Deletion of the transcriptional regulator GntR affects apoptosis and autophagy in *Brucella abortus*-infected RAW 264.7 cells

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Research

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Abstract

Background: Brucellosis is an important zoonotic disease caused by the pathogen *Brucella*. Regulating apoptosis and autophagy is the prerequisite for the intracellular survival of *Brucella*. GntR is an important transcriptional regulator of *Brucella* that can regulate the expression of many target genes, and then play a regulatory role in many cell processes, including regulation apoptosis and autophagy. Therefore, understanding the relationship between GntR and apoptosis and autophagy is crucial to comprehending the pathogenic mechanism of *Brucella*.

Methods: In the present study, we described the influence of GntR on apoptosis and autophagy after the infection of RAW 264.7 cells with *Brucella*. We constructed the GntR mutant strain (2308ΔGntR) of *Brucella abortus* 2308 (S2308). Following the infection of the RAW 264.7 cells with S2308 and 2308ΔGntR, apoptosis and autophagy were detected.

Results: Western blot analysis and flow cytometry analysis indicated that the apoptosis rate of the 2308ΔGntR-infected group was remarkably higher than that of the S2308-infected group. Confocal laser microscopy experiments indicated the presence of the P62 protein as punctate aggregates in the 2308ΔGntR group.

Conclusion: These results showed that 2308ΔGntR promoted apoptosis and inhibited autophagy in the RAW 264.7 cells during *Brucella* infection.

Introduction

Brucella spp. are intracellular parasites that cause the a worldwide zoonotic disease called brucellosis in animals and humans [1]. Brucellosis brings enormous economic burdens for developing countries [2]. Macrophages, dendritic cells, and embryonic trophoblasts are the target cells of *Brucella* [3, 4]. Furthermore, several other cells are susceptible to *Brucella* infection, and they include epithelial cells, human leukemic monocyte cells, osteoclasts, brain microvascular endothelial cells, and hepatic stellate cells [5–8]. *Brucella can* inhibit the maturation of phagosomes and prevent macrophages from forming phagolysosomes [9].

Apoptosis is a programmed cell death process, which is an essential physiological process that regulates tissue mass and architecture during normal tissue development [10]. Apoptosis is an active form of cell death characterized by various morphological and biochemical features [11]. *Francisella tularensis* could induce apoptosis in murine macrophages [12]. *Brucella* could also regulate apoptosis in macrophages [13]. Autophagy is a major protein degradation system. Autophagy is ubiquitous in eukaryotic cells, and it can preserve the homeostasis of the intracellular environment [8]. Autophagy is also a vital intracellular mechanism that prevents microbial infection [8, 14]. Autophagy participates in cellular functions as well [15]. Therefore, apoptosis and autophagy play a crucial role in regulating a series of important cell life activities.

Bcl-2 is an inhibitor of apoptosis. Increased levels of Bcl-2 expression in cells could prevent apoptosis [16]. Stimulating factors could inhibit Bcl-2 expression and induce apoptosis [17]. Bax can join to Bak at the outer membrane of the mitochondria, collapse the mitochondrial membrane potential, and consequently trigger the cell apoptosis [18]. The outbalance ratio of Bcl-2 and proapoptotic Bax plays a pivotal role in apoptosis [19]. The imbalance of Bcl-2 and proapoptotic Bax leads to apoptosis [19]. Caspase-3 and − 8 are the key regulators of the apoptotic response, and they interact with the calpain family, a group of cysteine proteases, in the course of diseases [20]. Caspase-3 and − 8 are activated in the death receptor pathway of apoptosis. Caspase-3 and − 8 overexpression could enhance apoptotic activity [21]. Autophagy is an intracellular bulk degradation system. P62 is an autophagic substrate [22]. When autophagy is enhanced, the P62 substrate is cleared [23]. However, when the inhibitor 3-methyladenineis used to inhibit autophagy, the P62 substrate accumulates [24]. P62 can be used as a marker of autophagy [25]. Microtubule-associated protein light chain 3 (LC3) is a mammalian homolog of yeast Atg8. Upon induction of autophagy, LC3 is conjugated to phosphatidylethanolamine [26]. Therefore, LC3 has been used as a specific marker to monitor autophagy.

GntR is a transcriptional regulator. It is involved in the regulation of biological functions in many pathogens, including stress response and nutrient uptake [27, 28]. GntR is an important virulence factor in *Brucella* [29]. In addition, GntR affects the expression of the virulence genes of *Brucella* and the secretion of inflammatory cytokines during infection [29, 30]. However, the intracellular functions of GntR, apoptosis, and autophagy remain unknown.

In the present study, the roles of GntR in apoptosis and autophagy were detected. This study aimed to determine the function of GntR in apoptosis and autophagy during *Brucella* infection and to provide new ideas and clues for further elucidating the mechanisms of the intracellular survival and pathogenesis of *Brucella*.

Materials And Methods

Bacterial strains and cell line

B. abortus 2308 strain (S2308) was obtained from the Center of Chinese Disease Prevention and Control (Beijing, China). The 2308Δ*gntR* mutant and 2308Δ*gntR*-C complementary strain were constructed and kept in our research laboratory [30]. All *Brucella* strains were cultured in tryptic soy agar (TSA) or broth (TSB) (Difco, MI, USA) at 37°C in 5% CO_2 (v/v). The murine macrophage RAW 264.7 line was obtained from the Cell Resource Center (Beijing, China). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Rockville, MD, USA) at 37°C with 5% CO_2 (v/v). All experimental procedures were performed in Biosafety Level 3 Laboratory (BSL-3).

