Autophagy downstream of endosomal Toll-like receptor signaling in macrophages is a key mechanism for resistance to *Leishmania major* infection

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Leishmaniasis is a disease caused by infection by protozoan parasites of the genus *Leishmania*. In mammals, these parasites survive and replicate in macrophages and parasite elimination by macrophages is critical for host resistance. Endosomal Toll-like receptors (TLRs) have been shown to be crucial for resistance to *Leishmania major* in vivo. For example, mice in the resistant C57BL/6 genetic background that are triple-deficient for TLR3, -7, and -9 (TLR3/-7/-9) are highly susceptible to *L. major* infection. TLR3/-7/-9 mice are as susceptible as mice deficient in MyD88 or UNC93B1, a chaperone required for appropriate localization of endosomal TLRs, but the mechanisms are unknown. Here we found that macrophages infected with *L. major* undergo autophagy, which effectively accounted for restriction of parasite replication. Signaling via endosomal TLRs was required for autophagy because macrophages deficient for TLR3, -7, and -9, UNC93B1, or MyD88 failed to undergo autophagy, which effectively accounted for restriction of parasite replication. Signaling via endosomal TLRs was required for autophagy because macrophages deficient for TLR3, -7, and -9, UNC93B1, or MyD88 failed to undergo *L. major*-induced autophagy. We also confirmed that Myd88-/-, Tlr3/-7/-9/-, and Unc93b1-/-/- cells were highly permissive to *L. major* replication. Accordingly, shRNA-mediated suppression of Atg5, an E3 ubiquitin ligase essential for autophagosome elongation, in macrophages impaired the restriction of *L. major* replication in C57BL/6, but did not affect parasite replication in Myd88-/-/- or Unc93b1-/-/- macrophages. Rapamycin treatment reduced inflammatory lesions formed in the ears of *Leishmania*-infected C57BL/6 and Tlr3/-7/-9/- mice, indicating that autophagy operates downstream of TLR signaling and is relevant for disease development in vivo. Collectively, our results indicate that autophagy contributes to macrophage resistance to *L. major* replication, and mechanistically explain the previously described endosomal TLR-mediated resistance to *L. major* infection.

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Leishmaniasis is a disease caused by infection by protozoan parasites of *Leishmania* genus. It is transmitted by the bite of phlebotomine sand flies and it represents a major public health concern in many countries of Africa, Asia, and Latin America (1). During the blood meal, flagellated promastigotes of *Leishmania* are inoculated into the vertebrate host by the sand fly bite and are internalized by host macrophages into phagolysosome-like vesicles called parasitophorous vacuoles, where they differentiate into amastigotes and proliferate (reviewed by Ref. 2). In macrophages, *Leishmania* parasites interact with pattern recognition receptors (PRRs), leading to transcriptional regulation of genes that facilitate macrophage activation and recruitment of additional immune cells to the site of infection. The Toll-like receptors (TLRs) family of PRRs is probably the most studied and best characterized family of PRR (3). Thus far, 13 members of the TLR family were identified in mammals. They are distinguished by their ligand specificity and subcellular location. TLR1, -2, -4, -5, and -6 are located at the cell surface, whereas TLR3, -7, -8, -9, -11, and -12 are located in endosomal membranes. TLRs signal through myeloid differentiation primary response gene 88 (MyD88)-dependent and -independent pathways, leading to activation of mitogen-activated protein kinases (MAPks) and ultimately inducing the translocation of nuclear factor κB (NF-κB) to the nucleus. At the nucleus, NF-κB promotes the transcription and synthesis of proinflammatory cytokines and proteins related to antimicrobial responses (reviewed by Ref. 4). The importance of TLRs in host defense against *Leishmania* is highlighted by observations that disruption in MyD88 abolished the resistance of C57BL/6 mice to *Leishmania major* infection (5–8). Several studies addressed the role of specific TLRs on host protection against *Leishmania* infection. *Leishmania* lipophosphoglycan was shown to stimulate human and murine macrophages to produce reactive oxygen and nitrogen species by activating TLR2 (5, 9, 10). Macrophages from *Tlr4*−/− mice were more permissive for *Leishmania* (Viannia) panamensis replication and *Tlr4*−/− mice had a higher parasite burden and delayed healing of skin lesion compared with control mice in experimental models of *L. major* infection (11–13).

