Countervailing, time-dependent effects on host autophagy promote intracellular survival of *Leishmania*

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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 down-regulation 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.

The leishmaniases are a group of vector-borne infectious diseases that are primarily endemic to tropical and sub-tropical regions of the world. The populations affected tend to be from lower socioeconomic strata, and there are projected to be approximately 12 million people affected worldwide.

Beyond this, there are an estimated 350 million people across 88 countries living at risk for contracting leishmaniasis. 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 range 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. The main etiological agent for human visceral leishmaniasis is *Leishmania donovani*.

Among various phagocytic cells, *Leishmania* primarily targets macrophages. Like all *Leishmania* species, *L. donovani* has a digenetic life cycle, transitioning from the motile promastigote form within the sandfly 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. For example, we showed that *Leishmania* infection of both murine and human macrophages hijacks the PI3K\(^3\)/Akt pathway, leading to the inactivation of glycogen synthase kinase-3\(^\beta\) (GSK-3\(^\beta\)) and the induction of IL-10 production, via enhanced activity of the transcription factor cAMP-response element-binding protein.

In this 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.

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whether *Leishmania* infection modulates host cell autophagy via an mTOR-dependent pathway and, importantly, how 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 (8). More than 30 autophagy-related proteins (ATGs) have been identified. Among 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 (9).

Autophagy can be regulated via multiple signaling pathways. Broadly, the two commonly defined pathways are either mTOR-dependent or mTOR-independent. As mentioned previously, 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 (8). For example, inositol-lowering agents, such as lithium, induce autophagy independent of any change in mTOR activity (10).

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* (11).

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 (12). Another study reported the accumulation of LC3-II in human bone marrow cells during *L. donovani* infection (13). Furthermore, induction of autophagy in infected macrophages has been linked to increased growth and parasite load of *Leishmania amazonensis* (14, 15). Most recently, it has been found that *Leishmania major* uses macrophage autophagy to inhibit T-cell responses and prevent parasite clearance (16). 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 (17, 18). It has also been shown that autophagy induction through endosomal Toll-like receptors plays a role in macrophages conferring resistance against *L. major* infections (19).

In this 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 alternative 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 survival.

**Results**

**Infection by *L. donovani* initially inhibits and then induces host macrophage autophagy in a biphasic 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-I (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 may be detected by Western blot assay or by immunofluorescence microscopy as punctate vesicular LC3-II (20). We used both readouts to investigate the induction of autophagy in response to *L. donovani* infection in PMA-differentiated THP-1 cells that have been extensively used as a model system to study human visceral leishmaniasis (21).

To examine whether *L. donovani* induces autophagy, THP-1 cells were infected with stationary phase *Leishmania* promastigotes, and autophagy was monitored by Western blottings using LC3-II-specific antibodies over 48 h. As shown in Fig. 1, expression of LC3-II early after infection (2–8 h) was not affected relative to control cells. However, over the course of longer infection (24–36 h), there was a clear increase in LC3-II expression in infected cells.

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 the lysosomal degradation inhibitors pepstatin A and E64d (9) and were assessed by Western blotting 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 (22). Indeed, the results presented in Fig. S1 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 *per se* 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 an increased autophagic flux. Conversely,
increased levels of p62 are indicative of inhibition of autophagy (23–27). As shown in Fig. S2, late Leishmania infection resulted in the depletion of p62 levels, compared with control, confirming an induction of autophagic flux. Lysosome-mediated autophagic degradation was confirmed by treating control and infected cells with pepstatin A and E64d, as shown in Fig. S2. Treatment with pepstatin A and E64d, in Leishmania-infected cells, resulted in the restoration of p62 levels, as expected. Taken together, the LC3-II and 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 (Fig. S3, lanes 1 and 2). The results showed the accumulation of p62, indicative of active autophagic inhibition (Fig. S3, lanes 1 and 2). To test the possibility that 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 (29, 30). As shown in Fig. 2, A and B, Leishmania infection nearly completely abrogated rapamycin-induced autophagy at 6 h post-infection. dTHP-1 cells treated with chloroquine (12.5 μM for 2 h) 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 Fig. S3, lanes 3 and 4. Notably, Leishmania did not affect accumulation of LC3-II in response to chloroquine (Fig. 2, A and B), which acts through a distinct pathway from that of rapamycin (30, 31). 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 the dTHP-1 cells. For this assay, we also used carboxyfluorescein succinimidyl ester (CFSE)-positive 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 the 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 Fig. 2, C and D. As expected, based on the Western blotting results in Fig. 2, A and
during the first 6 h infection with *Leishmania* was associated with inhibition of rapamycin-induced autophagosome formation (Fig. 2, C and 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 (Fig. 2) and induction of host autophagy as infection progresses (Fig. 1) show dynamic biphasic regulation of host autophagy in response to *Leishmania*.

