

## Full Length Research Paper

# Role of autophagy in inhibition of brucella-containing vacuole-lysosome fusion in murine macrophages

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This study aimed to investigate the role of autophagy in the inhibition of Brucella-containing vacuole (BCV) and lysosome fusion in *BRUCELLA SUIIS* 1330 infected murine macrophage RAW264.7 cells. Autophagosome induction was observed following *B. SUIIS* 1330 infection. The autophagic pathway was inhibited pharmacologically by 3-methyladenine (3-MA) treatment or by RNAi-mediated depletion of the autophagy related gene, LC3. Compared with controls, suppression of the autophagic pathway was associated with a significant decrease in *B. SUIIS* 1330 internalization ( $P<0.05$ ) and reduction in the detection of *B. SUIIS*-containing vacuole and lysosome fusion by fluorescence colocalization. These results implicate autophagy in the inhibition of BCV-lysosome fusion which favors the survival of *B. SUIIS* 1330 in murine macrophage RAW264.7 cells.

**Key words:** *Brucella suis* 1330, autophagy, BCV-lysosome fusion, colocalization.

## INTRODUCTION

*Brucella* are Gram-negative, facultative intracellular bacteria, which are endemic in many developing countries. These pathogens cause brucellosis which is one of the five most common bacterial zoonosis globally and significant cause of human suffering and economic losses. Animals and humans are infected via small injuries, aerosols or ingestion of contaminated dairy products. Professional phagocytes are the primary target of *Brucella* infection (Baldwin and Winter, 1994; Liautard et al., 1996; Price et al., 1990; Smith and Ficht, 1990), where the bacteria survive and replicate efficiently with

minimal disturbance by the immune system (Jones and Winter, 1992).

In contrast to other intracellular pathogens, *Brucella* species do not produce exotoxins, antiphagocytic capsules or thick cell walls, resistance forms or fimbriae and do not display antigenic variation. Furthermore, plasmids or lysogenic phages that have the potential to transfer virulence genes have not been identified (Ugalde, 1999). The inhibition of *Brucella*-containing vacuole (BCV)-lysosome fusion has been proposed as a mechanism by which *Brucella abortus* achieves intracellular survival (Frenchick et al., 1985; Arenas et al., 2000; Frenchick et al., 1985; Harmon et al., 1988; Liautard et al., 1996; Oberti et al., 1981; Pizarro-Cerda et al., 1998 a, b). Typically, avoiding the fusion of BCVs and lysosomes makes this pathogen exceedingly well adapted to the intracellular environment through the ability to control its own intracellular trafficking (Arenas et al., 2000). However, many aspects of the mechanism underlying the inhibition of BCV-lysosome fusion remain unclear.

The accumulation of recent evidence indicates that autophagy is one of the most remarkable components of

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**Abbreviations:** BCV, Brucella-containing vacuole; CFU, colony forming units; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; GFP, green fluorescent protein; MOI, multiplicity of infection; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffered saline; 3-MA, 3-methyladenine; MDC, monodansylcadaverine; TRD, Texas red-dextran.

the intracellular host cell defense machinery that bacteria confront upon cell invasion (Gutierrez et al., 2004; Ogawa et al., 2005). However, several pathogens subvert the autophagic pathway and manipulate this process at the molecular level as a strategy to establish persistent infection (Otto et al., 2004; Romano et al., 2007; Schnaith et al., 2007). It has been reported that *B. abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of non-professional phagocytes (Cerdeira et al., 1998). In this study, the role of autophagy in inhibition of BCV-lysosome fusion was investigated in *B. suis* 1330 infected murine macrophages.

## MATERIALS AND METHODS

### Reagents

N-Hydroxysuccinimidyl ester 5-(and-6)-carboxyfluorescein and Texas red-dextran (TRD) (molecular weight of 70,000) were purchased from Molecular Probes (Eugene, Oreg. USA) Wortmannin (WM) was purchased from Sigma Aldrich (St. Louis, Missouri, USA). Polyclonal antibodies for  $\beta$ -actin and LC3 were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

### Cell culture

Murine macrophage RAW264.7 cells and LC3 depleted RAW264.7 cells were routinely cultured at 37°C in a 5% CO<sub>2</sub> atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

### Plasmids, bacterial strains, culture, and preparation

The plasmids pGFP-LC3 and *B. suis* 1330-GFP (1330-GFP) for constitutive expression of green fluorescent protein (GFP) were kindly provided by Dr. Yin Han (Department of College of Medicine, Lan Zhou University, China). These were prepared as described previously (Ouahrani-Bettache et al., 1999). Virulent *B. suis* 1330 and 1330-GFP were cultured in Brucella broth or on Brucella agar plates.

