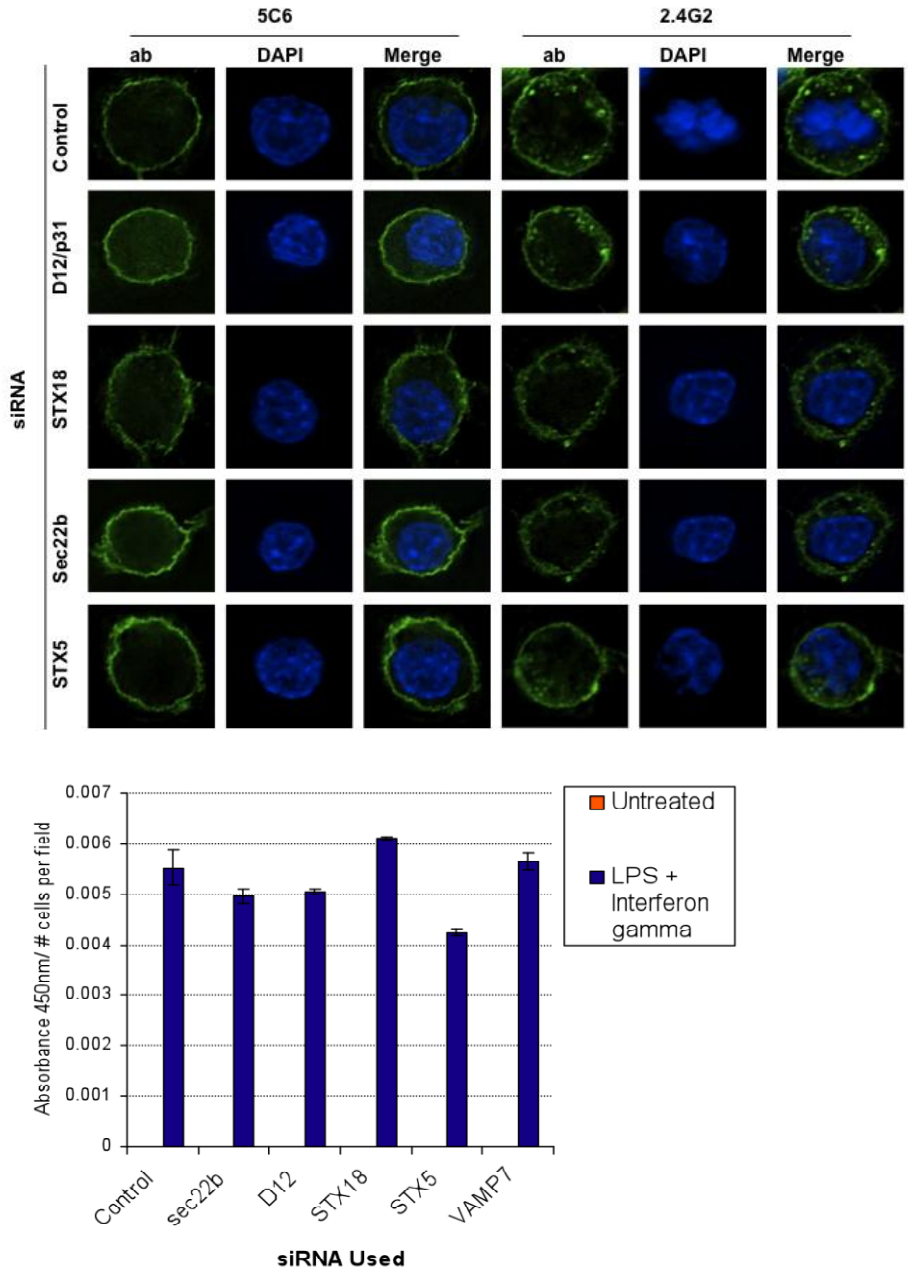

Figure 2-10. Knockdown of individual ER/Golgi SNAREs does not affect surface marker localization or IL-6 secretion. RAW264.7 cells were transfected with either control, D12, sec22b, STX18 or STX5 siRNA and processed for immunofluorescence labeling with DAPI and either anti-CR3 antibody (5C6) or anti-FcR antibody (2.4G2). Representative images are shown for each condition above. Secretion of IL-6 after treatment with LPS/Interferon-gamma was also assessed by ELISA. IL-6 data is representative of at least 3 experiments. In all samples tested, IL-6 was undetectable in the untreated cells.