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 (5, 28). To investigate this possibility, cells were first treated with Akt1/2 inhibitor for 4 h; subsequently, the cells were infected with *Leishmania* 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 Fig. 3 show that prior treatment with the 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).

*L. donovani* induces host autophagy at later stages of infection despite actively attenuating mTOR-dependent autophagy

As shown in Fig. 1, *Leishmania* induced autophagy at later stages of infection, and it was of interest to ask whether this involved the use of the classical mTOR pathway or possibly an alternative 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 Thr-389 and the phosphorylation of the eIF4E inhibitor, 4E-BP1, at multiple sites (32). As shown in Fig. 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 a positive control did down-regulate mTOR activity as expected (Fig. 4). These results indicate that neither degradation of mTOR nor inhibition of host mTOR activity was likely to be the mechanism by which *Leishmania* induced delayed host cell autophagy.

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 (Fig. 5, A and B), and these results were confirmed by confocal microscopy (Fig. 5, C and 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 (Fig. 6 A). These results, along with the findings reported in Fig. 2 and Fig. S3, 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 the Akt1/2 inhibitor (Sigma). 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 Fig. 6 B, where it is also shown that *Leishmania*-induced autophagy was significantly enhanced in the presence of the Akt inhibitor. These findings provide support for a model in which *Leishmania* uses the PI3K–Akt–mTOR pathway to down-regulate classical autophagy, while at the same time using an alternative mTOR-independent pathway to induce autophagy in response to infection.

**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 (33). One of the
first of these to be reported is linked to an inositol-signaling pathway where elevated levels of IP₃ 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 (10). Our interest in this mTOR-independent pathway arose from our previous finding of reduced IP₃ levels in cells infected with *L. donovani* (34). Thus, we hypothesized that *Leishmania* might engage an mTOR-independent pathway by reducing the concentrations of IP₃ leading to induction of delayed autophagy. IMPase is the key enzyme required to generate free inositol that is essential for the inositol-signaling pathway to function (35). Therefore, we measured the enzymatic activity of IMPase in *Leishmania*-infected cells and found that it was significantly reduced (Fig. 7). These findings identify one potential mTOR-independent pathway that may be used for the induction of delayed autophagy by *Leishmania.*

**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 under “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 Fig. 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).

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, we elected to study two autophagy-related proteins. One protein is part of the two-conjugation system, autophagy protein 5 (ATG5), although 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 phosphatidylethanolamine to form LC3-II and for the elongation of autophagic membranes (36). ATG9A is important for adding membrane to the autophagosome during its formation (37). We used siRNAs to down-regulate ATG5 or ATG9A. Treatment of cells with specific siRNAs prior to infec-

Figure 6. 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.
Leishmania exploits host cell autophagy

Figure 7. Leishmania 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 **p** values are 0.0001.

Figure 8. Rapamycin pre-treatment of host macrophages results in the reduced survival of Leishmania. 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 “Experimental procedures.” On day 5, transformed motile promastigotes were counted. A, histogram shown is the result of L. donovani 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.

Discussion

Diverse intracellular microbes, including Leishmania (38–41), Yersinia (42), Coxiella (43), Mycobacterium (44) and others, have evolved mechanisms to modulate numerous macrophage functions to facilitate their survival within host cells. One of these mechanisms, used by Leishmania, is to create an immunosuppressive environment by promoting the production of the cytokine IL-10 (45). In a recent study, we examined the molecular mechanism used by Leishmania to induce host IL-10 production and defined a key role played by the PI3K/Akt pathway (5). Given that this pathway also regulates mTOR-dependent autophagy (46, 47) and the importance of the latter to host defense (48), in this study we investigated what impact activation of the host PI3K/Akt pathway by L. donovani might have on autophagy.

