COUNTERVAILING, TIME-DEPENDENT EFFECTS ON HOST AUTOPHAGY
PROMOTE THE INTRACELLULAR SURVIVAL OF LEISHMANIA

by

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Abstract

Autophagy is essential for cell survival under stress and has also been implicated in host defense. Here, we investigated the interactions between *Leishmania donovani*, the main etiological agent of visceral leishmaniasis, and the autophagic machinery of human macrophages. Our results revealed that during early infection—and via activation of the Akt pathway—*Leishmania* actively inhibits the induction of autophagy. However, by 24 h, *Leishmania* switched from being an inhibitor to an overall inducer of autophagy. These findings of a dynamic, biphasic response were based on the accumulation of lipidated light chain 3 (LC3), an autophagosome marker, by Western blotting and confocal fluorescence microscopy. We also present evidence that *Leishmania* induces delayed host cell autophagy via a mechanism independent of reduced activity of the mechanistic target of rapamycin (mTOR). Notably, *Leishmania* actively inhibited mTOR-regulated autophagy even at later stages of infection, whereas there was a clear induction of autophagy via some other mechanism. In this context, we examined host inositol monophosphatase (IMPase), reduced levels of which have been implicated in mTOR-independent autophagy, and we found that IMPase activity is significantly decreased in infected cells. These findings indicate that *Leishmania* uses an alternative pathway to mTOR to induce autophagy in host macrophages. Finally, RNAi mediated downregulation of host autophagy protein 5 (ATG5) or autophagy protein 9A (ATG9A) decreased parasite loads, demonstrating that autophagy is essential for *Leishmania* survival. We conclude that *Leishmania* uses an alternative pathway to induce host autophagy while simultaneously inhibiting mTOR-regulated autophagy to fine-tune the timing and magnitude of this process and to optimize parasite survival.
Lay Summary

The leishmaniases are a collective group of diseases that primarily affect people residing in tropical regions. The most severe form, visceral leishmaniasis, is caused by the parasite *Leishmania donovani*. This pathogen infects the host’s innate immune cells; particularly, it resides in the phagolysosomes of macrophages, wherein it can exploit cellular mechanisms. Interestingly, one such mechanism that macrophages can employ to clear pathogens, such as leishmania, is autophagy. This cellular process regulates cell homeostasis and provides a defense against invading pathogens.

This study has found that *Leishmania donovani*, during an early infection period, can prevent macrophages from inducing autophagy. Conversely, during a late infection period, it can promote macrophage autophagy. Interestingly, these opposing responses allow for the survival of parasites within the macrophage. Moreover, *Leishmania donovani* appears to utilize specific pathways to activate and inhibit autophagy. These findings show that leishmania is well apt in manipulating its host for its benefit.
Preface

Chapter 2 is a version of research work that has been recently published. Thomas, S.A., Nandan, D., Kass, J. and Reiner, N.E. (2017) Countervailing, time-dependent effects on host autophagy promotes intracellular survival of leishmania. Journal of Biological Chemistry. 293, 2617-2630. My responsibilities in contributing to this article included planning the experimental design, performing the experiments, conducting the statistical analysis using the appropriate software and drafting the manuscript. The experiments in sections “2.2.3 Leishmania donovani induces host autophagy at later stages of infection despite actively attenuating mTOR-dependent autophagy” and “2.2.4 Host IMPase activity is reduced in response to leishmania infection” were also conducted by Dr. Devki Nandan. He was also involved in the preparation of the manuscript, experimental design and data interpretation. Dr. Neil Reiner was the supervisory author of the article and was involved in the project’s conceptualization, experimental design, data interpretation and the manuscript preparation.

Chapter 3 is a discussion based on the results of the aforementioned article. I was responsible for contributing to this portion of the thesis, with the supervision from Dr. Neil Reiner.

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List of Abbreviations

4E-BP1  Eukaryotic translation initiation factor 4E-binding protein 1
Akt  Protein kinase B
Akti  Akt Inhibitor
APC  Antigen presenting cell
ATG  Autophagy-related protein
ATG12  Autophagy-related protein 12
ATG14  Autophagy-related protein 14
ATG16L  Autophagy-related protein 16
ATG3  Autophagy-related protein 3
ATG4B  Autophagy-related protein 4B
ATG5  Autophagy-related protein 5
ATG7  Autophagy-related protein 7
ATG8  Autophagy-related protein 8
ATG9A  Autophagy-related protein 9A
BSA  Bovine serum albumin
cAMP  Cyclic adenosine monophosphate
cDNA  Complementary DNA
CFSE  Carboxyfluorescein diacetate succinimidyl ester
CR3  Complement receptor 3
CREB  cAMP response element-binding protein
DNA  Deoxyribonucleic acid
dTHP-1  Differentiated human acute leukemia cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FKBP12</td>
<td>FK506 binding protein 12</td>
</tr>
<tr>
<td>FVCO1</td>
<td>FYVE and coiled-coil domain containing 1</td>
</tr>
<tr>
<td>FOXO-1</td>
<td>Forkhead box protein O1</td>
</tr>
<tr>
<td>GP63</td>
<td>Metalloprotease protein 63</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3 beta</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HOPS</td>
<td>Homotypic fusion and protein sorting complex</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IMPase</td>
<td>Inositol monophosphatase</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible Nitric oxide synthase</td>
</tr>
<tr>
<td>IPPase</td>
<td>Inositol polyphosphate 1-phosphatase</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-associated protein 1A/1B</td>
</tr>
<tr>
<td>LIR</td>
<td>LC3-interacting regions</td>
</tr>
<tr>
<td>LPG</td>
<td>Lipophosphoglycan</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular traps</td>
</tr>
</tbody>
</table>
NF-κB  Nuclear factor kappa B  
NGS  Normal goat serum  
NTD  Neglected tropical disease  
p-4EBP1  Phosphorylated eukaryotic translation initiation factor 4E-binding protein 1  
p-Akt  Phosphorylated protein kinase B  
p-S6K  Phosphorylated ribosomal S6 kinase  
p62  Sequestosome 1  
PAS  Pre-autophagosome structure  
PBS  Phosphate buffer saline  
PCR  Polymerase chain reaction  
PI3K  Phosphoinositide 3 kinase  
PLEKHM1  Pleckstrin homology domain-containing protein family member 1  
PMA  Phorbol myristate acetate  
PMSF  Phenylmethylsulfonyl fluoride  
Rab24  Ras-related protein 24  
Rab5  Ras-related protein 5  
Rab7  Ras-related protein 7  
Rab8B  Ras-related protein 8B  
RILP  Rab-interacting lysosomal protein  
S6K  Ribosomal S6 Kinase  
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis  
SHP-1  Src homology region 2 domain-containing phosphatase-1  
siRNA  Small interfering RNA
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SNAP29</td>
<td>Synaptosomal-associated protein 29</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor-attachment protein receptors</td>
</tr>
<tr>
<td>SQSTM-1</td>
<td>Sequestosome 1</td>
</tr>
<tr>
<td>STX17</td>
<td>Syntaxin 17</td>
</tr>
<tr>
<td>TBK-1</td>
<td>TANK-binding kinase 1</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper 1 cell</td>
</tr>
<tr>
<td>THP-1</td>
<td>Human acute leukemia cell</td>
</tr>
<tr>
<td>TOR</td>
<td>Target of rapamycin</td>
</tr>
<tr>
<td>VAMP8</td>
<td>Vesicle-associated membrane protein 8</td>
</tr>
<tr>
<td>VL</td>
<td>Visceral leishmaniasis</td>
</tr>
<tr>
<td>VSP34</td>
<td>Class III PI 3-kinase</td>
</tr>
</tbody>
</table>
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Chapter 1: Introduction

1.1 Leishmaniases

Leishmaniasis is one of several neglected tropical diseases recognized by the World Health Organization [1], [2]. Neglected tropical diseases (NTDs) comprise a diverse group of infections, with the causative pathogens ranging from bacteria to parasites to fungi. The commonality among these diseases is that they primarily affect populations residing in impoverished, tropical/subtropical regions [3]. The combination of the temperate climate and low sanitary conditions for people living in these areas, make it an ideal circumstance for the propagation of these diseases [4]–[6][3]. Leishmaniasis, as one such disease, is caused by the etiological agent from the genus, *Leishmania*. This parasitic protozoan alternates its life stages between a sandfly vector and a mammalian host. In addition, the parasite itself interchanges between the motile, promastigote stage and the non-motile, amastigote stage [4]–[6]. The three most common clinical manifestations of leishmaniasis are: cutaneous, mucocutaneous and visceral. While all three have debilitating effects on the hosts, the former two are less severe than the latter. Manifestations from cutaneous and mucocutaneous leishmaniasis are severe ulcers to either the skin or oral cavity, respectively. However, the far more fatal form, visceral leishmaniasis, results in internal damage of the organs, including the liver and spleen [4].

Collectively, the leishmaniases affect twelve million people globally, with 350 million people at risk of contracting the disease. Moreover, the visceral form of leishmaniasis is fatal, if left untreated; yet, the current treatment options for leishmaniasis prove to have toxic side effects for patients (as reviewed in [7]). These issues, associated with
leishmaniases, make finding proper treatment and preventative options critical for the well-being of those affected and at risk.

1.1.1 Historical Context

As numerous parasitic infections have plagued humans for years, it has, over time, resulted in the co-evolution of both groups. In particular, the protozoan parasites of the Leishmania genus have been documented throughout paleoparasitological findings and historical texts. Recent excavations of fossilized ancient sandflies have identified paleoleishmania species residing within these vectors [8], [9]. The earliest known historical records, for parasitic infections, date back to Ancient Egypt, in 1500 BCE [10]. The Papyrus Ebers are a comprehensive collection of medical records from Ancient Egypt; this historical document sheds light on parasitic diseases, as well as other ailments, that may have afflicted the Ancient Egyptians. It makes reference to intestinal *Ancylostoma duodenale* or Old-World hookworm. And interestingly, it also makes references to skin lesions that may be indicative of the superficial cutaneous leishmaniasis [11], [12]. Recent reports have also noted the presence of *Leishmania donovani* DNA from ancient Egyptian mummies [13]. Similarly, other paleoparasitological studies from the New World were able to identify leishmania species from mummified and skeletal remains, found in Columbia and Chile. Moreover, the studies were able to note the physical disfigurements in skeletal structures that are indicative of cutaneous leishmaniasis [14].

Despite the ancient accounts and intact remains of persons potentially afflicted by leishmania, it was not until the emergence of modern medicine, around the beginning of
the 20\textsuperscript{th} century, that leishmania were identified as disease causing agents. Old world visceral leishmaniasis, or kala-azar (translated: “black fever” in Hindi), was first noted in Colonial India (1824-1862), in the areas of present day Bangladesh and West Bengal [10]. Later on, clinicians, including Ronald Ross, were able to characterize the disease by the skin discoloration, enlarged spleens and severe fever afflicting the patients. However, at the time, it was believed to be a virulent and severe form of malaria [15], [16]. It was not until May 1903 that William B. Leishman, a pathologist, discovered ovoid shaped aggregates from the spleen of a patient suffering from splenomegaly and fever, near Calcutta. His continued observations of these bodies led him to state that it was a residual form of trypanosomes [17]. Remarkably, in a parallel, yet independent finding, Charles Donovan, a professor, found similar bodies from the spleens of patients in Madras. However, unlike Leishman, he did not conclude that these bodies were degenerated trypanosomes [18]. After further analysis of both men’s discoveries, Ronald Ross was able to conclusively identify a new parasitic species and proposed its scientific name as \textit{Leishmania donovani}. Even with the identification of a new species, the mode of transmission for the parasite was still ambiguous. Two decades later, the Sergent brothers were able to denote the role of sandflies, from the \textit{Phlebotomus} genus, as a vector [10].