The abbreviations used are: PRR, pattern recognition receptor; TLR, Toll-like receptor; BMDM, bone marrow-derived macrophage; ANOVA, analysis of variance.
Endosomal TLRs triggers anti-Leishmania autophagy

Because Leishmania survives and replicates inside parasitophorous vacuoles, it is believed that TLRs expressed in that compartment act as important molecules for parasite recognition and host protection. It has been shown that Thr9−/− mice are more susceptible to L. major during the acute phase of infection, despite ultimately resolving the lesions (14). Although Leishmania parasites do not express double-stranded RNA, the natural ligand for TLR3, TLR3 accounts for nitric oxide and TNF-α production by macrophages infected with Leishmania donovani (15). Further studies demonstrated that the combined signaling of the endosomal TLR3, -7, and -9 was essential for host resistance to L. major infection (16). Similarly, C57BL/6 mice mutant for UNC93B1, a chaperone that mediates translocation of nucleic-sensing TLRs from the endoplasmic reticulum to endosomes, and thus do not have functional signaling of TLR3, TLR7, and TLR9 (17), are more susceptible to L. major infection due to a combined defect in TLR3, TLR7, and TLR9 function (16).

Despite the requirement of endosomal TLRs in host protection against L. major was previously defined, the mechanisms on how these receptors mediate parasite elimination in macrophages remains to be elucidated. Here we studied the importance of endosomal TLRs for the control of L. major replication by macrophages. We found that macrophages from Unc93b1−/− or Tlr3/7/9−/− mice were more susceptible to L. major infection due a defect in activation of antimicrobial autophagy. Endosomal TLRs effectively triggered anti-microbial autophagy in macrophages and lead to restriction of parasite replication in vivo. Collectively, our data suggest that autophagy is a critical immune mechanism operating in macrophage autonomous resistance against L. major infection, which could explain the previously described role of endosomal TLR in host resistance to Leishmaniasis.

Results

L. major induces autophagy in murine macrophages

Autophagy is the major self-catabolic pathway for lysosomal degradation of cytoplasmic macromolecules and organelles, being essential in periods of stress and nutrient deprivation. The key event in autophagy is the formation of the autophagosome, crescent-shaped slivers of membrane that wrap cytoplasmic targets (18). During infection, autophagy can capture and degrade intracellular microbes in a process known as xenophagy (19). Accumulating evidence has shown that ligand-mediated engagement of TLR triggers autophagy in macrophages (20–22). Because it has been previously demonstrated that endosomal TLR signaling is essential to protect the host against infection by L. major (16), we sought to investigate whether the engagement of endosomal TLRs by L. major triggers autophagy and if autophagy triggered by endosomal TLRs contributes to resistance to L. major infection. To address these questions, we first looked at whether L. major induces autophagy in bone marrow-derived macrophages (BMDMs) by studying the formation of autophagosomes. We used the autophagosomal marker LC3 fused with the green fluorescent protein (GFP-LC3) and quantified autophagosomes as a read-out for autophagy activation (23). During autophagy, GFP-LC3 is recruited to the autophagosome membrane, leading to formation of cytoplasmic aggregates containing LC3 (herein called GFP-LC3 dots). BMDMs were transduced with lentivirus vector encoding GFP-LC3 and infected with L. major (at a multiplicity of infection of 10) for 24 h or treated with rapamycin (1 μM) for 5 h and fixed. Infected cells were stained with rabbit polyclonal antibody anti-Leishmania. GFP-LC3 dots (green) and L. major (red) are shown in the fluorescence micrographs. Scale bar: 10 μm. B, GFP-LC3-transduced BMDMs were infected with L. major at the indicated time points or treated with rapamycin for 5 h. The number of GFP-LC3 dots per cell was scored by fluorescence microscopy. C, BMDMs obtained from C57BL/6 mice were infected with L. major as described in A and stained with a monocolonal antibody against endogenous LC3. The number of autophagosomes per cell was scored by fluorescence microscopy. For B and C, bars represent mean ± S.E. of triplicate samples (100 infected cells were scored per sample). D, BMDMs were infected with L. major for 24 h and the expression of endogenous LC3-I and LC3-II was assessed by immunoblotting. Actin immunoblotting was used as a loading control. Autophagic flux was assessed by treating the cells with bafilomycin A1 (100 μM) for 24 h. Data are from one representative experiment repeated three times. *p < 0.05; t-test.

