YALE JOURNAL OF BIOLOGY AND MEDICINE 92 (2019), pp.511-521.

REVIEW

YALE JOURNAL OF BIOLOGY AND MEDICINE
YJBM
CELEBRATING 90 YEARS 1928–2018

The *Leishmania* Parasitophorous Vacuole Membrane at the Parasite-Host Interface

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The *Leishmania* parasitophorous vacuole membrane (LPVM†) sits at the interface of the parasite and its host. Evidence shows that molecules from the endocytic pathway as well as molecules from the secretory pathway are localized in the LPV and displayed on LPVM. In the review, we discuss our current understanding of the composition of the LPVM.

INTRODUCTION

Leishmaniasis is a neglected tropical disease caused by protozoan parasites of the genus *Leishmania*. Manifestations of leishmaniasis range from self-limited, singular cutaneous lesions to persistent, disseminated, or mucocutaneous lesions, or visceral disease, the latter of which includes infection of the spleen, bone marrow, and liver and is fatal if left untreated. The World Health Organization has estimated that 350 million people are at risk of leishmaniasis worldwide, and approximately 1.3 million new cases occur each year, with up to 30,000 deaths [1,2]. *Leishmania* parasites exhibit a dimorphic life cycle, existing as flagellated promastigotes in their sand fly vector, transforming into their amastigote form upon infection of mammalian hosts. When an infected sand fly takes a blood meal, infectious metacyclic promastigotes are injected into the host, where parasites are taken up by professional phagocytic cells of the host's immune system, including neutrophils and macrophages. Phagocytosed parasites resist degradation within these cells and establish themselves within compartments known as *Leishmania* parasitophorous vacuoles (LPVs) [3,4]. The LPV is an intracellular compartment that is derived in part from the host cell endocytic pathway. The LPV membrane (LPVM) represents a crucial component for establishing and maintaining infection, as it exists at the interface between host and pathogen. The display of molecules derived from the host's secretory pathway on the LPVM has led to the proposition that the LPV is a hybrid compartment, comprised of elements from at least two pathways within the host cell [5,6]. It is well established

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†Abbreviations: LPV, *Leishmania* parasitophorous vacuole; LPVM, *Leishmania* parasitophorous vacuole membrane; ER, endoplasmic reticulum; EE, early endosome; LE, late endosome; MVB, multivesicular body; PI4P, phosphoinositol-4-phosphate; $PI_{3,4}P_2$, phosphoinositol-3,4-bisphosphate; $PI_{3,4,5}P_3$, phosphoinositol-3,4,5-trisphosphate; LFR1, *Leishmania* ferric reductase 1; LIT1, *Leishmania* ferrous importer 1; LAMP1/2, lysosomal-associated membrane proteins 1 & 2; EEA1, early endosome antigen 1; Nramp1, natural resistance-associated macrophage protein 1; SLC38A9, arginine transporter; CD36, scavenger receptor; AAP3, *Leishmania* high-affinity arginine transporter; NOX2, membrane-bound catalytic subunit of NADPH oxidase complex; VAMP8, vesicle-associated membrane protein 8; SNARE, soluble, NSF-sensitive attachment receptor protein; Arg, arginine.

Keywords: organelles

that parasites of the *L. mexicana* complex reside in large, communal vacuoles that can take up a significant portion of the host cell's internal volume; whereas, other species, such as *L. donovani* and *L. infantum*, reside and segregate into individual vacuoles following parasite division. In addition to those morphological differences between LPVs of different *Leishmania* species, some temporal differences in the interactions of *Leishmania* species and their host cells have been reviewed in several excellent reviews [3,4].

Not unlike other intracellular pathogens that reside in membrane-enclosed compartments, *Leishmania* face challenges that include nutrient acquisition, deleterious host cell activation, and host cell apoptosis that can compromise their survival. Although much has been learned about the activities within LPVs, much more is still unknown about the molecular composition of its limiting membrane, the LPVM. Our goal here is to review what is known about the composition of the LPVM. Based on published studies of the activities associated with LPVs, we will sometimes infer conclusions about the composition of the LPVM. Greater understanding of LPV biology will increase our insight into how these parasites hijack normal host processes to survive.

THE MOLECULAR COMPOSITION OF THE LPVM

LPVs are dynamic compartments that undergo maturation while engaging in selective interactions with endocytic organelles resulting in transient displays of molecules on the LPVM. This transiency explains in part the observations that molecules that are primarily associated with early compartments of the endocytic pathway, such as early endosome antigen 1 (EEA1), Rab5, and transferrin receptor, have been found to be displayed on maturing LPVMs [7]. Soon after internalization of *Leishmania* parasites and the subsequent maturation of LPVs, these compartments become inaccessible to most particles that are fed to the macrophages, which is evidence of their evolving molecular composition.