Flow cytometry

The apoptosis was detected by flow cytometry, as previously described but with several modifications [31]. Briefly, monolayers of RAW 264.7 macrophages of 1×10^6 cells/well were cultured in six-well plates for 24 h at 37°C under 5% CO_2 and then infected with *Brucella* strains at a multiplicity of infection (MOI) of 100. Culture plates were centrifuged at 350 × *g* for 5 min at room temperature, and incubated at 37°C for 45 min. After washing twice with the medium without antibiotics, the infected cells were incubated for 60 min in the presence of 50 μg/mL gentamicin to kill the extracellular bacteria. Then, the cultures were placed in fresh DMEM containing 25 μg/mL gentamicin (defined as time zero) and incubated at 37°C. The control group comprised uninfected cells. At 24 h post-infection, the cells were washed with PBS, centrifuged at 900 × *g* for 5 min, and then resuspended with 500 μL of binding buffer mixed with 5 μL of fluorescein isothiocyanate (FITC)-labeled Annexin V (Annexin V-FITC) and 5 μL of propidium iodide (PI). After incubation in the dark at room temperature for 15 min, the cell samples were detected by flow cytometry (Life Technology, USA). All assays were performed thrice.

Confocal microscopy

The expression of P62 protein in the cell was observed by confocal microscopy, as previously described with several modifications [13]. Briefly, RAW 264.7 cells were infected with S2308 or 2308ΔGntR as described above. The control group comprised uninfected cells. At 24 h post-infection, the cells were fixed in 4% paraformaldehyde (PFA) for 10 min. Then, the cells were washed for 5 min with 1 mL of PBS containing 2 mg glycine. The cells were blocked with 1 mL of PBS containing 1% (v/v) bovine serum albumin (BSA) and 0.1% (v/v) Triton X-100 for 1 h. The cells were washed thrice with PBS and added with primary (rabbit anti-mouse P62 polyclonal antibody) antibody (Bioworld, Minneapolis, USA). Then, the cells were placed overnight at 4°C. Thereafter, the cells were washed thrice with PBS and added with secondary (green fluorescently tagged goat anti-rabbit IgG antibody) antibody (Bioworld, Minneapolis, USA) for 1 h at 37°C. Subsequently, the cells were washed thrice with PBS, and fluorescence was observed by confocal microscopy (ZEISS, Germany).

Macrophage infection and RNA extraction

Murine macrophage RAW 264.7 cells were used to detect the apoptosis and autophagy of S2308, 2308Δ*gntR* and 2308Δ*gntR*-C. The RAW 264.7 cells were infected with *Brucella* as described above. At 4, 8, 12 and 24 h post-infection, 1 mL TRIzol (Invitrogen, Carlsbad, CA, USA) was added to the cells for each well. RNA was also isolated from the uninfected cells as a negative control. Residual DNA in the samples was removed using DNase I (Promega, Madison, WI, USA). RNA concentration and purity were determined spectrophotometrically using an ND 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The total RNA from the *Brucella*-infected cells was extracted as previously described [32, 33]. All assays were performed in triplicate and repeated at least thrice.

Quantitative real time-PCR (qRT-PCR)

QRT-PCR was used to detect the expression levels of apoptosis-associated genes (caspase-3, caspase-8, Bax, and Bcl-2) and autophagy-associated genes (LC3-I, LC3-II, and p62), as previously published

procedures with several modifications [34]. The cDNA was generated from the total RNA by using a random hexamer primer and the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The apoptosis- and autophagy-associated genes were selected. The primers used to amplify these genes are listed in Table 1. Samples were run in triplicate and amplified in a 20 μL reaction containing 2 × SYBR Premix Ex Taq II (Takara, Japan), 100 nM forward and reverse primers, and 1 μL of cDNA target. The mix was incubated for 5 min at 95°C, and then 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s were performed on a Roche LightCycler 480 II system (Roche, Basel, Switzerland). The relative transcriptional levels were determined by the methods of $2^{-\Delta\Delta Ct}$, as described previously [35]. The expression of β-actin was used as a reference gene. All assays were performed in triplicate and repeated at least thrice.

Western blot (WB) analysis

To determine the expression levels of caspase-3 and P62 in the RAW 264.7 cells, we analyzed caspase-3 and P62 protein lysates by WB as previously described with several modifications [13, 36]. Briefly, the RAW 264.7 cells were infected with S2308 and 2308ΔGntR following the steps as described above. The control group comprised uninfected cells. At 24 h post-infection, the cells were lysed in ice-cold RIPA lysis buffer (Solarbio Science and Technology, Beijing, China) for 20 min and centrifuged at 13,400 × *g* for 20 min at 4°C. Protein lysates (50 μg total protein/lane) were separated by 12% SDS-PAGE and electrotransferred to a nitrocellulose (NC) membrane by using a semi-dry trans-blot cell (Bio-Rad, Hercules, CA, USA) at 200 Ma for 1 h in transfer buffer (100 Mm Tris-HCl, 150 Mm NaCl, 0.05 % Tween 20, Ph 7.2). Membranes were incubated in blocking solution (5% nonfat milk in Tris-buffered saline Tween-20 [TBST]) for 1 h at room temperature. Membranes were washed thrice with the TBST buffer. Subsequently, the membranes were incubated with primary (rabbit anti-mouse caspase-3 and P62 polyclonal antibody) antibodies (Bioworld, Minneapolis, USA) at 37°C for 1 h. The membrane was washed with TBST thrice and incubated with secondary (goat anti-rabbit horseradish peroxidase-labeled IgG antibody) antibodies (SBA, Birmingham, Al, USA) for 1 h in 5% milk/TBST at 37°C. After three washes, bound conjugate was visualized using an enhanced HRP-DAB substrate color kit (Tiangen Biotech Co. Ltd., Beijing, China). Western blott analysis was repeated thrice.

