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Salmonella enterica serovar Typhi plasmid pR_{ST98} enhances intracellular bacterial growth and S. typhi-induced macrophage cell death by suppressing autophagy

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Objectives: Plasmid pR_{ST98} is a hybrid resistance-virulence plasmid isolated from *Salmonella enterica* serovar Typhi (*S. typhi*). Previous studies demonstrated that pR_{ST98} could enhance the virulence of its host bacteria. However, the mechanism of pR_{ST98}-increased bacterial virulence is still not fully elucidated. This study was designed to gain further insight into the roles of pR_{ST98} in host responses.

Methods: Human-derived macrophage-like cell line THP-1 was infected with wild-type (ST8), pR_{ST98}-deletion (ST8-ΔpR_{ST98}), and complemented (ST8-c-pR_{ST98}) *S. typhi* strains. Macrophage autophagy was performed by extracting the membrane-unbound LC3-I protein from cells, followed by flow cytometric detection of the membrane-associated fraction of LC3-II. Intracellular bacterial growth was determined by colony-forming units (cfu) assay. Macrophage cell death was measured by flow cytometry after propidium iodide (PI) staining. Autophagy activator rapamycin (RAPA) was added to the medium 2 h before infection to investigate the effect of autophagy on intracellular bacterial growth and macrophage cell death after *S. typhi* infection.

Results: Plasmid pR_{ST98} suppressed autophagy in infected macrophages and enhanced intracellular bacterial growth and *S. typhi*-induced macrophage cell death. Pretreatment with RAPA effectively restricted intracellular bacterial growth of ST8 and ST8-c-pR_{ST98}, and alleviated ST8 and ST8-c-pR_{ST98}-induced macrophage cell death, but had no significant effect on ST8-ΔpR_{ST98}.

Conclusions: Plasmid pR_{ST98} enhances intracellular bacterial growth and *S. typhi*-induced macrophage cell death by suppressing autophagy.

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Introduction

Typhoid fever is an acute and life-threatening infectious disease caused by a facultative gram-negative pathogen *Salmonella enterica* serovar Typhi (*S. typhi*). According to estimations by the World Health Organization (WHO), 22 million cases occur each year, 5% of which are fatal.¹ In result of preventive use of antimicrobial drugs and vaccination programs, the incidence of typhoid fever has decreased in recent years. Nonetheless, typhoid fever is still a serious public health problem in developing countries.² Worse, the treatment of infected patients has become complicated as *S. typhi* acquires a considerable variety of genes by plasmid movement between bacteria through conjugation, such as genes for antibiotic-resistance and virulence.

Plasmid pR_{ST98} is a large 159 kb plasmid isolated from *S. typhi* strains from an outbreak of typhoid fever in the mid to late 1980s in China. Patients infected with *S. typhi* carrying pR_{ST98} had more severe disease with a higher rate of complication and mortality. Studies of our laboratory indicated that pR_{ST98} is a hybrid resistance-virulence plasmid carrying both antibiotic-resistance and virulence genes. Using polymerase chain reaction (PCR), Southern blot, and DNA sequence analysis, *Salmonella* plasmid virulence (*spv*) homologous genes, which were previously considered to be absent from *S. typhi*, were identified on pR_{ST98}.³ It has been reported that the presence of *spv* genes greatly enhance the virulence of its host.⁴

Although *spv* homologous genes on pR_{ST98} were identified, the mechanism of pR_{ST98} enhancing its host virulence was still not fully elucidated. In order to gain further insight into the roles of pR_{ST98} in host responses, autophagy, intracellular bacterial growth, and apoptosis were examined in human-derived macrophage-like cell line THP-1 infected with wild-type (ST8), pR_{ST98}-deletion (ST8-ΔpR_{ST98}), and complemented (ST8-c-pR_{ST98}) *S. typhi* strains in the present study.

Materials and methods

Bacterial strains and culture

Wild-type *S. typhi* strain harboring pR_{ST98} ST8 was obtained from the blood of patients during a typhoid fever outbreak in Suzhou, Jiangsu Province, China. pR_{ST98}-deletion *S. typhi* strain ST8-ΔpR_{ST98} and pR_{ST98}-complemented *S. typhi* strain ST8-c-pR_{ST98} were constructed in this study's laboratory. Bacteria were grown to mid-logarithmic phase at 37°C in Luria-Bertani (LB) broth, harvested by centrifugation, washed with phosphate-buffered saline (PBS), and resuspended in RPMI 1640 medium prior to the addition to macrophages.

Cell culture

THP-1 cells were maintained in RPMI 1640 with L-glutamine and 10% fetal bovine serum. Prior to infection, cells were treated with 50 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma) to induce differentiation into adherent macrophage-like cells. On the following day, medium and nonadherent cells

were removed and replaced with fresh complete medium. Cells rested for two days following chemical differentiation to ensure that they reverted to a resting phenotype before infection.