The characteristics of the LPV and its LPVM have long been studied. This review focuses primarily on the contributions of the host cell to the LPV, since very little is known regarding the identity and characteristics of specific parasite molecules that are secreted into the LPV and that are displayed on the LPVM. Doubtless, *Leishmania* parasites release parasite-derived molecules that affect normal host cell functions. Work by Silverman and colleagues demonstrated that parasite-derived proteins are distributed throughout the host cell [8]. However, much more remains to be discovered on the parasite's molecular contributions to the complex host-pathogen interactions.

Earlier reviews provided many details of the characteristics of the LPV and of the composition of the LPVM [7,9,10]. Briefly, LPVs were shown to exhibit many of the characteristics of phagolysosomes that maintain extensive interactions with organelles of the endocytic pathway. Consequently, molecules derived from the endocytic pathway were found to be displayed on the LPVM. These molecules include the lysosome-associated membrane proteins 1 & 2 (LAMP1 & LAMP2), and the proton pump v-H^+ ATPase [9]. A selection of other molecules that have been demonstrated to be present within the lumen of the LPV or on the LPVM include MHC class II, the $gp91^{phox}$ subunit of the NADPH oxidase complex, macrosialin, and the cathepsin proteases B, D, H, and L, and a variety of other hydrolases of lysosomal origin [11,12].

Correspondingly, the molecular components of the vesicle fusion machinery that mediate fusion of endocytic vesicles, including members of the Rab GTPase family and soluble NSF attachment protein receptor (SNARE) proteins, have been shown to be displayed on the LPVM (Figure 1) [7,11,13]. VAMP7 is one example of a SNARE that might interact with the LPV. In previous studies, VAMP7 was shown to be actively recruited to phagosomes harboring *Coxiella burnetii*, an obligate intracellular bacterial pathogen [14]. *C. burnetii* has long been known to inhabit a similar intracellular niche as *Leishmania* parasites, even living in cohabitation within the LPV [15-17]. Since these two pathogens exist in similar compartments within host cells, an overlap may exist in what vesicular fusion machinery can be found on the membranes of these compartment, including VAMP7. VAMP8 is one host SNARE that has been shown to be excluded from LPVs, due in part to the action of GP63, a parasite-derived metalloprotease, that directly cleaves VAMP8 [18]. This cleavage also results in preventing the complete assembly of the NADPH oxidase complex on the LPVM.

In addition to molecules from the endocytic pathway, host molecules from the secretory pathway have also been shown to appear in the LPV. Calnexin, a transmembrane ER-protein folding chaperone, was shown to be recruited to the LPVs of *L. donovani* and *L. pifanoi* as early as minutes post-infection and was shown to remain at the LPVM through at least 24 hours. Additionally, SEC22b was shown to be recruited very early in infections with both *L. donovani* and *L. pifanoi*, and likewise was shown to be retained at the LPVM throughout infection [5]. SEC22b is an R-SNARE that is normally found on vesicles trafficking between the ER and Golgi [19]. Since SNAREs are the machinery by which membrane fusion is facilitated, the presence of SEC22b and other SNAREs could provide insight into the origin of vesicles that are recruited by *Leishmania* to the LPV. Interactions with host secretory pathway components has been observed in other intracellular parasitic infections.

