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Phagocytosis is inhibited by autophagic induction in murine macrophages

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ABSTRACT

Recent studies have demonstrated that communication takes place between the autophagic and phagocytic pathways, indicating that the convergence of these two pathways plays an important role in the innate immune response against intracellular microbes. The present study investigated the effect of autophagic induction on the phagocytic capacity of murine macrophages. Autophagy induced by physiological and pharmacological means was shown to reduce the phagocytic capacity of murine macrophages, regardless of cell origin or the nature of the phagocytosed particles themselves. This autophagic inhibitory effect on phagocytosis was shown to be an early and reversible event that results in no loss of cell viability. Furthermore, the data presented herein demonstrate that the induction of autophagy does not affect a macrophage's capacity to recognize and bind to particles, indicating that autophagy does not inhibit the particle recognition process, even though particle internalization is suppressed. The findings herein support the notion that phagocytosis and autophagy may be interdependent and complementary processes.

1. Introduction

Originally, autophagy was described as a mechanism that maintains cellular homeostasis [1] by way of a natural physiological process that leads to the acquisition of energy in response to stress, as well as being the process responsible for the renewal of cellular organelles [2]. Autophagy leads to the degradation of proteins, subcellular domains and organelles, which is an evolutionarily conserved process occurring naturally in eukaryotic cells and is inducible in response to a variety of stress situations [3]. To date, the known autophagic processes are subdivided into different pathways, including chaperone-mediated autophagy, microautophagy and macroautophagy. Both micro- and macroautophagy are intracellular processes involving macromolecular degradation in eukaryotic cells. In the microautophagic process, the transfer of cytosolic components into the lysosomal compartment occurs by direct invagination of the lysosomal membrane, while in the macroautophagic process, cytosolic components are sequestered into an autophagosome prior to fusion with lysosomes [4].

Macroautophagy, also known as the autophagic process, is triggered in response to starvation conditions and regulated by the activity of the serine–threonine kinase, the mammalian target of rapamycin (mTOR). This enzyme is activated under nutrient-rich conditions. By contrast, mTOR activity is blocked by rapamycin, triggering the formation of autophagosomes under nutrient-rich conditions [5]. Furthermore, the autophagic pathway occurs in an orderly manner. Initially, a portion of the cytoplasm and organelles, such as endoplasmic reticulum (ER), mitochondria and peroxisomes, are sequestered by a double-layered membrane which leads to the formation of an autophagosome. Autophagosomes then fuse with lysosomes, losing their inner membranes, and these vesicles become acidic due to the acquisition of proton ATPases in the outer membranes. Subsequently, autophagosomes acquire other proteins, such as lysosomal enzymes, and this organelle is then called an autophagolysosome, or autolysosome, which is where the degradation of sequestered material occurs [6,7]. The origin of the autophagosome double membrane is unknown. Evidence indicates that this structure originates from ribosome-devoid regions of the ER [6]. However, it has been recently suggested that the plasma membrane may contribute to the formation of the autophagosome membrane [8].

The autophagic process has also been described as a mechanism that plays a role in the innate and adaptive immune systems

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involved in intracellular infection by microorganisms. This cellular mechanism may protect host cells and promote the establishment of infection, depending on the nature of the pathogen [9–11]. These data suggest that the course of infection depends not only on the initial steps of host cell–pathogen interaction, but also on subsequent events in which pathogen-induced vacuoles interact with autophagic vesicles.

Phagocytosis is the primary mechanism by which unicellular eukaryotes obtain nutrients [12]. In metazoans, phagocytosis is important not only for cell nutrition, but is also involved in internalization, killing and removal of foreign particles. In mammals, it is a mechanism involved in tissue remodeling during organism development, senescent cell removal and inflammation. Additionally, phagocytosis is one of the most important processes involved in the mammalian defense against infection by intracellular microorganisms [12,13].

Recent studies have observed crosstalk between the autophagic and phagocytic pathways [14,15]. Fusion between nascent phagosomes and autophagosomes has been recently described [16]. This phenomenon suggests that autophagy may play a complementary role during phagocytosis. Furthermore, it has recently been demonstrated that in the macrophage cell line J774, the induction of autophagy enhances bacterial uptake prior to phagocytosis [17].

The present study aimed to evaluate possible connections between autophagic and phagocytic pathways by determining the effect of autophagic induction on the phagocytic capacity of murine macrophages. We hypothesized that the induction of autophagy prior to phagocytosis would alter the phagocytic capacity of macrophages.