Figure 1. L. major induces autophagy in murine BMDMs. A, BMDMs obtained from C57BL/6 mice were transduced with lentivirus vector encoding GFP-LC3 and infected with L. major (at a multiplicity of infection of 10) for 24 h or treated with rapamycin (1 μM) for 5 h and fixed. Infected cells were stained with rabbit polyclonal antibody anti-Leishmania. GFP-LC3 dots (green) and L. major (red) are shown in the fluorescence micrographs. Scale bar: 10 μm. B, GFP-LC3-transduced BMDMs were infected with L. major at the indicated time points or treated with rapamycin for 5 h. The number of GFP-LC3 dots per cell was scored by fluorescence microscopy. C, BMDMs obtained from C57BL/6 mice were infected with L. major as described in A and stained with a monocolonal antibody against endogenous LC3. The number of autophagosomes per cell was scored by fluorescence microscopy. For B and C, bars represent mean ± S.E. of triplicate samples (100 infected cells were scored per sample). D, BMDMs were infected with L. major for 24 h and the expression of endogenous LC3-I and LC3-II was assessed by immunoblotting. Actin immunoblotting was used as a loading control. Autophagic flux was assessed by treating the cells with bafilomycin A1 (100 μM) for 24 h. Data are from one representative experiment repeated three times. *p < 0.05; t-test.
downward shift in relationship to LC3-I and LC3-I to LC3-II conversion is usually used to assess autophagosome formation by immunoblotting (23). Confirming our microscopy analysis, we observed that infection with L. major triggered autophagy in macrophages, as we found an increased LC3-I to LC3-II conversion (Fig. 1D). This data supports a previous report in the literature indicating that L. major induces LC3 conversion in macrophages (24).

LC3 is associated with both the outer and inner autophagosomal membrane. During the fusion between autophagosomes with lysosomes to form autolysosomes, the LC3-containing outer membrane is delivered to cytosol, whereas the LC3-containing inner membrane is degraded by lysosomal enzymes, a process known as autophagic flux. If autophagic flux is interrupted, an accumulation of LC3-II in cytosol is observed (23, 25). Thus, the increased LC3-II expression detected in L. major-infected cells could indicate either an active induction of autophagy or an inhibition of autophagic flux. To distinguish both processes, we treated L. major-infected BMDMs with bafilomycin A1, an endosomal acidification inhibitor, which blocks autophagic flux (23). We found that upon bafilomycin A1 treatment, L. major-infected cells showed an accumulation of LC3-II in the cytosol as compared with L. major-infected untreated cells (Fig. 1D), suggesting the occurrence of activation, rather than inhibition of autophagic flux. This indicates that the increased number of autophagosomes and LC3-II found in L. major-infected cells were due to induction of autophagy, rather than inhibition of autophagic flux. Overall, these data show that macrophages actively trigger autophagy in response to L. major infection.

**Afterpage contributes to resistance against L. major infection in BMDMs**

To determine whether autophagy accounts for the restriction of L. major replication in BMDMs, we inhibited autophagy by knocking down Atg5, an E3 ubiquitin ligase essential for autophagosome elongation (26). BMDMs were transduced with lentiviral vectors coding two independent shRNA sequences targeting Atg5. The Atg5 knockdown was confirmed by immunoblotting (Fig. 2A). Atg5 knockdown in BMDMs did not affect the phagocytosis of L. major as evaluated at 1 h after infection (supplemental Fig. S1). In contrast, Atg5 knockdown resulted in an increased percentage of infected cells (Fig. 2B) and in the average number of amastigotes per cell (Fig. 2C) at 48 h post-infection compared with cells transduced with scramble shRNA (see representative images of L. major-infected cells stained with Giemsa at Fig. 2E). Furthermore, we evaluated the frequency of the number of intracellular amastigotes in shRNA-transduced BMDMs. For each condition, we determined the percentage of macrophages containing 0, 1–2, 3–5, 6–10, or more than 10 intracellular amastigotes. We observed that the inhibition of autophagy in BMDMs significantly increased the proportion of cells presenting from 6 to 10 or more than 10 intracellular parasites, whereas the percentage of uninfected cells or cells presenting low numbers of intracellular amastigotes, such as 1–2 parasites per cell, were decreased (Fig. 2D).