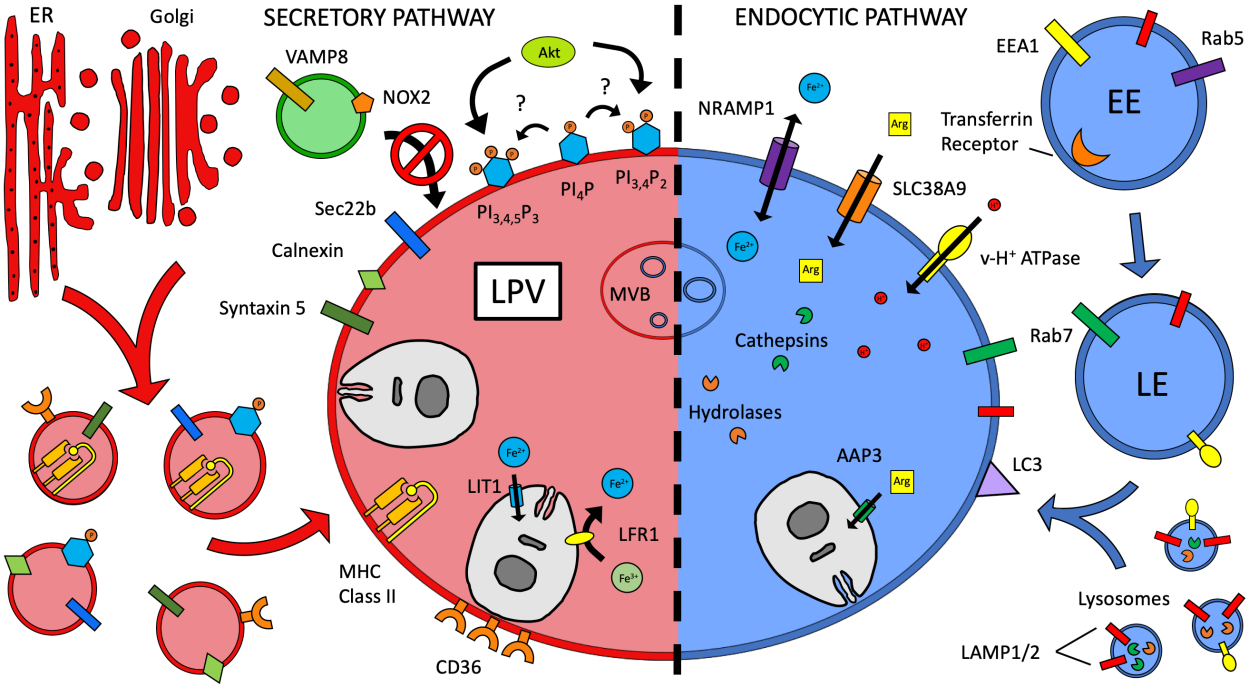

Figure 1. **Model of the composition of the *Leishmania* Parasitophorous Vacuole membrane**. Shown here is a representative image of an LPV formed by *L. amazonensis*, a member of the *L. mexicana* complex. Like phagolysosomes, the LPV engages in numerous interactions with endocytic pathway compartments leading to the display of endocytic pathway molecules on the LPVM. Some of the molecules whose presence on the LPVM has been confirmed include LAMPs1/2, Rab7, Nramp1, v-H+ ATPase and SLC38A9. Evidence also exists that secretory pathway molecules are diverted to the LPVM. Some of the molecules from the secretory pathway include calnexin, SEC22b, syntaxin 5, CD36, immature MHC class II molecules, and the phosphoinositide PI4P. In addition to molecules in the LPV lumen derived from the endocytic and secretory pathways, the LPV also contains multivesicular/membranous structures whose origin is presently not known. Some molecules such as VAMP8 have been shown to be excluded from the LPV at least in part, through the actions of parasite-derived molecules. PI4P has been confirmed to be displayed on the LPVM, while the display of other phosphoinositides is currently under investigation.

Abbreviations: LPV, *Leishmania* parasitophorous vacuole; LPVM, *Leishmania* parasitophorous vacuole membrane; ER, endoplasmic reticulum; EE, early endosome; LE, late endosome; MVB, multivesicular body; PI4P, phosphoinositol-4-phosphate; $PI_{3,4}P_2$, phosphoinositol-3,4-bisphosphate; $PI_{3,4,5}P_3$, phosphoinositol-3,4,5-trisphosphate; LFR1, *Leishmania* ferric reductase 1; LIT1, *Leishmania* ferrous importer 1; LAMP1/2, lysosomal-associated membrane proteins 1 & 2; EEA1, early endosome antigen 1; Nramp1, natural resistance-associated macrophage protein 1; SLC38A9, arginine transporter; CD36, scavenger receptor; AAP3, *Leishmania* high-affinity arginine transporter; NOX2, membrane-bound catalytic subunit of NADPH oxidase complex; VAMP8, vesicle-associated membrane protein 8; SNARE, soluble, NSF-sensitive attachment receptor protein; Arg, arginine.

Brucella, *Legionella*, and *Chlamydia* are three bacterial pathogens that reside within host cells that have all been shown to interact with and disrupt their respective host cell secretory pathways. *Toxoplasma gondii*, an obligate, intracellular apicomplexan parasite, has also been shown to subvert host cell secretory pathway components during the course of infection [20-23].

NUTRIENT ACQUISITION AND LPVM CHARACTERISTICS

The nutritional preferences of intracellular amastigotes are distinguishable from those of extracellular promastigotes. To survive within host cells, *Leishmania* parasites that are sequestered in LPVs must be able to appropriate nutrients required for growth, survival, and replication. Like phagosomes, LPVs are accessible to endocytic pathway compartments that presumably give them access to a variety of carbon sources including sugars, amino acids, and lipids. The studies of McConville and colleagues have shown that not only do intracellular

amastigotes prefer sugars as a primary carbon source, but they can also slow down their metabolism when growing in an infected host [24,25]. Intracellular amastigotes are dependent on sugars as a major carbon source *in vivo* but are also able to utilize other nutrients taken from the host for survival. Furthermore, intracellular survival of *L. major* is dependent on host cells endocytosing and degrading glycosaminoglycans [25]. Zivanovic and colleagues investigated the molecular composition of LPVs using Surface-Enhanced Raman Scattering (SERS) as a novel approach to examine the composition of the LPV from a molecular standpoint. As might be expected, various proteins, carbohydrates, and peptides were identified from spectra. Interestingly, they found a strong contribution from a variety of lipids, including various sterols and phosphoinositides, indicating a high abundance of these molecules in LPVs [26].