\textbf{1.1.2 Life Cycle & Transmission}

Leishmania have dimorphic life stages that are dependent on the cyclic transmission between mammalian reservoirs and the sandfly vector. There are eighty-one species of sandflies that are vectors for twenty-nine species of leishmania. Sandflies
from the genus *Phlebotomus* are responsible for the transmission of leishmaniasis in the Old World, whereas the genus *Lutzomyia* is responsible for transmission in the New World [19]. In the extracellular, motile promastigote stage, the parasites reside in the alimentary lumen of female sandflies. Promastigotes are characterized by elongated bodies with one or two flagella. It is in the gut of the sandfly that promastigotes replicate from slightly ovoid flagellates, known as procyclic promastigotes, to needle-like, infective flagellates, known as metacyclic promastigotes [20], [21]. When a sandfly obtains a blood meal from a mammalian host, such as a human or canine, it can inject the metacyclic promastigotes into the bloodstream of the host. From here, the leishmania are in an infective state, where they are able to infiltrate innate immune cells, particularly neutrophils and macrophages. Once residing in the phagolysosomes of macrophages, the promastigotes can transform into the non-motile, ovoid amastigotes, where they can replicate. The replication process, in turn, can result in the rupture of the host cells, furthering the dissemination of the parasites into nearby immune cells. Moreover, depending on the species, leishmania can spread throughout the skin and within visceral organs. In a subsequent blood meal by the sandfly, the amastigotes are taken up and can revert to the promastigote stage within the vector [4], [20]–[22].

### 1.1.3 Disease Manifestations

The clinical manifestations for leishmaniases are dependent on the species, the host, the host’s immune response and the parasite. The three most notable manifestations of the disease are: the cutaneous, mucocutaneous and visceral leishmaniasis [23].
Cutaneous leishmaniasis is the most prevalent form of the disease. It is reported that approximately 1 million new cases of cutaneous leishmaniases occur each year in endemic regions [2]. The severity of the manifestations can vary from asymptomatic to the development of superficial, ulcerative lesions to the dissemination of ulcers throughout subcutaneous regions of the body. Generally, one of the early signs of localized cutaneous leishmaniasis is the presence of a localized erythema, at the puncture site. Within six months, ulcers may develop at the localized or multiple areas. Interestingly, the possibility for self-curing can also occur within months after the disease [24]–[26]. Diffuse cutaneous leishmaniasis can develop in patients with a compromised immune system. It can be readily seen in people with human immunodeficiency virus (HIV) infection [27]. Despite the process of self-curing, the residual scar formations can leave patients with severe disfigurement.

Mucocutaneous leishmaniasis is the least prevalent form that usually occurs in patients who have recovered from cutaneous leishmaniasis. It has been reported that between 3-5% of cases occur after cutaneous leishmaniasis and lesions can be observed within 2 to 50 years [28]–[30]. Primarily, the mucosal membranes of the nasal and oral cavities are affected. Dissemination of the parasite results in lesion formation at those sites.

Visceral leishmaniasis (VL), or kala-azar, is the most severe form of the disease and the lack of treatment can be fatal for the patient(s). Globally, there are about 400,000 new cases and 20,000 deaths reported annually, with 300,000 people at risk in affected regions [2]. The clinical manifestations are denoted by hepatosplenomegaly, severe fever and anemia. The etiological agents for visceral leishmaniasis are *Leishmania donovani,*
*Leishmania infantum* or *Leishmania chagasi*. The progression of VL can vary from between 2 weeks to 18 months, after infection. Generally, inflammation of the viscera can occur within a few months; however, the development lesions, in this area, can take years, post infection [28], [31].

### 1.1.4 Populations affected by the leishmaniases

As mentioned above, ~ twelve million people are affected by the leishmaniases and 350 million people are at risk of infection [2]. The populations affected by these diseases are primarily those that reside in impoverished, warm climate regions. The temperate weather and the limited sanitation allows for the development of sandfly species [32], [33]. This in turn allows for the propagation of the disease.

The socioeconomic impact that leishmaniases have on these populations has been well documented. Recent studies have shown that despite the subsidies from governmental and non-profit organizations for leishmaniasis treatment, families can incur immense medical expenses that can exceed the annual household income [34]–[36]. The rates of infection among men and women are very similar. However, it has been found that women are less likely to receive the appropriate treatment needed; this can be due to factors including the lack of financial resources to see a physician [37], [38]. Moreover, scarring and facial disfigurement can further stigmatize women. Such women may be excluded from their communities, have their marital status affected and, in some cases, be withheld from pursing an education [38]–[41].

While the impact has been seen largely in impoverished, resident populations, it can also be seen in transient populations that have temporarily resided in the affected
areas. Recent studies have noted the emergence of cutaneous leishmaniasis in tourists. Some of the tourist-friendly regions intersect with areas affected by leishmaniasis. As such, tourists are at risk of infection and may not be aware of the risks posed [42], [43]. There have also been cases reported of refugees, from endemic regions, that have carried the disease in to new regions [43], [44]. Another group that has been surprisingly affected by leishmaniasis infections is the foreign military personnel. Between 2001 and 2016, the US Armed Forces reported 2040 incidences of leishmaniasis, with the majority of those being cutaneous leishmaniasis [45].

1.1.5 Diagnosis & Treatment

Molecular diagnostic tools combined with clinical assessments are readily followed for the diagnosis of leishmaniasis in patients. Due to the fact that some forms of leishmaniasis have similar clinical signs as other tropical infectious diseases, various laboratory techniques can be utilized for a precise diagnosis. For the diagnosis of cutaneous and mucocutaneous leishmaniasis, a common technique used is the observation of Giemsa-stained biopsy samples for the presence of the parasites. Additionally, molecular techniques such as PCR amplification of parasite DNA, western blotting of specific proteins or ELISA detection of antibodies against the parasite can be utilized. However, due to the low adaptive immune response to cutaneous or mucocutaneous leishmaniasis, the detection of antibodies is not sensitive and is rarely used. With visceral leishmaniasis, the aforementioned techniques can be used. In addition, as a humoral response can be elicited, so alternative methods, such as rapid testing using an immunochromatographic assay for rK39 or direct agglutination test can
used. Notably, the tests can also be used in the field, with a sensitivity of 85-100% [28], [29], [36], [46]–[48].

Treatment options for leishmaniasis are currently both limited in efficacy and may be toxic as well. These limitations can make treatment difficult and even more challenging when combined with high drug costs. In addition, the growth in drug resistance has limited the use of some of these drugs. One of the most readily used treatments for visceral leishmaniasis is liposomal amphotericin. This intravenous medication has mild toxicity and currently does not have documentation in terms of parasite resistance. Moreover, it has at least a 96% efficacy against the disease [49]. Pentavalent antimonies are also used as a first line of defense. Despite the cost effectiveness and ease in administration, antimonies have been shown to have high parasitic resistance and significant toxicities [46]. Other drugs that have been used either alone or in combination include paromomycin, pentamidine and miltefosine. These treatments are low in cost and have an ease in administration; however severe toxicity and parasite resistance is still of concern in using them [49].

With a large demographic of people at risk of contracting these diseases, paired with the limits of the treatments, research has been slowly emerging in developing a vaccine against the leishmaniases. Currently, there are no pharmaceutical vaccinations against leishmaniasis in humans. The challenges associated with creating this vaccine include, the inability to illicit a proper cellular immune response, varied responses in hosts and issues related to cost [50]. The approved, marketed vaccines are against canine leishmaniasis. The first-generation vaccines used killed or attenuated parasites, while the second-generation vaccines used recombinant parasite proteins. The third-generation
vaccines utilized the DNA encoding parasitic proteins. These vaccines have been relatively successful in preventing canine leishmaniasis [51].

1.1.6 Pathogenesis

Leishmania, similar to many parasitic organisms, are well versed in manipulating and modulating its resident host; conversely, innate immune cells, including macrophages, have an arsenal of protective mechanisms, against invading pathogens. Mechanisms of pathogenesis can vary with the species of leishmania and, as such, they provide insight into the complexity of these parasites’ responses [52]. Entry of the parasites into the host's bloodstream begins the cascade in the innate immune response. The first responders to the site tend to be neutrophils. These granulocytes are able to engulf the leishmania promastigotes. Neutrophils utilize mechanisms such as releasing harmful reactive oxygen species or antimicrobial peptides for pathogen removal [53]. However, studies have shown that leishmania can evade these mechanisms [54], [55]. Moreover, leishmania can use the ineffective neutrophils as “Trojan horses” to, ultimately, be phagocytized by other antigen presenting cells (APCs) [56], [57]. Recently, it has been found that neutrophils can releases neutrophil extracellular traps (NETs) that ensnare promastigotes; however, because of the lipophosphoglycan (LPG) surface on the parasites, the antimicrobial mechanisms are unable to kill them [58].

Natural killer (NK) cells can also play a role in the innate response. These cells are recruited to the site and survey infected antigen presenting cells. Moreover, NK cells are known to provide an initial source of cytokines, such as interleukin-12 (IL-12) and interferon-gamma to promote a T helper 1 (Th1) response [53]. This response can prime
and activate other APCs like macrophages, to kill intracellular leishmania. It has been shown that NK cells can stimulate infected macrophages to control the dissemination of *Leishmania infantum* and *Leishmania major*, through the production of cytotoxic effectors [59], [60].

Leishmania species are able to interact with several components of the host’s innate immune system; however, they primarily reside in macrophages and dendritic cells, the major antigen presenting cells. Macrophages provide an ideal niche for leishmania, yet paradoxically, are also responsible for the clearance of these parasites from the body [61]. Upon phagocytosis by these antigen presenting cells or even prior to, a plethora of cell signaling pathways can be activated. The complement receptor mediated uptake of promastigotes is known to elicit anti-inflammatory responses, promoting survival of the parasites. The third complement receptor (CR3) has been well studied in its role for promastigote uptake by macrophages. Together with other corresponding receptors, such as mannose receptors, CR3 works to recognize the parasites [62], [63]. However, mannose receptor mediated uptake has been found to induce inflammatory responses, aiding in clearance of parasites. The production of reactive oxidative species and pro-inflammatory cytokines, such as interferon-gamma, has been shown to inhibit the survival of leishmania [64]. However, as aforementioned, leishmania is able to counter these defenses by avoiding oxidative damage and manipulating cell signaling [64].

The intricacies of macrophage cell signaling, particularly against pathogen invasion, have been a focus of interest. Leishmania has been shown to activate numerous pathways, including the MAPK and SHP-1 [65], [66]. Of particular interest is the activation
of the phosphoinositide 3-kinase (PI3K) pathway. Recent research has found that leishmania is capable of activating this notable pathway in macrophages [67]. There are three major classes of PI3-Kinases, yet it is the Class I PI3K that has been shown to play a role in leishmania pathogenesis. Recent findings have shown that mutations or pharmacological inhibitors of the kinase’s catalytic subunits can confer resistance to leishmania infection. *In vivo* knockdowns of the p110 delta (p110δ) catalytic subunit generated mice with resistance against both Leishmania major and Leishmania donovani. Moreover, the mice exhibited a faulty expansion of regulatory T cells, resulting in sustained pro-inflammatory responses of macrophages [68]–[70]. Similarly, knockouts or pharmacological inhibition of the p110 gamma (p110γ) catalytic unit prevented the progression of a *Leishmania mexicana* infection [71], [72]. This response can be seen in both cutaneous and visceral forms of leishmaniasis, making it a promising pharmaceutical target.