To determine the expression levels of LC3-II/I in the RAW 264.7 cells, we analyzed the LC3 protein lysates by WB, as previously described with several modifications [8, 37]. The RAW 264.7 cells were infected with S2308, 2308ΔGntR of 2308ΔGntR-C as described previously. At 24h post-infection, the samples (50 μg total protein/lane) were loaded onto SDS-PAGE gels in this experiment. The primary (rabbit anti-mouse LC3 antibody) antibody (Bioworld, Minneapolis, USA) and secondary (goat anti-rabbit horseradish peroxidase-labeled IgG antibody) antibody (SBA, Birmingham, Al, USA) were purchased from Cell Signaling Technology and used at dilutions of 1:1,000 and 1:2,000, respectively. The bound conjugate was visualized using an enhanced HRP-DAB substrate color kit (Tiangen Biotech Co. Ltd., Beijing, China). Western blot analysis was repeated thrice.

Statistical analysis

QRT-PCR data were analyzed using the Roche LightCycler480 1.5 software (Roche, Basel, Switzerland), and the relative quantification was obtained using the $2^{-\Delta\Delta Ct}$ method. The expression levels of the apoptosis- and autophagy-related genes were expressed as mean ± standard deviation (SD). Statistical analysis was performed with Student's unpaired *t*-test. The differences between groups were analyzed by analysis of variance (ANOVA) followed by Tukey's honestly significant difference post-test by comparing all the groups. The results were analyzed by the Fisher test and expressed in percentages. SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA) was used to analyze the differences between groups. *P* values of < 0.05 were considered statistically significant.

Results

Apoptosis in Brucella-infected cells

At 24 h after the S2308 and 2308ΔGntR infection of the RAW 264.7 cells, the percentages of apoptotic cells were detected by flow cytometry. The percentages of apoptotic cells of the S2308-infected group and 2308ΔGntR-infected group were 3.30% ± 0.50% and 16.80% ± 1.30%, respectively (Fig. 1). The results indicated that the percentages of apoptotic cells of the S2308-infected group were significantly lower than those of the 2308ΔGntR-infected group ($P < 0.05$). Hence, S2308 can inhibit apoptosis in RAW 264.7 cells.

GntR affects the expression levels of apoptosis-associated genes in *Brucella*-infected cells

To test the effect of GntR on apoptosis-associated genes, we analyzed the expression levels of caspase-3, caspase-8, Bax, and Bcl-2 in *Brucella*-infected cells. Following 2308ΔGntR infection, the relative expression levels of caspase-3, caspase-8, and Bax in the RAW 264.7 cells were significantly higher than those in the S2308-infected cells ($P < 0.01$, Fig. 2A, 2B, and 2C). The relative expression level of Bcl-2 in the 2308ΔGntR-infected cells was significantly lower than that in the S2308-infected cells ($P < 0.05$, Fig. 2D). The ratio of Bax/Bcl-2 in the 2308ΔGntR-infected cells was significantly higher than that in the S2308-infected cells (Fig. 2E). These results indicated that the 2308ΔGntR mutant affected the expression levels of the apoptosis-associated genes in the RAW 264.7 cells.

GntR affects the expression level of caspase-3 protein in *Brucella*-infected cells

We used WB to analyze the expression levels of caspase-3 protein. The results showed that the expression levels of caspase-3 protein in the 2308ΔGntR-infected cells were significantly higher than those in the S2308-infected cells ($P < 0.01$, Fig. 2F). Hence, the 2308ΔGntR mutant affected the expression levels of caspase-3 protein in the RAW 264.7 cells.

Localization expression levels of P62 protein in *Brucella*-infected cells

To further observe the expression of P62 protein in the cell, we conducted an experiment using a confocal laser scanning microscope. The focal points of P62 protein at 24 h post infection were observed by

confocal microscopy. P62 showed punctate aggregates (Fig. 3A). P62 protein was dispersed in the control cells while the experimental groups showed punctate aggregates (Fig 3A). After S2308 and 2308ΔGntR infection, the P62 punctate aggregates became increasingly significant (Fig 3A), indicating that cell autophagy was inhibited after the infection of *Brucella*. Especially under 2308ΔGntR infection, the intracellular P62 punctate aggregates (Fig 3A) increased, indicating that autophagy was inhibited. Hence, 2308ΔGntR inhibited autophagy in the RAW 264.7 cells.

2308ΔGntR affects the expression levels of autophagy-associated genes

The relative expression levels of p62, LC3-II, and LC3-I were computed using the $2^{-\Delta\Delta Ct}$ method. Following the infection of the RAW 264.7 cells with S2308 and 2308ΔGntR, the relative expression levels of p62 and LC3-I in the 2308ΔGntR-infected cells were slightly higher than those in the S2308-infected cells (Fig. 4A and 4B). No significant difference was observed between the 2308ΔGntR- and S2308-infected groups ($P > 0.05$). The relative expression levels of LC3-II in the 2308ΔGntR-infected cells were significantly lower than those in S2308-infected cells ($P < 0.01$, Fig. 4C). The ratio of LC3-II/LC3-I in the 2308ΔGntR-infected cells was significantly lower than that in the S2308-infected cells ($P < 0.01$, Fig. 4D). Hence, the 2308ΔGntR mutant affected the expression levels of the autophagy-associated genes in the RAW 264.7 cells.