Autophagy is a conserved, dynamic process in which intracellular components are packaged within autophagosomes that ultimately fuse with lysosomes leading to degradation of vesicular cargo. Classical regulation of autophagy involves mTOR, which functions as a negative regulator (8). Various growth factors can modulate mTOR activity, and a major signal cascade for its regulation is via the PI3K/Akt pathway (49).

Several species of Leishmania have been observed to induce host autophagy, but the molecular mechanism(s) involved in the autophagy response have only recently begun to emerge (12–19). For example, two recent studies reported a link between Leishmania-induced autophagy and a differentially expressed set of host miRNAs, including MIR-30A-3p in infected cells (17, 18).

To study Leishmania-induced autophagy further, in this study, we established a robust model of autophagy induction in response to L. donovani infection (Fig. 1). This involved delayed kinetics with induction of macrophage autophagy requiring at least 24 h to appear. Strikingly, we found that the apparent unresponsiveness of host cells at earlier time points (6 h) was not simply a passive encounter, but rather it was due to active inhibition of autophagy by Leishmania. This conclusion is based on the finding that during early stages of infection Leishmania was able to completely abrogate rapamycin-induced autophagy (Fig. 2 and Fig. S3). These results suggested that as a survival strategy, Leishmania may actively inhibit early stage autophagy possibly by using the PI3K/Akt pathway to activate mTOR. In fact, further investigation showed the clear involvement of this pathway in the inhibition of autophagy at early stages of Leishmania infection. This is based on the finding that pre-treatment of host cells with the Akt1/2 inhibitor impeded the ability of Leishmania to attenuate rapamycin-induced autophagy, at early stages of infection (Fig. 3). However, this does not preclude the engagement of other autophagy regulatory pathways. For example, recent findings have shown the role of miRNAs in autophagy regulation, particularly MIR-30A-3p, in Leishmania-infected macrophages (18). As a corollary, it was of interest to investigate whether early induction of host autophagy would be deleterious to Leishmania survival.
To examine this possibility, first autophagy was induced using rapamycin for 2 h, followed by L. donovani infection for 24 h. At the end of the experiment, the viability of internalized parasites was tested by transforming them into motile promastigotes (Fig. 8). This showed that early induction of autophagy was in fact deleterious to Leishmania and suggested that attenuation of autophagy at early stages of infection is a pathogen survival strategy. Although rapamycin has been used extensively in the literature as an inducer of autophagy by inhibiting mTOR, there is the potential for off-target effects that may have affected the survival of intracellular Leishmania. However, rapamycin did not directly affect the growth and replication of L. donovani promastigotes in culture (data not shown). This is not surprising as the L. donovani TOR homolog shares only 23% homology with human mTOR. The infectiveness of rapamycin on growth and proliferation of L. major promastigotes in culture has also been previously reported (50). To the best of our knowledge, the ability of L. donovani to actively inhibit classical mTOR-regulated autophagy at early stages of infection is a novel strategy that promotes pathogen survival (Figs. 2 and 3 and Fig. S3).