Arginine salvage within LPVs is another crucial activity for amastigote survival. *Leishmania* parasites are dependent on arginine for a plethora of cellular activities, notably protein synthesis and the synthesis of polyamines and other metabolic precursor molecules. Upon infection, *Leishmania* induce an arginine deprivation response (ADR) in response to the limited availability of arginine within host cells [27]. An important difference arises between *Leishmania* and host cells in that arginine is considered non-essential for host cells due to their ability to break down citrulline into arginine [28]. In contrast, arginine is an essential, required nutrient for *Leishmania* parasites. Sensing the deprivation of arginine triggers an upregulation of a high-affinity arginine transporter, AAP3, by the amastigotes, which is able to provide enough arginine to satisfy the parasites' requirement. Depletion of arginine within the host cell alters normal host metabolic and signaling responses, in a manner beneficial to *Leishmania* parasites [27]. Classically activated pro-inflammatory macrophages express high levels of inducible nitric oxide synthase (iNOS), which then utilizes arginine in the cytosol to generate NO [28]. Alternatively activated anti-inflammatory macrophages express high levels of arginase and trigger a net influx of arginine into the cell. Two important host molecules that regulate arginine availability within the cell are the cationic amino acid transporter (CAT1) and SLC38A9, an arginine/glutamine transporter. CAT1 works to import arginine from the extracellular milieu to the cytosol, and SLC38A9 is a transporter found on the membranes of phagosomes, and therefore also on LPVMs [29]. Host cells respond to low levels of arginine by upregulating the expression of CAT1, in a similar manner to which *L. donovani* upregulates AAP3 [30]. CAT1 imports arginine into the host cell, and SLC38A9 transports arginine into the lumen of the LPV, where *Leishmania* parasites, through AAP3, are able to utilize arginine for their metabolism. Because of this, AAP3 plays an important role in acquisition of arginine for parasites within the LPV.

Additionally, arginase activity within host cells has been associated with parasite load at infection sites. Arginase breaks down arginine into polyamines, and inhibition of arginase resulted in reduced pathology and increased control of parasitic infection *in vivo* [31]. This link between host activity regarding arginine import and metabolism illustrates a key pathway that *Leishmania* parasites exploit in order to acquire essential nutrients. Triggering a depletion of arginine within host cells alters the activation state of macrophages, decreases NO production at the LPVM, and upregulates the amastigote protein AAP3, which is capable of providing enough arginine for parasite needs within the LPV.

Iron is another essential nutrient for *Leishmania* parasites. Iron availability has been implicated in the transformation of promastigotes into amastigotes [32]; therefore, both the sources and the regulation of iron within infected host cells are important in understanding how *Leishmania* species establish and maintain themselves during an infection. Promastigotes have been shown to be induced to transform into an amastigote-like form in culture, based on changes in pH and temperature, to replicate the conditions found within LPVs in *in vivo* infections [33-35] . Recently, iron uptake has also been associated with inducing transformation of promastigotes into amastigote-like forms, independent from changes in temperature and pH [32]. The requirement of iron for the transformation of promastigotes into amastigotes highlights a crucial difference between the two forms of the *Leishmania* life cycle. This iron-dependent transformation seems to involve production of reactive oxygen species (ROS), which are usually thought to be deleterious to invading microbes. *Leishmania* parasites therefore must also be able to regulate the fine balance between iron availability and the host's generation of ROS. Increased ROS production may also lead to a different signaling cascade within the parasites that results in a change in gene transcription, causing the transformation from promastigote to amastigote [32].

Leishmania parasites exploit a variety of host molecules to increase the host's intracellular iron supply, and subsequently to hijack this supply of iron to satisfy nutritional requirements. When macrophages are infected with *L. amazonensis*, host expression of ferroportin (Fpn1) is inhibited in a TLR-4–dependent manner. Ferroportin is currently the only known cellular iron exporter expressed in mammalian systems and is an important player in regulating iron homeostasis. By limiting expression of ferroportin, *L. amazonensis* limits release of intracellular iron pools from its host cell [36]. In addition, hepcidin (Hamp) expression is upregulated in a TLR-4–dependent manner. Hepcidin is a peptide hormone that also regu-

lates iron export in macrophages by inducing degradation of ferroportin [37]. By both limiting host expression of ferroportin and by increasing its degradation, *L. amazonensis* establishes an intracellular environment where iron is retained and accumulated. Because iron availability is required for establishing and maintaining an infection, the mature LPVM must contain certain molecules responsible for iron transport.

One host molecule implicated in iron movement within the cell that localizes to the LPVM is Nramp1 (natural resistance associated with macrophage protein 1). Nramp1 has been suggested to belong to a class of molecules that confer nutritional immunity to mammalian cells [38]. The functions of Nramp1 has been obtained from studies of its role in iron mobilization in *Leishmania* and other intracellular infections including *Mycobacterium* and *Salmonella* [39]. Contradictory studies have shown that Nramp1 can pump iron both from the lumen of phagosomal compartments out into the cytosol, and from the cytosol into the phagosomal lumen [40,41]. In addition, at least one study has also indicated that the direction of iron pumping might be pH dependent [42]. Although the Nramp1 mechanism has been controversial, these studies agree that Nramp1 localizes to the LPVM or phagosomal membrane. The functions of Nramp1 can be limited by mutations in the Nramp1 gene. This conclusion was elegantly shown in studies of the Nramp1 gene in the widely used mouse RAW264.7 macrophage that expresses $Nramp1^{D169}$, a mutant allele of Nramp1 that is non-functional. Transfection of $Nramp1^{G169}$ resulted in greater accumulation of iron within phagosomes that harbored *Mycobacteria* [43]. Interestingly, a secretory peroxidase has been identified in *L. donovani*, which has been shown to down regulate Nramp1 in host cells, in addition to playing a role in limiting the levels of ROS species in activated macrophages [44]. By controlling and limiting the expression of Nramp1, *Leishmania* parasites can further control access to their host cell iron supply.