To further evaluate the role of autophagy in macrophage resistance to L. major infection, we used a L. major strain stably expressing GFP (L. major-GFP) to measure parasite replication by flow cytometry as previously described (27). Consistent with our Giemsa staining data, we observed a higher percentage of GFP+ cells (Fig. 2F) and an increased mean fluorescence intensity (Fig. 2G) at 48 h post-infection of BMDMs with L. major-GFP. Taken together, these data suggest that autophagy represents an important mechanism used by macrophages to restrict L. major replication.

**Autophagy triggered by L. major is dependent on endosomal TLRs**

It was previously shown that endosomal TLR3, -7, and -9 fully accounts for resistance against L. major infection in vivo (16) and it is known that the engagement of TLRs triggers autophagy in macrophages (20–22). As we found that L. major induces autophagy in BMDMs and autophagy contributes to control L. major replication in vitro, we raised the question whether the function of endosomal TLRs plays any role in autophagy activation by L. major in BMDMs. We used Unc93b1−/− mice, which lack a chaperone that mediates translocation of nucleic-sensing TLRs from the endoplasmic reticulum to endolysosomes and thus, have defective signaling of endosomal TLRs including TLR3, -7, and -9 (17). We found that BMDMs from Unc93b1−/− mice presented defective autophagy activation in response to L. major infection (Fig. 3A). The defect in autophagy activation found in Unc93b1−/− resembled the one in Myd88−/− BMDMs, which have a global defect in TLRs signaling (Fig. 3A). All groups tested responded equally to rapamycin treatment. Because Unc93b1−/− cells are defective for TLR3, -7, and -9 signaling, we sought to confirm these findings by evaluating autophagy in BMDMs obtained from mice triple deficient for TLR3, -7, and -9 (Tlr3/7/9−/−). We found that Tlr3/7/9−/− BMDMs had a defective activation of autophagy similar to Unc93b1−/− BMDMs (Fig. 3A). These data suggest that signaling through endosomal TLRs accounts for autophagy activation by L. major in BMDMs.

Because we found that autophagy is an important mechanism controlling intracellular replication of L. major in BMDMs, and BMDMs defective in multiple endosomal TLRs fail to trigger autophagy during L. major infection, we tested whether macrophages deficient in multiple endosomal TLRs signaling were more permissible to L. major replication. To address this question, we first evaluated the replication of L. major in Unc93b1−/− and Myd88−/− BMDMs. We found a higher percentage of infected cells and an increased number of intracellular amastigotes in Myd88−/− and Unc93b1−/− BMDMs as compared with cells obtained from C57BL/6 mice (Fig. 3, A and B). The susceptibility of UNC93B1 mutant BMDMs was comparable with Myd88−/− cells, supporting the hypothesis that endosomal TLRs signaling mediate the MyD88-dependent resistance to L. major infection, as previously shown (16). In addition, both Unc93b1−/− and Myd88−/− BMDMs had a higher percentage of cells harboring from 3 to 5 or 6 to 10 intracellular parasites (Fig. 3D). In contrast, Unc93b1−/− and Myd88−/− BMDMs showed a lower percentage of uninfected cells (absence of intracellular para-
sites) compared with cells from C57BL/6 mice (Fig. 3D). We also evaluated the replication of *L. major* in *Tlr3/7/9*−/− BMDMs. As expected, *Tlr3/7/9*−/− BMDMs were more susceptible to *L. major* replication as compared with wild-type macrophages (Fig. 3, E–G).

