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Inhibition of autolysosome formation in host autophagy by *Trypanosoma cruzi* infection

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ABSTRACT

Autophagy has emerged as an essential component of the defense system against intracellular pathogens. We demonstrated that *Trypanosoma cruzi*, an intracellular protozoan parasite, was not eliminated by the host's autophagic machinery despite exposure to the host cell cytoplasm. Puncta of microtubule-associated protein 1 light chain 3 (LC3), an autophagy marker, and LC3-II, a lipidated form of LC3, were significantly increased after infection with *T. cruzi*, indicating that the parasite activated the early steps of host autophagy and induced autophagosome formation. However, autolysosomes were not observed in the infected cells. In addition, *T. cruzi* was not enwrapped by autophagosomes, suggesting that the parasite has mechanisms to allow it to evade autophagic capture. The results of this study indicate that host autophagy is incomplete following *T. cruzi* infection.

1. Introduction

Trypanosoma cruzi is an intracellular protozoan parasite that causes Chagas' disease. It affects 8 million people, mostly in Latin America (World Health Organization, 2012). The parasite has a complex life cycle with two main developmental stages in mammalian hosts, a trypomastigote and an amastigote stage. The non-proliferative trypomastigote form has a flagellum and spreads to tissues within the host *via* the bloodstream (de Souza, 1984). After invasion, the trypomastigote form is enclosed in a host membrane vesicle, known as a parasitophorous vacuole (TcPV). The TcPV is subsequently disrupted, and the parasite transforms into the amastigote form in the cytosol (Romano et al., 2009). Amastigotes then transform into trypomastigotes that can infect new cells (de Souza, 1984).

Autophagy is a major degradation system conserved among eukaryotic cells from yeast to mammals. Under nutrient starvation or certain cellular stresses, autophagy is induced, and the isolated autophagic double membrane, called a phagophore, is formed. Phagophores engulf organelles or cytosolic components and generate autophagosomes. Subsequently, autolysosomes are generated by the fusion of the outer membranes of the autophagosomes and lysosomes to degrade the cytoplasm-derived contents of autophagosomes, together with their inner membranes. These processes require many autophagy-related (Atg) proteins. When the autophagy pathway is activated, microtubule-associated protein 1 light chain 3 (LC3, a homolog of yeast Atg8; known as an autophagy marker) is localized with Atg12-Atg5-Atg16L complex on the surface of the phagophore. Atg12-Atg5-Atg16L complex is essential for the formation of phagophores and promotes the conversion of an immature LC3-I form to a mature, membrane associated LC3-II form. LC3-II mediates phagophore expansion. Consequently, the phagophore membrane is completely enclosed, and autophagosome formation is achieved (Nakatogawa et al., 2009; Mizushima et al., 2011; Gammoh et al., 2013). Autophagosomes are matured by a SNARE (*N*-ethylmaleimide-sensitive factor attachment protein receptor) protein, syntaxin 17 (Stx17), which is inserted into the autophagosomal membrane. The maturing autophagosome interacts with a lysosomal SNARE protein, vesicle-associated membrane protein 8 (VAMP8), to generate an autolysosome (Itakura et al., 2012; Itakura and Mizushima, 2013).

Abbreviations: Atg, autophagy-related protein; LC3, microtubule-associated protein 1 light chain 3; SNARE, *N*-ethylmaleimide-sensitive factor attachment protein receptor; Stx17, syntaxin 17; SQSTM1, sequestosome 1; TcPV, *T. cruzi* parasitophorous vacuole; VAMP8, vesicle-associated membrane protein 8.

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It is known that autophagy is essential not only for cell survival and maintenance of homeostasis, but also for elimination of intracellular pathogens. Group A *Streptococcus* and *Mycobacterium tuberculosis* are eliminated by the host autophagic machinery (Campoy and Colombo, 2009), while *Shigella flexneri* and *Listeria monocytogenes* are not captured by autophagosomes (Ogawa et al., 2011; Travassos et al., 2010; Campbell-Valois et al., 2015; Birmingham et al., 2007). *Burkholderia pseudomallei* secretes the protein BopA, which inhibits host autophagic degradation by an unknown mechanism (Devenish and Lai, 2015). *Brucella abortus* produces proteins that inhibit fusion of autophagosomes and lysosomes (Campoy and Colombo, 2009). Protozoan parasites use the host autophagic machinery to proliferate. *Toxoplasma gondii,* an intracellular parasite, utilizes host autophagy for its own nutrition and promotes its replication (Gao et al., 2014; Wang et al., 2009). It has been reported that LC3 protein is recruited to *T. cruzi* invasion sites (Romano et al., 2009), but it is unclear whether the host autophagic pathway is promoted or inhibited during infection. As the parasites are not eliminated from infected cells, we hypothesized that host autophagy is inhibited by infection. To clarify this hypothesis, we examined whether functional autophagic vesicles are formed and found that *T. cruzi* infection induces host autophagosome formation but that autolysosome formation is inhibited.

2. Materials and methods

2.1. Parasites and cells

Human fibrosarcoma cells, HT1080 (Japan Health Sciences Foundation, Tokyo, Japan), were used as the *in vitro* host. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; BioWest, Nuaillé, France) at 37 °C in the presence of 5% CO_2. *T. cruzi* trypomastigotes (Tulahuen strain) were maintained and propagated by continuous passage in HT1080 cells, as described previously (Nakajima-Shimada et al., 2000).