While the generation of an intracellular environment with an increased amount of iron is beneficial to establishing *Leishmania* infections, the parasites must still be able to both bring iron to the LPV and successfully transport it across the LPVM to make it available for the growing parasites. The last step in the procurement of iron for growing amastigotes within the LPV is the ability of the parasites themselves to uptake iron from their intraluminal microenvironment. The two molecules LFR1 and LIT1 are known to be expressed by the parasites themselves [45]. LFR1 is responsible for the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), while LIT1 is responsible for the import of ferrous ions from the LPV lumen into the parasite, where the iron is then available for usage [32,46]. Cumulatively, *Leishmania* species require iron for different cellular functions, including differentiation into amastigote forms. To do this, parasites increase iron import into host cells, while decreasing iron export, which results in a higher amount of available iron to the parasites within LPVs.

LIPIDS AND THE LPVM

Cholesterol is one important lipid that is found in lipid rafts on host cell plasma membranes. Caveolae, a subset of cholesterol-rich membrane lipid rafts, have been implicated in the uptake and survival of promastigotes within macrophages, highlighting one way that cholesterol and other lipids are trafficked to the LPV. However, an important difference between promastigotes and amastigotes of *L. chagasi* was observed regarding uptake through lipid rafts. A transient disruption of lipid rafts in host cell membranes affected promastigote uptake, but not amastigote uptake by macrophages. This finding is indicative of a difference in needs of *Leishmania* parasites regarding both the availability and origin of cholesterol during an infection [47]. Interestingly, fluorescently tagged cholesterol on the plasma membrane of host cells was found incorporated into the parasites' membranes, representing an important role that cholesterol hijacking and sequestration may be playing in *Leishmania* infections. Free cholesterol accumulates around LPVs, and this sequestration of cholesterol was significantly correlated with increased expression of proteins required for cholesterol biosynthesis in host cells, which suggests that *Leishmania* parasites exert a multifaceted control over the production and trafficking of cholesterol within infected cells [48]. What remains to be revealed is a deeper understanding of how and why *Leishmania* parasites control host cholesterol trafficking, and what, if any, parasite effector molecules may be responsible for this appropriation of nutrient availability.

One strategy employed by *Leishmania* to prevent host cell apoptosis, is by engaging sustained Akt activation [49]. This serine-threonine kinase is a downstream molecule in the Phosphatidyl-inositol-3-kinase (PI3K) signaling pathway [50]. Akt is a signaling molecule that is important in regulating the balance between cell survival signals and apoptotic signals. PI3K signaling is initiated in response to several stimuli, which then results in PI3K phosphorylation at the 3-position of the inositol ring of phosphatidyl-inositol lipids. The rate limiting step in the activation of PI3K/Akt signaling is the availability of the phosphoinositide. Once phosphorylation of the proper phosphoinositides occurs, Akt is then able to bind to these lipids, which initiates phosphorylation of Akt. The LPVM was recently shown to display phosphoinositides, specifically PI4P [51]. The current gap in knowledge regarding the display of phosphoinositides on the LPVM is the origin of these lipids as well as the kinases and poten-

tially, the phosphatases that modify them. PI4P can serve as the precursor for $PI_{3,4}P_2$. A significant reservoir of PI4P is the Golgi apparatus, with minor stores in the plasma membrane. While it is possible that some PI4P molecules may become incorporated in the LPVM during phagocytosis of parasites, the continued downstream activation of PI3K/Akt signaling seems to suggest a sustained recruitment of available PI4P to the LPVM. One possibility is that host PI4P in the Golgi is actively transported to the LPV, where it undergoes phosphorylation and conversion to $PI_{3,4}P_2$ (Figure 1). In this scenario, either a host-derived PI3 kinase or a parasite-derived kinase would be expected to perform this function. Neither of these molecules have been shown to be associated with LPVMs.