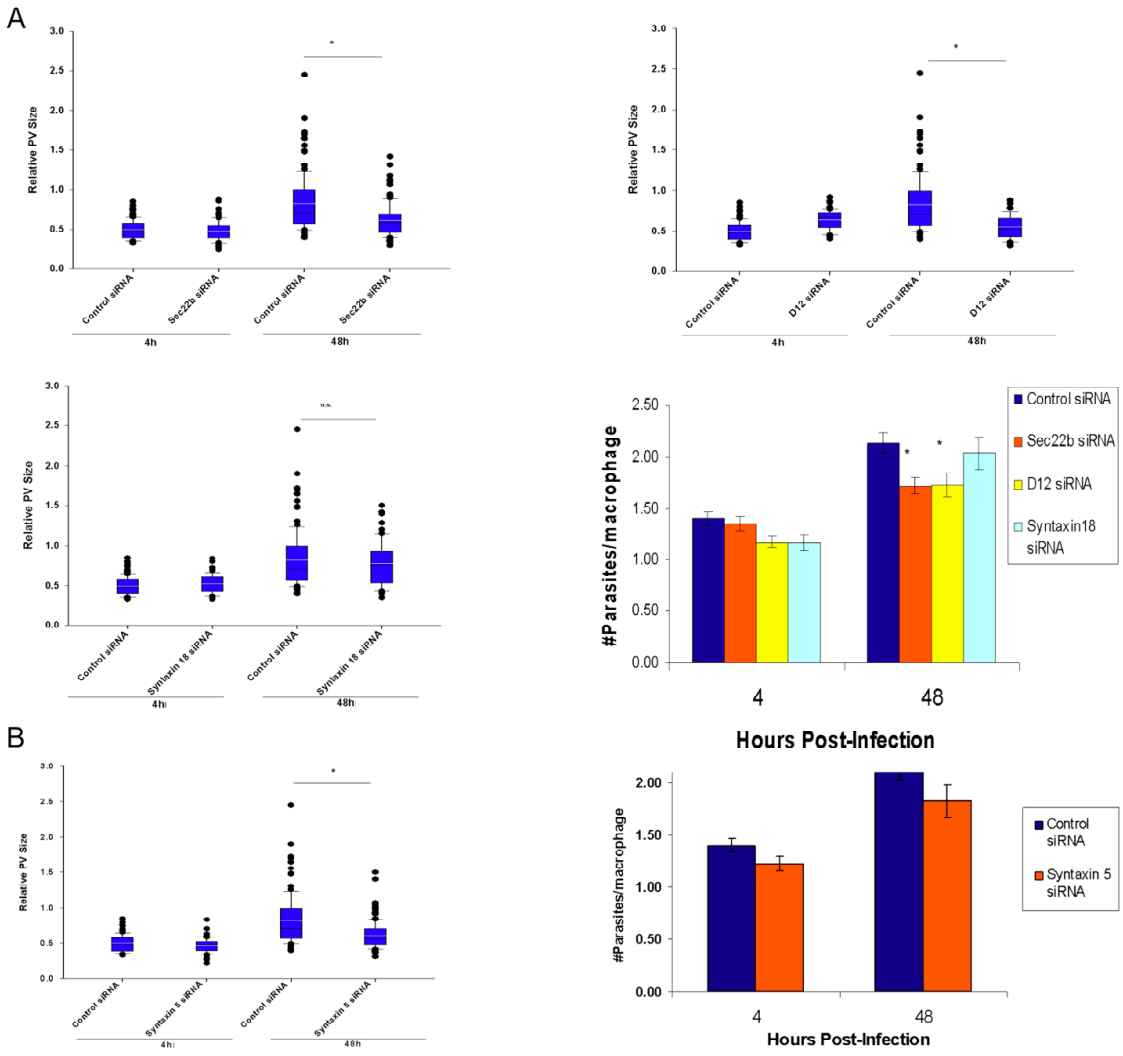

Figure 2-11. Knockdown of ER/Golgi SNAREs limits PV distention and parasite replication. RAW264.7 cells were transfected with siRNA targeted to sec22b, D12, STX18, or STX5 or with control siRNA. Transfected cells were then infected with *Leishmania amazonensis* and the infection was terminated at 4 and 48 hours post infection. Cells were then processed for immunolabeling with LAMP1 antibody and DAPI. PV size relative to host cell nucleus (box plots) and number of parasites per infected cell (bar charts) were assessed. In (A) PV size and number of parasite per infected cell is shown for knockdown of the ER SNAREs; whereas, in (B) data is for the knockdown of the ER/Golgi SNARE STX5. In the PV size box plots, white lines represent mean PV size and black lines represent median PV size. Significance is denoted by an (*) and is shown only when the p value is less than 0.05.

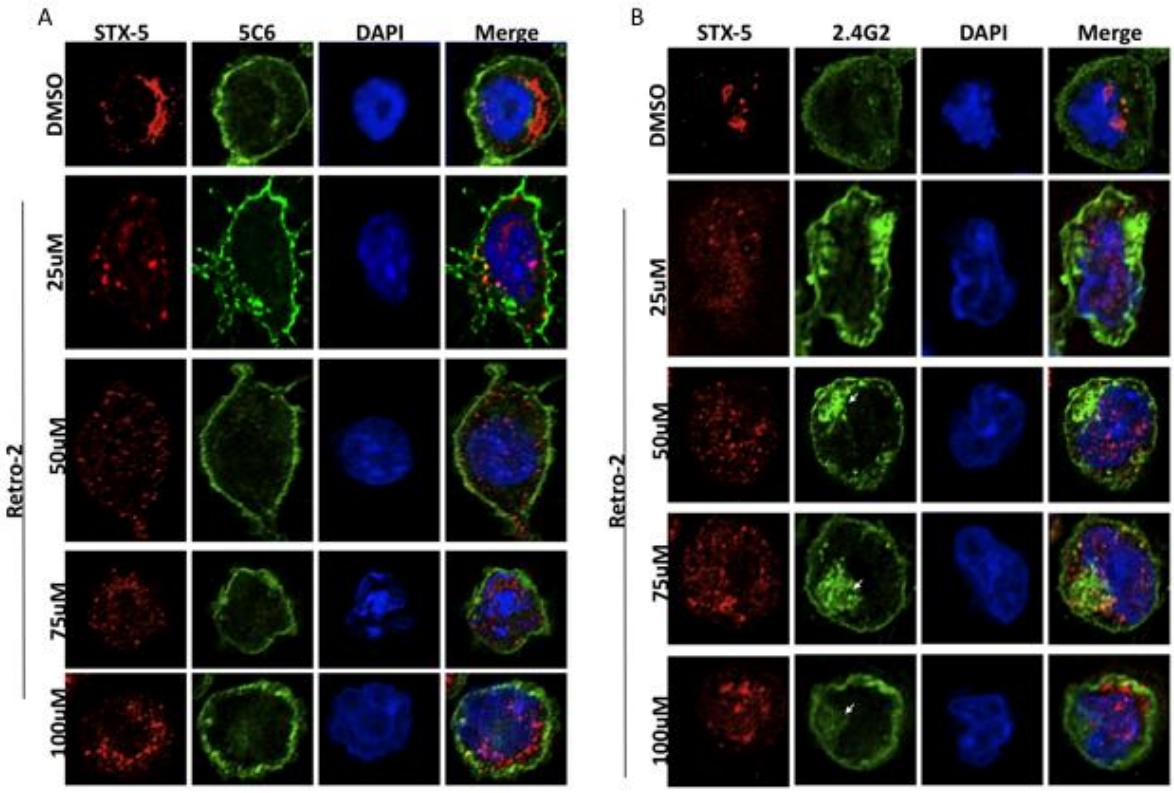

Figure 2-12. Effect of retro-2 on RAW264.7 surface markers. RAW264.7 cells were treated with DMSO or retro-2 (dissolved in DMSO) at increasing concentrations for 24 hours. Cells were then processed for immunofluorescence labeling with anti-STX5, anti-CR3 (5C6) or anti-FcR (2.4G2) antibodies and DAPI staining. White arrows indicate retention of FcR in an internal compartment. Images are representative of a least 3 experiments.