In the present study, we evaluated whether 2308ΔGntR could inhibit the expression of LC3-II. The ratio of LC3-II/I was decreased in the 2308ΔGntR-infected cells (Fig. 4E and 4F). Furthermore, the complemented strain 2308ΔGntR-C promoted autophagy (Fig. 4E and 4F). Collectively, these results indicated that 2308ΔGntR affected autophagy in the RAW 264.7 cells.

GntR affects expression levels of P62 protein in *Brucella*-infected cells

We used WB to analyze the expression levels of P62 protein. The results showed that the P62 protein expression levels in the 2308ΔGntR-infected cells were significantly higher than those in the S2308-infected cells ($P < 0.05$, Fig. 3B and 3C). Hence, 2308ΔGntR affected the expression levels of P62 protein in the RAW 264.7 cells.

Discussion

Brucellosis is a worldwide zoonosis, which remains prominent in many countries. The transcriptional regulatory system is one of the main virulence systems of *Brucella.* The transcriptional regulator GntR of *Brucella* plays a key role in the pathogenic process of *Brucella* [29]. GntR modulates various biological processes, including cytotoxicity [30], secretion of inflammatory cytokines [30], antibiotic production [38], antibiotic resistance [39], and plasmid transfer [40]. Transcriptional regulator GntR regulates the expression levels of many genes in *Brucella* [41]. However, the cellular functions of GntR are still unknown. In the present study, we demonstrated that the transcriptional regulator GntR participated in apoptosis and autophagy during *Brucella* infection.

Apoptosis plays an important role in the pathogenicity of pathogens. *Brucella* may inhibit the apoptosis of host cells to reproduce in cells. However, the specific mechanism remains unclear. In this study, we used 2308ΔGntR mutant and parental strain S2308 to infect RAW 264.7 cells. Our results were the same as those of previous studies [13, 34]. The percentages of apoptotic cells of the 2308ΔGntR-infected cells were significantly higher than those of the S2308-infected ones. Wild-type *B. abortus* 2308 is a smooth *Brucella*, which inhibits apoptosis for its long-term survival in host cells. We confirmed that the virulence of 2308ΔGntR was lower than that of the parental strain [29]. The survival time of the 2308ΔGntR mutant strain in the host cells was short [29]. These results may be related to apoptosis.

In this study, the ratio of Bax/Bcl-2 in the 2308ΔGntR-infected group was significantly higher than that in the S2308-infected group. In addition, the mRNA expression levels of the apoptosis-related genes caspase-3 and caspase-8 were up-regulated in 2308ΔGntR. Hence, 2308ΔGntR promoted apoptosis in the RAW 264.7 cells. The activation of caspase-3 indicated apoptosis [42]. Our results suggested that 2308ΔGntR enhanced caspase-3 protein expression. Moreover, the results indicated that *Brucella* transcriptional regulator GntR was involved in apoptosis and that the 2308ΔGntR mutant promoted apoptosis in the RAW 264.7 cells.

Autophagy is a cellular pathway that plays an important in development and survival. Autophagy has an important significance in *Brucella* intracellular trafficking. In epithelial cells, *B. abortus* is transited through autophagosomes [43]. Thus, the regulation of autophagy may be a crucial target of *B. abortus*. We detected the localization expression levels of P62 protein by confocal laser scanning microscopy. We found that the P62 punctate aggregates in the 2308ΔGntR-infected cells became more significant than those in the S2308-infected cells. The lack of P62 degradation indicates that 2308ΔGntR inhibits autophagy in RAW 264.7 cells.

Autophagy is related to microbial infection [44]. LC3 is a major marker of autophagy and a *Brucella*-induced autophagic response is related to LC3 [45]. After 2308ΔGntR infected the cells in this work, the ratio of LC3-II/LC3-I was decreased. These results indicated that 2308ΔGntR inhibited autophagy in the RAW 264.7 cells. Furthermore, P62 protein expression levels increased after 2308ΔGntR infected the cells. In sum, these results indicated that the *Brucella* transcriptional regulator GntR was involved in autophagy. They also confirmed that the 2308ΔGntR mutant inhibited autophagy in the RAW 264.7 cells.

In sum, we found that the mRNA relative expression levels of caspase-3, caspase-8, Bax, p62, and LC3-I and the ratio of Bax/Bcl-2 increased more significantly in 2308ΔGntR than in S2308. Moreover, the mRNA relative expression levels of Bcl-2 and LC3-II and the ratio of LC3-II/LC3-I decreased more significantly in 2308ΔGntR than in S2308. WB, flow cytometry, and confocal laser microscopy indicated that 2308ΔGntR promoted apoptosis and inhibited autophagy in the RAW 264.7 cells during *Brucella* infection. These results indicated that GntR was involved in the apoptosis and autophagy in the *Brucella*-infected cells. These results provide new ideas and clues for further elucidating the molecular mechanism of *Brucella* pathogenicity.

Declarations

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Author's contributions

ZL and HZ designed the experiments. ZL, SW, LX, JZ, JH and HZ performed the experiments and analyzed the data. ZL and HZ contributed reagents/materials/analysis tools. ZL, SW, HZ and JZ wrote and revised the paper.