AUTOPHAGY

Cellular autophagy is a process by which cells regularly degrade and recycle cellular constituents, including proteins, lipids, and other molecules within an intracellular compartment. Several different pathways of autophagy exist, including canonical autophagy, in which a double membrane forms an autophagosome around the matter to be degraded. In this pathway, autophagy-related (Atg) proteins, like LC3, are responsible for assembling the autophagosome, which in turn fuses with lysosomes to facilitate degradation of its contents [52]. In addition to canonical autophagy, a second type of autophagy, also known as xenophagy, occurs in response to recognition of a foreign particle or pathogen by host receptors. This recognition is not limited to just the cytosol; pathogens within intracellular compartments may also be recognized through the binding of host receptor to a molecule of pathogen origin expressed on the vacuole membrane, or in instances where the pathogen's vacuole has been damaged, and therefore exposed to cytosolic components of the host cell. Once the foreign particle has been identified through receptor binding of autophagy machinery, an autophagosome forms, and destruction is likewise mediated through the fusing of lysosomes [53].

Leishmania species have long been known to exploit host cell autophagy as a means of stealing nutrients required for survival [10]. LPVs of *L. mexicana* acquire nutrients through at least two pathways – directly from the cytosol of their host cell or through the fusing of autophagosomes. Lucifer Yellow, a small, anionic molecule, was shown to be directly imported from the host cytosol into the lumen of the LPV, which implies that the activity of one or more anion transporters working on the LPVM. In addition to the more direct provision of small ionic molecules via transport from the host cytosol, *L. mexicana* acquires other nutrients from the host in a manner that is diminished by treatment of compounds that affect autophagy [10]. The relationship between parasite and host autophagy pathway has been further characterized and *L. amazonensis* was demonstrated to induce autophagy upon infecting macrophages, both *in vivo* and *in vitro* [54]. Levels of LC3II increased upon infection with *L. amazonensis*, and higher expression of LC3II was further associated with a greater infection index. Furthermore, when autophagy was induced via cellular starvation, the infection index increased when compared to control infections where autophagy in host cells was not induced prior to infection, implying *L. amazonensis* infections are more successful when autophagy increases in host cells [54]. Likewise, an increase in parasite load of *L. amazonensis* infection of BALB/c macrophages was found to be correlated with induction of autophagy of host cells [55], further linking increased host autophagy with more robust *Leishmania* infections.

Both *L. amazonensis* and *L. major* have been shown to induce autophagy in host cells, with both species' LPVMs displaying LC3 during the course of infection [56]. LC3 is an autophagic membrane marker, which is found on compartments within the cell where autophagic degradation takes place. The presence of LC3 on LPVMs seems to indicate a process by which *Leishmania* parasites are hijacking normal host autophagy, in order to acquire vital nutrients that would otherwise be recycled by the host cell. The viability of both *L. amazonensis* and *L. major* within host cells increases when autophagy is induced, and decreases when autophagy is halted, suggesting that internal fitness of these parasites is directly linked with the availability of host nutrients via the autophagy pathway. One difference arises when comparing *L. amazonensis* and *L. major* with regard to hijacking host autophagy. While earlier time points in infection showed similar percentages of $LC3^+$ LPVs of both species, after 24 hours of infection, over 40 percent of *L. amazonensis* LPVs still showed LC3 on the membranes, but only 18 percent of *L. major* LPVs were positive for LC3. If *Leishmania* parasites are exploiting host autophagy to acquire nutrients, then when autophagy is induced in host cells, one might expect the infection burden and parasite viability to increase. Indeed, Dias *et al*. showed that autophagic induction exerted a positive influence on infections, with *L. major* benefitting to a greater extent than *L. amazonensis*. However, the inhibition of host autophagy did not have an effect on infections with either *L. amazonensis* or *L. major*.

A third form of autophagy is known as LC3-associated phagocytosis (LAP). LAP may also be triggered in response to an intracellular infection. LC3 is recruited to foreign particles that are contained within a phagosome or endosome with a single membrane, in a NOX2-dependent manner [57]. NOX2 is the catalytic subunit of the NADPH oxidase complex, responsible for the generation of ROS and is membrane bound. Although sharing some

similarities with canonical autophagy, LAP culminates in LC3 being expressed on the cytosolic side of the single membrane, which has been thought to lead to quicker fusion of lysosomes for degradation [53].

L. major was shown to avoid destruction within host cells by LAP. After internalization of *L. major* promastigotes, recruitment of both NOX2 and LC3 to phagosomes was impaired in a manner dependent on GP63 [58]. This recruitment of NOX2 and subsequent recruitment of LC3 to the nascent phagosome containing *L. major* was shown to be a result of the down-modulation of host VAMP8, an R-SNARE. VAMP8 can be found on membranes throughout the endocytic pathway, as well as the *trans*-Golgi and the plasma membrane [19]. GP63 was shown to cleave and therefore exclude VAMP8 from LPVs [18]; by doing so, *L. major* is able to limit and control the downstream recruitment of both NOX2 and LC3 to avoid degradation associated with LAP as an antimicrobial response by the macrophage [13]. This association, or lack thereof, between the LPVM and VAMP8 represents an important part of the relationship between LPVs and host. *Leishmania* parasites are able to control what host cell components are able to interact with their LPV, and disruption of interactions with host SNAREs is a likely mechanism through which these parasites are able to effect this control over vesicular fusion with the LPV.