2. Materials and methods

2.1. Macrophage obtainment and cultivation

All experiments were performed according to the standards of the Ethics Committee on Animal Experimentation at the Oswaldo Cruz Foundation – CPqGM/FIOCRUZ. Thioglycolate-elicited inflammatory peritoneal macrophages from CBA mice were obtained and cultivated in 24-well plates containing 12-mm glass slides at a concentration of 2×10^5 macrophages per well as previously described [18].

2.2. Parasites

The *Leishmania amazonensis* (strain MHOM/Br88/Ba-125) promastigotes used in this study were maintained in axenic culture for up to seven passages, suspended in Schneider's (Gibco) complete medium supplemented with 10% inactivated fetal calf serum and 50 μg/mL of gentamicin (Sigma). All parasites were washed, counted and added to macrophage cultures at a ratio of 10:1.

2.3. Induction of autophagy

To induce physiological autophagy (Starvation), macrophages were incubated in EBSS nutrient-poor medium (Earl's Balanced Salt Solution, Sigma) as previously described [19]. To induce pharmacological autophagy, macrophages were incubated in nutrient-rich medium (complete DMEM) in the presence of 50 μg/mL of rapamycin (Sigma), a drug that inhibits mTOR kinase activity [5].

2.4. Autophagic effect on phagocytosis

To assess the *in vitro* effects of autophagic induction on the phagocytic capacity of murine macrophages, the cells were incubated under autophagic conditions as described above. Next, heat inactivated yeast particles (*Saccharomyces cerevisiae*, Sigma) or *L. amazonensis* stationary phase promastigotes were added to macrophage cultures at a ratio of 10:1 particles or parasites per cell. Then the macrophage cultures were fixed in ethanol at 99% for 15 min, stained with H&E and a minimum of 400 cells were counted using a bright-field light microscope. The percentage of macrophages that phagocytosed particles or parasites was calculated as the percentage of phagocytosis and the number of particles or parasites per macrophage was quantified. Cell viability was tested using the trypan blue exclusion technique which showed that 95–99% of cells incubated under both physiological and pharmacological autophagic conditions suffered no loss of cell viability.

2.5. Transmission electron microscopy

To evaluate the ultrastructural aspects of the cells incubated under physiological and pharmacological autophagic conditions, macrophage suspensions were distributed in 6-well plates at a concentration of 2×10^6 per well. At the end of the incubation period, the cells were processed as previously described [20]. The samples were embedded in Polybed epoxy resin (Polysciences, Warrington, PA, USA) and after polymerization, ultrathin sections were obtained. The sections were contrasted and then observed using a transmission electron microscope (JEOL JEM 1230 at 80 kV).

2.6. Fluorescence microscopy

Macrophages were incubated in physiological and pharmacological autophagic conditions for 2 h at 37 °C 5% CO_2 and fluorescein isothiocyanate-labeled zymosan particles (FITC-zymosan, Sigma) were added at a ratio of 10 particles per macrophage (10:1). The culture plates were centrifuged (500*g*) at 4 °C for 5 min and then incubated for 10 min at 4 °C, a temperature at which cells recognize and bind to particles, but under which internalization is infeasible [21] and differentiation between particle binding and particle internalization is observable. After this time period, cultures were washed to remove non-adherent particles and fixed in 4% paraformaldehyde (Sigma) for 20 min. The macrophage cultures were then stained with DAPI (Vectashield, Burlingame, CA, USA), which marks nuclei in blue, and 0.16 μg/mL of rhodamine–phalloidin (Sigma) to observe polymerized actin in fixed cells. Particle binding was identified by colocalization of zymosan-FITC stained in green with actin cups labeled in red, a phenomenon that is quantifiable due to the presence of yellow-colored assemblies in plasma membrane. The percentage of cells displaying colocalization of actin cups with zymosan-FITC was determined by counting at least 600 cells in five fields using Image-Pro Plus 6.0.

2.7. Statistical analysis

The graphs and statistical analyses were done using the program GraphPad Prism, version 5.00 – Incorporate GraphPad Software. The bar graphs represent the means ± SE (standard error) of a set of experiments done at least in triplicate and repeated four times. The one-way ANOVA and Tukey post-test were used for comparison between three or more groups. Differences were considered statistically significant when $p \leqslant 0.05$.