Bacterial infection and drug treatment

Mid-logarithmic phase growth cultures of *S. typhi* strains ST8, ST8-ΔpR_{ST98}, and ST8-c-pR_{ST98} were added to THP-1 macrophages at a multiplicity of infection of 10:1. Culture plates were centrifuged at 1500 rpm for 10 min and incubated at 37°C for 30 min (0-h time point). The medium was then removed, washed three times with PBS and replaced with medium containing 100 mg amikacin per mL to kill the remaining extracellular bacteria. After 2 h of further incubation at 37°C, the medium in culture plates was replaced with medium containing 10 mg amikacin per mL to prevent intracellular growth of bacteria released from infected THP-1 cells. To induce autophagy, autophagy activator rapamycin (RAPA) was added to the medium 2 h before infection.

Assessment of macrophage autophagy by flow cytometry

At 0.5 h post-infection, THP-1 cells were harvested and manipulated as previously described.⁵ Cells were washed with PBS and permeabilized with 0.025% digitonin (Sigma). Following this, the cells were washed three times in PBS, pelleted by centrifugation at 1,000 rpm for 5 min, and fixed in 4% paraformaldehyde (Sigma) for 20 min at room temperature (RT). After two washes in PBS, the cells were incubated for 30 min at RT with the anti-LC3 antibody (Sigma, 1:200), washed three times in PBS, and incubated for 1 h at RT with a secondary anti-rabbit antibody (Sigma, 1:500). After washing in PBS, the cells were analyzed by flow cytometer.

Measurement of intracellular bacterial growth

At 6 h post-infection, THP-1 cells were harvested and washed with PBS. 300 μL 0.1% Triton X-100 (prepared in distilled water) was added to cells. After 5 min, cell lysates were collected and serially diluted 10-fold in PBS, and aliquots were plated onto LB agar to enumerate bacterial colony-forming units.

Assessment of macrophage cell death by flow cytometry

At 6 h post-infection, THP-1 cells were harvested and washed with PBS. Cells were fixed in 70% ethanol at 4°C for 24h, treated with RNaseA at 37°C for 30 min and stained with propidium iodide (PI) in the dark for 30 min. The cells were analyzed by flow cytometer (FC500, Beckman Coulter – Brea, CA, USA).

Statistical analysis

Data were presented as mean ± standard deviation (SD). Experimental results were analyzed for their significance by Student's t-test using the Statistical Package for the Social Sciences (SPSS) software. A p-value < 0.05 was considered to be statistically significant.

Results

Macrophage autophagy

To this moment, the most reliable marker for monitoring autophagosome formation is the detection of the membrane-associated fraction of LC3-II. In the present study, digitonin was used to extract the membrane-unbound LC3-I protein from cells, followed by flow cytometric detection of the membrane-associated fraction of LC3-II. Compared with macrophages infected with ST8 (2.95 ± 0.17) and ST8-c-pR_{ST98} (3.02 ± 0.17), a significant increase in fluorescence of macrophages infected with ST8- Δ pR_{ST98} (7.50 ± 0.19) was observed ($p < 0.01$), suggesting that pR_{ST98} suppressed the autophagy of infected macrophages (Fig. 1).

Intracellular bacteria growth

Macrophages were lysed and plated on agar at 10-fold series dilutions. As show in Fig. 2, ST8 and ST8-c-pR_{ST98} showed more viable counts than ST8- Δ pR_{ST98} ($p < 0.01$), suggesting that ST8 and ST8-c-pR_{ST98} replicated more quickly than ST8- Δ pR_{ST98} in infected macrophages. Pretreatment with RAPA effectively restricted intracellular growth of ST8 and ST8-c-pR_{ST98} ($p < 0.01$), but had no significant effect on ST8- Δ pR_{ST98} ($p > 0.05$).

Macrophage cell death

Dead population appeared as a sub-G1 peak. As show in Fig. 3, the percentage of macrophage cell death induced by ST8, ST8- Δ pR_{ST98} and ST8-c-pR_{ST98} was $69.39 \pm 1.63\%$, $52.11 \pm 1.98\%$, and $68.38 \pm 1.10\%$. Macrophages infected with ST8