Next, we sought to determine whether the higher susceptibility of *Myd88*−/− and *Unc93b1*−/− BMDMs was due to the impaired autophagy activation. To address this question, we evaluated the effect of autophagy inhibition on *L. major* replication in *Unc93b1*−/− or *Myd88*−/− BMDMs by knocking down Atg5. We found that whereas Atg5 knockdown resulted in an increased replication of *L. major* in BMDMs from C57BL/6 mice, it had no effect on parasite multiplication in *Myd88*−/− or *Unc93b1*−/− BMDMs (Fig. 4, A and B). Our data indicates that inhibition of autophagy increased parasite replication in C57BL/6, but not in *Tlr3/7/9*−/− BMDMs. Thus, we tested whether induction of autophagy with rapamycin inhibits *L. major* replication in BMDMs and rescue the capacity of *Tlr3/7/9*−/− BMDMs to control *L. major* replication. Initially, we determined whether the highly pure rapamycin R-5000 induces autophagy in macrophages. To test this, we evaluated the phosphorylation of rpS6, a downstream effector of mammalian target of rapamycin (mTOR) that is hypophosphorylated in autophagy-inducing conditions (28). When BMDMs were treated with increasing concentrations of rapamycin, the phosphorylation of rpS6 was reduced, compared with untreated cells (Fig. 5A). Thus, we used 0.1, 1, and 10 μM rapamycin to treat macrophages infected with *L. major* and evaluated the parasite replication after 48 h of infection. We found that although treatment with rapamycin of *L. major*-infected BMDMs from C57BL/6 mice had no effect in the percentage of infected cells (Fig. 5B), the number of intracellular...
amastigotes was significantly reduced compared with untreated cells (Fig. 5C). Treatment with rapamycin restored the ability of \textit{Tlr3/7/9}−/− BMDMs to restrict \textit{L. major} replication (Fig. 5, B and C). Collectively, our data suggest that endosomal TLR signaling favors the induction of autophagy, which represents a critical process for restriction of \textit{L. major} replication in macrophages.

**Autophagy contributes to reduction of lesion development in vivo**

To address the role of endosomal TLRs-induced autophagy in the control of \textit{L. major} infection in vivo, we infected mice with \textit{L. major} by intradermal inoculation of parasites in the ear and treated with rapamycin. The treatment started 3 weeks post-inoculation and consisted of one daily dose of rapamycin given by intraperitoneal route over 10 days. The size of Leishmaniasis lesions was measured weekly. We first determined whether the R-5000 rapamycin induces autophagy in macrophages infected with \textit{L. major}. By evaluating the phosphorylation of rpS6, we found that rpS6 was hypophosphorylated in response to rapamycin treatment (Fig. 6A). We also found that \textit{L. major} infection resulted in a hypophosphorylation of rpS6, confirming our data that \textit{L. major} infection induces autophagy in BMDMs. Experiments performed with \textit{Tlr3/7/9}−/− cells confirmed that TLR3, -7, and -9 are required for autophagy induction in response to \textit{L. major}, but not in response to

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**Figure 3. BMDMs deficient for endosomal TLRs have defective autophagy response and are more susceptible to \textit{L. major} infection.** A, BMDMs were transduced with lentivirus vector encoding GFP-LC3 and infected with \textit{L. major} (10 parasites per cell) or treated with rapamycin (1 μM) for 5 h. The number of GFP-LC3 dots per cell was scored by fluorescence microscopy. B–G, BMDMs were infected with \textit{L. major} (10 parasites per cell) and 48 h after infection the number and frequency of intracellular parasites were evaluated by Giemsa staining. Bars represent mean ± S.E. of triplicate samples (100 infected cells per sample). Data are from one representative experiment repeated three (A–D) or two (E–G) times. *, \(p < 0.05\); t test (B, C, E, and F), and two-way ANOVA (A, D, and G).

**Endosomal TLRs triggers anti-Leishmania autophagy**

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![Figure 4. Autophagy triggered by endosomal TLRs accounts for BMDMs resistance to L. major. BMDMs were transduced with lentivirus vector encoding a shRNA sequence specific for Atg5 or shRNA control (scramble) followed by infection with L. major for 48 h (10 parasites per cell). A and B, percentage of infected cells (A) and the average number of intracellular parasites (B) evaluated by Giemsa staining. Bars represent mean ± S.E. of triplicate samples (100 infected cells per sample). Data are representative of one independent experiment repeated three times. *p < 0.05; two-way ANOVA.](image)

rapamycin, as we found increased rpS6 phosphorylation in L. major-infected Tlr3/7/9−/− compared with C57BL/6 (Fig. 6A). These data demonstrate that rapamycin can potently induce autophagy in infected C57BL/6 and Tlr3/7/9−/− BMDMs. We further performed in vivo infections and treatment with rapamycin. The measurement of ear lesions in L. major-infected mice indicates that C57BL/6 presented smaller lesions compared with Tlr3/7/9−/− mice (Fig. 6B, C), confirming previous findings (16). The induction of autophagy by treatment with rapamycin reduced the lesion sizes by ~50% either in C57BL/6 or Tlr3/7/9−/− mice, suggesting that the rescue of autophagy function by treatment with rapamycin in susceptible Tlr3/7/9−/− mice restored their ability to control lesion progression. In addition to lesion size, we also assessed the parasite burden in lesions. However, we did not detect differences in the number of parasites in ear lesions or in draining lymph nodes of C57BL/6 or Tlr3/7/9−/− mice treated with vehicle or rapamycin (Fig. 6D). Importantly, the reduction in the lesion size supports the hypothesis that autophagy operates downstream of endosomal TLRs activation and contributes to the control of the inflammatory lesion progression in vivo.