3. Results and discussion

In order to evaluate the effect of autophagy on the phagocytic capacity of murine macrophages, cell cultures were incubated under physiological autophagic conditions as described in Section 2. Transmission electron microscopy was performed to evaluate

whether morphological alterations were present in macrophages subjected to starvation conditions. In addition to less electron-dense cytoplasm, numerous double membrane vacuoles characteristic of mature autophagosomes [22] were observed in more than 90% of the cells (Fig. 1A and B), confirming that autophagy was induced in peritoneal inflammatory CBA macrophages.

A reduction in yeast particle phagocytosis by macrophages incubated under autophagic conditions was detected, in comparison to control cells incubated in complete DMEM. Phagocytosis inhibition was detected within 15 min of the exposure of particle to cells which had been previously incubated for 2 h under physiological autophagic conditions. A maximum inhibition level of 70% was obtained at the 60-min interval of cell–particle interaction (Fig. 2A, $p < 0.05$, $n = 4$, ANOVA) and the average number of internalized particles per macrophage was reduced by 40% at a 60-min interval during starvation medium incubation, in comparison to controls (Fig. 2B, $p < 0.05$, $n = 4$, ANOVA). To verify whether this inhibitory effect was related to incubation under starvation conditions, the macrophages were incubated under pharmacological autophagic conditions, i.e. in a nutrient-rich medium in the presence of rapamycin, an mTOR inhibitor. Similar to physiological autophagic induction, pharmacological autophagic induction induced morphological alterations on a cellular level in over 90% of the cells (Fig. 1C), as well as down modulation of the phagocytosis of yeast particles (Fig. 2A and B). These findings indicate that it is the autophagic process itself which triggers an inhibitory effect on the phagocytosis of yeast particles by macrophages, regardless of incubation conditions.

To test whether this inhibitory effect occurs in other phagocytes as well as in inflammatory macrophages from CBA mice, further experiments were performed using non-inflammatory CBA macrophages and the RAW cell line. The induction of physiological autophagy reduced phagocytosis by 95% (±0.50) in resident peritoneal cells, by 90% (±1.63) in bone marrow-derived macrophages, and by 90% (±7.07) in RAW cells. Similarly, the induction of pharmacological autophagy reduced phagocytosis by 85% (±5.12) in resident peritoneal cells, by 60% (±5.31) in bone marrow-derived macrophages, and by 80% (±10.31) in RAW cells. To further investigate the universal nature of this effect, BALB/c and C57BL/6 inflammatory peritoneal macrophages were submitted to physiological and pharmacological autophagic conditions as described above. Physiological autophagic induction reduced phagocytosis by 18% (±13.23) in BALB/c and 35% (±10.44) in C57BL/6 macrophages. Similarly, pharmacological autophagic induction reduced phagocytosis by 80% (±8.41) in BALB/c and 80% (±8.30) in C57BL/6 macrophages. Taken together, these data demonstrate that autophagy inhibits particle uptake by macrophages of different origin, suggesting that autophagic induction affects classical phagocytosis by professional phagocytes in a generalized manner.

To determine the length of time necessary for autophagy to inhibit phagocytosis, CBA macrophages were incubated under either physiological or pharmacological autophagic conditions for time intervals ranging from 0 to 120 min. Yeast particles were then added to all cell groups and each macrophage culture was incubated for an additional 30 min. Cultures exposed to pharmacological autophagic conditions exhibited a reduction of approximately 60% in the percentage of phagocytosis in as promptly as 15 min, a level which was maintained at later intervals. By contrast, the cultures incubated in starvation medium presented a gradual reduction in phagocytosis ranging from 25% at time 0 to 60% at