Availability of data and materials

All the data generated or analyzed during this study are included in the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have agreed to submit this manuscript.

Competing interests

The authors declare that no competing interest exists in this study.

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Tables

Table 1 Primers used in this study.

Primer		5’-3’ sequence
Caspase-3-RT-F	Forward	TTCCACGCAAAGAAACAGAT
Caspase-3-RT-R	Reverse	GGAGGACCGTCAGATTAGAT
Caspase-8-RT-F	Forward	TGCCGTGGAGAGAAACAA
Caspase-8-RT-R	Reverse	ATGAAAAGTGAGCCCCTG
Bcl-2-RT-F	Forward	GACTTCTCTCGTCGCTACCG
Bcl-2-RT-R	Reverse	ACAATCCTCCCCCAGTTCAC
Bax-RT-F	Forward	GCCTTTTTGCTACAGGGTTT
Bax-RT-R	Reverse	TGCTGTCCAGTTCATCTCCA
P62-RT-F	Forward	TCTTTGGACCCCCGTGTGA
P62-RT-R	Reverse	TCTCACAGATACCCCACGACCA
LC3-I-RT-F	Forward	CCGACCGCTGTAAGGAGG
LC3-I-RT-R	Reverse	GCCGGATGATCTTGACCAAC
LC3-II-RT-F	Forward	GAACAAAGAGTGGAAGATG
LC3-II-RT-R	Reverse	GCCGTCTGATTATCTTGA
β-actin-RT-F	Forward	AGCCTTCCTTCTTGGGTATGG
β-actin-RT-R	Reverse	CCTGTCAGCAATGCCTGGGTA