Consistent with these findings, the downstream targets of PI3K, Protein kinase B (Akt) and the mammalian target of rapamycin (mTOR), have been a focus of interest for their roles not only in cancer biology, cell growth and differentiation, but also in infectious diseases [73]. In particular, the activation of the PI3K pathway can affect apoptosis and autophagy [74], [75]. Akt has a central role as a regulator of several cell signaling pathways and is one of the prime kinases activated, downstream of PI3K (as reviewed in [76]). Interestingly, recent research has implicated this kinase in leishmania pathogenesis. Through the phosphorylation of Akt, *Leishmania amazonensis* can activate NF-κB for the translocation of the p50 homodimer to the nucleus of macrophages, where it can repress expression of the nitric oxide synthase (iNOS) gene. As the infection can
limit the generation of nitric oxide by inhibiting this enzyme, the parasites are able to persist within macrophages [77]. Moreover, *L. amazonensis* has been shown to promote up-regulation of interleukin-10 (IL-10), while decreasing interleukin-12 (IL-12). This in turn shifts the intracellular milieu from a pro-inflammatory macrophage to an anti-inflammatory one [78]. In keeping with this, *Leishmania mexicana* is able to activate Akt in dendritic cells. It is theorized that activation of this pathway can prevent apoptosis and allow for amastigotes to replicate within the host [79]. Similar responses have also been seen with *L. donovani* infection where in its phosphorylated or activated state, Akt is able to phosphorylate and deactivate glycogen synthase kinase β (GSK3β). This removes the brakes on the IL-10 transcription factor CREB, resulting in up-regulation of IL-10, an anti-inflammatory cytokine [80]. Moreover, another study found that through the same pathway and same etiological agent, β-catenin (a downstream protein) can remain activated and translocate into the nucleus. This decreases the pro-inflammatory responses and improves anti-apoptotic responses [81]. In addition, the same study found that, independent of inactive GSK3β, phosphorylated Akt can inactivate the nuclear protein, FOXO-1 resulting in its translocation to the cytoplasm. FOXO-1 has been implicated in pro-apoptotic responses; thus, its export, into the cytoplasm, can inactivate it and prevent apoptosis. These proteins, β-catenin and FOXO-1, allow for an overall pro-leishmania environment [81]. The effect that Akt plays in providing a propitious environment for leishmania makes it a potential target.

Further down the PI3K/Akt pathway is a kinase aptly named after the molecule that targets it, known as the mammalian target of rapamycin (mTOR). Rapamycin (or sirolimus) is a macrolide secreted by the soil dwelling bacterium, *Streptomyces*
hygroscopicus. It was first identified in the mid to late 1970’s to be a potent antifungal drug, particularly against *Candida albicans* [82]–[85]. Researchers were able to identify two proteins, TOR1 and TOR2, that interacted with rapamycin in yeast cells [86]. Years later, the conserved, homologue protein, mTOR, was identified in mammalian cells, as well as the mode of action for rapamycin [87]–[91]. Interestingly, TOR proteins, in both yeast and mammalian cells form complexes with other proteins. In mammalian cells, the complexes are known as mTORC1 and mTORC2. The second complex is insensitive to rapamycin, while the first complex can be completely inhibited by the drug. Rapamycin binds to the FK506 binding protein 12 (FKBP12), which is associated with mTOR and allosterically inhibits the complex [90], [92]. Given that mTOR regulates a myriad of cellular targets, its inhibition or its activation can affect numerous cell functions, including cell growth, mRNA translation and autophagy [93]–[98]. In light of its functionality, there are a few studies that have shown leishmania’s ability to manipulate macrophage mTOR signaling and even the protein itself. Jaramillo et al. identified a new role for the leishmania virulence factor, GP63 [99]. This surface metalloprotease has been studied for aiding in the intracellular survival of leishmania; interestingly, it has been known to cleave several host proteins [100]–[104]. The researchers found that an early *Leishmania major* infection resulted in the cleavage of macrophage mTOR, through a GP63-dependent manner. Moreover, this degradation of mTOR resulted in a global downregulation of macrophage protein translation [99]. Contrary to these findings, other researchers observed an up-regulation in mTOR activity using the same host-pathogen model and they did not observe a decrease in host mTOR expression [105], [106]. Since mTOR is a negative regulator of autophagy, it was concluded that the induction of autophagy, observed with
Leishmania major infection, was not due to its cleavage or degradation [105], [106]. In line with these results, *Leishmania donovani* can also activate mTOR to repress IL-12 and promote IL-10 secretion. This effectively allows for a pro-parasitic environment within the macrophages [107]. As with the previous kinases, leishmania is able to modulate mTOR activity to make its intracellular environment advantageous for survival.

1.2 Autophagy

The process known as autophagy has long been considered a form of cellular recycling. The term was first coined during the *Ciba Foundation Symposium on Lysosomes* in 1963 and its etymology is derived from the Greek language for “self-eating” [108]. Autophagy is the intracellular mechanism for the degradation of cellular components, through the use of lysosomal enzymes (as reviewed in [109]). This process was discovered by Christian de Duve, who identified the role of lysosomes and these vesicles’ association with distinguishable, double membrane vesicles [110]. The identification of autophagy not only led to Duve’s 1974 Nobel Prize in Physiology and Medicine, but also became the gateway into exploring and understanding this research topic [111]. Over the years, the focus has shifted from understanding a generalized mechanism to its implications in physiology.

1.2.1 Biogenesis of autophagosomes

There are over thirty autophagy proteins that play specific roles throughout autophagic flux. Some of the key proteins are known as autophagy-related proteins (ATG; AuTophaGy-related proteins). The initiation of autophagosome formation has been a
long-debated phenomenon. However, it has been suggested that the endoplasmic reticulum maybe one of the membrane donors for the formation of the pre-autophagosome structure (PAS). In particular activation of phosphatidylinositol-3-kinase (PI3K) has been shown to play a role in the initiation of these structures. Class III PI3K, particularly Vps34, activation is necessary for initiation. The small GTPase Rab5 is known to activate this PI3K complex and the association of both are necessary for the appearance of early autophagosomal structures [112]. Recently, Class II PI3K has also been shown to contribute to the phosphatidylinositol-3-phosphate pool, which is also necessary for autophagosome biogenesis [113]. While the initiation and generation of the autophagic structures is undertaken by Class III PI3K, the Class I PI3Ks contribute to autophagy regulation through its downstream effectors, Akt and mTOR (as reviewed in [114]).

The second step in the flux is elongation of the PAS. Expansion of the pre-vesicle allows for more or specific cargo to be encased. Individual autophagy proteins can aid in expansion of the autophagophore. The sources from which the membranes can be acquired include the Golgi complex and plasma membrane [115]–[117]. While the PAS can derive the membrane from different cellular components, the transmembrane, autophagy protein 9 (ATG9) is responsible for trafficking the donor membranes to the PAS [118], [119]. Additionally, complexes of autophagy proteins can aid in elongating the PAS. There are two distinct ubiquitin-like reactions that play a role in expansion. The first ubiquitin reaction is the conjugation of ATG5-ATG12-ATG16L, which elongates the PAS into a longer, cup-shaped, isolation membrane until it can eventually close to form the autophagosome [120], [121]. This ATG complex dissociates from structure, once it has
formed. The second ubiquitin reaction allows for the generation of the marker, microtubule-associated protein 1 light chain 3 (MAP1-LC3/ATG8). There are seven members of the LC3 family of proteins, with the most common being LC3B [122]. This form is vital for the progression of autophagy and as such is used as a well-accepted marker for monitoring autophagy [123]. The autophagy proteins ATG4, ATG7, ATG3 are responsible for converting the precursor protein (LC3-I) into its lipidated form (LC3-II). The carboxyl group, from the glycine residue, is cleaved by ATG4, resulting in the formation of LC3-I [124]. Subsequently, ATG7 and ATG3 can conjugate this protein with phosphatidylethanolamine, resulting in its anchoring to the autophagophore. This form is known as LC3-II. In parallel, the ATG5-ATG12-ATG complex can also aid in the attachment of the lipidated LC3. However, unlike the ATG5-ATG12-ATG16L complex, LC3-II remains on the vesicle until it is ultimately degraded by lysosomes (as reviewed in [125]–[128]). Interestingly, the LC3-II can decorate both the inner and outer facing membranes of the autophagophore. Having LC3-II present in the inner region allows for it to interact with various adaptor proteins. Recently, these adaptor proteins have been found to play a role in sequestering specific content to be degraded. The LC3-interacting regions (LIR) of these proteins can associate with the internal LC3-II and capture the cargo ([129], [130]).

The final steps to ensure closure and maturation of the autophagosomes, as well as the binding to late endosomes and lysosomes. The process for closure has been relatively elusive, in comparison to other steps in autophagy. Since the phagophore is a cup-shaped, three-dimensional structure, it requires scission events to ultimately close it off and form a spherical autophagosome [131], [132]. Most recently, it has been noted
that defects in ATG3 and ATG4B can result in the disruption of autophagosome closure. Both proteins play a role in lipidating LC3-I. The lack of proper LC3-II formation, in the ATG4B mutant and ATG3 knockout cells, affects the closure of autophagosomes; yet, interestingly, does not affect its size. If lysosomal or endosomal fusion proteins are recruited to the improperly formed autophagosomes, the cargo within will not be degraded and instead, may spill out into the cytoplasm [124], [133].

1.2.2 Migration & fusion of autophagosomes and lysosomes

The ability for autophagosomes, and corresponding vesicles, to migrate are just as crucial as is the formation of autophagosomes. Moreover, this migration process allows for early autophagosomes to mature into late autophagosomes. Autophagosome generation can occur anywhere within the cytoplasm and thus it can be dispersed throughout. However, late endosomes and lysosomes tend to aggregate closer to the perinuclear region. One study found that the lysosome location could determine the fusion of autophagosomes to lysosomes; in particular, the perinuclear position allows for optimal fusion [134]. Kinesin and dynein motor proteins are responsible for intracellular migration along microtubules. As dynein motor proteins migrate components towards the minus-end of the microtubules, it can move autophagosomes from peripheral areas towards the perinuclear region. Conversely, as kinesin motor proteins migrate components towards the plus-end, lysosomes and endosomes are able to move outwardly (as reviewed in [135]–[138]). In addition, there are several adaptor proteins, that associate with autophagosomes, and are necessary for its migration and maturation. The Rab family consists of GTPases that can regulate membrane trafficking within cells (as reviewed in
One such member is the small GTPase, Rab7. It is a multi-functional enzyme that plays a role in the maturation of autophagosomes, while also helping with anchoring it to kinesin and dynein proteins, via FYVE and coiled-coil domain containing 1 (FYCO1) and Rab-interacting lysosomal protein (RILP) [141], [142]. Rab7 is progressively recruited onto autophagosomes from early vacuoles to late vacuoles and it has been found to play a role primarily in the late autophagosomes. The loss of function, of this protein, has been linked to the accumulation of autophagosomes, impeding lysosome formation and disrupting lysosomal fusion [143]–[145]. In addition, Rab7 plays a role in selective autophagy by sequestering intracellular pathogens and promoting specific organelle degradation [146]–[148]. Other Rab proteins that play roles in autophagosomes maturation include Rab 8B and Rab 24. The former, in association with TANK-binding kinase 1 (TBK-1), can promote the clearance of intracellular pathogens and is necessary for the proper maturation of autophagosomes [149]. The latter primarily aids in maturation process of autophagosomes. Rab24 readily associates on the outer membrane of the autophagosomes [150], [151]. Moreover, it is needed for the clearance of the autophagosome cargo, under basal cellular conditions. Interestingly, it has also been found that LC3 plays a role in directing movement of autophagosomes to lysosomes and disruption of endogenous LC3 can prevent fusion [151]–[154].