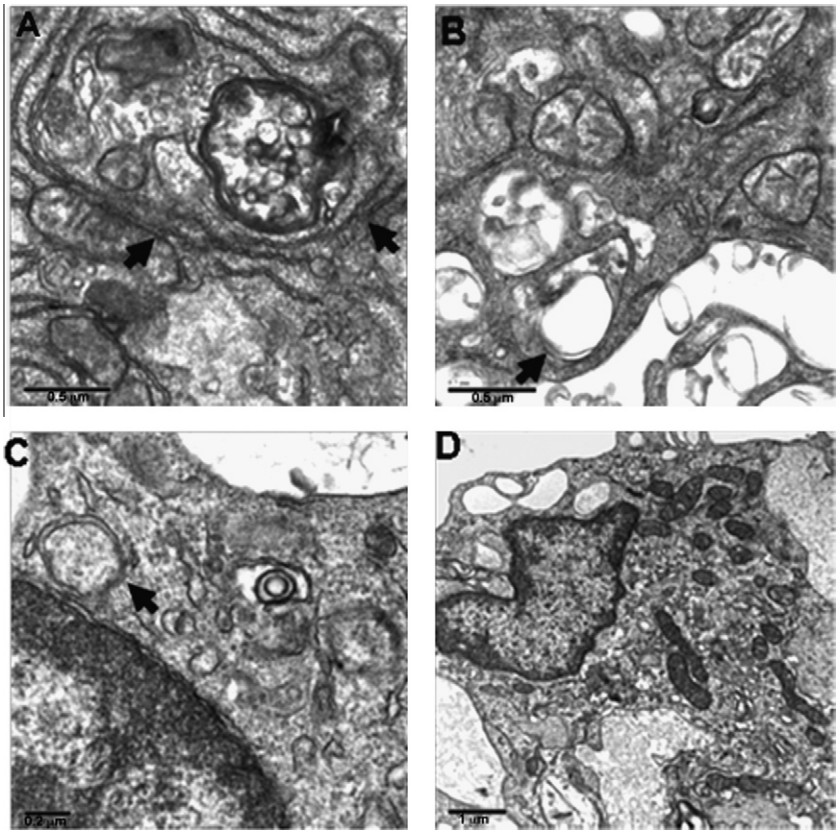

Fig. 1. Autophagic conditions induce morphological alterations in cells as detected by electron microscopy analysis. Inflammatory peritoneal CBA macrophages (2×10^5/mL) were incubated in EBSS, a nutrient-free medium used for physiological autophagic induction (A, B), or incubated in nutrient-rich DMEM complete medium in the presence of rapamycin (50 μg/mL) (C) to induce pharmacological autophagy, as described in Section 2. The control group was incubated in DMEM complete medium (D). Next, cells were fixed and morphology was assessed using electron microscopy. Pictures clearly depicted aspects of cells in the autophagic process (A–C). Arrows represent double membrane vacuoles in the cell cytoplasm.