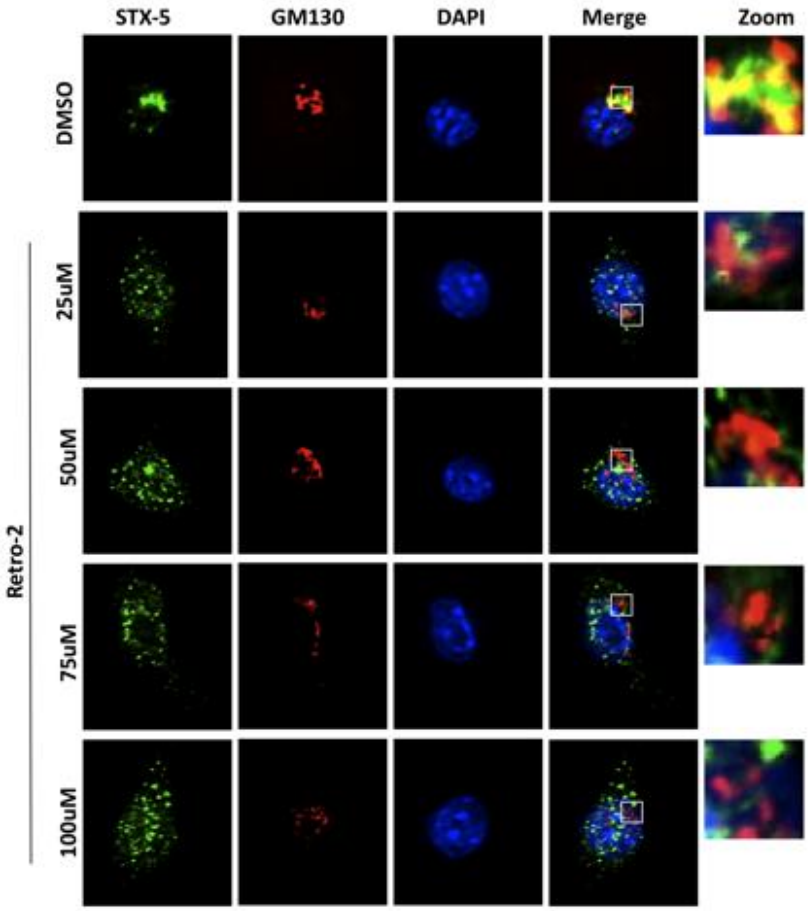

Figure 2-13. Effect of retro-2 on STX5 localization in RAW264.7 cells. RAW264.7 cells were treated with DMSO or retro-2 (dissolved in DMSO) at increasing concentrations.for 24 hours. Cells were then processed for immunofluorescence labeling with anti-STX5, anti-GM130 antibodies and DAPI. White squares indicate the area to be magnified. A yellow signal indicates colocalization of STX5 and GM130. Images are representative of at least 3 experiments.

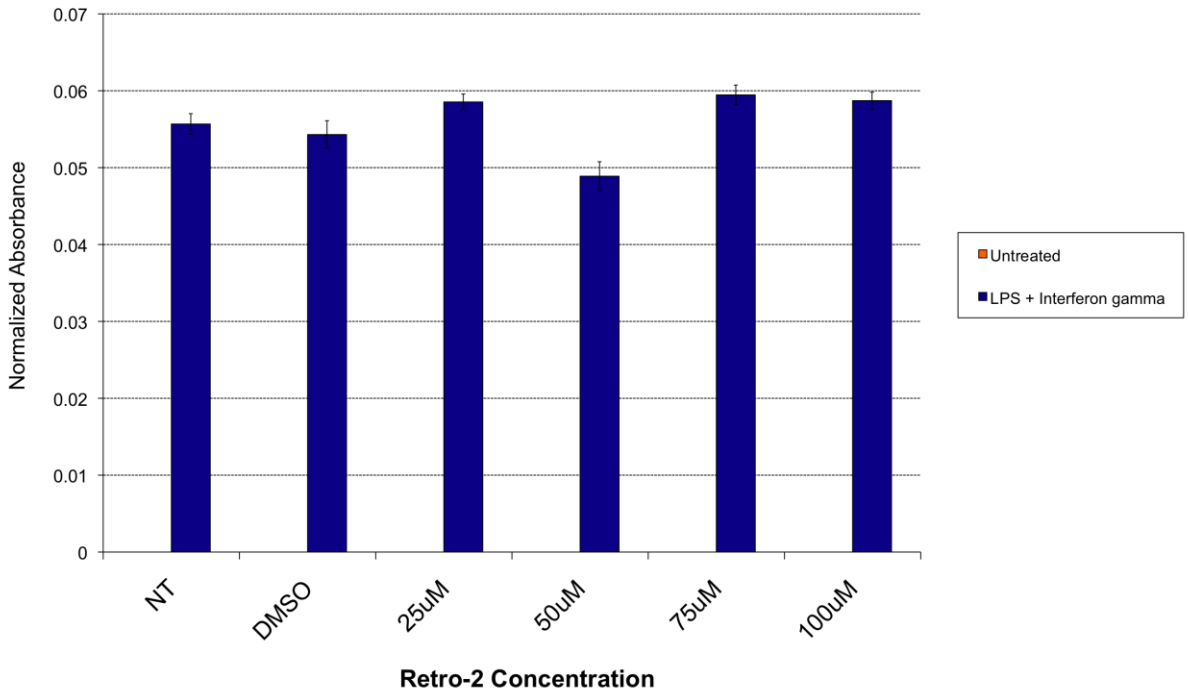

Figure 2-14. Secretion of IL-6 is not affected by retro-2 treatment. RAW264.7 cells were treated with DMSO or retro-2 (dissolved in DMSO) at increasing concentrations for 2 hours. Cells were then activated with LPS/IFNγ for 24 hours. Relative amounts of IL-6 in the supernatant were measured by ELISA. Data is representative of at least 3 experiments.