Figures

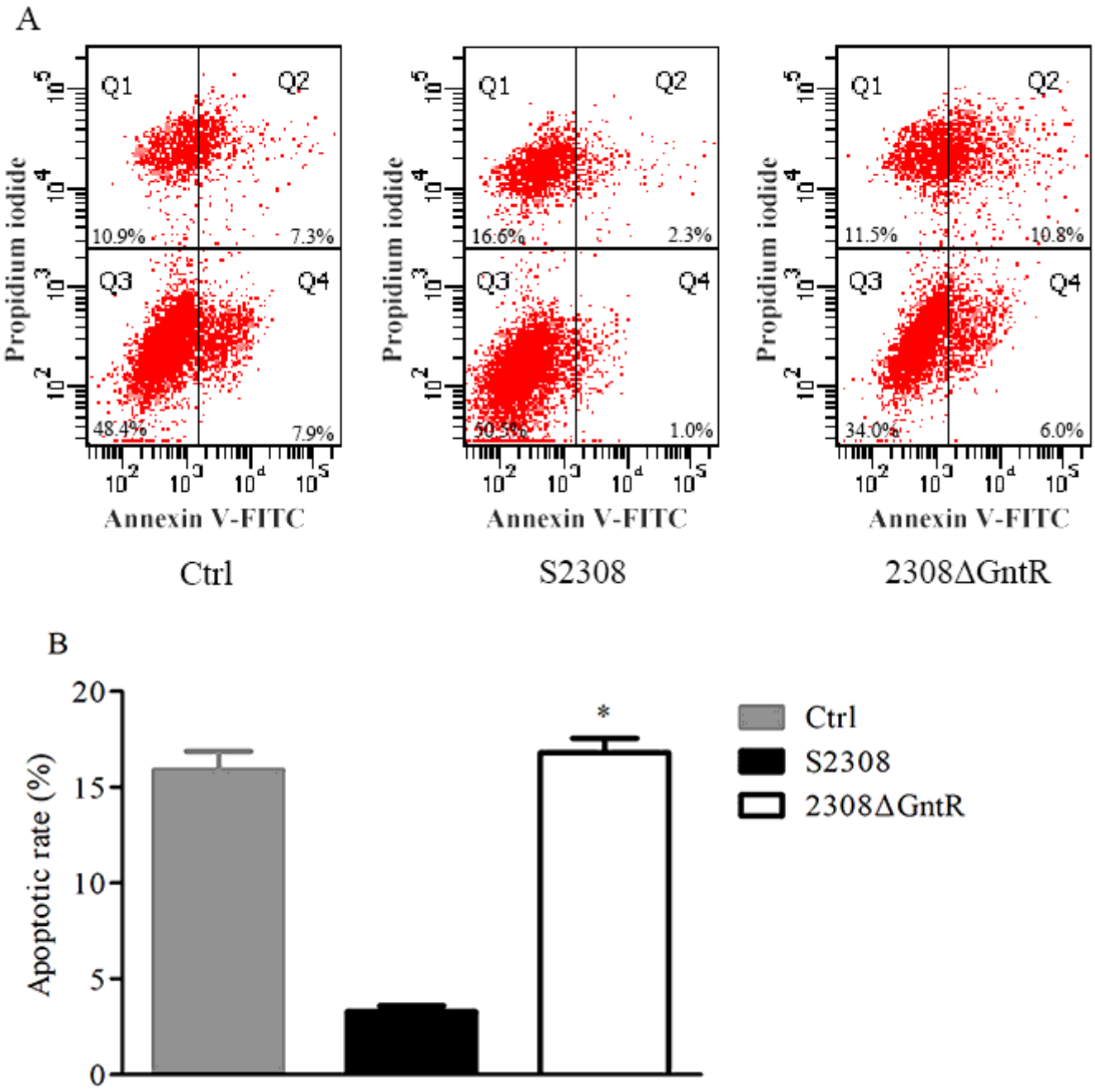

Figure 1

Flow cytometry to detect percentages of apoptotic cells of different treatment groups. RAW 264.7 cells were infected with S2308 and 2308ΔGntR at MOI of 100. At 24 h post-infection, cells were washed with PBS, and incubated with a mix of 500 μL of binding buffer, 5 μL of Annexin V-FITC, and propidium iodide at room temperature away from the dark for 15 min. The cells not infected with Brucella served as the control. S2308- and 2308ΔGntR-infected cell apoptosis was measured by flow cytometry (A). The percentages of apoptotic cells from independent experiments were statistically analyzed (B, apoptotic rate). Data are expressed as the means ± SD from at least three individual experiments. The percentages of apoptotic cells (in quadrants Q2 and Q4) of the S2308-infected cells were significantly lower than

those in the 2308ΔGntR-infected group. Significant differences between the mutant and parent strain are indicated by * (P < 0.05) (t-test).

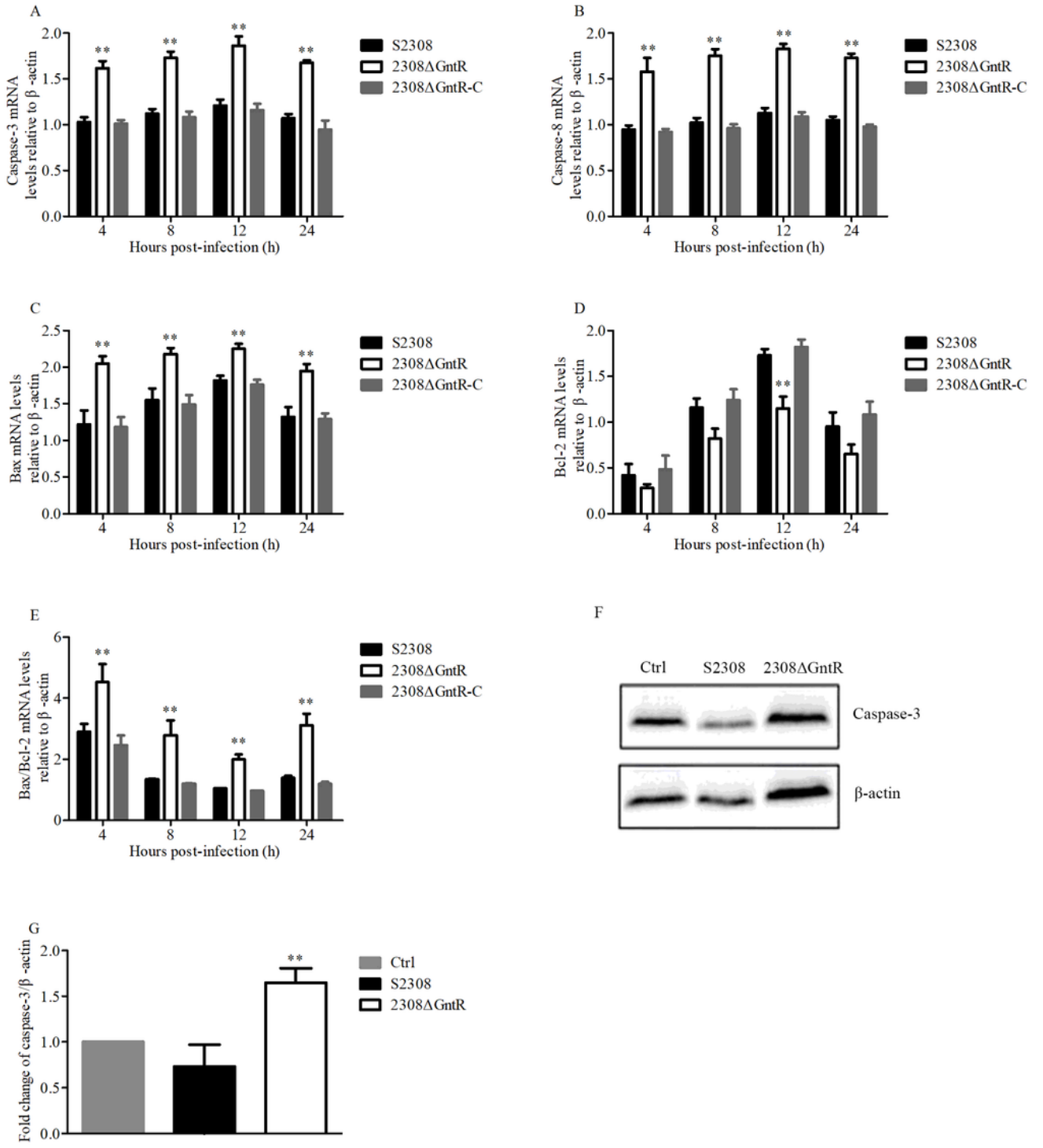

Figure 2

The mRNA levels of apoptosis-associated gene expression by qRT-PCR. RAW 264.7 cells were infected with S2308, 2308ΔGntR, or 2308ΔGntR-C at MOI of 100. At 4, 8, 12 and 24 h post-infection, the total RNA was extracted and reverse transcribed into cDNA, and the mRNA levels of caspase-3 (A), caspase-8 (B),

Bax (C), and Bcl-2 (D) were quantitated by qRT-PCR. The caspase-3 protein (F) expression level was determined by Western blot analysis. Densitometric analysis of the results from three independent experiments performed as described for panel F; caspase-3 (G). The relative expression levels of caspase-3, caspase-8, and Bax and the ratio of Bax/Bcl-2 in the 2308ΔGntR-infected group were significantly higher than those in the S2308-infected group. The relative expression level of Bcl-2 in the 2308ΔGntR-infected group was significantly lower than that in the S2308-infected group. Caspase-3 protein expression levels in the 2308ΔGntR-infected group were significantly higher than those in the S2308-infected group. Data are expressed as the means ± SD from at least three individual experiments. Significant differences between the mutant and parent strains are indicated by * ($P < 0.05$), ** ($P < 0.01$) (t-test).