The fusion of autophagosomes to lysosomes or endosomes requires the timely coordination of three protein groups: Rab proteins, membrane-tethering complexes and soluble N-ethylmaleimide-sensitive factor-attachment protein receptors (SNAREs). Specific Rab proteins are involved with membrane trafficking, both during the early and late stages of autophagy. These proteins are also involved with allowing cargo
sequestration during autophagosome formation and vacuole movement within the cell, after autophagosome formation. One of the primary and most researched Rab proteins, in regards to autophagy, is Rab7 [143]–[145]. As mentioned above, its versatility enables it to have multiple roles within the autophagic pathway, but it has a direct role in autophagosome-lysosome fusion [144], [155]. Rab7 can decorate autophagosomes, due to its fusion with late endosomes. In knocking down or inhibiting expression of Rab7, autophagosomes were unable to completely fuse with lysosomes and it highlighted the integral role that Rab7 plays in the autophagic flux. [143], [156].

Membrane-tethering proteins are another group that function to connect vesicular bodies to each other and to initiate the process of fusion. Homotypic fusion and protein sorting complex (HOPS) are one such membrane-tethering protein. Activation of Rab7 recruits its downstream targets, pleckstrin homology domain containing protein family member 1 (PLEKHM1) and Rab-interacting lysosomal protein (RILP); these proteins can subsequently recruit HOPS and promote fusion of autophagosomes to either late endosomes or lysosomes [157].

The final interactive proteins needed to further the bridge between vesicular bodies are the SNARE proteins. The interactions of SNARE proteins that are located on autophagosomes, endosomes and lysosomes can favorably release energy needed for fusion to occur (as reviewed in [158], [159]). The three prominent SNARE proteins involved with autophagosome-lysosomal fusion are: syntaxin 17 (STX17), synaptosomal-associated protein 29 (SNAP29) and vesicle-associated membrane protein 8 (VAMP8). Upon the progression of autophagic flux, STX17 accumulates on autophagosomes where it can bind to components of HOPS and to SNAP29. Studies have proven the need for
both STX17 and SNAP29 for optimal fusion between autophagosomes and lysosomes [160]–[163]. On the surface of lysosomes, the SNARE protein, VAMP8 is recruited and binds to the STX17-SNAP29 complex on the autophagosomes. Interestingly, the autophagy protein, ATG14, coordinates the binding of the STX17-SNAP29 complex to VAMP8 and enhances STX17-SNAP29-VAMP8 mediated fusion [164], [165].

The mechanisms for vesicle migration and fusion require the synchrony of several effector proteins. While much has been found in regard to these processes, there is still more needed to understand the underlying mechanisms to enable autophagic flux.

1.2.3 Autophagy & disease

Early on, the primary role for autophagy was observed to be the clearance of long-lived organelles and aggregated proteins that were readily degraded and recycled within the cell. As such, research had focused on the homeostatic role of autophagy. However, recent studies have looked at alternative roles that autophagy can play within the cell and even for the manifestations of different diseases. Autophagy has been shown to be involved, for example, in neurodegenerative diseases, certain cancer types, and cardiac diseases (as reviewed in [166]).

Autophagy was first reported to promote the pathogenesis of neurodegenerative diseases, including Alzheimer’s, Parkinson’s, and Huntington disease. These diseases have a fundamental commonality, in which protein aggregates can be observed within the neural cells. Initial studies stated that the aggregates were protective against cellular death [167]–[169]. However, recent studies have found that the aggregation of proteins can be detrimental for neural cells. Mutations in proteins, such as tau, α-synuclein and
huntingtin fragments, can make them aggregate prone [170]. The clearance of these bodies would primarily be through autophagy. Thus, defects or impediments in the autophagic process can hinder the aggregate clearance and promote pathogenesis [171], [172]. Under similar circumstances, dysfunction in autophagy has been linked to cardiac disease progression. Since cardiomyocytes are consistently contractile cells, they require basal autophagy to maintain and recycle their internal milieu. Interestingly, autophagy acts as a double-edged sword with cardiomyocytes. During cardiac ischemia, autophagy can be cytoprotective by providing necessary energy for cells; however, during cardiac reperfusion, autophagy can enhance cell death [173]–[175].

Of particular interest is the role that autophagy plays in infectious diseases. As autophagy is a method for the clearance of intracellular material, it was reasonable for researchers to explore its function as an immune defense mechanism (as reviewed in [176]). Here again, autophagy may act a double-edged sword when it comes to the interactions between hosts and pathogens. On the one hand, autophagy can promote killing of intracellular pathogens when it is activated and, on the other hand, the host’s autophagic mechanisms can be manipulated by pathogens to ensure their survival.
Chapter 2: Countervailing, time-dependent effects on host autophagy promote intracellular survival of leishmania

2.1 Background

As new light has been shed on the multifaceted role of autophagy, there has been a greater understanding of its interaction with the innate immune system and its role in the clearance of various microorganisms. Research has identified numerous microorganisms that are directly affected by host autophagy [143], [177], [178]. Furthermore, several microorganisms, ranging from prokaryotes to eukaryotes have been shown to manipulate host cell autophagy [148], [179]–[181]. Depending on the species of both leishmania and the host, there can be improved survival or better clearance of the parasite [106], [182]–[188].

Through the interactions between parasites and host macrophages, leishmania has been shown to activate a multitude of host cell pathways to promote its survival. One key regulatory pathway that is activate by leishmania is the PI3K/Akt pathway [80], [81], [189], [190]. Downstream of this pathway is the mammalian target of rapamycin complex 1 (mTORC1/mTOR). This kinase is a master regulator of an array of cell processes including protein synthesis and cell growth [191]. Another vital process regulated by mTOR is the activation of state of autophagy. The link between intracellular pathogens and the activation of the host’s autophagy, as a clearance mechanism, has been of recent interest. Conversely, it has also been found that pathogens can exploit this mechanism as a means for survival, proliferation, and nutrient acquisition [176], [181], [192], [193].

Taken together, we hypothesized that through the PI3K/Akt pathway, Leishmania
*donovani* may regulate macrophage autophagy. In addition, we hypothesized that leishmania could utilize or facilitate this induction for its own benefit and survival.

### 2.1.1 Objectives

The leishmaniases are a group of vector-borne infectious diseases that are primarily endemic to tropical and sub-tropical regions of the world [2]. The populations affected tend to be from lower socioeconomic strata and there are projected to be in the range of 12 million people affected worldwide [194]. Beyond this, there are estimated to be 350 million people across 88 countries living at risk for contracting leishmaniasis [195]. A multitude of factors, including poor sanitary conditions, lack of vector control, rapid environmental changes, increased travel, and resistance to commonly used drugs have contributed to rising incidence rates.

Depending on the leishmania species, the severity and form of leishmaniasis ranges from the relatively limited cutaneous leishmaniasis to a progressive, lethal form of visceral leishmaniasis that involves the liver, spleen, and bone marrow. Cutaneous leishmaniasis can be characterized by superficial lesions and ulcers that cause moderate to severe disfigurement. Visceral leishmaniasis, in contrast, results in internal organ damage that can be fatal when left untreated. It has been estimated that the incidence rate of visceral leishmaniasis is in the range of 200,000-400,000 cases per year [2]. The main etiological agent for human visceral leishmaniasis is *Leishmania donovani*.

Amongst various phagocytic cells, leishmania primarily target macrophages. Like all leishmania species, *L. donovani* has a digenetic life cycle, transitioning from the motile promastigote form within the sand-fly gut to the non-motile amastigote form inside
macrophages. Both life cycle stages have evolved to use multiple strategies to resist host microbicidal functions and to evade the immune system [196]. For example, we showed that leishmania infection of both murine and human macrophages, hijacks the PI3K/Akt pathway [80] leading to the inactivation of glycogen synthase kinase-3β (GSK-3β) and the induction of IL-10 production, via enhanced activity of the transcription factor CREB [80].

In the present study, we sought to characterize other macrophage functional programs that might be affected downstream of the PI3K/Akt pathway in infected cells. One candidate of particular interest because of its pleiotropic regulatory properties is the mammalian target of rapamycin (mTOR), which is positively regulated by Akt. mTOR is a conspicuous kinase that functions as a master regulator of numerous cellular processes, including autophagy [191], [197]. It was this context that prompted us to ask whether leishmania infection modulates host cell autophagy via an mTOR-dependent pathway, and importantly, whether this impacts intracellular survival.

Autophagy encompasses a spectrum of conserved, catabolic processes in which cellular debris is removed and degraded. The most commonly addressed form is macroautophagy, herein referred to as autophagy. It is characterized by the active degradation of cytoplasmic constituents that are engulfed by double membrane structures, known as autophagosomes. These distinctive structures ultimately fuse with lysosomes to form autophagolysosomes. It is at this stage that the intravesicular contents are degraded [127]. More than 30 autophagy related proteins (ATGs) have been identified. Amongst these, the lipid conjugated protein marker, microtubule-associated protein 1 light chain 3b (LC3-II)/ATG8, associates with autophagosomes and can be
detected using various techniques. In fact, LC3-II has been used extensively as an indicator of autophagy in a wide variety of cells and tissues [198].

Autophagy can be regulated via multiple signaling pathways. Broadly, the two commonly defined pathways are either mTOR-dependent or mTOR-independent. As previously mentioned, PI3K/Akt activates mTOR leading to inhibition of cellular autophagy and this is considered to be the classical pathway for regulation. In addition to this pathway, mTOR-independent regulation of autophagy has also been recently studied [127]. For example, inositol-lowering agents, such as lithium induce autophagy independent of any change in mTOR activity [199].

Autophagy has long been considered to be a major recycling mechanism used by the cell. However, recent research has found that it has other functions, including roles in innate immunity and antimicrobial defense. Notably, autophagy in macrophages attenuates survival of numerous pathogens such as *Mycobacterium tuberculosis*, *Shigella flexneri*, *Listeria monocytogenes*, and *Toxoplasma gondii* [176].

Current knowledge around host autophagy and leishmania pathogenesis is a focus of interest. One early study suggested that the transfer of dextran, from macrophage cytosol to *Leishmania mexicana* phagosomes, occurred via autophagy [188]. Another study reported the accumulation of LC3-II in human bone marrow cells during *L. donovani* infection [187]. Furthermore, induction of autophagy in infected macrophages has been linked to increased growth and parasite load of *Leishmania amazonensis* [182], [185]. Most recently, it has been found that *Leishmania major* uses macrophage autophagy to inhibit T-cell responses and prevent parasite clearance [183]. The molecular mechanism(s) involved in leishmania-mediated regulation of host autophagy have begun
to emerge. For example, recent evidence suggests that select host microRNAs (miRNAs) may participate in regulation of host autophagy in response to leishmania infection [106], [186]. It has also been shown that autophagy induction through endosomal Toll-like receptors plays a role in macrophages conferring resistance against \textit{Leishmania major} infection. [184].

In the present study, we report that leishmania actively inhibits the induction of host classical autophagy via the early and sustained activation of mTOR. In striking contrast, as infection progressed, significant induction of autophagy was seen at later stages of infection. In fact, we present evidence that leishmania engages an alternate, mTOR-independent signaling pathway to induce host autophagy while at the same time maintaining tonic control of this process via activated mTOR. Our findings suggest a model in which leishmania infection brings about countervailing, time-dependent effects on host autophagy through distinct pathways thereby promoting intracellular parasite survival.
2.2 Results

2.2.1 Infection by *Leishmania donovani* initially inhibits and then induces host macrophage autophagy in a bi-phasic manner

Autophagosomes are formed from a cup-shaped double-membrane sac called the isolation membrane in the cytoplasm. During maturation of autophagosomes, specific insertion of lipidated LC3-1 (also known as LC3-II) occurs, which remains associated with autophagosomes. Thus, accumulation of LC3-II has been extensively used as a marker of autophagy, and increased levels of LC3-II can be detected by Western blotting or by immunofluorescence microscopy as punctate vesicular LC3-II [200]. We used both readouts to investigate the induction of autophagy in response to *L. donovani* infection in PMA differentiated THP-1 cells which have been extensively used as a model system to study human visceral leishmaniasis [201].