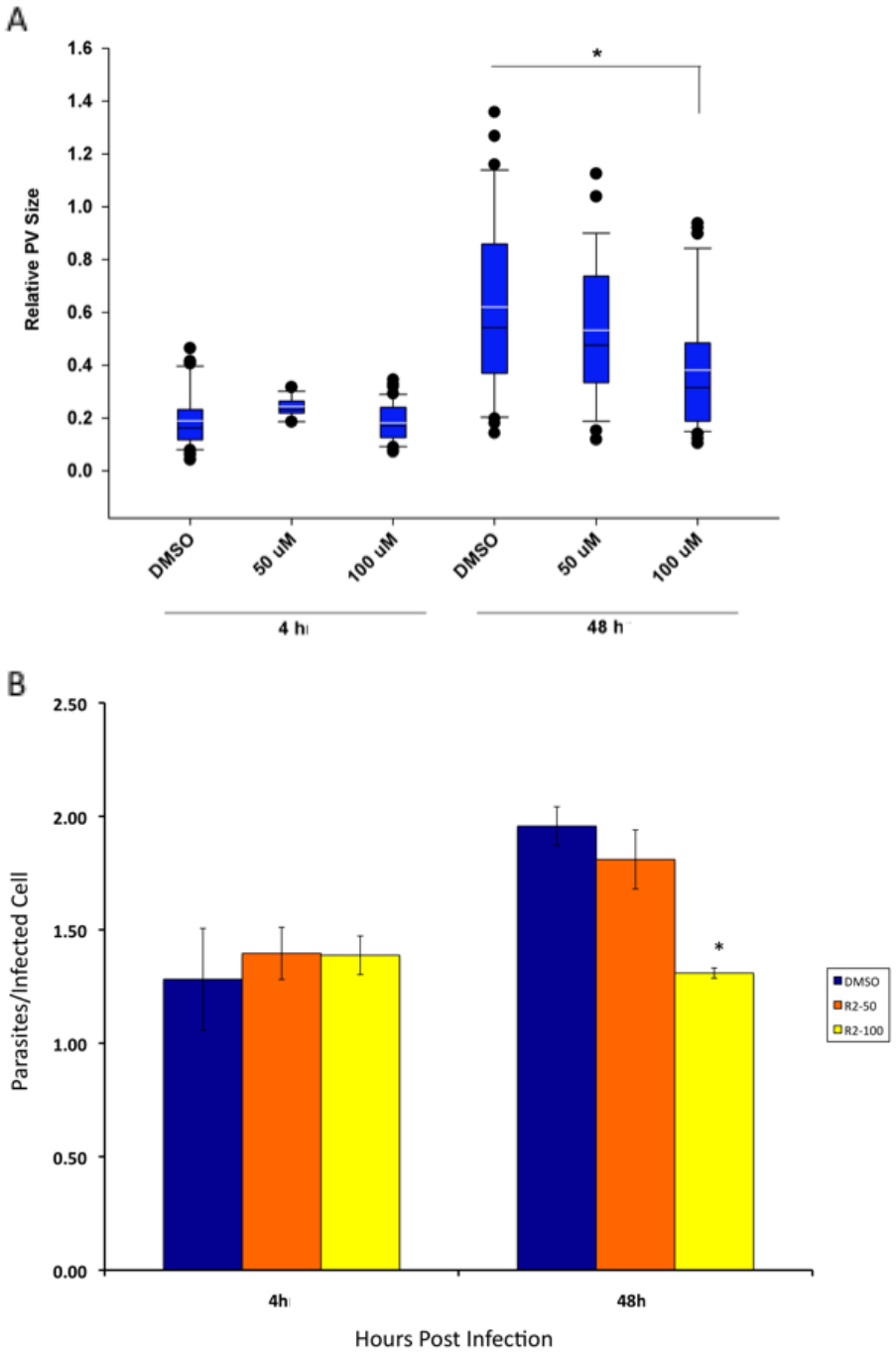

Figure 2-15. Retro-2 treatment of RAW264.7 cells results in reduced PV size and parasite replication. RAW264.7 cells were infected for 2 hours followed by treatment with DMSO or retro-2 (dissolved in DMSO) at increasing concentrations. Infections were stopped at 4 and 48 hours post infection and processed for immunofluorescence labeling with anti-LAMP1 antibody and DAPI. Relative PV size was determined and is shown in Panel (A). White lines indicate mean PV size and black lines indicate median PV size. Panel (B) shows the number of parasites per infected cell at the indicated time points. The (*) indicates significance as determined by a p value of less than 0.05. Data is representative of at least 3 experiments.