### Brucella infection

RAW264.7 cells were infected with Brucella at a multiplicity of infection (MOI) of 100. Infected cells were then incubated at 37°C. Thirty minutes post-infection, culture medium was removed and the cells were rinsed with phosphate buffered saline (PBS). Fresh medium, supplemented with 40  $\mu$ g/ml gentamicin was then added for 1 h to eradicate extracellular bacteria.

### RNA interference of LC3

Oligonucleotide sequences synthesized for RNAi were as follows:

#### LC3-1

5'-  
GATCCGGACCTTCAAGCAGCGCCGGAGGTGTGCTGTCCCTCC  
GGCGCTGCTTGAAGGTCTTTTA-3'5'-  
AGCTTAAAGACCTTCAAGCAGCGCCGGAGGGACAGCACACC

TCCGGCGCTGCTTGAAGGTCCG-3'

#### LC3-2

5'-  
GATCCGGCAGCGCCGGAGCTTTGAACAGTGTGCTGTCCCTGTT  
CAAAGCTCCGGCGCTGCTTTTA-3'  
5'-  
AGCTTAAAGCAGCGCCGGAGCTTTGAACAGGACAGCACACT  
GTTCAAAGCTCCGGCGCTGCCG-3'

The two complementary oligonucleotides were annealed and subcloned into pSuper\_neo vector (Oligoengine, USA). RNAi vectors were transfected into RAW264.3 cells using Lipofectamine 2000 and stable clones were selected by the addition of G418. Stable clones were screened by RT-PCR and expression of LC3 was confirmed by Western blotting using LC3-specific antibodies.

### Labeling of autophagic vacuoles

For labeling of autophagic vacuoles with MDC, RAW 264.7 cells were split onto glass coverslips following siRNA 48 h transfection. After 4 h of Brucella infection, cells were incubated with 0.05 mM monodansylcadaverine (MDC) (1:1 DMSO/ethanol) in the culture media at 37°C for 30 min. Cells were then washed three times with PBS (pH 7.4), fixed with freshly prepared 4% paraformaldehyde solution in PBS for 15 min at room temperature and analyzed by fluorescence microscopy (MDC excitation wavelength, 380 nm; emission filter, 525 nm) (Axioplan, Zeiss, Germany).

### Lysosome labeling

Lysosomes were labeled by fluid-phase pinocytosis with TRD 10  $\mu$ g/ml, pulsed for 16 h and chased for 1.5 h before infection with bacteria.

### Evaluation of phagosome-lysosome fusion

Measurements were made following lysosomal labeling and infection. Cells were fixed for 20 min with 3% paraformaldehyde and washed in PBS. Coverslips were mounted in Mowiol medium and examined by confocal fluorescence microscopy (Leica DMB). Fusion was evaluated by the colocalization of the markers, GFP and TRD at 4, 12 and 24 h post-infection. The percentage fusion was analyzed in 100 to 200 cells for each strain at each time point. Results were calculated from two independent experiments.

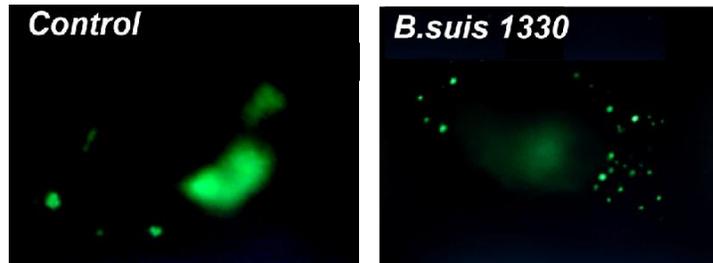
### Drug treatment

Cells were co-incubated in 24 well plates with the autophagy inhibitor 3-methyladenine (3-MA) at the indicated concentrations. Following infection, the treated cells were incubated at 37°C with 5% CO<sub>2</sub>. To evaluate Brucella internalization, after 30 min of infection, fresh medium, supplemented with the same concentration of drug and gentamicin (80  $\mu$ g/ml) was added to eradicate extracellular bacterium. Cells were lysed after 45 min and CFUs were counted after three days.