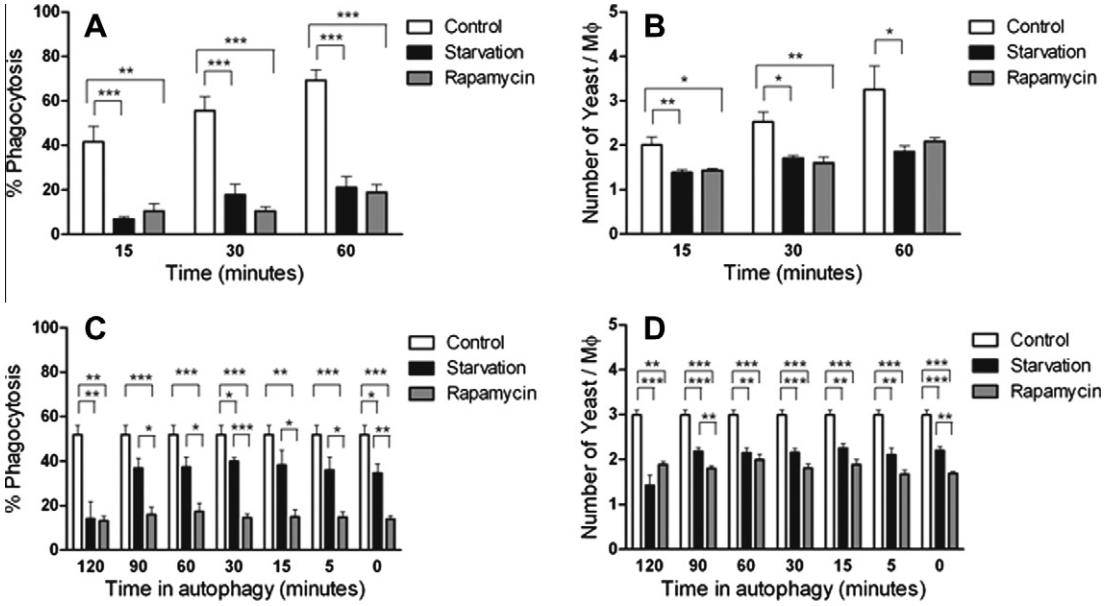

Fig. 2. Prior induction of autophagy inhibits the phagocytic capacity of murine macrophages early after interaction. For quantitative microscopic observations, CBA macrophages (2×10^5/mL) were incubated in physiological (Starvation) and pharmacological (Rapamycin) autophagic conditions as described in Fig. 1. In parallel, the control group was incubated in DMEM complete medium (Control). Two hours later, macrophages were incubated in the presence of yeast, fixed and stained with H&E. The percentage of yeast phagocytosis (A), as well as the number of yeast particles per macrophage (B) were quantified. To determine the length of time necessary for autophagy to inhibit phagocytosis, cells were further incubated for seven time intervals, ranging from 0 (time 0) to 120 min (0–5–15–30–60–90–120 min). Control cells were incubated in the nutrient-rich DMEM complete medium. Next, yeast particles were added to cultures (10:1) for 30 min, and after fixation the percentage of phagocytosis (C) and the number of particles per macrophage were quantified (D). The bars shown here correspond to the average results of four independent experiments performed in quadruplicate ±SE and the differences between cultures submitted to autophagic conditions in comparison to control cells were considered significant when $p < 0.05$ (ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

120 min, which was similar to the levels obtained in pharmacological autophagic induction (Fig. 2C, $p < 0.05$, $n = 4$, ANOVA). The number of internalized particles per cell was also reduced by 20–30% under both autophagic conditions at all intervals (Fig. 2D, $p < 0.05$, $n = 4$, ANOVA). These data demonstrate that the inhibitory effect on phagocytic capacity occurs at relatively early stages of both types of autophagic induction.

To establish whether autophagy affects phagocytosis directly or indirectly, an assay was conducted to test the reversibility of the autophagic inhibitory effect. CBA macrophages were incubated in nutrient-poor medium for 2 h, then washed and divided into two groups. The first group was kept in the starvation medium, while the second was placed into a nutrient-rich medium, and both groups were further incubated for an additional 30 or 60-min period. All cultures were subsequently incubated in the presence of yeast particles for 30 min and phagocytosis levels were then quantified. As expected, the macrophages cultured in starvation medium throughout the entire incubation period presented a significantly reduced percentage of phagocytosis in comparison to control cells (Fig. 3). By contrast, the cultures initially incubated in nutrient-poor medium and then shifted to a nutrient-rich medium, phagocytosed particle at levels similar to control macrophages incubated only in nutrient-rich medium (Fig. 3A and B, $p < 0.05$, $n = 4$ ANOVA). These data clearly demonstrate that the autophagic inhibitory effect on phagocytosis is completely reversible, thus reinforcing the notion that a direct correlation exists between autophagic induction and the inhibition of phagocytosis by macrophages.

In order to further investigate the correlation between autophagic induction and phagocytosis, we assessed whether this effect is dependent on particle recognition by incubating other inert particles in the experimental autophagic induction model. Macrophages were incubated under autophagic conditions for 2 h and subsequently incubated in the presence of inert particles, such as zymosan and latex beads, as well as live *L. amazonensis* promastigotes. Autophagic induction was observed to inhibit the phagocytosis of zymosan and latex beads (data not shown). Additionally, phagocytosis of *L. amazonensis* promastigotes and the number of parasites per cell (data not shown) were significantly reduced by exposure to autophagic conditions. These data demonstrate that autophagy affects the phagocytosis of several varieties of large particles, indicating that this effect is not dependent on any specific receptors involved in particle recognition.

To confirm that macrophage receptors do not play a role in the autophagic inhibition of phagocytosis, we tested whether particle opsonization by serum-derived soluble factors has an influence on this inhibitory effect. Yeast particles were incubated in the presence of fetal bovine serum for 30 min, then washed and added to macrophage cultures which were previously incubated in a nutrient-poor medium. These macrophage cultures showed similar reductions in the percentage of phagocytosis of non-opsonized or opsonized yeast particles (data not shown), which further supports the notion that particle–receptor recognition does not play a role in the autophagic inhibitory effect on phagocytosis.