**Discussion**

Engagement of TLRs by pathogen-associated molecules is possibly the most studied immune mechanism for pathogen recognition and activation of immune responses (3, 29). An efficient immune response against Leishmania relies on the differentiation of IFN-γ-producing CD4 lymphocytes of Th1 type in a mechanism dependent on the IL-12 secretion by macrophages (30–32). It was demonstrated that the combined signaling of endosomal TLR3, -7, and -9, but not individual receptors, accounts for the production of IL-12 by L. major-infected macrophages and host resistance to Leishmaniasis in a murine model of L. major infection (16). However, the cellular mechanism by which endosomal TLRs signaling mediates macrophage resistance to L. major infection was unclear. In this work, we identified autophagy as a major mechanism operating downstream of endosomal TLR to induce resistance to L. major infection. We found that L. major replicates more efficiently in BMDMs deficient for UNC93B1, a molecule essential to mediate signaling via endosomal TLR3, -7, and -9 (17). Unc93b1−/− BMDMs are defective in autophagy activation in response to L. major infection. Consistent with these findings, Tlr3/7/9−/− triple knock-out BMDMs are also defective in autophagy activation in response to L. major infection and more permissive for L. major replication in vitro. Experiments performed in vivo also support the importance of TLR3, -7, and -9 and autophagy to control lesion development and parasite replication. Although we found that rapamycin contributed to reduce lesion development, it did not affect parasite replication. It is possible that a longer treatment with rapamycin would be required to promote restriction of parasite replication. Alternatively, it is possible that autophagy promotes the reduction of the inflammatory process in the lesion site, leading to a reduction of the lesion size and swelling, but did not affect parasite replication in vivo. Interestingly, autophagy was shown to negatively regulate inflammasome activation (33–35). Thus, we believe that rapamycin-mediated induction of autophagy in vivo plays a dual role in parasite replication by promoting autophagy-induced restriction of parasite burden or inhibiting the inflammasome-mediated restriction of L. major replication, as previously reported (27). Nonetheless, the hypothesis of a dual effect of rapamycin in vivo should be considered for further investigation related to the use of autophagy-inducing compounds in infections with Leishmania spp. Regardless of the effect of rapamycin in vivo, autophagy induction in macrophages is critical for restriction of parasite replication. It still remains to be determined how autophagy limits the intracellular replication of L. major in macrophages. Early studies performed with Leishmania mexicana indicated that autophagy facilitated the delivery of large molecules to the parasitophorous vacuoles (36). This model is consistent with autophagy being important to promote parasite nutrition and replication. In agreement with these findings, studies performed with Leishmania amazonensis, which belongs to the same complex as L. mexicana, show that induction of autophagy in BALB/c macrophages (but not C57BL/6) resulted in increased intracellular parasite replication (37). The authors of that study concluded that the effect of autophagy in parasite replication was dependent on host genetic background and possibly the species of Leishmania. Previously, the demonstration that Leishmania parasites trigger autophagy in macrophages was achieved using the detection of LC3-I to LC3-II conversion by immunoblot. In this sense, it was shown that L. donovani, a species related to the development of visceral Leishmaniasis, triggered autophagy in human macrophages (38). Using the same method, another study further reported induction of autophagy in macrophages and in vivo in mouse infected with L. amazonensis (39). Furthermore, it was shown that L. major triggers LC3-I to LC3-II conversion in BMDMs (24). Thus, despite that the induction of autophagy in response to Leishmania have been convincingly demonstrated in macrophages, the effect of autophagy on the outcome of the infection is still obscure. This is possibly because many in vitro studies used inducers or inhibitors of autophagy that could potentially interfere on both macro-
phages and parasites autophagy function. Herein, we unequivocally demonstrated using genetic approaches that autophagy contributes to restrict L. major replication in BMDMs. We used shRNA to knockdown Atg5 in macrophages, ensuring in this way that the autophagy function in L. major was intact during infection. Collectively, our data identify autophagy as a key process for macrophage restriction of L. major replication. This process is induced in response to stimulation of endosomal TLRs in macrophages and may represent a major effector function induced upon TLRs activation that operates in host resistance to L. major resistance.