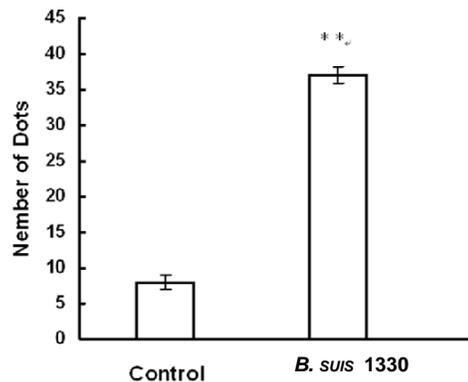
### MTT Assay

Cells were seeded in 96-well plates. After 24 h, the medium was changed and 3-MA (10 mM) was added with further incubation up

A



B



**Figure 1.** Autophagosome formation in murine macrophage RAW264.7 is induced by *B. suis* 1330 infection. (A) RAW264.7 cells transfected with pGFP-LC3 were infected with Brucella at a multiplicity of infection (MOI) of 100 for 30 min. Extracellular bacteria were eradicated by gentamicin (40  $\mu$ g/ml) treatment for 1 h. Infection was visualized by laser scanning confocal microscopy. (B) A greater accumulation of GFP-LC3 was detected in the *B. suis* 1330 infected cells compared with control cells ( $P<0.01$ ). Results were quantified as the average number of GFP-LC3 particles inside the cytoplasm of 100 cells. Measurements were performed by three independent analysts in a blinded fashion.

to 48 h. The incubation medium in test wells was replaced with 50  $\mu$ l 3-(4, 5-Dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) solution and the cells were incubated for 4 h at 37°C. The MTT solution was then discarded and replaced with 150  $\mu$ l DMSO to dissolve the precipitate completely at room temperature. The optical density was then measured spectrophotometrically at 570 nm.

## RESULTS AND DISCUSSION

### ***B. suis* 1330 infection triggers autophagosome formation in murine macrophage RAW264.7**

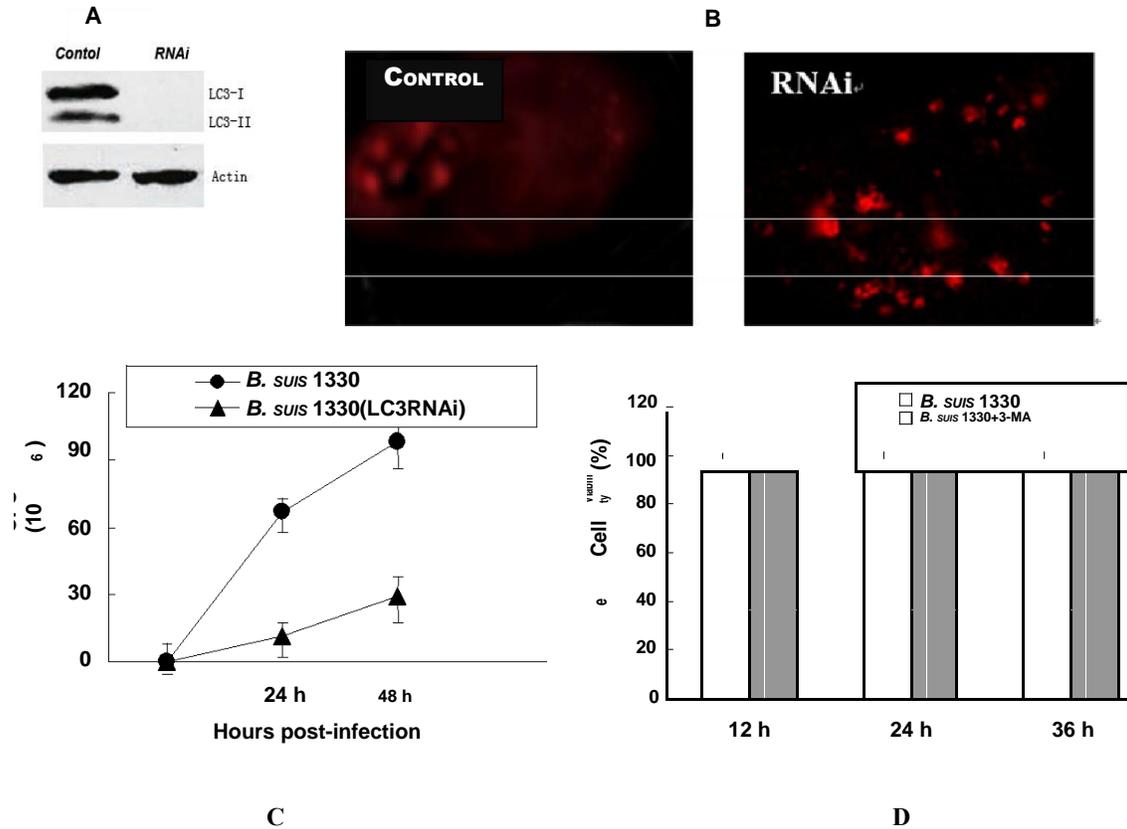
The induction of autophagy by *B. suis* 1330 infection was investigated by transfection of RAW264.7 cells with pGFP-LC3 and visualization by laser scanning confocal microscopy (Figure 1A). Punctate accumulation of GFP-LC3 proteins in *B. suis* 1330 infected cells was observed and quantified as the average number of GFP-LC3 particles in the cytoplasm of 100 cells. Measurements