Nevertheless, recent reports and the present findings make it clear that Leishmania does activate autophagic machinery in host cells, albeit with delayed kinetics (Fig. 1), in the case of L. donovani (13, 18). In respect to the latter, it was of interest to establish whether delayed autophagy involves mTOR-dependent cell signaling or an alternative signaling pathway that is mTOR-independent. To address the mechanism of late stage (24 h) autophagy induction by L. donovani, we examined whether this correlated with a decrease in mTOR activity based upon phosphorylation of S6 kinase and 4EBP-1 as surrogate markers (32, 51–53). Surprisingly, Leishmania infection did not lead to a decrease in mTOR activity (Fig. 4). Recently, proteolytic inactivation of mTOR in L. major-infected bone marrow-derived macrophages by Leishmania metalloprotease glycoprotein 63 was reported (54). Therefore, we examined mTOR levels from L. donovani-infected cells by Western blotting. In contrast to the findings by Jaramillo et al. (54), L. donovani-infected dTHP-1 cells showed no evidence of proteolytic inactivation of mTOR (Fig. 4, bottom panel). This result indicates that unlike the case for L. major and murine bone marrow-derived macrophages, proteolytic degradation of mTOR was not likely to be involved in the induction of autophagy in dTHP-1 cells infected with L. donovani. Taken together, these results show that Leishmania does not use an mTOR-dependent pathway to induce late stage autophagy. In fact, Leishmania appears to inhibit mTOR-dependent autophagy at late stages of infection. This interesting result is supported by three findings. First, Leishmania markedly enhanced phosphorylation of host Akt at a late stage of infection, which would be expected to inhibit mTOR-dependent autophagy (Fig. 6A). Second, and consistent with the latter, Leishmania-induced autophagy was significantly increased in the presence of the Akt1/2 inhibitor (Fig. 6B). Third, cells infected with Leishmania for 24 h remained resistant to rapamycin-induced autophagy (Fig. 2). These findings so far suggest the possibility that Leishmania uses an alternative mTOR-independent pathway to induce delayed host autophagy.

In addition to regulation of autophagy by mTOR, various mTOR-independent pathways have been reported (8, 55). These pathways are sensitive to chemical perturbations, including one that is sensitive to cellular concentrations of inositol (10). It has recently been shown that an inositol-signaling path-
way negatively regulates mTOR-independent autophagy (56). Interestingly, inositol-lowering agents, such as lithium chloride, induce autophagy without affecting mTOR activity. We hypothesized that *Leishmania* may induce delayed autophagy by impeding the phosphoinositol-signaling pathway. Support for this hypothesis came from our previous finding of reduced IP$_3$ levels in cells infected with *L. donovani* (34). In this report, we selected IMPase for study as this key enzyme is involved in inositol recycling and *de novo* synthesis of inositides (8, 56). We reasoned that if the activity of this enzyme is reduced in *Leishmania*-infected cells, then this would result in reduced cellular inositides and lead to induction of autophagy. In fact, this turned out to be the case. *Leishmania* significantly attenuated IMPase activity in infected cells (Fig. 7) supporting a model in which *Leishmania* induces autophagy at late stages of infection by modulating an mTOR-independent inositol-signaling pathway. However, this finding does not rule out the possibility that *Leishmania* also uses other mTOR-independent pathway(s) in addition to the inositol-signaling pathway to induce delayed host autophagy.

An important question that arises from these findings is whether host autophagy is involved in defense against *Leishmania* or whether it functions to promote parasite survival. Several reports focused on pathogens other than *Leishmania* have drawn opposing conclusions in this regard. For example, in *Helicobacter pylori* (57), *L. monocytogenes* (58), and *M. tuberculosis* infections (59, 60), autophagy appears to limit growth of these pathogens. In contrast, *T. gondii* (48), hepatitis C virus (61), and *Coxiella burnetii* (62) appear to take advantage of autophagy to support infection. In the context of *Leishmania* infection, it should be pointed out that the impact of host autophagy on parasite survival appears to depend upon the host and strain. For example, induction of autophagy correlated with increased parasite loads of *L. amazonensis* in BALB/c but not in C57BL/6 mice (14). A recent study using *L. major*-infected bone marrow-derived macrophages showed host autophagy was detrimental to pathogen survival (17). In contrast, it was recently reported that in a stationary phase, the *L. major* inoculum containing a significant number of apo-optosis-like parasites activated human macrophage autophagy machinery, thereby dampening T-cell responses and promoting parasite survival (16). In this study, inhibition of autophagy by down-regulating either ATG5 or ATG9A (both are essential constituents of autophagosome formation) reduced the survival of *Leishmania* inside the infected macrophages. This provided unambiguous evidence to show that autophagy is beneficial for the survival of *L. donovani* in human macrophages at a late stage of infection. In fact, *Leishmania*-induced autophagy in host macrophages could be an important determinant of nutrient supply where survival is largely dependent on host resources. In support of this suggestion, acquisition of host cell macromolecules by *L. mexicana* involved an autophagy-like mechanism (12).