Another recent study explored the changing nature of the relationship between *Leishmania* and host autophagy throughout the course of an infection. Like *L. major*, very little recruitment of LC3 to the phagosome occurred at early time points of infection with *L. donovani*. However, by 24 hours, LC3 recruitment to the LPV markedly increased, suggesting an important change during the course of infection, with regard to autophagic induction [59]. GP63 is displayed on the cell surface of *Leishmania* promastigotes but not expressed after differentiation into amastigotes [60], which may explain this shift in LC3 recruitment during the course of infection. Early on, avoiding deleterious effects of the NADPH oxidase complex seems to be of primary importance, while after transformation into amastigotes has occurred, hijacking nutrients from the host autophagy pathway becomes more important. Furthermore, *L. donovani* was shown to actively inhibit autophagy early in infection through the activation of the host PI3K/Akt signaling pathway. mTOR is an important regulator of host autophagy, and is positively regulated by Akt; when activated, mTOR acts to inhibit the autophagy pathway. During *L. donovani* infections, Akt was shown to be continually activated, which indirectly resulted in the active inhibition of host autophagic processes. This inhibition of autophagy through mTOR may be responsible for the lack of LC3 recruitment to the LPV at early time points of infection. Interestingly, *L. donovani* was also shown to induce autophagy at later time points, despite the active inhibition through mTOR signaling, implying that an mTOR-independent process is responsible for this later induction of host autophagy [59].

New studies have shown the potential for an anti-PDL-1 antibody to be used as a therapeutic treatment against *L. donovani* infections. Blocking PDL-1 resulted in an inhibition of autophagy, as measured by impaired recruitment to LPVs of autophagy markers beclin-1 and LC3. Additionally, accumulation of p62, a selective autophagy-associated protein that binds to ubiquitinated proteins and damaged cellular components, was observed. p62 targets these proteins and cellular components to the autophagosome where they are degraded and recycled [61]. Levels of p62 are inversely proportional to levels of autophagic flux; when p62 accumulates, autophagic flux is diminished. These observations due to anti-PDL-1 treatment indicate a potential target to disrupt *Leishmania* interactions with host autophagy.

Taken together, *Leishmania* parasites have a complex and well-regulated relationship with host autophagic processes. Early in infection, autophagy is actively inhibited and avoided, potentially as an evasion mechanism to prevent microbicidal activities from degrading parasites before they establish themselves within the mature LPV environment. Later, after differentiation into the amastigote form within established LPVs, *Leishmania* have altered metabolic needs and induce host autophagy as a means by which to acquire the nutrients necessary for survival and replication. Interestingly, induction of autophagy has also been shown to decrease arginase activity and therefore also the production of NO by host cells. This decrease in NO production is thought to be the primary reason why *L. major* benefitted more than *L. amazonensis* upon autophagic induction of host cells, since *L. major* is more sensitive to the effects of host NO [62].

AVOIDANCE OF REACTIVE OXYGEN

One mechanism of pathogen destruction used by phagocytic cells of the immune system is the assembly of the NADPH oxidase subunits, to form the NADPH oxidase complex on both the plasma membrane, and the membranes of phagosomes. The NADPH oxidase complex catalyzes the production of superoxide radicals, which then kill ingested microorganisms. In *Leishmania* infections, the complete assembly of NADPH oxidase on the LPVM is inhibited, leading to a diminished production of superoxide radicals [63,64]. In *L. pifanoi* (a member of the *L. mexicana* complex) infections, the percentage of LPVs positive for NADPH oxidase complexes was significantly lower in LPVs containing amastigotes than promastigotes. Furthermore, only the 65-kDa premature form of the $gp91^{phox}$ subunit of the NADPH

complex was present on LPVs [65]. This observation was interpreted to mean that as *Leishmania* parasites differentiate, the exclusion of mature NADPH oxidase complex subunits from the LPV increases. Lipophosphoglycan (LPG) is a surface glycolipid that has been shown to play important roles in establishing *Leishmania* infections. Descoteaux and colleagues showed that LPG inhibits the recruitment of both $p47^{phox}$ and $p67^{phox}$ subunits of the NADPH oxidase complex to the LPVM in *L. donovani* infections; however, LPG does not impair the formation of complexes that contain these two subunits [66]. These authors concluded that it is the remodeling of the LPV and selective exclusion of NADPH oxidase subunits from the LPVM that are the crucial elements of the evasion of superoxide destruction, as opposed to a general inhibition of the host cell NADPH oxidase complex formation. Even though NO can exert its effects from a distance, several groups have evaluated the localization of iNOS in *Leishmania*-infected cells. NO can be produced through treatment with TNFα or IFNγ that induce the activation of iNOS and has been shown to be recruited to the membranes of other pathogen-containing compartments [67,68]. Inhibition of iNOS production of NO promotes intracellular growth of *Leishmania* in macrophages [69]. An important difference arises when comparing *Leishmania* species and their ability to resist IFNγ – induced killing by host cells. *L. amazonensis*, residing in larger LPVs harboring multiple parasites, are significantly more resistant to IFNγ – induced killing, when compared to *L. major*, a species that dwells in smaller LPVs containing individual parasites [62].