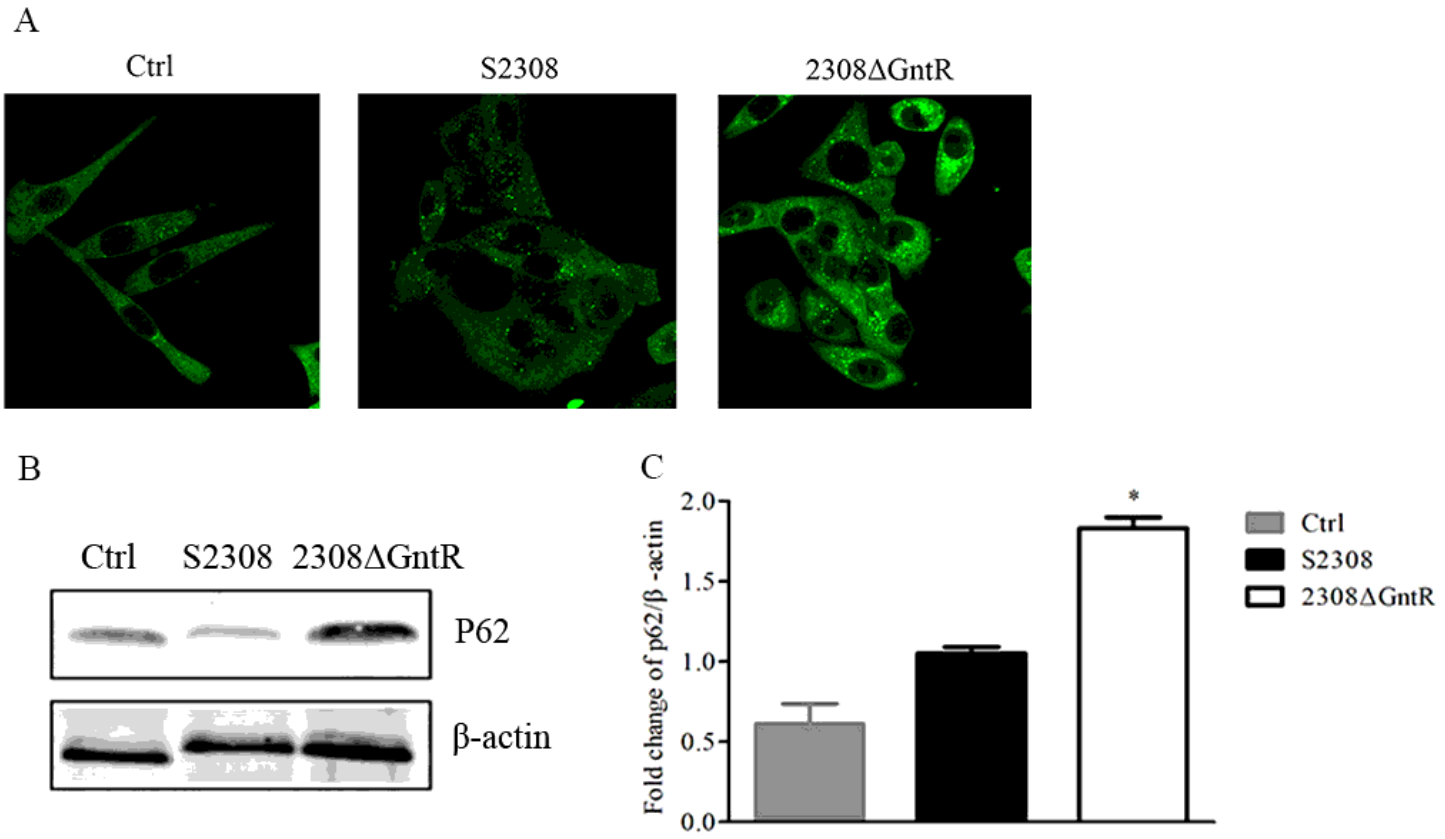

Figure 3

Detection of autophagy levels. Confocal laser microscopy to detect cell autophagy in different groups (A). Cells were seeded into a confocal dish and infected with S2308 and 2308ΔGntR. At 24 h post-infection, cells were fixed in 4% paraformaldehyde. Cells were washed thrice with PBS and added with P62 primary antibodies and fluorescently tagged secondary antibodies. The expression of P62 protein in the cell was observed by confocal microscopy. P62 showed punctate aggregates in the S2308-infected group and the P62 punctate aggregates became increasingly significant in the 2308ΔGntR-infected group. Western blot analysis was performed to detect the expression of P62 protein (B). RAW 264.7 cells were infected with S2308 and 2308ΔGntR at MOI of 100. At 24 h post-infection, the cells were placed on ice and lysed by RIPA buffer for 20 min. The lysate was collected and subjected to Western blot detection. Densitometric

analysis of results from three independent experiments performed as described for panel A; P62 (C). The P62 protein expression levels in the 2308ΔGntR-infected group were significantly higher than those in the S2308-infected group. Data are expressed as the means ± SD from at least three individual experiments. Significant differences between the mutant and parent strain are indicated by * (P < 0.05).