A highly novel finding of this study is that *Leishmania* regulates host autophagy in a biphasic, time-dependent manner. Active attenuation of host autophagy by *Leishmania* at early stages of infection appears to be beneficial to the survival of as yet not fully differentiated organisms (Fig. 8). In contrast, the delayed kinetics of a robust autophagic signal, which occurred around 24 h post-infection, suggest the possibility that this response may have been elicited by increased nutrient demand. This was not supported by a decrease in mTOR activity, which has been shown in many studies to be the mechanism of autophagic stimulation by nutrient deprivation. An alternative possibility to consider is that delayed onset, *Leishmania*-induced autophagy may result from an active event brought about by infection. This could be linked to active inhibition of host IMPase resulting in up-regulation of mTOR-independent autophagy.

Autophagy was initially characterized as a non-selective bulk degradation pathway induced by nutrient deprivation (8, 63). However, it is now becoming clear that the recycling of damaged organelles, removal of dysfunctional protein aggregates, and elimination of intracellular pathogens are highly selective processes that require cargo recognition by the specialized autophagy machinery (64, 65). Therefore, it is reasonable to propose that *Leishmania* prefers cargo-selective autophagy, induced by inhibition of host IMPase activity for its optimal intercellular growth and survival. Support for this hypothesis will require characterization of autophagosomes and their cargo induced in response to *Leishmania* infection.

In summary, our results indicate that *Leishmania* uses dual strategies to exert countervailing effects on host autophagy. This presumably enables fine-tuning of autophagy to optimally promote parasite survival. On the one hand, *Leishmania* inhibits mTOR-dependent autophagy at both early and late stages of infection, most likely by sustained activation of host Akt and mTOR (Fig. 10). On the other hand, *Leishmania* infection induces mTOR-independent autophagy, at later stages of infection, likely by down-regulating host IMPase activity (Fig. 10). It is tempting to speculate that *Leishmania* might derive a number of advantages through this bidirectional, time-dependent regulation of host autophagy. First, inhibition of autophagy at early stage infection by activating host Akt would provide an opportunity for *Leishmania* promastigotes to begin to differentiate into amastigotes to adapt the harsh conditions of phagolysosomes. Second, sustained activation of host Akt would contribute to inhibition of apoptosis, which is a potent pathogen clearance mechanism. In this context, it is known that *Leishmania* confers host cell resistance to apoptosis by activating Akt (28, 66). Third, activation of mTOR-independent autophagy, in the background of sustained activation of the PI3K-Akt pathway, would allow for fine-tuning of autophagy for optimal acquisition of essential nutrients from the host.

The molecular triggers used by *Leishmania* to either resist (early infection) or promote (later infection) autophagy are not completely known. Recently, we and others have shown that *Leishmania* contains specialized secretory vesicles, termed exosomes, that release multiple parasite-encoded proteins into the host cell during infection (67, 68). It is possible that one or more of these exported proteins regulates host cell autophagy. Currently, work is in progress to investigate whether *Leishmania* exosomes have the potential to regulate host autophagy. In fact, our initial findings show that incubation with *Leishmania*
exosomes activates macrophage Akt. This should be a fruitful area for ongoing studies. In addition, our host-parasite system presents a novel opportunity for the coordinated investigation of apoptosis, autophagic signals, and *Leishmania* pathogenesis.

**Experimental procedures**

**Antibodies and reagents**

Primary antibodies for LC3-II, SQSTM-1/p62, phospho-p70S6 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 form 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 protease inhibitor, pepstatin A, PMA, leupeptin, aprotinin, normal goat serum (NGS), bovine serum albumin (BSA), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma.

**L. donovani culture**

Sudan strain S2 promastigotes were incubated and cultured at 26 °C, in M199 media (Sigma) with 10% heat-inactivated fetal calf serum (Gibco), 20 mM HEPES (Sigma), 100 units/ml penicillin/streptomycin (Sigma), 100 μM adenosine (Sigma), 2 mM L-glutamine (Gibco), 6 μg/ml hemin (Sigma), and 10 μg/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.