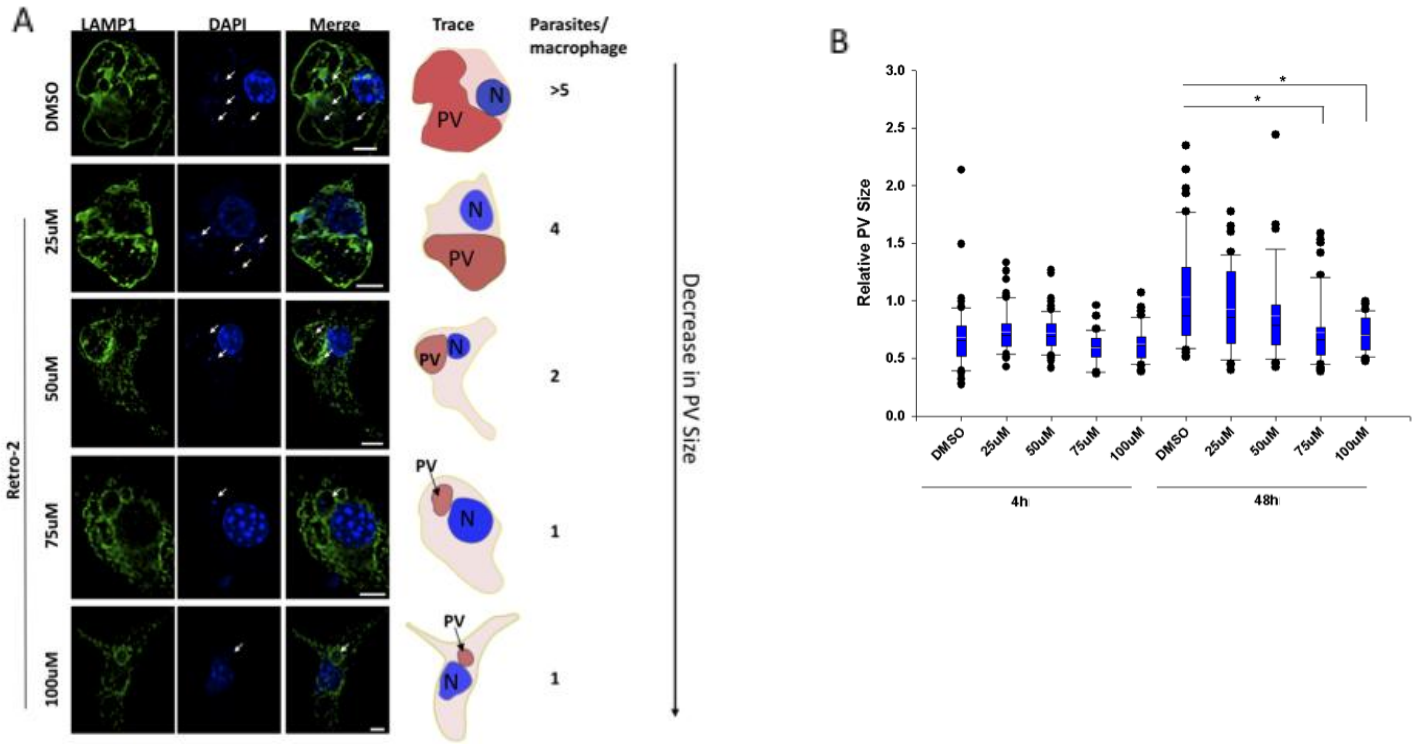

Figure 2-16. Retro-2 treatment blocks PV distention in primary macrophages. PECs were infected with *Leishmania amazonensis* for 2 hours. Cells were washed and then treated with DMSO or retro-2 (dissolved in DMSO) at increasing concentrations. Infections were stopped at 4 and 48 hours post-infection and processed for immunolabeling with anti-LAMP1 antibodies and DAPI. In (A) representative images of infected cells at 48 hours show the effect of retro-2 on PV size. Traces are used to show the contours of the PV. Panel (B) shows the measurements of PV size relative to the host cell nucleus for each condition. White lines show the mean PV size and black lines show the median PV size. An (*) is used to show significance. A p value of less than 0.05 is considered significant. Data is representative of at least 3 experiments.

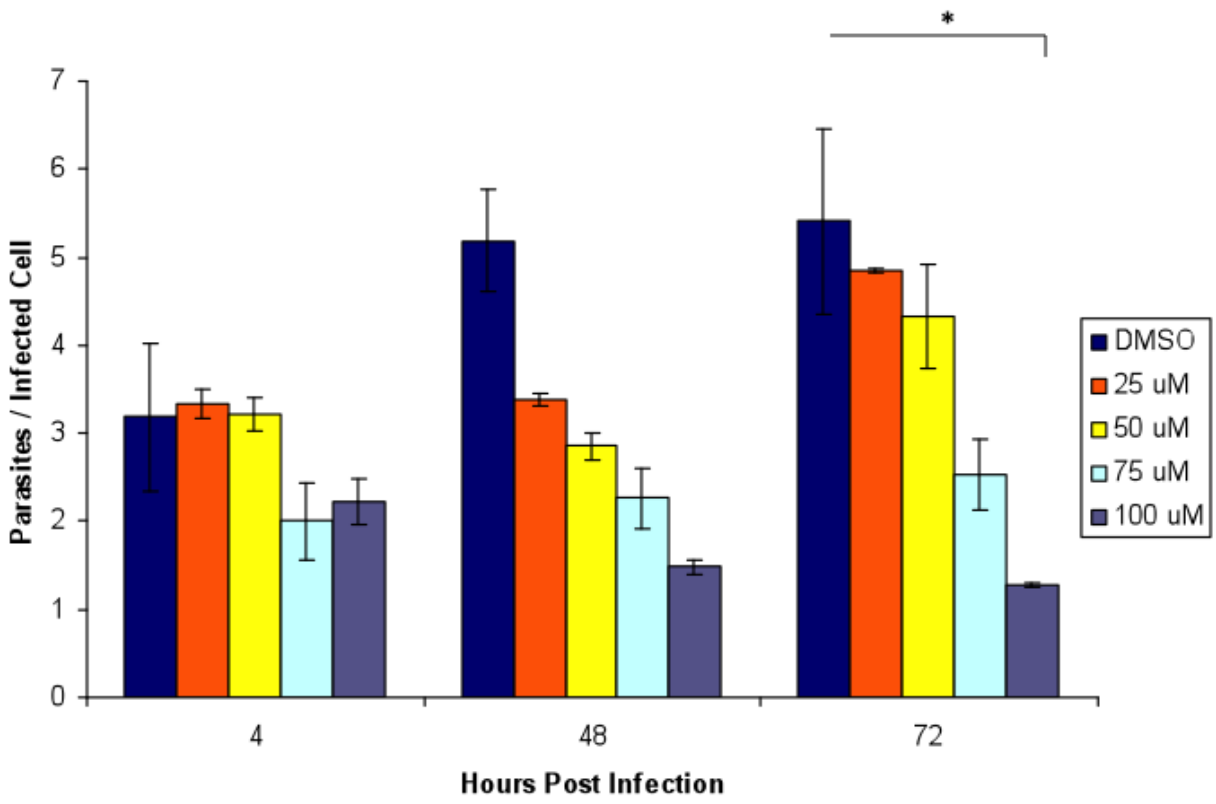

Figure 2-17. Retro-2 treatment inhibits *Leishmania amazonensis* replication in primary macrophages. PECs were infected for 2 hours before DMSO or retro-2 (dissolved in DMSO) was added at increasing concentrations. Infections were stopped at the indicated timepoints and the number of parasites per infected cell was determined. At least 50 infected cells were counted for each condition. An (*) indicated significance as determined by a p value of less than 0.05.