To examine whether *L. donovani* induces autophagy, THP-1 cells were infected with stationary phase leishmania promastigotes and autophagy was monitored by Westerns using LC3-II specific antibodies over 48 h. As shown in Figure 2.1, expression of LC3-II early after infection (2 h – 8 h) was not affected relative to control cells. However, over the course of longer infection (24 h – 36 h), there was a clear increase in LC3-II expression in infected cells.
Figure 2.1. *Leishmania donovani* induces delayed onset host macrophage autophagy. dTHP-1 cells were incubated with *L. donovani* (L.d.) promastigotes at an m.o.i. of 20:1. At the indicated time points, control uninfected and infected cells were washed with HBSS. Whole cell lysates were collected and analyzed by immunoblotting with antibodies specific for LC3-II. The same membrane was stripped and reprobed with actin for loading controls. The histograms shown are the means ± S.D. of the densitometric analyses of the 8-, 12-, 24-, 36-, and 48-h time points of three independent experiments. Data are presented as mean ± S.D.; *p* < 0.05; ns = not significant.

These increased levels of LC3-II could have been due either to enhanced *de novo* synthesis (autophagy induction) or to inhibition of turnover of LC3-II, due to a potential infection-induced blockade of the autophagic flux. To address this question, infected cells were treated or not with lysosomal degradation inhibitors pepstatin A and E64d [198] and assessed by Western blot for LC3-II levels. If autophagic flux was normal in infected cells, then their levels of LC3-II should have been increased in the presence of lysosomal degradation inhibitors because the transit of LC3-II through the autophagic pathway would have been blocked [202]. Indeed, the results presented in Appendix A show that each alone –infection or inhibitor treatment– were equipotent and together they were
additive in boosting LC3-II levels. These results—assuming near complete reduction in flux by inhibitor treatment—support the conclusion that infection promotes the induction of autophagy with the attendant increases in de novo synthesis of LC3-II. To further strengthen these results, we also monitored levels of Sequestosome-1 (SQSTM-1/p62), an alternative autophagy marker, during leishmania infection. p62 is a ubiquitin receptor protein that can bind to specific cargo and sequester it in autophagosomes. The degradation of p62, through the autophagy pathway, has been extensively used as an indicator of increased autophagic flux. Conversely, increased levels of p62 are indicative of inhibition of autophagy [203]–[207]. As shown in Appendix B, late leishmania infection resulted in the depletion of p62 levels, compared to control, confirming an induction of autophagic flux. Lysosomal mediated, autophagic degradation was confirmed by treating control and infected cells with pepstatin A and E64d, as shown in Appendix B. Treatment with pepstatin A and E64d, in leishmania infected cells, resulted in the restoration of p62 levels, as expected. Taken together, the LC3-II & p62 results show that leishmania induces host autophagy at a late stage of infection. We also investigated the level of p62 at the early stage of infection (Appendix C, lanes 1 & 2). The results showed the accumulation of p62, which is indicative of a blockade in autophagic flux.

2.2.2 Leishmania donovani actively inhibits rapamycin-induced host autophagy during early infection

As shown in Figure 2.1, leishmania did not induce host autophagy at early time points post infection (2-8 h). This could have been passive, due to “silent entry” of leishmania into host cells or to leishmania actively attenuating host autophagy during early infection. We favored the second possibility since it is known that leishmania activates the host
PI3K pathway and activation of PI3K is inhibitory for autophagy through its positive effects on mTOR [190]. Moreover, the accumulation of p62 during the early stage of leishmania infection was indicative of active autophagic inhibition (Appendix C, lanes 1 & 2). To test the possibility that leishmania actively inhibited host autophagy in the early stages of infection, dTHP-1 cells were infected with leishmania for 6 h, followed by treatment with the well characterized autophagy inducer rapamycin for 2 h [208], [209]. As shown in Figure 2.2 A & B, leishmania infection nearly, completely abrogated rapamycin induced autophagy at six hours post infection. dTHP-1 cells treated with chloroquine (12.5 µM for 2h) were used as positive controls to confirm LC3-II induction. These findings were strengthened by monitoring levels of p62 under the conditions above, as shown in Appendix C, lanes 3 & 4. Notably, leishmania did not affect accumulation of LC3-II in response to chloroquine (Figure 2.2 A & B), that acts through a distinct pathway from that of rapamycin [209], [210]. These findings further indicate that the inhibitory effect of leishmania infection on autophagy is selective.

To confirm inhibition of rapamycin-induced autophagy by leishmania using an orthogonal readout, we examined autophagosome formation using confocal microscopy and LC3-II specific immunostaining of LC3-II puncta within dTHP-1 cells. For this assay, we also used CFSE (+) leishmania for direct detection of organisms inside macrophages. Cells were infected with *L. donovani* for 6 h and then treated with rapamycin for 2 h followed by intracellular LC3-II staining. Cells treated with rapamycin alone for 2 h were used as positive controls. Non-infected cells at the 6 h time point were used to determine baseline number of autophagosomes. The images taken were analyzed for LC3-II positive puncta and the mean immunofluorescence index was determined for 100 cells
over three independent experiments. Representative images and quantification of puncta are shown respectively in Figure 2.2 C & D. As expected based on the Western results in Figure 2.2 A & B, during the first six hours, infection with leishmania was associated with inhibition of rapamycin induced autophagosome formation (Figure 2.2 C & D). Taken together, these results clearly show that host autophagy is inhibited during early stage infection with leishmania. Inhibition of host autophagy during early infection (Figure 2.2) and induction of host autophagy as infection progresses (Figure 2.1) shows dynamic biphasic regulation of host autophagy in response to leishmania.
Figure 2.2. *Leishmania donovani* actively attenuates rapamycin-induced autophagy in macrophages at an early stage of infection. dTHP-1 cells were incubated with *L. donovani* promastigotes for 6 h. Subsequently, the cells were incubated either chloroquine (12.5 \( \mu \)M) or rapamycin (12.5 \( \mu \)g/ml) for 2 h. Whole-cell lysates were then collected and analyzed by immunoblotting for LC3-II. The same membrane was stripped and reprobed for actin as a loading control. A, Western blot for LC3-II and actin levels. B, densitometry analysis of three independent experiments. Data are presented as mean S.D.; ns, not significant; *, \( p < 0.05 \). C, immunofluorescence and mean fluorescence index of LC3 puncta, during an early infection. dTHP-1 cells were incubated with CFSE-positive *L. donovani* promastigotes (green) for 6 h. The cells were then washed and given new media containing rapamycin (12.5 \( \mu \)g/ml) for 2 h. Uninfected control and infected cells with and without rapamycin were then fixed and stained for LC3-II (red) and the nuclei (blue). Confocal microscopy was performed to acquire images using LSM 790 Zeiss microscope, and immunofluorescence analysis was done using ImageJ software. The zoomed confocal photomicrographs are shown to improve clarity of LC3-II decorated autophagosomes. D, quantification of puncta from 100 dTHP-1 cells from each group over three independent experiments is shown as a histogram. Data are presented as mean + S.D.; ns, not significant; *, \( p < 0.05 \).
It was of interest to investigate the role of the regulatory pathway(s) that leishmania targets to inhibit host autophagy. As it is well established that leishmania activates host Akt, it was reasonable to link the involvement of this pathway in the active inhibition of host autophagy [80], [190]. To investigate this possibility, cells were treated with Akt 1/2 inhibitor for 4 h prior to leishmania infection, for 6 h. Subsequently, the cells were treated with rapamycin for 2 h. At the end of the experiment, the level of LC3-II was determined by Western blot assay. The results in Figure 2.3 show that the prior treatment with Akt inhibitor prevented leishmania’s ability to inhibit rapamycin-induced autophagy. These findings directly link the utilization of the host Akt pathway to inhibit autophagy by leishmania. It should be pointed out that the concentration of Akt inhibitor used to pre-treat cells did not affect the internalization of leishmania (data not shown).

**Figure 2.3. Leishmania donovani uses the Akt pathway to inhibit autophagy during an early stage infection.** dTHP-1 cells were pretreated with an Akt1/2 inhibitor for 4 h. Subsequently, cells were washed with HBSS. The cells were then either infected with *L. donovani* (*L.d.*) promastigotes at an m.o.i. of 20:1 or rested for 6 h. Cells were then washed and incubated with rapamycin (12.5 μg/ml) for 2 h. Whole-cell lysates were collected and analyzed by immunoblotting for LC3-II. The same membrane was stripped and reprobed for actin as a loading control. Shown are the densitometry analyses of three independent experiments and a representative Western blot for LC3-II and actin levels. Data are presented as mean S.D.; *ns*, not significant; *, *p* 0.05.
2.2.3 *Leishmania donovani* induces host autophagy at later stages of infection despite actively attenuating mTOR-dependent autophagy

As shown in Figure 2.1, leishmania induced autophagy at later stages of infection and it was of interest to ask whether this involved use of the classical mTOR pathway or possibly an alternate, mTOR-independent mechanism. We investigated whether delayed induction of autophagy by leishmania correlated with down-regulation of mTOR, an important negative regulator of classical autophagy. Two direct downstream sequelae of mTOR activation include the phosphorylation of S6 kinase at T389 and the phosphorylation of the eIF4E inhibitor, 4E-BP1, at multiple sites [211]. As shown in Figure 2.4, infection of dTHP-1 cells did not affect either the abundance of mTOR or lead to any changes in the phosphorylation states of either host 4E-BP1 or S6 kinase. In contrast, rapamycin when used as positive control did downregulate mTOR activity as expected (Figure 2.4). These results indicate that neither degradation of mTOR nor inhibition of host mTOR activity were likely to be the mechanism by which leishmania induced delayed host cell autophagy.