were performed by three independent analysts in a blinded fashion. Results revealed a greater accumulation of GFP-LC3 in the *B. suis* 1330 infected cells compared with control cells ( $P<0.01$ ) (Figure 1B).

It has been reported that *B. abortus* induces autophagy in infected HeLa cells and macrophage cells (Arenas et al., 2000; Gorvel and Moreno et al., 2002) In this study, autophagosome formation was observed in target cells infected with *B. suis* 1330. These results indicate a relationship between autophagy and *B. suis* 1330 infection, although the precise mechanism remains to be elucidated.

### ***B. suis* 1330 induced autophagosome formation is suppressed by the autophagy inhibitor 3-MA or the depletion of LC3**

The effect of autophagy on *B. suis* 1330 replication



**Figure 2.** *B. suis* 1330 -induced autophagosome formation is suppressed by the autophagy inhibitor 3-MA or the depletion of LC3. The autophagic pathway in *B. suis* 1330 infected murine macrophage RAW264.7 cells was inhibited pharmacologically by exposure to 3-MA and by RNAi-mediated knockdown of endogenous LC3. (A) The expression of both LC3-I and LC3-II was determined by Western Blotting, and was found to be significantly lower in the knockdown RAW264.7 cells compared with the untreated control group. Detection of  $\beta$ -actin served as a loading control. (B) LC3 depletion markedly suppressed *B. suis* 1330 induced autophagosome formation evaluated by MDC staining following exposure to *B. suis* 1330 for 4 h. (C) Relative bacterial number (CFU) was clearly reduced following 3-MA pretreatment and RNAi-mediated LC3 knockdown. (D) Cell viability was not affected by pharmacological inhibition of autophagy or depletion of LC3. Cell viability was evaluated by MTT at 12, 24 and 36 h post-infection.

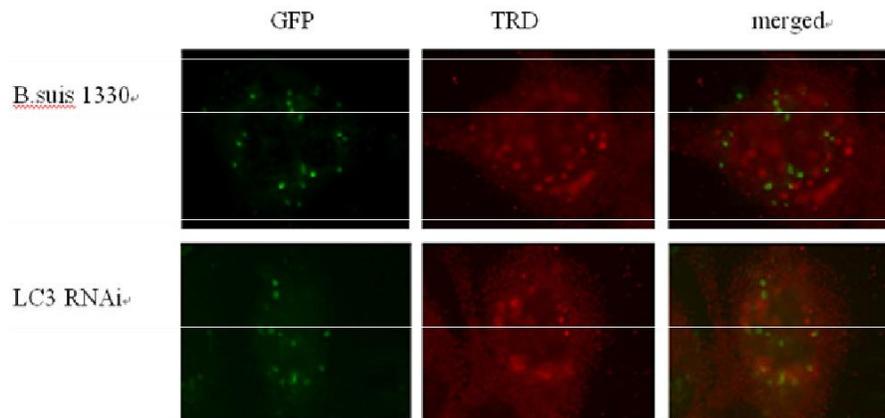
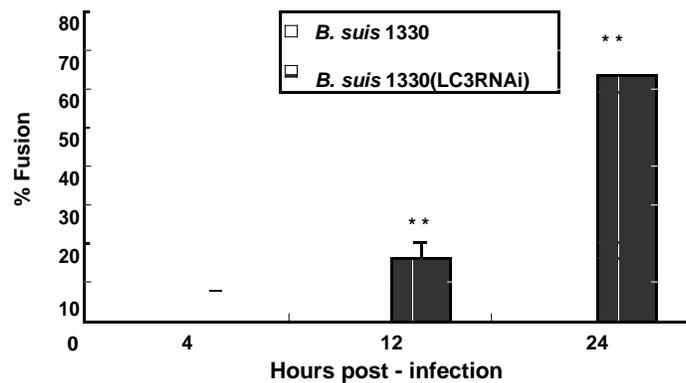
efficiency in *B. suis* 1330 infected murine macrophage RAW264.7 cells was investigated by suppression of the autophagic pathway. This was achieved by exposure of the cells to the autophagy inhibitor 3-MA and by knockdown of endogenous LC3. The expression of both LC3-I and LC3-II, determined by Western Blotting, was found to be significantly lower in the LC3-depleted RAW264.7 cells compared with the untreated control group. Detection of  $\beta$ -actin served as a loading control (Figure 2A). Autophagosome formation in LC3 depleted or 3-MA treated cells was evaluated by MDC staining following exposure of these cells to *B. suis* 1330 for 4 h. LC3 depletion markedly suppressed *B. suis* 1330 induced autophagosome formation (Figure 2B) and relative bacterial number (CFU) was clearly reduced following 3-MA pretreatment and RNAi-mediated LC3 knockdown (Figure 2C). Cell viability was assessed by MTT assay and was not found to be affected markedly by