Fluorescence microscopy analysis was performed to provide morphological evidence that autophagy does not influence particle–receptor interaction. Macrophages were incubated under autophagic conditions and then allowed to interact with FITC-zymosan for 10 min at 4 °C to distinguish particle recognition from the internalization step of phagocytosis. After fixation, macrophage cultures were labeled with rhodamine–phalloidin to visualize actin cups, which are involved in the early stages of phagocytosis [13]. When comparing cell cultures incubated under both physiological (Fig. 4B) and pharmacological autophagic conditions (Fig. 4C) to control cultures (Fig. 4A), the percentage of colocalization of zymosan-FITC (stained in green) with actin cups (labeled in red) (Fig. 4D, $p > 0.05$, $n = 4$, ANOVA), as well as the number of particles colocalized with actin cups (Fig. 4E, $p > 0.05$, $n = 4$, ANOVA) were found to

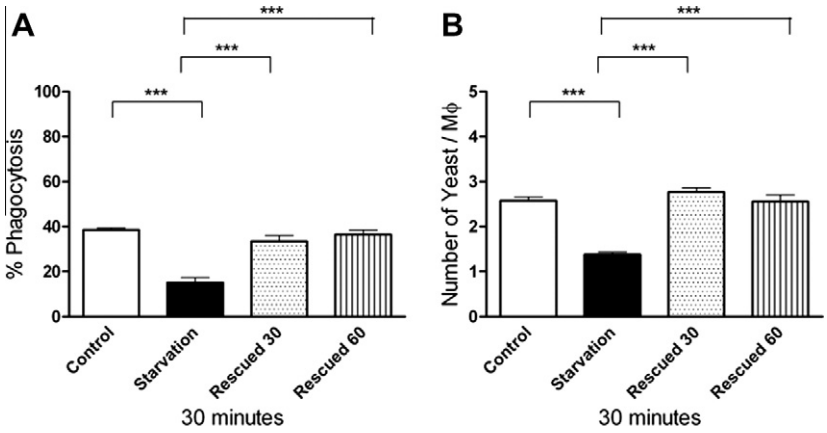

Fig. 3. The autophagic inhibition of phagocytosis is reversible. CBA macrophages (2×10^5/mL) were incubated in nutrient-poor medium for 2 h, then washed and divided into two groups. The first group remained in the starvation medium, while the second group was placed into a nutrient-rich medium, and both groups were further incubated for an additional 30 and 60 min. All cultures were incubated in the presence of yeast particles (10:1). The control group was incubated in DMEM complete medium (Control) during all the experimental intervals. The percentage of phagocytosis (A) and the number of yeast particles per cell (B) were quantified. The bars here correspond to the average results from four independent experiments performed in quadruplicate ±SE (ANOVA, $^{***}p < 0.001$).