**Experimental procedures**

**Mice and BMDM preparation**

Male or female mice were used for generation of BMDMs. The strains used were C57BL/6, Myd88-/-/- (40), Tlr5/-/-/-, and Unc93b1-/-/- mice (17). Genetically modified strains were in the C57BL/6 genetic background. Mice were bred and maintained at the animal facilities of the University of São Paulo (Ribeirão Preto, Brazil). The care of the mice was in compliance with the institutional guidelines on ethics in animal experiments approved by CETEA (Comissão de Ética em Experimentação Animal da Faculdade de Medicina de Ribeirão Preto, approved protocol number 089/2013 and 130/2016), which follows the Brazilian national guidelines recommended by CONCEA (Conselho Nacional de Controle em Experimentação Animal). BMDMs were generated in vitro using L929 cell-conditioned medium as previously described (41). BMDMs were cultured in RPMI 1640 (Sigma) medium supplemented with 10% FBS and 5% L929 cell-conditioned medium.

**Leishmania parasites and BMDM infection**

Promastigotes of L. major (LV39) were cultured at 25 °C without CO_2_ in 199 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 units/ml of penicillin (Sigma), 100 μg/ml of streptomycin (Sigma), 2 mM L-glutamine (Sigma), 40 mM HEPES (Sigma), 0.1 mM adenine (Sigma), 5 mg/ml of hemin (Sigma), and 1 mg of biotin (Sigma). L. major promastigotes constitutively expressing the green fluorescent protein (L. major-GFP) were kindly provided by Dr. Angela Kayse Cruz (FMRF-USP). L. major-GFP was cultured as above, with the addition of 32 μg/ml of hygromycin B (Sigma). Stationary phase L. major promastigotes were prepared from 5-day cultures. BMDMs were seeded in 24-welled plates (Corning) and left to attach overnight before infection. BMDMs were infected at a ratio (parasite per cell) indicated in figure legends. Where indicated, BMDMs were infected with L. major for 12 h and infected cells were incubated with crescent concentrations of rapamycin R-5000 (LC Laboratories, MA). L. major replication was determined at 48 h after infection in cells stained with Giemsa. At least 100 cells per well were counted for each condition. Alternatively, L. major replication was evaluated by flow cytometry (FACSCanto II, BD Biosciences) after infection of BMDMs with L. major-GFP.

**BMDM transduction with lentivirus**

Recombinant viral particles were obtained by transfecting HEK293 cells with the lentiviral vector pH-RS-NCSGWΔNotI-GFP-LC3 and the helper plasmids pCMVΔ8.91 and pMDG (42). Lentivirus-containing supernatant was added onto bone marrow cells previously incubated for 3 days with L929 cell-conditioned medium (LCCM), followed by an overnight incubation at 37 °C in 5% CO_2_. The next day, the supernatant was replaced with fresh lentiviral supernatant followed by overnight incubation at 37 °C in 5% CO_2_. Then, the supernatant was replaced with fresh media and cells were incubated for an additional 48 h before being used in autophagy assays.

**Atg5 knock down by shRNA**

Lentiviral particles were generated by transfecting HEK293T cells with the helper plasmids pMD2.G and psPAX2, in addition to plasmids codifying specific sequences for Atg5 or control (scramble) shRNA. The sequences used were: Atg5 sequence 1: 5’-CCGGGGATCTGAGCCTCCAGATAGACTCGAGTTATCCTGGTGAgCTGACTGAGTTATCCTGAGTTATCCTGGTGACAGATGTTTTCG-3’; Atg5 sequence 2: 5’-CCGGGCAAGTACTGCTGATCCTGCTTTATGACTCGAGTTATCCTGGTGACAGATGTTTTCG-3’. The transduction of BMDMs was performed as described above. Expression of the Atg5 protein was systematically evaluated by immunoblotting.