![Figure 2.4. *Leishmania donovani* does not inhibit macrophage mTOR activity to induce autophagy at a late stage of infection. dTHP-1 cells were incubated with *L. donovani* promastigotes for 24 h. Whole-cell lysates from uninfected and infected cells were analyzed with the indicated antibodies. In parallel, whole-cell lysates from rapamycin (100 nM) treated dTHP-1 cells were used as controls for S6 kinase and 4EBP1 activities (surrogate markers for mTOR activity).]
To examine this question further, cells infected for 24 h were then treated with rapamycin and analyzed for LC3-II levels. Interestingly, the levels of LC3-II in infected cells, plus/minus rapamycin were equivalent (Figure 2.5 A & B) and these results were confirmed by confocal microscopy (Figure 2.5 C & D). The finding that infection and treatment with rapamycin were not additive suggests, as one possibility, that the induction of autophagy in response to leishmania may have reached its maximal potential. Alternatively, the lack of summation raised the possibility that leishmania is still able to inhibit classical mTOR regulated autophagy through 24 h of infection –as it had through 6 h of infection- by activating host Akt. To examine this possibility, we measured levels of phospho-Akt at 24 h post infection and found these to be strikingly high (Figure 2.6 A). These results, along with the findings reported in Figure 2.2 and Appendix C, strongly suggest that leishmania actively inhibited classical mTOR-dependent autophagy at both early and late stages of infection. To examine this directly, cells were infected for 24 h and then treated with Akt 1/2 inhibitor (Sigma-Aldrich). If leishmania actively inhibited classical autophagy by activating the Akt-mTOR pathway, then inhibition of Akt should prevent mTOR activation, thereby removing this brake on induction of host autophagy. This prediction is shown to be correct in Figure 2.6 B where it is also shown that leishmania-induced autophagy was significantly enhanced in the presence of Akt inhibitor. These findings provide support for a model in which leishmania uses the PI3K-Akt-mTOR pathway to downregulate classical autophagy, while at the same time using an alternate mTOR-independent pathway to induce autophagy in response to infection.
Figure 2.5. *Leishmania donovani* induces host macrophage autophagy at 24 h post-infection while inhibiting rapamycin-induced autophagy. dTHP-1 cells were incubated with *L. donovani* promastigotes for 24 h. Subsequently, the cells were incubated with either chloroquine (12.5 μM) or rapamycin (12.5 μg/ml) for 2 h. Cells were then washed with HBSS, and whole-cell lysates were collected and analyzed by immunoblotting for LC3-II levels. The same membrane was stripped and reprobed with anti-actin as a loading control. A, Western blot for LC3-II and actin levels. B, densitometry analysis of three independent experiments. Data are presented as mean S.D.; ns, not significant; *, p 0.05. C, immunofluorescence of LC3-II puncta at 24 h infection. dTHP-1 were incubated with CFSE-positive *L. donovani* promastigotes (*green*) for 24 h. The cells were then washed and given new media containing rapamycin for 2 h. Non-infected control and infected cells with and without rapamycin were then fixed and stained for LC3 (*red*) and the nuclei (*blue*). The cells were imaged using LSM 790 Zeiss microscope, and immunofluorescence analysis was done using ImageJ software. D, puncta quantitation was done on 100 dTHP-1 cells from each group over three independent experiments. Data are presented as mean S.D.; ns, not significant; *, p 0.05.
Figure 2.6. *Leishmania* donovani induces host Akt phosphorylation & autophagy is enhanced in the presence of an Akt inhibitor. A. *L. donovani* infection induces phosphorylation of host Akt. dTHP-1 cells were incubated with *L. donovani* promastigotes for 24 h. Whole-cell lysates from uninfected and infected cells were analyzed with the indicated antibodies. Histogram shows densitometry analysis of three independent experiments. Data are presented as mean S.D.; **, *p* 0.01. B, *Leishmania*-induced autophagy is enhanced in the presence of Akt inhibitor. Twelve h post-*L. donovani* infection, cells were treated with Akt inhibitor for 24 h. In parallel, uninfected cells treated with Akt inhibitor alone and infected cells without Akt inhibitor were used as controls. At the end of the experiment, whole-cell lysates were analyzed for the levels of LC3-II. Actin levels were also analyzed as loading controls. The histogram shown is a densitometric analysis of relative LC3-II levels in three independent experiments. Data are presented as mean S.D.; *, *p* 0.05; **, *p* 0.01.

### 2.2.4 Host IMPase activity is reduced in response to leishmania infection

Apart from the regulation of autophagy by mTOR, various mTOR-independent autophagy pathways have been described that are sensitive to chemical perturbations [212]. One of
the first of these to be reported is linked to an inositol signaling pathway where elevated levels of Inositol (1,4,5)P$_3$ (IP$_3$) inhibit the generation of autophagosomes and negatively regulate autophagy. Conversely, inositol-lowering agents, such as mood-stabilizing drugs like lithium induce autophagy without inhibiting mTOR activity [199]. Our interest in this mTOR-independent pathway arose from our previous finding of reduced IP3 levels in cells infected with *L. donovani* [213]. Thus, we hypothesized that leishmania might engage an mTOR-independent pathway by reducing the concentrations of IP3 leading to induction of delayed autophagy. Inositol monophosphatase (IMPase) is the key enzyme required to generate free inositol that is essential for the inositol signaling pathway to function [214]. Therefore, we measured the enzymatic activity of IMPase in leishmania infected cells and found that it was significantly reduced (Figure 2.7). These findings identify one potential -mTOR-independent pathway- that may be used for the induction of delayed autophagy by leishmania.

**Figure 2.7. Leishmania donovani infection attenuates host IMPase activity.** dTHP-1 cells were incubated with *L. donovani* promastigotes for 24 h. Uninfected and infected cells were washed, and cell lysates were collected. Whole-cell lysates containing equal amounts of proteins were analyzed for IMPase activity using inositol 1-phosphate as a substrate as described under “Experimental procedures.” Inorganic phosphate liberated from inositol 1-phosphate was measured using malachite green assay. The data show an average of two independent experiments. The histogram shows the data for three independent experiments. Data are presented as mean S.D.; ****, p 0.0001.
2.2.5 Dynamic regulation of host cell autophagy by leishmania and impact on survival

To address the biological relevance of bi-directional regulation of host cell autophagy by leishmania, we investigated whether attenuation of autophagy during early infection (< 12 h) is beneficial to leishmania. Here, we used various concentrations of rapamycin for 2 h to induce autophagy in dTHP-1 cells prior to infection. After 2 h of rapamycin treatment, cells were infected with *L. donovani* for 24 h. At the end of the experiment, infected cells were extensively washed, and internalized parasites were released from the infected cells by mild treatment with SDS as described in Experimental Procedures. The effect of SDS was neutralized by adding leishmania growth media and freed amastigotes were allowed to transform in motile promastigotes and counted. The parasite rescue results presented in Figure 2.8 clearly show that pre-treatment of host cells with rapamycin to induce autophagy prior to infection was inhibitory to survival of parasites in a concentration dependent manner. These findings suggest that early inhibition of host autophagy by leishmania is beneficial to promastigotes as they are not yet fully equipped to survive inside the hostile environment of phagolysosomes. It should be pointed out that rapamycin concentrations up to 25 µg/ml did not affect internalization of promastigotes and were not toxic to promastigotes in culture (data not shown).
Figure 2.8. Rapamycin pre-treatment of host macrophages results in the reduced survival of *Leishmania donovani*. dTHP-1 were pre-treated with indicated concentrations of rapamycin for 2 h and subsequently incubated with *L. donovani* promastigotes for 6 h. Macrophages were washed and given new media containing the indicated concentrations of rapamycin for 24 h. At the end of the experiment, infected cells with and without rapamycin were washed, and internalized parasites were released by lysing of cells using a mild concentration of SDS, followed by transfer to the transformation medium as described under “Chapter 3: Research Methodology.” On day 5, transformed motile promastigotes were counted. A, histogram shown is the result of *Leishmania* growth in rapamycin treated infected cells normalized to untreated infected cells in three independent experiments performed in duplicate. Data are presented as mean S.D.; **, p 0.005; ****, p 0.0001. B, in parallel, cells treated with various concentrations of rapamycin were confirmed for the induction of LC3-II.

Next, we investigated the possibility that once infection is established, the induction of autophagy by leishmania may confer a parasite survival advantage. To address this question, we elected to study two autophagy related proteins. One protein is part of the two conjugation system, autophagy protein 5 (ATG5), while the other, autophagy protein 9A (ATG9A), is not. ATG5 is an important protein for autophagic activity and is essential for autophagosome formation. It is required for LC3-I conjugation to PE to form LC3-II and for the elongation of autophagic membranes [128]. ATG9A is important for adding membrane to the autophagosome during its formation [215]. We used siRNAs to downregulate ATG5 or ATG9A. Treatment of cells with specific siRNAs prior to infection resulted in significantly decreased ATG5 or ATG9A levels (Figure 2.9 A & C). Cells transfected with scrambled siRNAs were used as negative controls. Cells that were down-regulated for either ATG5 or ATG9A and control cells were then infected with *L. donovani* for 24 h. At the end of the experiment, the infected cells were lysed for parasite rescue. The results presented in Figure 2.9 B & D show that reduced ATG5 or ATG9A levels,
respectively, correlated with decreased survival of leishmania. Taken together, these results indicate that delayed induction of host cell autophagy by leishmania is critical to optimal intracellular survival.

Figure 2.9. ATG5 or ATG9A knockdown in host macrophages results in reduced survival of *Leishmania donovani*. THP-1 cells were transfected with indicated siRNAs for ATG5 or ATG9A for 48 h. Control and ATG knockdown cells were differentiated with PMA, and subsequently incubated with *L. donovani* promastigotes for 24 h. At the end of the experiment, infected cells were washed, and internalized parasites were released by lysing cells using a mild concentration of SDS, followed by transfer to the transformation medium as described under “Chapter 3: Research Methodology.” On day 5, transformed motile promastigotes were counted (*B* for ATG5 and *D* for ATG9A). In parallel, cells treated with siRNAs were confirmed for low ATG5 protein levels and low LC3-II protein levels (*A* for ATG5 and *C* for ATG9A). Data are presented as mean S.D.; *, *p* 0.05; **, *p* 0.01.
2.3 Research Methodology

2.3.1 Antibodies and reagents
Primary antibodies for LC3-II, SQSTM-1/p62, phospho-p70-S6 kinase (Thr-389), phospho-4E-BP1 (Thr-70), mTOR, Akt, phospho-Akt (Ser-473), ATG5, and ATG9A were obtained from Cell Signaling Technology. The primary antibody for actin was obtained from Sigma. For immunoblotting, the secondary antibodies, peroxidase-conjugated affinity purified anti-rabbit IgG (H&L) (goat), were obtained from Applied Biomaterials, and Alexa Fluor 680 goat anti-rabbit IgG (H&L) antibodies were obtained from Life Technologies, Inc. For immunofluorescence, the secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (H&L), was obtained from Life Technologies, Inc. For the staining of *L. donovani* parasites, CellTrace™ CFSE from Life Technologies, Inc., was used.

Rapamycin, chloroquine diphosphate salt, Akt1/2 inhibitor (A6730), E64d pro-tease inhibitor, pepstatin A, PMA, leupeptin, aprotinin, normal goat serum (NGS), bovine serum albumin (BSA), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma.

2.3.2 Leishmania donovani culture
Sudan strain S2 promastigotes were incubated and cultured at 26 ºC, in M199 media (Sigma) with 10% heat-inactivated fetal calf serum (Gibco), 20 mM HEPES (Sigma), 100 units/ml penicillin/streptomycin (Sigma), 100 mM adenosine (Sigma), 2 mM L-glutamine (Gibco), 6 mg/ml hemin (Sigma), and 10 mg/ml folic acid (Sigma). The promastigotes were passaged every 3 days and kept in culture for a maximum of 15–20 passages. To maintain the virulence and infectivity of the promastigote strain, fresh amastigotes were isolated from infected Syrian Golden hamster spleens, followed by *in vitro* transformation into promastigotes.
2.3.3 THP-1 cell culture

THP-1 cells, obtained from ATCC (TIB-202TM), were incubated and cultured at 37 °C, 5% CO₂ in RPMI 1640 media (HyClone) containing 10% heat-inactivated fetal calf serum (Gibco), 10 mM HEPES (Sigma), 100 units/ml penicillin/streptomycin (Sigma) and 2 mM L-glutamine (Gibco). Cells in suspension were passaged every 2–3 days to maintain a density between 3 10⁵ and 8 10⁵ cells/ml. For differentiation, THP-1 cells were treated with 10 ng/ml PMA for 16 –18 h. After adherence, cells were washed three times with Hanks’ balanced salt solution (HBSS) (Sigma) and given fresh media not containing PMA. The cells were rested for 6 h before being used experimentally. For infections, day 5 stationary promastigotes were used at an m.o.i. of 20:1.