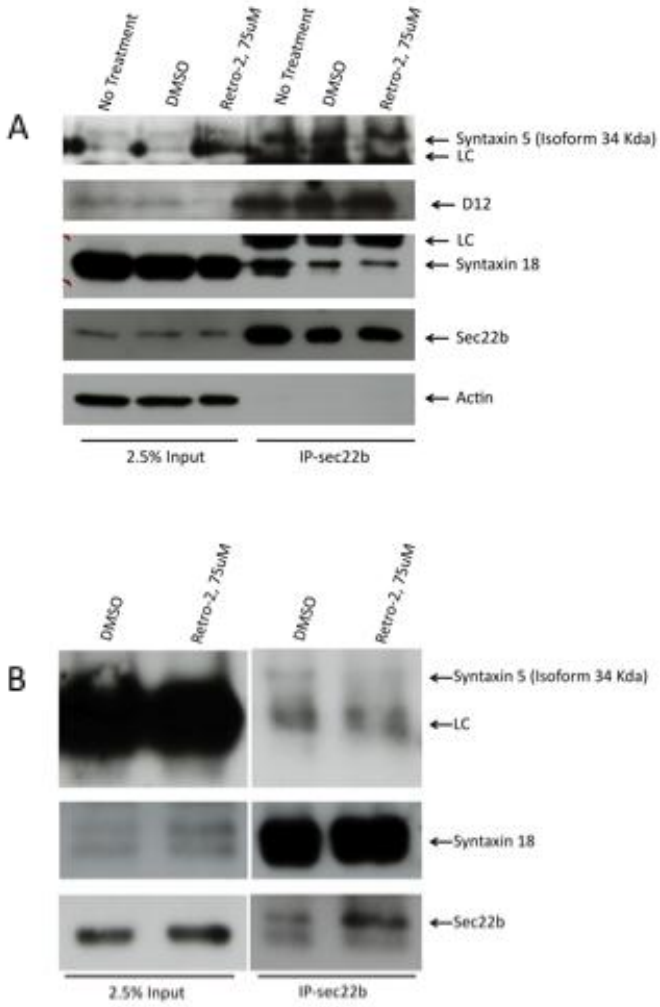

Figure 2-18. STX5 and sec22b do not interact at the PV in retro-2 treated RAW264.7 cells. Cells were infected for 2 hours before treating with retro-2 at 75 μM for 16 hours. Cells were then incubated with n-ethylmaleimide (NEM) at 1 mM for 15 minutes in serum-free media. After NEM incubation, cells were lysed and co-immunoprecipitation using sec22b antibody was performed on (A) whole cell lysate or (B) the enriched PV fraction. Co-immunoprecipitate was run on SDS-PAGE and transferred to PVDF membrane. The blot was probed with antibodies to STX5, D12, STX18, sec22b and actin. The figures above are representative of at least 3 experiments.

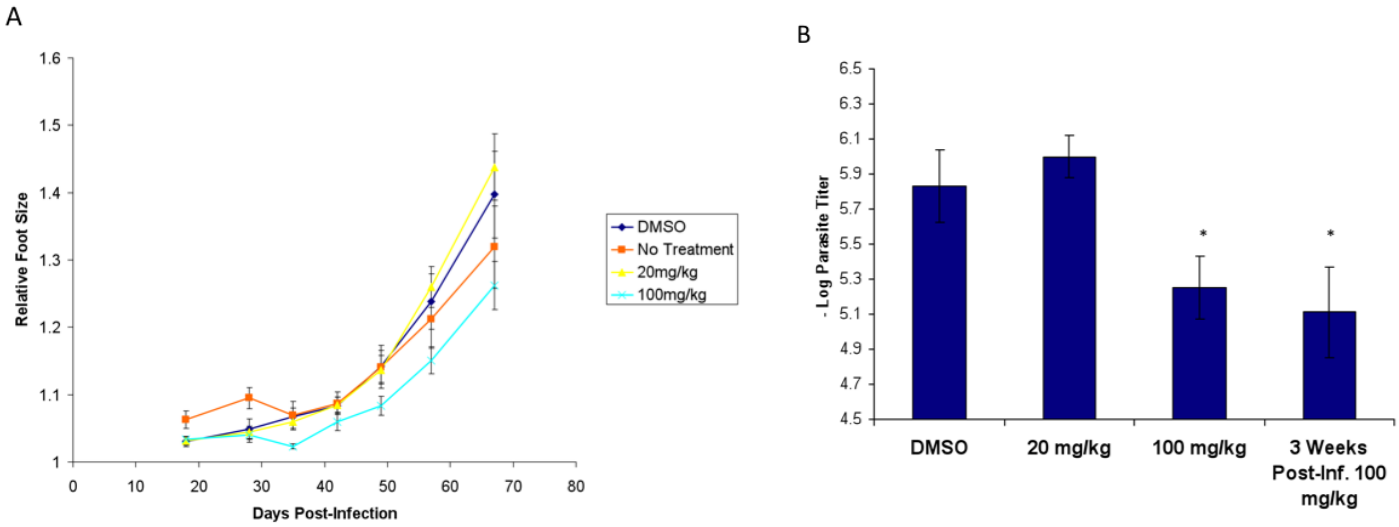

Figure 2-19. Retro-2 limits experimental *Leishmania amazonensis* infection. Balb/c mice received either DMSO control, 20 mg/Kg or 100 mg/Kg of retro-2 (dissolved in DMSO/saline) intra-peritonealy 24 hours after infection with stationary stage promastigotes at the footpad. A separated group of mice received 100 mg/Kg of retro-2 3 weeks post infection. Lesion size was measured at the indicated timepoints post infection and the mean size is plotted in panel (A). After a 9 week infection the parsite titer at the footpad was assessed by limiting dilution assay. The mean parasite titer is shown in panel (B) for each group assessed. Each group consisted of 8-12 mice. Siginificance, as determined by a p-value of less than 0.05, is denoted by an (*).