**GFP-LC3 dots assay**

For immunofluorescence of endogenous LC3, infected cells were fixed in 10 min with methanol at −20 °C and permeabi-
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Figure 6. Rapamycin treatment in vivo contributes to reduce the inflammatory lesions induced by L. major. A, BMDMs were left untreated (control), treated with rapamycin R-5000 (30 μM) for 1 h (rapam.), or infected with L. major (10 parasites per cell) (L.m.) for 24 h, or infected with L. major for 24 h and treated with rapamycin for 1 h (L.m. + rapam.). Cell lysates were collected and subjected to immunoblotting. B, mice were infected with L. major (10^6 parasites per mouse) by intradermal inoculation in ear. At 3 weeks post-infection, mice were treated daily with one dose of rapamycin R-5000 (5 mg/kg) (rapamycin) or with vehicle control (vehicle) by intraperitoneal route during 10 days. Ear thickness was measured weekly using a digital gauge caliper. After 5 weeks of infection, animals were euthanized and ears and retromaxillary lymph nodes (LN) were collected and parasite burden was evaluated. For B and D, data represent mean ± S.E. from 4–5 animals per group. In D, each dot represents a single animal. Data are representative of one independent experiment repeated three times. *, p < 0.05; t test.

Western blot

To evaluate rpS6 phosphorylation in uninfected BMDMs, cells were treated with rapamycin R-5000 (LC Laboratories) with the indicated concentrations for 24 h and cell lysates were collected as described below. To evaluate rpS6 phosphorylation in L. major-infected BMDMs, cells were infected with L. major at a ratio of 5 parasites per cell for 24 h, treated or not with rapamycin R-5000 (30 μM) for 1 h, and cell lysates were collected as indicated below. For LC3-I to LC3-II conversion assays, BMDMs were infected with L. major at a ratio of 10 parasites per cell and treated or not with baflomycin A1 (100 nM) (Sigma) for 24 h or rapamycin (Sigma) (1 μM) for 5 h. Cells were lysed in a buffer containing 100 mM NaCl, 20 mM Tris (pH 7.6), 10 mM EDTA (pH 8), 0.5% SDS, and 1% Triton X-100 with protease inhibitor mixture (Roche) and incubated 20 min on ice. Laemmli sample buffer was added to lysates and samples were boiled for 8 min. Proteins were then separated by SDS-PAGE in 15% polyacrylamide resolving gel and transferred to nitrocellulose membranes. Primary antibodies used were: anti-Atg5 (1:1000; Abcam; catalog number 108327), anti-LC3 (1:1000; Sigma; catalog number L7543), anti-rpS6 (1:1000; Cell Signaling; catalog number 2217), anti-phospho (Ser-235/236)-rpS6 (1:1000; Cell Signaling; catalog number 2211), and anti-actin (1:1000; Santa Cruz; catalog number sc-47778).

Mouse infection and treatment with rapamycin

Female, 6–8-week-old C57BL/6 or Tlr3/7/9−/− mice were used for infection. L. major LV39 (MRHO/SU/59/P) was cultivated in Schneider’s Insect Medium (Gibco) supplemented with 20% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 mM l-glutamine, and 2% human male urine. Mice were infected in the left ear through intradermal inoculation of 10^6 L. major axenic promastigotes. The ear thickness was measured weekly using a digital gauge caliper. After 5 weeks of infection, animals were euthanized and ears and retromaxillary lymph nodes were collected. The material was mashed in 70-μm cell strainers (Corning, Corning, NY), resuspended in Schneider’s complete medium, and serial dilutions were made in 96-well plates. After 7–10 days, parasite titers were determined as previously described (43). For treatment of infected mice, rapamycin R-5000 stock solution was prepared through suspension in 100% ethanol (Mallinckrodt Baker) at a concentration of 20 mg/ml. Aliquots were stored at −80 °C. Before treatment, rapamycin was diluted in PBS containing 5.2% (v/v) PEG-400 (Lab Synth, SP, Brazil) and 5.2% (v/v) Tween 80 (Sigma) to a concentration of 5 mg/ml. Animals received 5 mg/kg of rapamycin or vehicle by intraperitoneal route daily for 10 days, starting 3 weeks post-infection.

Statistical analysis

Statistical differences between groups were made using PRISM software (version 6.0, GraphPad, San Diego, CA). Two-tailed unpaired Student’s t test was used for single comparisons; analysis of variance (ANOVA) test was used for multiple comparisons.

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