LPVM CHARACTERISTICS AND POTENTIAL TARGETS FOR CONTROL OF INFECTION

LPVs have been shown to interact with host secretory pathway components, including the ER and vesicles associated with the ER-Golgi intermediate compartment (ERGIC). Calnexin, an important transmembrane chaperone protein with a luminal calcium-binding domain found in the ER, was shown to be recruited to LPVMs of *L. mexicana* complex species and *L. donovani* [5]. Additionally, SEC22b, a membrane-associated SNARE was also found to be recruited very early to LPVMs. SNAREs are especially important in the context of parasitic hijacking, because they are part of the machinery that facilitates and regulates vesicular fusion within a cell. Other studies evaluated the effect of disruption of fusion between vesicles of the secretory pathway and LPVs. When a variety of SNARE proteins from the early secretory pathway of host cells were targeted, including SEC22b and syntaxin 5 (STx5), the size of communal LPVs that harbor parasites in the *L. mexicana* complex, including *L. amazonensis* was significantly diminished. In addition to diminished LPV size, parasite replication was shown to be significantly reduced, associating vacuole size and parasite proliferation [70]. These studies were followed by evaluation of the small molecule Retro-2, which inhibits retrograde trafficking in cells by inducing the mis-localization of STx5 [71,72]. Retro-2 treatment caused a blockade in LPV development and also resulted in a significant reduction in parasite burden in infected cells *in vitro* as well as *in vivo* while affecting neither host ER morphology, nor host secretion and exhibiting no toxicity to animals [71].

Another important host molecule found to be localized at the LPVM is CD36. CD36 is a scavenger receptor found primarily on the plasma membrane. A novel experimental model of infecting *Drosophila* flies with *L. amazonensis* was utilized to explore the role of CD36 during infection. Using this novel infection model, a homolog scavenger receptor of CD36 was knocked down, and reduced phagocytosis and increased parasite burden were observed, which indicated that CD36 was playing some role in *Leishmania* infections [73]. CD36 was implicated in LPV expansion and maturation in *L. amazonensis* infections in C57BL/6 bone marrow-derived macrophages (BMDMs). BMDMs lacking CD36 were unable to support *L. amazonensis* proliferation. CD36 was observed on the LPVM co-localizing with Rab7a as early as 1 hour post-infection. In cells infected with the promastigote form, CD36 recruitment to the LPVM increased coincident with the transformation of the parasite into the amastigote form within the LPV. Although it wasn't specifically addressed, it is probable that LPVs harboring amastigotes acquired CD36 by interception of secretory pathway traffic. As CD36 accumulated in the LPVM, it became concentrated at sites where the amastigote was in contact with the membrane. Even after 24 hours post-infection, CD36 remained localized at the posterior pole of the parasite, indicating a stable interaction and prolonged association between *L. amazonensis* and host CD36 during an infection. These observations are reminiscent of earlier studies that showed the concentration and eventual ingestion/degradation of MHC class II molecules by *Leishmania* parasites in the LPV (Figure 1) [12]. The role that CD36 is playing during infection has yet to be fully defined and characterized, but when CD36 is knocked out, LPV size is significantly decreased at 24 hours post-infection, which seems to suggest the recruitment and retention of CD36 is involved with LPV development and expansion, two critical processes for the intracellular establishment and success of *L. amazonensis* [73].

Another association with host cell processes can be seen with *LYST/Beige* in *Leishmania* infections. Host cells upregulate transcription of *LYST/Beige*, a known

regulator of lysosome size, in response to infection with *L. amazonensis*. In cells deficient in *LYST/Beige*, *L. amazonensis* LPV expansion increases. Contrastingly, when *LYST/Beige* was overexpressed, LPV expansion was limited, suggesting an important role *LYST/Beige* plays in response to infection. Furthermore, intracellular survival of *L. amazonensis* was diminished when LPV size was limited, and increased when LPV size was expanded, further linking LPV development with the ability of *L. amazonensis* to survive and replicate within host cells [62].

In conclusion, the LPV and its limiting membrane, are critical for the establishment of infection by *Leishmania* parasites within their mammalian host. The LPVM is at the interface between host-pathogen interactions. Though this review has focused on host-derived molecules in the LPV and on the LPVM, it is quite likely that parasite-derived molecules also appear on the LPVM. Much less is known about the identity or characteristics of such parasite molecules, even though they may be responsible for the strict control *Leishmania* parasites exert on which vesicles fuse with the LPV. Greater understanding of the molecular components from both host and parasite that characterize the LPVM is important for an in-depth understanding and for a more complete picture of how parasites work to hijack host resources, prevent degradation, and halt apoptosis in their host cells. Further research is needed to identify and characterize both effector molecules from *Leishmania* parasites, and host factors that together are responsible for the interactions between host and pathogen.

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