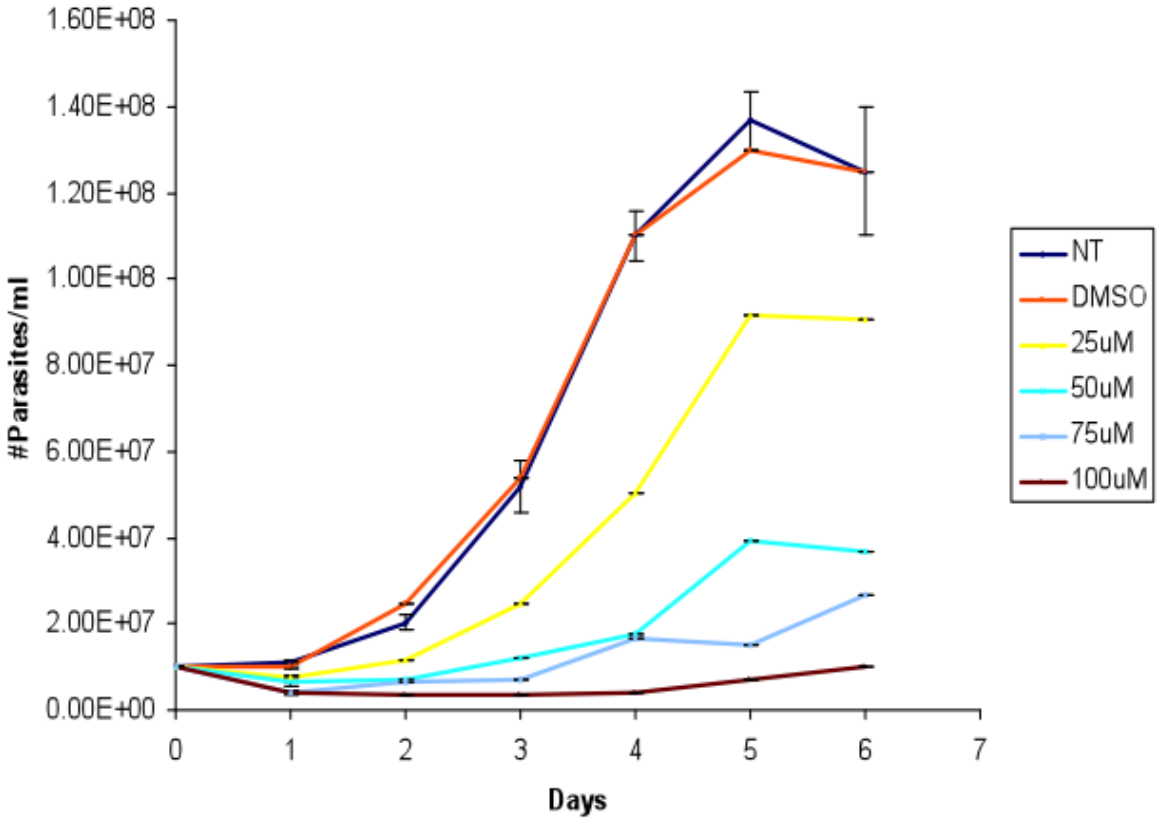

Figure 2-20. Retro-2 inhibits replication of parasites in axenic culture. *Leishmania amazonensis* promastigotes were cultured with the indicated amounts of retro-2 (dissolved in DMSO) in Schneider's medium. NT indicates No Treatment. DMSO alone serves as a control as the retro-2 used was dissolved in DMSO. At the indicated time points, small aliquots of the culture were removed and a parasite count was performed. Cultures were grown in triplicate and the experiment was performed twice.

CHAPTER 4
DISCUSSION

It is generally accepted that the *Leishmania* PV is a fusogenic compartment that interacts with organelles of the endocytic pathway (Antoine et al., 1998b; Courret et al., 2002). The large, communal vacuoles in which *L. amazonensis* resides has made it a model system for studying PV biogenesis and fusion with host cell compartments. Indeed, it has been shown that the *L. amazonensis* PV fuses so extensively with the lysosomal compartment that it is virtually depleted in infected host cells (Alexander and Vickerman, 1975; Barbieri et al., 1985; Barbiéri et al., 1990). More recently, the interaction of *Leishmania* PVs with another host cell compartment, the ER, is beginning to be appreciated. Immunofluorescence and ultrastructural techniques have been used to show the presence of various ER molecules including calnexin, glucose-6-phosphatase and sec22b on the PV (Kima and Dunn, 2005; Gueirard et al., 2008; Ndjamen et al., 2010). In this sense, the PV must be considered a hybrid compartment with a broader range of interactions in host cells.

In the present study, we have extended studies of the ER interaction with the PV. Here, the ER SNARE molecules D12, STX18 and STX5 have been shown to also be present on the PV. Our interest in these molecules began with the finding that sec22b is present on the PV membrane (Ndjamen et al., 2010). Sec22b is a SNARE molecule that functions in membrane fusion events in the ER as well as in the ER Golgi intermediate compartment. Importantly, SNAREs are required for almost all membrane fusion events in eukaryotic cells (Jahn and Scheller, 2006). Therefore, the presence of sec22b on the PV membrane suggests that it may be involved in the interaction between the ER and the PV. This is supported by studies of the *Legionella* containing

vacuole (LCV). The LCV, which is an ER-derived vacuole, also recruits sec22b (Kagan et al., 2004). Moreover it has been shown that by inhibiting sec22b function, the delivery of ER-derived vesicles to the LCV is compromised (Kagan et al., 2004). Also, in a somewhat related system, the overexpression of dominant negative sec22b blocks the delivery of ER components to the membrane of latex-bead phagosomes (Cebrian et al., 2011). The presence of the ER SNARE sec22b, as well as D12, STX18 and STX5, suggest that these SNAREs play a role in the interaction of the ER with the PV.

We chose to target the function of the ER SNAREs found on the PV as a means of assessing the contribution of the ER to PV biogenesis and parasite replication. There are several reports that discuss the effect of targeting ER SNAREs on host cell function. Particularly relevant in the study of ER and ER/Golgi SNAREs is the effect of SNARE function disruption on the secretory pathway. It has been reported that sec22b, STX18 and STX5 are required for constitutive secretion by mammalian cells (Gordon et al., 2010; Okayama et al., 2012). Knockdown or overexpression of wild-type D12, on the other hand, has no effect on constitutive secretion (Okumura et al., 2006). In our system, knockdown using siRNA or overexpression of dominant negative sec22b, D12 and STX18 had no effect on trafficking along the secretory pathway as assessed by the expression of several surface markers and the secretion of IL-6 upon activation with LPS/IFNγ. However, targeting these SNAREs did have a significant effect of PV growth and parasite replication. The observation that the functioning of the secretory pathway was unaffected by targeting these SNAREs is supported by evidence that SNARE redundancy has evolved as a mechanism to ensure the functioning of vital cell functions (Bock et al., 2001). One explanation for why targeting individual ER SNAREs affects