2.3.4 Western blotting

PMA differentiated THP-1 (dTHP-1) cells were washed with HBSS and lysed in ice-cold cell lysis buffer (20 mM Tris-HCl, pH 6.8, 1% Triton X-100, 1 mM EDTA, 0.15 M NaCl, 1 mM sodium orthovanadate, 5 mM NaF, 5 mg/ml aprotinin, 5 mg/ml leupeptin, and 2 mM PMSF). Lysates were boiled in equal volumes of 4 Laemmli loading buffer for 7 min.

Whole-cell lysates were separated by SDS-PAGE and transferred to appropriate transfer membranes (Bio-Rad). For LC3-II and phospho-4EBP1, whole-cell lysates were subjected to Tris/Tricine, 15% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. For SQSTM-1/p62, phospho-p70-S6 kinase, ATG5, and ATG9A, whole-cell lysates were subjected to Tris-glycine, 10% SDS-PAGE and transferred to nitrocellulose membrane. For mTOR, whole-cell lysates were subjected 4 –20% gradient SDS-PAGE and transferred to nitro-cellulose membrane. Transferred proteins were probed with appropriated antibodies, according to the manufacturer’s instructions. Protein bands were
either observed on Blue X-ray film (Carestream) using ECL Select™ Western blotting detection reagent from GE Healthcare (RPN2235) for enhanced chemiluminescence or using Odyssey CLX Imaging System (LI-COR Biosciences) for infrared fluorescence.

2.3.5 Determination of intracellular parasite burden

For parasite infection rate and burden, infected dTHP-1 cells were briefly fixed using ice cold 2% paraformaldehyde in phosphate-buffered saline (PBS), for 15 min and protected from light. The fixed cells were washed twice with PBS and placed onto Prolong™ Diamond Antifade Mountant with DAPI (Life Technologies, Inc.). DAPI was used to stain macrophage and parasite nuclei to determine the number of infected macrophages. Cell images were acquired at 40 using Zeiss Axioplan 2 imaging microscope. At least 100 cells were counted for each condition to determine the average number of parasites per macrophage and percent macrophages infected.

2.3.6 Confocal microscopy

For the infection, stationary phase (day 5) *L. donovani* promastigotes were spun down, washed twice with PBS, and resuspended in 1 ml of PBS with 6 ml of 1 mM CellTrace™ CFSE. After a 30-min incubation at 37 °C, parasites were spun down and resuspended in 10% fetal calf serum in PBS. Promastigotes were then spun down and resuspended into PBS to infect dTHP-1 at an m.o.i. of 20:1.

For the fixation, cells were washed once with HBSS and twice with PBS. Then the cells were fixed with ice-cold 2% paraformaldehyde in PBS for 15 min, protected from light. The cells were then washed twice with PBS. The cells, with the respective treatments, were stained for LC3-II and DAPI. The cells were blocked with 5% NGS, 0.3% Triton X-
100 in PBS for 1 h. The primary antibody for LC3-II was used at a 1:400 dilution in 1% BSA, 0.3% Triton X-100 in PBS, overnight at 4 °C. The secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (H L), was used at a 1:250 dilution in 1% BSA, 0.3% Triton X-100 in PBS for 2 h. The cells were placed onto Prolong™ diamond antifade mountant with DAPI. Cells were imaged using Zeiss LSM 780 confocal microscope under 63 magnification and Zen software. For the analysis of LC3-II puncta, ImageJ macro plugin software was used as described previously (69). The analysis was done on 100 cells over three independent experiments.

2.3.7 Parasite rescue and transformation assay
For this assay, dTHP-1 cells were infected with L. donovani promastigotes at an m.o.i. of 20:1. After the desired period of infection, cells were extensively washed with HBSS to remove non-internalized parasites. Controlled lysing of infected cells was performed using 0.01% SDS as described previously (70). Quantification of the infection was performed through transformation of live, rescued Leishmania amastigotes to log phase promastigotes in M199 media by incubating the plates in 26 °C for 48 h. The evaluation of their growth was performed by manual counting of transformed promastigotes using trypan blue solution (0.4% w/v in PBS) and a hemocytometer. Counts were taken from each group in triplicates.

2.3.8 IMPase assay
IMPase activity was assayed by measuring the conversion of inositol 1-phosphate into inositol and inorganic phosphate. The release of inorganic phosphate was then measured using malachite green (71). Control and Leishmania-infected cells were extensively
washed with warm HBSS to remove serum and non-internalized parasites. Then, cells were washed three times with hypotonic buffer (20 mM Tris-HCl, pH 7.8), and the plate was placed on ice. Cells were then dislodged and disrupted in ice cold extraction buffer (50 mM Tris-HCl, pH 7.8, 250 mM KCl, 3 mM MgCl$_2$ supplemented with aprotinin, leupeptin, and PMSF) by passing several times through a 22-gauge needle. The resulting cell extracts were left on ice for 10 min and then clarified at 10,000 g for 10 min at 4 °C. Equal amounts of proteins from control and infected cells were assayed for phosphatase activity using 0.4 mM inositol 1-phosphate (Sigma) as the substrate, at 37 °C for 30 min, and the reaction was stopped with malachite green reagent. Inorganic phosphate present in each well was calculated by reading the OD$_{620}$ against a standard curve. Enzyme activity was then calculated by subtracting the inorganic phosphate formed in wells with cell extract and inositol 1-phosphate from inorganic phosphate formed in corresponding wells with cell extract not containing inositol 1-phosphate.

2.3.9 siRNA knockdown
The oligoribonucleotides targeting the cDNA sequence of human ATG5 and human ATG9A, as well as non-specific control siRNA, were obtained from OriGene (catalog no. SR306286 for ATG5 and SR312320 for ATG9A). THP-1 cells in 24-well dishes were transfected with non-specific or ATG5 siRNA or ATG9A siRNA (50 pmol/well) using HiPerFect transfection re-agent (Qiagen) according to the manufacturer’s instruction. After 48 h of transfection, the cells were differentiated with 10 ng/ml PMA and infected with L. donovani (m.o.i. 20:1) for 24 h before preparation of whole-cell lysates or of controlled lysis for parasite rescue.
2.3.10 Statistical analysis
The data of three independent experiments were determined using a paired \( t \) test on
GraphPad Prism 6.0 software. The values were considered statistically signification at *,
\( p \leq 0.05 \); **, \( p \leq 0.01 \); ****, \( p \leq 0.0001 \).
Chapter 3: Thesis Discussion

For decades, autophagy was unequivocally seen as a generalized, cellular degradation process that occurred within eukaryotes. Its presence in the majority of eukaryotes, ranging from yeast to *C. elegans* to mammalians cells, made it a *bona fide* mechanism for maintaining homeostasis [216]. Most recently, several diverse forms and functions of autophagy have been identified and studied; these branches of autophagy give insight into the complexity that is beyond the simplified cellular degradation process. Of particular interest, in this study, we observed the role that autophagy plays in innate immunity, specifically looking at the interactions between human macrophages and the parasite, *Leishmania donovani*.

Intracellular microorganisms are notorious for their ability to manipulate host cell function. Therefore, altering of the host’s autphagic pathway or machinery is not beyond the scope of these organisms’ abilities. Microbes, such as *Mycobacterium tuberculosis*, *Toxoplasma gondii* and *Plasmodium berghei*, are able to modulate macrophage function, particularly pertaining to autophagy [148], [179]–[181]. Moreover, several leishmania species are, similarly, able to affect host autophagy. Current research has found that *Leishmania mexicana* and *Leishmania amazonensis* are able to utilize the cargo of autophagosomes and increase their survival within macrophages [182], [185], [188]. Conversely, the induction of autophagy has been found to be detrimental for *Leishmania major* survival [106], [183]. The disparity between these findings may be related to different species of leishmania or other factors, including macrophage cell type.

In recent years, research has shifted towards understanding the molecular mechanisms underlying this regulation. Some of these mechanisms, including
Leishmania donovani altering the microRNA profiles in macrophages to increase the expression of autophagy specific microRNAs or the induction of autophagy through endosomal Toll-like receptors to protect, macrophages against Leishmania major [184], [186].

Our research augments the current and emerging evidence into understanding the molecular mechanisms that govern autophagy during an infection. Firstly, we present evidence for time dependent regulation of autophagy (Figure 2.1). The early time points of infection did not evoke an autophagic response and this could have been due to either active inhibition or to a passive response. However, Leishmania donovani was able to attenuate rapamycin-induced autophagy (Appendix A, B, C and Figure 2.2) pointing towards active inhibition. Rapamycin is a potent mTORC1 inhibitor that can readily induce autophagy. It associates with the immunophilin protein, FKBP12 to form a complex and allosterically binds to mTORC1. The result of this binding is the inactivation of mTORC1 enzymatic activity thereby promoting the up-regulation of autophagy [191], [217]. As such, it is apparent that during this early period, Leishmania donovani can actively attenuate autophagy, in an mTOR-dependent manner. Interestingly, previous research has shown that Leishmania donovani activates Protein kinase B (Akt), within macrophages [80], [81], [190]. Both Akt and mTORC1 are salient kinases involved in numerous cellular signaling pathways, particularly for autophagy. The upstream activation of Akt leads to activation of mTORC1 activity [218]. However, there are multiple pathways that intersect into and can affect these proteins. Evidently, Leishmania donovani seems to use the Akt/mTOR axis to modulate macrophage autophagy (Figure 2.3). By blocking the activation of Akt with an Akt inhibitor (Akti), Leishmania donovani
was no longer able to inhibit rapamycin-induced autophagy. These results add to current knowledge that this pathway is important for the survival of the intracellular parasite [107]. While these results do not obviate the regulation by other mechanisms or signaling pathways, it strongly indicates that it has a role in the parasite’s survival. The most recent findings have shown that *Leishmania donovani* up-regulates the expression of MIR30A-3p in macrophages, early on, to prevent the induction of autophagy. MIR30A-3p is a known micro RNA that inhibits autophagy [186]. As mentioned, it may be possible for leishmania to synergistically use mechanisms to assure autophagic inhibition during early infection. Furthermore, this blockade is necessary for an established infection to ensue and promote the survival of intracellular leishmania. By amplifying macrophage autophagy and subsequently infecting macrophages with leishmania promastigotes, it was apparent that macrophages primed for an induction in autophagy were able to clear intracellular parasites (Figure 2.8). In turn, this shows the deleterious effects of early autophagic induction on leishmania survival.

With that being stated, it was intriguing to explore the effect of autophagy induction during the later stage of infection. It has shown that species of leishmania are capable of inducing autophagy, particularly *Leishmania donovani* which can have a delayed induction [182], [186]–[188]. Our results were able to corroborate these findings as noted by the time dependent increase in autophagic activity (Figure 2.1). However, the mechanistic basis for this induction still remained unclear. Autophagic regulation, through various cell signaling pathways, have been extensively studied [219], [220]. In general, these cell signaling pathways fall under two broad categories, either mTOR-dependent or mTOR-independent. Classical regulation is mediated by the mTOR-dependent pathway
and has been extensively studied for its role in cellular nutrient sensing, particularly during starvation. The PI3K/Akt/mTOR pathway is one such example of classical autophagic regulation and thus it was reasonable to propose that *Leishmania donovani* may use it not only for early inhibition of autophagy, but also for induction of late stage autophagy. To address this, we observed mTOR activity by monitoring the downstream targets of the kinase. Reduced phosphorylation of mTOR should correspond to reduced phosphorylation of ribosomal protein S6 kinase-1 (p70S6K) and translational initiation factor 4E binding protein-1 (4E-BP1). The former target protein phosphorylates the 40S ribosome component, ribosomal protein S6, to promote cell growth, while the latter binds to eukaryotic initiation factor 4A (eIF4A) to repress its function and thereby inhibit protein translation [221]–[224]. Interestingly, the results showed that during a 24-hour infection, *Leishmania donovani* did not alter the activity of mTOR (Figure 2.4). Previous research had shown that in murine macrophages, *Leishmania major* was able to proteolytically degrade mTOR by the metalloprotease protein 63 (gp63) [99]. This degradation could result in the increase in autophagic activity; however, with *Leishmania donovani*, there was no such degradation of the kinase (Figure 2.4). In fact, it was apparent that the infection, during this late stage, was still actively inhibiting mTOR-dependent autophagy, without altering the levels of mTOR. During the 24-hour infection, *Leishmania donovani* markedly enhanced Akt phosphorylation in macrophages (Figure 2.6 A). Moreover, the potent repression of Akt phosphorylation, by an Akt 1/2 inhibitor, amplified the autophagic induction in leishmania-infected macrophages (Figure 2.6 B). In addition to this, macrophages infected for 24 hours were able to confer resistance to rapamycin-induced autophagy (Figure 2.5). These findings strongly indicate that *Leishmania donovani*
actively represses the mTOR-dependent induction of autophagy. Moreover, the PI3K/Akt/mTOR axis has been implicated in several other cellular processes, including the regulation of apoptosis [225], [226]. The multifarious effects by this pathway may explain the necessity, for *Leishmania donovani*, to not only inhibit mTOR dependent autophagy, but also prevent apoptosis and promote the up-regulation of an anti-inflammatory environment [81], [107].