2.2. T. cruzi *infection and autophagy induction*

Exponentially growing HT1080 cells (3×10^4 cells) were inoculated and incubated overnight in DMEM with 10% FBS, then infected with *T. cruzi* trypomastigotes (3×10^5 parasites).

For induction of autophagy, cells were washed once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_3 \cdot 12H_2O$, 1.8 mM KH_2PO_3, pH 7.4), then cultured in either amino acids free DMEM without FBS for 2 h, or in glucose free DMEM with 10% FBS for 9 h.

2.3. Immunocytochemistry

HT1080 cells were grown on a coverslip with a diameter of 12-mm in a 24-well plate. After *T. cruzi* infection or nutrient starvation, the cells were fixed with 4% paraformaldehyde, washed with PBS three times, and then immersed in permeabilization buffer (2 mM Tris–HCl, 1 mM EDTA, 0.1% Triton X-100, pH 8.0) for 5 min. The cells were washed with PBS and blocked with 1% BSA (Sigma-Aldrich, St. Louis, MO) in PBS for 1 h. After blocking, cells were stained with primary antibodies for 1 h, washed three times with PBS, followed by staining with fluorescent dye-conjugated secondary antibodies for 1 h. The following primary antibodies were used: mouse anti-LC3 monoclonal antibody (1:50, MBL, Aichi, Japan), rabbit anti-Atg16L polyclonal antibody (1:1000, MBL), and rabbit anti-mCherry polyclonal antibody (1:1000, GeneTex, Irvine, CA). The following secondary antibodies were used: Alexa 568-conjugated donkey anti-rabbit IgG antibody (1:3000, Life Technologies, Carlsbad, CA), and Alexa 488-conjugated donkey anti-mouse IgG antibody (1:1000, Life Technologies). Cellular and parasite nuclei were stained with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan). The cover slips were mounted on the slides in PermaFluor reagent (Thermo Fisher Scientific, Waltham, MA). Image acquisition was performed by an FSX100 fluorescence microscope (Olympus, Tokyo, Japan). For quantification of LC3 puncta, one hundred cells were randomly selected from multiple fields of view and the numbers of dots stained with anti-LC3 antibodies were counted. The infectivity and intracellular growth of *T. cruzi* amastigotes was determined by counting the numbers of parasite nuclei stained with Hoechst 33342. The co-localization of LC3 and Atg16L or LC3 and Stx17 was determined by analyzing one hundred cells using FSX-BSW software (Olympus). Pearson's correlation coefficient values were calculated using Image J software (NIH).

2.4. Immunoblot analysis

HT1080 cells (1×10^5 cells) were cultured in 60-mm dishes overnight, and then either infected with *T. cruzi* (1×10^6 parasites) or subjected to amino acid starvation. After washing with PBS, cells were harvested with cell scrapers, resuspended in ice-cold PBS supplemented with protease inhibitor cocktail (Thermo Fisher Scientific), and lysed *via* ultrasonication on ice. The protein concentration was determined using a protein assay dye reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instruction. The protein sample was mixed with the equal volume of 2 × SDS sample buffer (0.1 M Tris–HCl, pH6.8, 4% SDS, 12% β- mercaptoethanol, 20% glycerol) and heated at 95 °C for 5 min prior to electrophoresis on 15% SDS-polyacrylamide gels (XV PANTERA gel; DRC Corporation, Tokyo, Japan). For immunoblot analysis, proteins were transferred to polyvinylidenedifluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany) for 1 h. After blocking with 5% skim-milk in Tris-buffered saline (TBS; 137 mM NaCl, 2.7 mM KCl, 25 mM Tris–HCl, pH 7.4) overnight at 4 °C, the membranes were incubated with primary antibodies diluted with 1% skim-milk in TBS for 1 h at room temperature, washed with 0.1% Tween 20 with TBS (TBS-T), and followed by probing with the horseradish peroxidase (HRP)- or the alkaline phosphatase (AP)-conjugated secondary antibodies for 1 h. The following primary antibodies were used: rabbit anti-LC3 polyclonal antibody (1:1000, MBL), mouse anti-p62/SQSTM1 monoclonal antibody (1:1000, MBL), and mouse anti-GAPDH monoclonal antibody (1:2000, clone 3C2, Abnova, Taipei, Taiwan). We confirmed that the mouse anti-GAPDH antibody did not react with *T. cruzi* lysate. The following secondary antibodies were used: HRP-conjugated goat anti-rabbit IgG antibody (1:5000, Cayman, Ann Arbor, MI) and AP-conjugated goat anti-mouse IgG antibody (1:2000, Jackson Immuno Research, West Grove, PA). After washing with TBS-T, target proteins were detected with ImmunoStar LD (Wako) or Tropix® CDP-Star (Life Technologies) and visualized using a Molecular Imager® (Chemi Doc™ XRS+; Bio-Rad).

2.5. Plasmid, transfection, and isolation of stable transfectants

To construct a plasmid encoding mCherry-tagged Stx17, a DNA fragment encoding Stx17 was amplified from total RNAs derived from HT1080 cells by RT-PCR using KOD FX DNA polymerase (Toyobo, Osaka, Japan) and the following primer pairs, the forward primer (5′-GGACTCAGATCTCGAGATGTCTGAAGATGAAGAAAA-3′) and reverse primer (5′-GATCCCGGGCCCGCGAACTGCATTTCTTGTCAGTTT-3′). The obtained DNA fragments were subcloned into *Xho* I and *Kpn* I site of the pmCherry-N1 (Clontech, Mountain View, CA). The construct was transfected into HT1080 cells using Turbofect transfection reagent (Thermo Fisher Scientific). The transfected