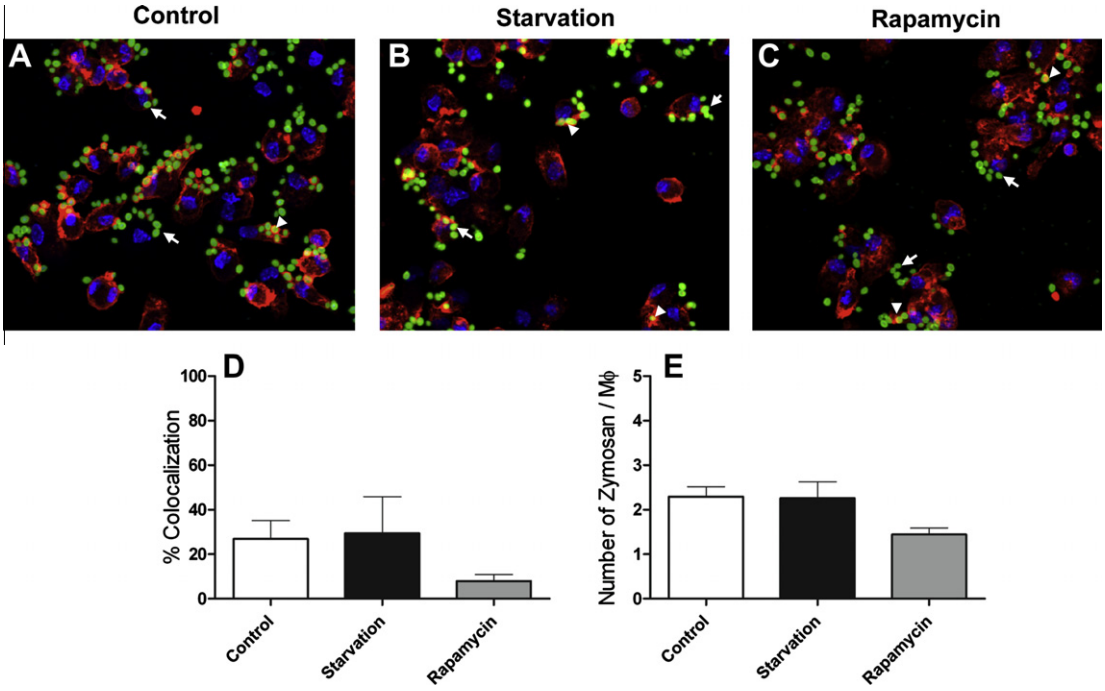

Fig. 4. Prior induction of autophagy inhibited particle internalization but did not alter particle binding to macrophages. CBA macrophages (2×10^5/mL) were incubated under physiological (Starvation) (B) and pharmacological (Rapamycin) autophagic conditions (C) and in parallel, control cells were incubated in DMEM complete medium (A). Macrophages were allowed to bind to zymosan-FITC (10:1) for 10 min at 4 °C, then fixed and processed for quantitative observation under fluorescence microscopy (A–C). Particle binding was identified by colocalization in yellow (arrow head) of zymosan-FITC stained in green (arrow) with polymerized actin filaments (actin cups) labeled with phalloidin in red. The percentage of colocalization of zymosan-FITC with labeled cells (D) and the number of particles colocalized with actin cups (E) were quantified. The bars shown here correspond to the average results from four independent experiments with control, rapamycin and starvation medium groups, performed in quadruplicate ±SE, which do not display statistically significant differences among these groups (ANOVA). Original magnification is 600×. Blue arrowheads indicate yeast particles associated with macrophage cell surfaces.

be similar among groups. These findings provide evidence that autophagic inhibition of phagocytosis occurs during a downstream step in which recognition and binding have already taken place, leading us to conclude that autophagic induction does not influence particle–receptor interactions during phagocytosis.

The present data is in apparent disagreement with previous findings [17], which showed that in macrophage cell line J774, incubated exclusively in starvation conditions, an enhancement of the phagocytosis of *Escherichia coli* and *Staphylococcus aureus* occurred. Unlike Martinet et al. (2009) we demonstrated that both physiological and pharmacological methods of autophagic induction provoke an inhibitory effect on the phagocytosis of large particles, such as latex beads (2 μm), zymosan, yeast and live *Leishmania* parasites. Further studies must be conducted to determine the precise mechanism involved in the induction of autophagy prior to phagocytosis. Since these two studies possess different methodologies, including the procedure used for quantification, the type and size of particles used and the time intervals of autophagic induction analyzed, it is possible that each study triggers autophagy in a different manner. Additionally, in apparent contrast

with the present results, the prior induction of autophagy was shown to not affect the phagocytosis of live *Coxiella burnetti* [10] or killed *S. aureus* [23]. However, both of these studies employed Chinese hamster ovary (CHO) cells, a non-macrophage cell line, which may indicate that the inhibitory autophagic effect on phagocytosis could be restricted to professional phagocytes.

Different lines of evidence describe the existence of interactions between autophagy and phagocytosis [16]. Using proteomic analysis, the autophagic protein LC3-II was identified in the membranes of latex bead-containing phagosomes [14]. In addition, LC3 recruitment to zymosan-containing phagosomes, as well as IgG bead-containing phagosomes, has been previously described [24]. Recent studies have demonstrated plasma membrane involvement as a membrane donor for the formation of autophagosomes [25,26]. These authors observed the formation of structures suggestive of pre-forming autophagosomes derived from the plasma membrane, requiring the recruitment of proteins from the endocytic pathway [8]. In the present study, one possible explanation for the loss of phagocytic ability in macrophages incubated under autophagic conditions may be the lack of available plasma membrane needed to perform particle internalization. The induction of autophagy may recruit and mobilize part of the plasma membrane for the formation of autophagosomes, thereby preventing the internalization of particles via the phagocytic pathway. Since the autophagic process has been demonstrated to be reversible, it is possible that when an autophagic stimulus is removed, the plasma membrane quickly become available again for the internalization process.

Alternatively, since the ER membrane has reportedly taken part in the biogenesis of both the autophagic [7] and phagocytic [27] pathways, another possible explanation of the inhibitory effect of autophagy on phagocytosis may originate from the insufficient amount of ER cisternal membrane available for both processes simultaneously.

Taken together, the present data clearly show that the induction of autophagy inhibits classical phagocytosis in murine macrophages via a mechanism that does not interfere with particle–receptor interaction. The results support the notion that phagocytosis and autophagy can be interdependent and complementary processes.

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