Notwithstanding these inhibitory effects, *Leishmania donovani* was still able to induce late stage autophagy and quite possibly through an mTOR-independent pathway. One such pathway that was germane to our research was the phosphoinositol signaling pathway. Activation of the membrane bound enzyme, phospholipase C results in the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3) [127], [227]. The latter acts as a vital second messenger eliciting the release of calcium ions from the endoplasmic reticulum and this mass influx of calcium can affect numerous signaling pathways including alternative mTOR-independent pathways (Kania, Roest, Vervliet, Parys, & Bultynck, 2017). Inositol triphosphate, in addition, can be dephosphorylated along the cyclical pathway by phosphatases, such as 5’-phosphatase and inositol polyphosphate 1-phosphatase (IPPase). Ultimately, after dephosphorylation, the resulting molecule is free inositol that is necessary for phosphoinositol signaling. Signaling by these free inositols results in the replenishment of IP3 and can thereby continue the phosphoinositol cycle [127].

The key phosphatase involved in the formation of free inositols is inositol monophosphatase (IMPase) and as such, was considered to be an important player in understanding the role of mTOR-independent autophagy [229], [230]. Numerous
publications have implicated the role of this enzyme with the induction of mTOR-independent autophagy. A recent publication found that carbamazepine, an antiepileptic medication, lowers the inositol levels to induce mTOR-independent autophagy. This induction has been shown to promote the clearance of *Mycobacterium tuberculosis* in macrophages [231]. However, the primary research focus, for mTOR independent autophagy, has been on neurodegenerative disease, such as Huntington disease. The findings have shown that mood stabilizing drugs, particularly lithium chloride, can inhibit the activity of IMPase, therein induce autophagy. By promoting autophagy, aggregates of proteins, such as alpha-synuclein, can be readily degraded [199], [232], [233]. In this way, by impeding IMPase activity, it is quite plausible that the perturbation in the cyclic signaling pathway would induce autophagy. Intriguingly, a past publication from the Reiner laboratory found that *Leishmania donovani* was able to lower IP$_3$ levels in macrophages [213]. Tying these two findings together, we investigated the activity of macrophage IMPase during *Leishmania donovani* infection. Our findings show that at 24 hours of infection, intracellular *Leishmania donovani* is capable of drastically reducing the activity of IMPase (Figure 2.7). This result indicates that there is a modulation of the phosphoinositol cycle allowing for the induction of autophagy, through an mTOR-independent pathway. Yet, as aforementioned, with *Leishmania donovani*, it is quite reasonable for it to harness the effects from more than one pathway to attain the appropriate response. In addition to the phosphoinositol cycle signaling pathway, calcium dependent autophagy can be affected by the depletion of intracellular IP$_3$. It can potentially skew the efflux of calcium ions from the endoplasmic reticulum to the cytosol of the macrophage, as the IP$_3$ receptor-mediated channels have a reduced activity. With
a decreased release of calcium ions into the cytosol, autophagy can be up-regulated [127], [228]. Interestingly, it has been shown that *Leishmania donovani* can perturb the intracellular calcium ion levels [234]. Taken together, it may be possible for *Leishmania donovani* to utilize other mTOR-independent pathways simultaneously to achieve its optimal survival.

Despite having identified the mechanisms of autophagic regulation, the question still remained as to whether or not autophagy was either advantageous or detrimental to the survival of the intracellular *Leishmania donovani*. Research pertaining to impact of host autophagy on various pathogens has been remarkably polarized. For example, the intracellular bacterium, *Listeria monocytogenes*, actively attempts to block host autophagic induction and it has been found that induction of autophagy results in clearance of the bacteria [235]–[238]. Conversely, the intracellular parasite, *Toxoplasma gondii*, actively promotes host autophagy to ensure nutrient uptake and its [180], [181]. Adding to that notion, even among different species of leishmania and macrophages, there is an apparent heterogeneity in the autophagic responses and any impact on pathogen survival. For example, *Leishmania amazonensis* induces autophagy to promote parasite load and survival in macrophages derived from BALB/c mice, yet this was not seen using C57BL/6 murine macrophages [182], [185]. *Leishmania major* infections, on the other hand, have been associated with parasite clearance via macrophage autophagy [106], [183], [184]. As discussed above, inducing autophagy prior to the establishment of infection with *Leishmania donovani* resulted in markedly reduced survival of intracellular parasites (Figure 2.8). However, hindering an autophagic
induction, during the late stage of infection, had also proved to be detrimental for the intracellular survival of *Leishmania donovani*.

Understanding the roles of individual components of the autophagic machinery has been the subject of extensive research, particularly considering that it tends to be conserved among eukaryotes. The autophagy-related proteins (ATGs; Autophagy-related proteins) play indispensable roles in the autophagic pathway [239]. Two such ATG proteins are autophagy related protein 5 (ATG5) and autophagy related protein 9 (ATG9). The distinct functions of both of these proteins, along with the fact that both proteins function independently of one another, make them ideal choice to study. ATG5, in conjugation with ATG12 and ATG16L, assists in the elongation of the pre-autophagosomal structure (PAS) and, more importantly, is responsible for anchoring the LC3-II molecules onto the double membrane. ATG9, on the other hand, has been shown to be the sole trafficker of membrane vesicles to the PAS, resulting in elongation of the autophagosome [125], [128], [240]–[243]. By down regulating both key proteins, there was a noted defect on the autophagic pathway and this resulted in markedly reduced parasite survival (Figure 2.9).

The duality involving *Leishmania donovani* and human macrophages, in relation to autophagy, makes this research nuanced and intriguing. On the one hand, inducing autophagy in the early stages of infection, through the Akt/mTOR axis, was devastating for the survival of *Leishmania donovani* (Figure 2.8); indeed, it is noteworthy that the parasites, at this time, actively abrogated the induction of autophagy (Figure 2.6). On the other hand, inducing autophagy in the later stages of infection, independent of the Akt/mTOR axis, is beneficial to the parasite survival. The carefully timed orchestration of
these two countervailing responses makes it clear that, *Leishmania donovani* has evolved mechanisms to can commandeer macrophage autophagy to optimize its survival.

This novel idea of having a controlled response, which is dependent on the host’s autophagic cell signaling pathway, makes this initial research topic a gateway into further understanding the subject. As stated before, the complexity of these autophagic pathways, whether it be an activating or inhibiting, makes studying it, in relation to an infectious disease, far more intriguing. From the data presented, there is a plausible selective response, suggesting that *Leishmania donovani* may be able to preferentially choose one pathway or several pathways for its intracellular growth. One such pathway that has the potential to be studied further is the calcium mediated autophagy pathways [127]. As it is known that *Leishmania donovani* can disrupt the egression of calcium from either the endoplasmic reticulum to the cytosol or the cytosol to extracellular milieu, it can be hypothesized that this affects autophagy through this calcium mediated pathway [234]. Moreover, as we have shown, in congruence with other research, *Leishmania donovani* can harness the appropriate autophagic response, during an early infection, from more than one autophagic pathway [186].

Another interesting point that my research raises is the topic of selective autophagy. As mentioned earlier, autophagy was initially generalized as the bulk degradation of intracellular components, during cell starvation [244]. However, further insight into autophagy has shown that there is an element of selectivity in the mechanism. The term was coined as selective autophagy; a process by which receptors, on the autophagophore, can recognize and bind to specific cargo to ensure its clearance. This blanket term includes the processes such as mitophagy and agrapha, which result in the
clearance of damaged mitochondria and protein aggregates, respectively [245]. As we have shown that *Leishmania donovani* preferentially regulates specific autophagic pathways, it is quite plausible that this regulation may also play a role in promoting the selective uptake of specific cargo. Recent protocols have allowed for the isolation of autophagosomes, which may be subjected to proteomic analysis [246]–[248]. Using this approach, isolating and analyzing the autophagosome cargo of macrophages infected with the parasite may give insight into how this promotes leishmania survival. Furthermore, research has found that proteins containing the LC3 interacting region (LIR) are critical for selective autophagy, particularly p62 [203], [249]. We have also shown there is a regulation of p62 levels in infected cells, which may also suggest a role for selective autophagy for leishmania survival.

Research into leishmania pathogenesis has been an ongoing topic as the parasite is well versed at manipulating and hijacking its resident host. Here, our research illuminates one such mechanism that is readily utilized by *Leishmania donovani*. It is able to, overall, biphasically regulate autophagy with early stage active inhibition and late stage activation. Moreover, it appears to selectively choose pathways for autophagic regulation that give optimal survival within the macrophages. While our research has given a fair amount of insight into leishmania pathogenesis and host autophagy, there is still much to be known and further studied, in this context.
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Appendix A. Host macrophage endogenous LC3-II markedly accumulates in the presence of E64d and pepstatin A during leishmania induced autophagy. dTHP-1 cells were incubated with *L. donovani* promastigotes for 24 h. Subsequently, cells were washed and treated with the cathepsin inhibitors, E64d + Pepstatin A (10 µg/mL per inhibitor) for 4 h. Whole cell lysates from uninfected and infected cells were analyzed for LC3-II levels. Actin levels were also analyzed as loading controls. The histogram shown is a densitometric analysis of relative LC3-II levels in three independent experiments.
Appendix B. Host macrophage endogenous p62 accumulates in the presence of E64d and pepstatin A during leishmania induced autophagy. dTHP-1 cells were incubated with *L. donovani* promastigotes for 24 h. Then, cells were washed and treated with the cathepsin inhibitors, E64d + Pepstatin A (10 µg/mL per inhibitor) for 4 h. Whole cell lysates from uninfected and infected cells were analyzed for p62 and actin levels. Densitometry analysis of three independent experiments. Data are presented as mean ± SD, ns = not significant, *p < 0.05, **p < 0.01.
Appendix C. *Leishmania donovani* actively counters rapamycin-induced p62 degradation at the early stage of infection. dTHP-1 cells were incubated with *L. donovani* promastigotes for 6 h. Subsequently, the cells were incubated with rapamycin (12.5 µg/mL) for 2 h. Whole cell lysates were then collected and analyzed by immunoblotting for p62. The same membrane was stripped and reprobed for actin as a loading control. (A) Western blot for p62 and actin levels. Densitometry analysis of three independent experiments. Data are presented as mean ± SD, ns = not significant, *p < 0.05, **p < 0.01.