PV biology but has a minimal effect on host cell function could be the upstream events required for membrane fusion. The partnering of SNAREs is one of the last events required for membrane fusion, the successful recruitment and tethering of vesicles/membranes is required to occur first (Jahn and Scheller, 2006). In a cell in which a single SNARE has been knocked down, the availability of redundant SNAREs is dependent on the localization of the SNAREs. Indeed, in the yeast system, inhibiting the ER-localized SNARE sec22p (sec22b is a sec22p homolog) has been shown to be compensated for by the upregulation of another SNARE, Yktp6, which is located further up in the secretory pathway (Liu and Barlowe, 2002). Therefore, in noncognate interactions such as the interaction ER SNAREs at the PV membrane, the spatial localization of SNAREs may limit the availability of redundant SNAREs. In addition, in other SNARE knockdown studies it has been shown that a very low residual expression level (approximately 10%) is sufficient to drive SNARE-mediated fusion (Bethani et al., 2009). Also of interest in the study from Bethani et al. is the observation that knockdown of SNAREs is accompanied by enhanced vesicle docking, suggesting that knockdown can be compensated for by enhanced docking (Bethani et al., 2009). In this sense, noncognate interactions such as vesicle docking at the PV may be more susceptible to SNARE knockdown; in that, the docking machinery may not be sufficient to compensate for SNARE knockdown. The implication is that SNAREs mediating secondary processes, such as the development of a pathogen-containing vacuole, can be targeted without affecting primary processes, such as constitutive secretion.

We have shown that the perturbation of at least one SNARE that functions in vesicle transport in the ER Golgi intermediate region, STX5, results in the control of *L.*

amazonensis in both an *in vitro* and *in vivo* setting. In a study seeking to identify molecules that could mitigate the toxic effects of ricin, Stechmann et al. identified a small molecule inhibitor of STX5 (Stechmann et al., 2010). In a recent study it was shown that ricin traffics to the *L. amazonensis* PV in a Brefeldin A-sensitive manner (Ndjamen et al., 2010); moreover, our studies presented here show that STX5 knockdown adversely affects PV growth and parasite replication. Therefore, we chose to study the effect of retro-2 on PV size and parasite replication. Interestingly, retro-2 was shown to inhibit the interaction of STX5 and sec22b at the PV membrane, while having no apparent effect on their interaction globally. In addition, although secretory pathway function, as assessed by surface marker localization and secretion of IL-6, was not affected, PV size and parasite replication were affected. These observations support the discussion above that secondary processes, such as PV development, are more susceptible to SNARE perturbation than primary processes. In *in vivo* infections, we showed a significant decrease in lesion size as well as parasite titer at the site of infection, without any apparent toxicity to the mouse, after a single dose of retro-2. These results imply that targeting SNARE function may have potential practical applications in the control of pathogens residing in membrane bound compartments. Indeed, as mentioned earlier, blocking sec22b function adversely affects the intracellular replication of *Legionella* that reside in a similar membrane-bound compartment.

Unexpectedly, STX5 also had an effect on the axenic growth of *L. amazonensis* parasites. *Leishmania* parasites do have SNAREs, including a STX5 orthologue (Besteiro et al., 2006). However, the SNARE repertoire was far smaller than in

mammalian cells implying that *Leishmania* parasites would be more susceptible to SNARE perturbation. Indeed, our observations were that retro-2 had no apparent toxic effect on RAW264.7 cells, PECs or in the mouse model; however, it did inhibit the axenic growth of *L. amazonensis* parasites. In this sense, targeting of single SNAREs may provide a strategy for controlling *Leishmania* infection.

Althogether, the growing evidence for a *Leishmania* PV interaction with the host cell ER; as well as, the work presented here places *Leishmania* parasites in a unique subset of intracellular pathogens – pathogens residing in ER-derived organelles. Several pathogens, including *Brucella, Legionella, Chlamydia* and *Toxoplasma* have been shown to reside in membrane bound compartments that interact with the ER. Moreover, the interaction is required for the establishment of a replicative niche. *Brucella*, for example, requires an interaction with ER exit sites (ERES) for the establishment of a replicative niche. Interestingly, *Brucella*, like *Leishmania*, resides in a compartment that interacts with both the endocytic pathway, including lysosomes, and the ER (Starr et al., 2008). *Legionella* containing vacuoles also share some features with *Leishmania* PVs in that the ER SNARE sec22b is displayed on the vacuole and is essential for the establishment of a replicative organelle (Kagan et al., 2004). Although *Brucella* seems to acquire its ER contribution from the ERES and COPII machinery (Celli et al., 2005), *Legionella* acquires its ER contribution from further up the secretory pathway and requires the COPI machinery (Kagan and Roy, 2002). Where exactly the *Leishmania* PV acquires its ER contribution from is unknown and will be interesting to learn. Other intracellular organisms have been shown to secrete effectors that promote an interaction of the pathogen-containing compartment with other host cell

compartments such as the ER. For example, *Legionella* has been shown to secrete the effector DrrA which has been shown to facilitate the tethering of ER-derived vesicles with the *Legionella* containing vacuole membrane (Arasaki et al., 2012). Whether or not *Leishmania* too promotes ER-PV interactions via effector(s) will also be interesting to learn. Indeed, the *Leishmania* molecule lipophosphglycan (LPG) has been shown to limit PV interaction with endocytic vesicles, while allowing for interactions with the PV – the mechanism is unclear (Gueirard et al., 2008). In light of these observations, targeting the ER-Pathogen containing vacuole interaction seems to be a strategy that may be employed for the control of this unique subset of pathogens.

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BIOGRAPHICAL SKETCH

Johnathan Anias Canton was born in Belize City, Belize in 1986 and grew up in the village of Boston in rural Belize. After completing his 6th form studies at St. John's College in Belize City, he attended the University of Florida and completed a Bachelor of Science degree in microbiology and cell science in the spring of 2008. Fascinated by the elegant interactions between pathogens and their host, he received a Ph.D. from the University of Florida in the summer of 2012 with a focus on the intracellular life of *Leishmania* parasites.