**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.
Leishmania exploits host cell autophagy

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 μg/ml aprotinin, 5 μg/ml leupeptin, and 2 mM PMSF). Cells 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, 10% SDS-PAGE and transferred to nitrocellulose membrane. For mTOR, whole-cell lysates were subjected 4–20% gradient SDS-PAGE and transferred to nitrocellulose membrane. Transferred proteins were probed with appropriate 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 (LI-COR Biosciences) for infrared fluorescence.

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 ProlongTM diamond antifade mountant with DAPI (Life Technologies, Inc.). DAPI was used to stain macrophage and percent macrophages infected. Cell images were acquired at ×40 using Zeiss Axiosplan 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.

Confocal microscopy

For the infection, stationary phase (day 5) L. donovani promastigotes were spun down and resuspended in 1 mL of PBS with 6 μl of 1 μM 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.

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.

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 MgCl₂, 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 OD₆₅₀ 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.

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 reagent (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.

Statistical analysis

The data of three independent experiments were determined using a paired t test on GraphPad Prism 6.0 software. The val-
ues were considered statistically signification at *, p < 0.05; **, p < 0.01; ****, p < 0.0001.

**Author contributions**—S. A. T., D. N., N. E. R. designed the research. S. A. T., D. N., and J. K. performed the experiments and analyzed data. S. A. T., D. N., and N. E. R. prepared and wrote the manuscript.