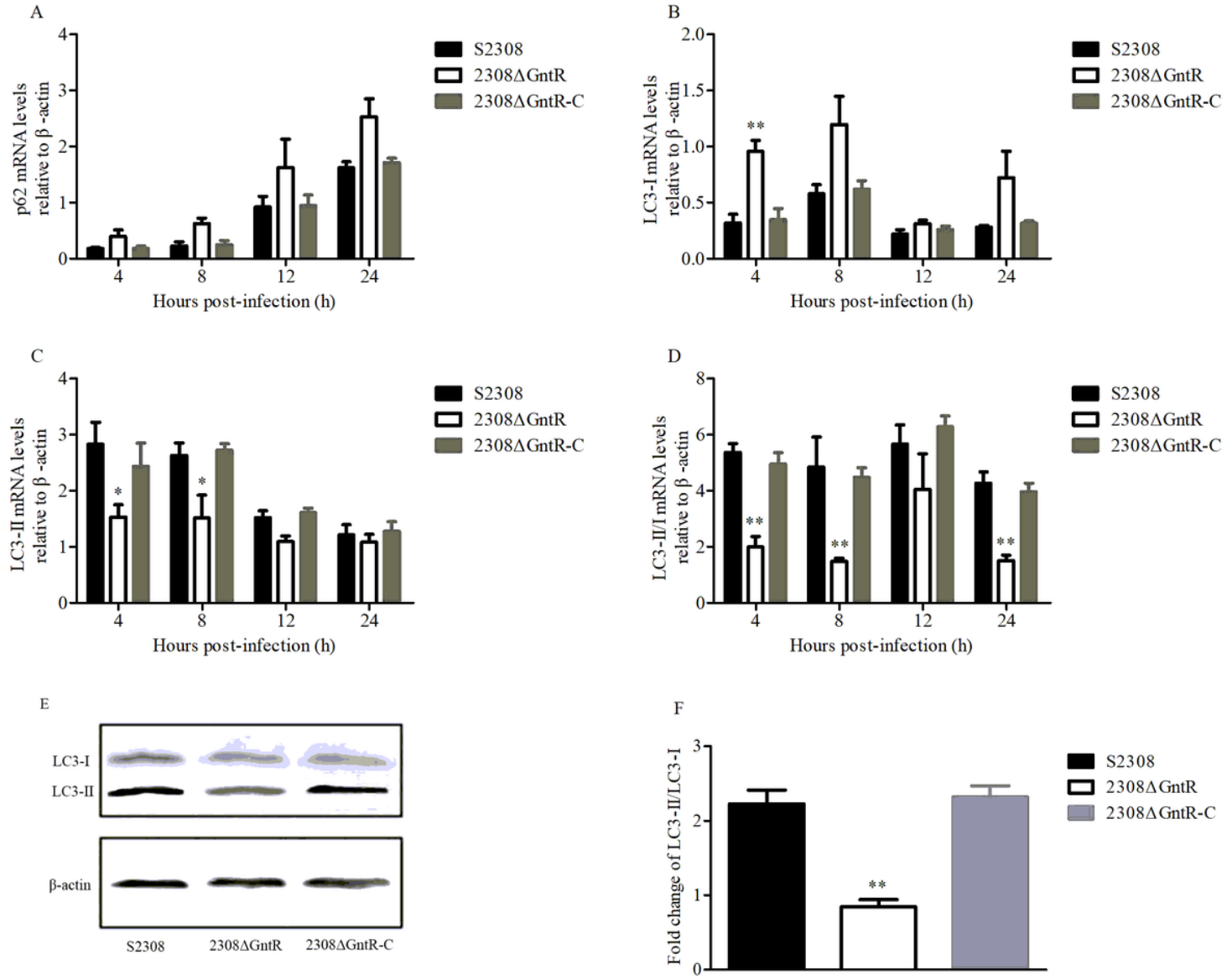

Figure 4

The mRNA levels of autophagy-associated genes expression by qRT-PCR. RAW 264.7 cells were infected with S2308, 2308ΔGntR or 2308ΔGntR-C at MOI of 100. At 4, 8, 12 and 24 h post-infection, the total RNA was extracted and reverse transcribed into cDNA, and the mRNA levels of p62 (A), LC3-I (B), and LC3-II (C) were quantitated by qRT-PCR. The relative expression levels of p62 and LC3-I in the 2308ΔGntR-infected group were significantly higher than those in the S2308-infected group. The relative expression levels of LC3-II and the ratio of LC3-II/LC3-I in the 2308ΔGntR-infected group were significantly lower than those in the S2308-infected group. RAW 264.7 cells were infected with S2308, 2308ΔGntR, or 2308ΔGntR-C at MOI of 100. At 24 h post-infection, the cells were placed on ice and lysed by RIPA buffer for 20 min. The lysate

was collected and the expression of LC3 protein was detected by Western blot analysis (E). Densitometric analysis of results from three independent experiments performed as described for panel E; LC3-II/LC3-I (F). The ratio of LC3-II/LC3-I in the 2308ΔGntR-infected group was decreased. Data are expressed as the means ± SD from at least three individual experiments. Significant differences between the mutant and parent strain are indicated by * ($P < 0.05$), ** ($P < 0.01$) (t-test).