pharmacological inhibition of autophagy or depletion of LC3 (Figure 2D).

In this study, reduction in *B. suis* 1330 was demonstrated by inhibition of the autophagic pathway in *B. suis* 1330 infected macrophage RAW264.7 cells by 3-MA treatment and RNAi-mediated LC3 depletion.

### Reduction in the detection of *B. suis*-containing vacuole and lysosome fusion by Fluorescence colocalization

Interactions between *B. suis*-containing vacuoles and lysosomes of the murine macrophage cell line RAW264.7 were investigated by confocal fluorescence microscopy. Lysosomes were first labeled by fluid-phase pinocytosis of TRD and then infected with bacteria which constitutively express a GFP. Cells were fixed at 4, 12

**A****B**

**Figure 3.** Reduction in the detection of *B. suis*-containing vacuole and lysosome fusion by fluorescence colocalization. Fusion between *B. suis*-containing vacuoles and lysosomes in RAW264.7 cells was investigated by confocal fluorescence microscopy. (A) Cells containing TRD-labeled lysosomes were infected with GFP-expressing bacteria and fixed at 4, 12 and 24 h post- infection. Fusion was evaluated by the colocalization of both markers. (B) The percentage of colocalization in RAW264.7 cells depleted of LC3 was much higher than that of wild-type RAW264.7 cells. Percentage fusion was analyzed in 100 to 200 cells for each strain at each time point. Results were calculated from two independent experiments.

and 24 h post-infection and examined. Fusion was evaluated by the colocalization of both markers (Figure 3A). Observation of a TRD ring around the internalized particle or partial association of TRD with phagosomes was considered to be indicative of fusion. The percentage of colocalization in RAW264.7 cells depleted of LC3 was much higher than that of wild-type RAW264.7 cells (Figure 3B). These results indicate that autophagy is involved in inhibition of phagosome-lysosome fusion in murine macrophage RAW264.7 cells.

Autophagy is an important host defense mechanism in the elimination of intracellular bacteria and protozoan pathogens, such as *Mycobacterium tuberculosis* (Gutierrez et al., 2004), and *Shigella* (Ogawa et al.,

2005), thus indicating a role for this pathway in innate immunity against intracellular bacteria. However, several studies indicate that many pathogens such as *Legionella pneumophila* and *Coxiella burnetii* (Horwitz et al., 1983; Swanson and Isberg, 1995) have evolved strategies to protect themselves against autophagy or to control the components of autophagy to their own benefit.

In this study, suppression of autophagy was associated with reduced bacterial internalization and BCV-lysosome formation, thus indicating intracellular bacterial survival is favored by *B. suis* 1330-induced autophagy. These data implicate autophagy in inhibition of BCV-lysosome fusion in murine macrophage RAW264.7 cells. These results are in accordance with the model reported by Qing-Ming

Qin1boratorio (Qing-Ming Qin et al., 2008), which demonstrate that early after internalization, *Brucella abortus* impairs fusion with lysosomes and traffics to autophagosome-like compartments, replicating in an ER-derived compartment. The precise relationship between autophagy and the inhibition of BCV-lysosome fusion and its role in the pathogenesis of *Brucella* remain to be elucidated.

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