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**References**

1. World Health Organization (2017) Leishmaniasis. Fact sheet 375 [www.who.int/mediacentre/factsheets/fs375/en/]
2. Alvar, J., Vélez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., and WHO Leishmaniasis Control Team (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7, e35671 CrossRef Medline
3. Kedzierski, L. (2010) Leishmaniasis vaccine: where are we today? J. Glob. Infect. Dis. 2, 177–185 CrossRef Medline
4. Kaye, P., and Scott, P. (2011) Leishmaniasis: complexity at the host-pathogen interface. Nat. Rev. Microbiol. 9, 604–615 CrossRef Medline
5. Nandan, D., Camargo de Oliveira, C., Moeenrezazhanlou, A., Lopez, M., Silverman, J. M., Subek, J., and Reiner, N. E. (2012) Myeloid cell IL-10 production in response to Leishmania involves inactivation of glycogen synthase kinase-3β downstream of phosphatidylinositol-3-kinase. J. Immunol. 188, 367–378 CrossRef Medline
6. Benjamín, D., Colomí, M., Moroni, C., and Hall, M. N. (2011) Rapamycin passes the torch: a new generation of mTOR inhibitors. Nat. Rev. Drug Discov. 10, 868–880 CrossRef Medline
7. Hemmings, B. A., and Restuccia, D. F. (2012) PI3K-PKB/Akt pathway. Cold Spring Harb. Perspect. Biol. 4, a011189 Medline
8. Ravikumar, B., Sarkar, S., Davies, J. E., Futter, M., García-Arencibia, M., Green-Thompson, Z. W., Jimenez-Sanchez, M., Koroluch, V. I., Lichtenberg, M., Luo, S., Massey, D. C., Menzies, F. M., Moreau, K., Narayanan, U., Renna, M., et al. (2010) Regulation of mammalian autophagy in physiology and pathophysiology. Physiol. Rev. 90, 1383–1435 CrossRef Medline
9. Mizushima, N., Yoshimori, T., and Levine, B. (2010) Methods in mammalian autophagy research. Cell 140, 313–326 CrossRef Medline
10. Sarkar, S., Floto, R. A., Berger, Z., Iamari, S., Cordenier, A., Pasco, M., Cook, L. J., and Rubinsztein, D. C. (2005) Lithium induces autophagy by inhibiting inositol monophosphatase. J. Cell Biol. 170, 1101–1111 CrossRef Medline
11. Deretic, V., Saitoh, T., and Akira, S. (2013) Autophagy in infection, inflammation and immunity. Nat. Rev. Immunol. 13, 722–737 CrossRef Medline
12. Schaible, U. E., Schlesinger, P. H., Steinberg, T. H., Mangel, W. F., Ko-...
transients is related to defective agonist-induced accumulation of inositol phosphates. J. Immunol. 148, 1188–1196 Medline
35. Majerus, P. W. (1992) Inositol phosphate biochemistry. Annu. Rev. Biochem. 61, 225–250 CrossRef Medline
36. Romanov, J., Walczak, M., Ibiricu, I., Schüchner, S., Ogris, E., Kraft, C., and Martens, S. (2012) Mechanism and functions of membrane binding by the Atg5-Atg12/Atg16 complex during autophagosome formation. EMBO J. 31, 4304–4317
37. Hurley, J. H., and Young, L. N. (2017) Mechanisms of autophagy initiation. Annu. Rev. Biochem. 86, 225–244 CrossRef Medline
38. Moore, K. J., and Matlashewski, G. (1994) Intracellular infection by Leishmania donovani inhibits macrophage apoptosis. J. Immunol. 152, 2930–2937 Medline
39. Olivier, M., Gregory, D. J., and Forget, G. (2005) Subversion mechanisms by which Leishmania parasites can escape the host immune response: a signaling point of view. Clin. Microbiol. Rev. 18, 293–305 CrossRef Medline
40. Naderer, T., and McConville, M. J. (2008) The Leishmania-macrophage interaction: a metabolic perspective. Cell. Microbiol. 10, 301–308 Medline
41. Shadab, M., and Ali, N. (2011) Evasion of host defence by Leishmania donovani. Cell. Microbiol. 13, 2617–2630
42. Ruckdeschel, K. (2002) Immunomodulation of macrophages by pathogenic Yersinia species. Arch. Immunol. Ther. Exp. 50, 131–137 Medline
43. Schimmell, C. G., Graham, J. G., Kurten, R. C., and Voth, D. E. (2014) Coxilfa burnetti type IV secretion-dependent recruitment of macrophage autophagosomes. Infect. Immun. 82, 2229–2238 CrossRef Medline
44. Hmama, Z., Peña-Díaz, S., Joseph, S., and Av-Gay, Y. (2015) Immunoevasion and immunosuppression of the macrophage by Mycobacterium tuberculosis. Immunol. Rev. 266, 220–232 CrossRef Medline
45. Cheekatla, S. S., Aggarwal, A., and Naik, S. (2012) mTOR signaling pathways. Mol. Biol. Int. 2011, 343961 Medline
46. Jung, C. H., Ro, S.-H., Cao, J., Otto, N. M., and Kim, D.-H. (2010) mTOR regulation of the IL-12/IL-10 signaling point of view. J. Clin. Invest. 121, 37–56 CrossRef Medline
47. Stolz, A., Ernst, A., and Dikic, I. (2014) Cargo recognition and trafficking in selective autophagy. Nat. Cell Biol. 16, 495–501 CrossRef Medline
48. Paulus, G. L., and Xavier, R. J. (2015) Autophagy and checkpoints for intracellular pathogen defense. Curr. Opin. Gastroenterol. 31, 14–23 CrossRef Medline
49. Kim, P. E. (2016) P38 signaling in Leishmania infections. Cell. Immunol. 309, 19–22 CrossRef Medline
50. Silverman, J. M., Clos, J., de Oliveira, C. C., Shrivani, O., Fang, Y., Wang, C., Foster, L. J., and Reiner, N. E. (2010) An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages. J. Cell Sci. 123, 842–852 CrossRef Medline
51. Lambertz, U., Silverman, J. M., Nandan, D., McMaster, W. R., Clos, J., Foster, L. J., and Reiner, N. E. (2012) Secreced virulence factors and immune evasion in visceral leishmaniasis. J. Leukocyte Biol. 91, 887–899 CrossRef Medline
52. McCoy, R. A., Rogers, S., Caldon, C. E., Lorca, T., Castro, A., and Burgess, A. (2014) Partial inhibition of Cdk1 in G2 phase overrides the SAC and decouples mitotic events. Cell Cycle 13, 1400–1412 CrossRef Medline
53. Jain, S. K., Sahu, R., Walker, L. A., and Tekwani, B. L. (2012) A parasite rescue and transformation assay for antileishmanial screening against intracellular Leishmania donovani amastigotes in THP1 human acute monocytic leukemia cell line. J. Vis. Exp. 2012, 4054 Medline
54. Baykov, A. A., Fvtsuschenko, O. A., and Avaeva, S. M. (1988) A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. Anal. Biochem. 171, 266–270 CrossRef Medline