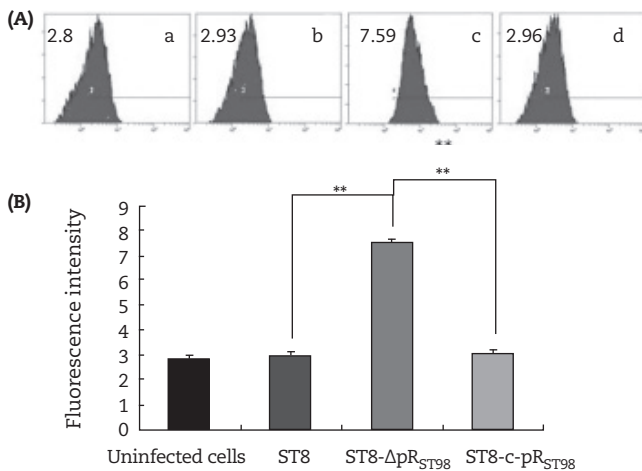


Fig. 1 - Assessment of macrophage autophagy by flow cytometry. (A.a) Uninfected cells. **(A.b)** THP-1 cells infected with *S. typhi* ST8. **(A.c)** THP-1 cells infected with *S. typhi* ST8- Δ pR_{ST98}. **(A.d)** THP-1 cells infected with *S. typhi* ST8-c-pR_{ST98}. **(B)** Mean fluorescence intensity of *S. typhi*-infected THP-1 cells. Statistical analysis was performed using Student's t-test (** $p < 0.01$).

and ST8-c-pR_{ST98} showed a significantly higher rate of cell death than macrophages infected with ST8- Δ pR_{ST98} ($p < 0.01$). Pretreatment with RAPA effectively alleviated ST8 ($69.39 \pm 1.63\%$ versus $58.82 \pm 1.88\%$; $p < 0.01$) and ST8-c-pR_{ST98} ($68.38 \pm 1.10\%$ versus $58.01 \pm 1.72\%$; $p < 0.01$)-induced macrophage cell death, but had no significant effect on ST8- Δ pR_{ST98} ($52.11 \pm 1.98\%$ versus $51.10 \pm 1.89\%$; $p > 0.05$).

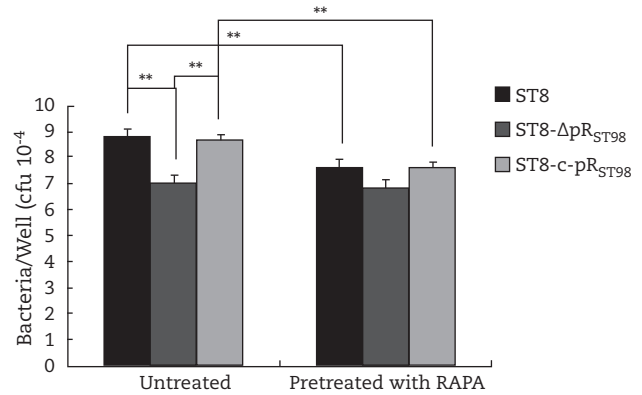


Fig. 2 - Intracellular bacteria growth in THP-1 cells. Statistical analysis was performed using Student's t-test ($p < 0.01$). RAPA, rapamycin.**

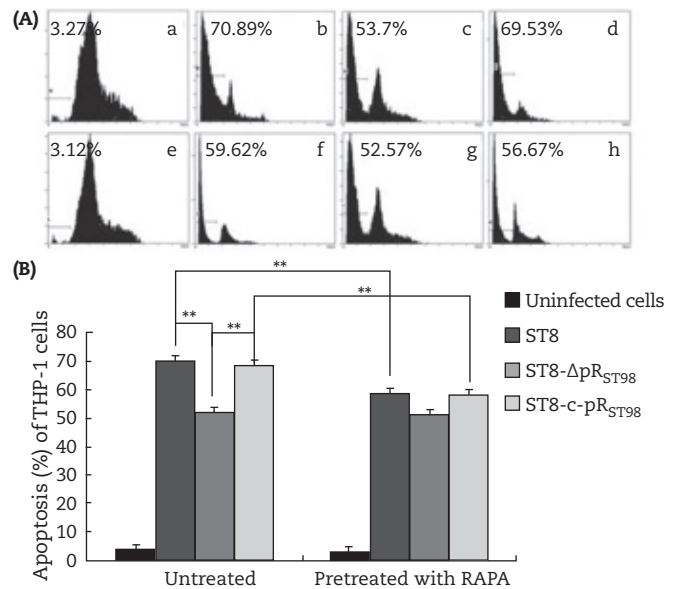


Fig. 3 - Assessment of macrophage cell death by flow cytometry. (A.a) Uninfected cells. **(A.b)** THP-1 cells infected with *S. typhi* ST8. **(A.c)** THP-1 cells infected with *S. typhi* ST8- Δ pR_{ST98}. **(A.d)** THP-1 cells infected with *S. typhi* ST8-c-pR_{ST98}. **(A.e)** Uninfected cells pre-treated with RAPA. **(A.f)** THP-1 cells infected with *S. typhi* ST8 after pre-treatment with RAPA. **(A.g)** THP-1 cells infected with *S. typhi* ST8- Δ pR_{ST98} after pre-treatment with RAPA. **(A.h)** THP-1 cells infected with *S. typhi* ST8-c-pR_{ST98} after pre-treatment with RAPA. Statistical analysis was performed using Student's t-test (** $p < 0.01$). RAPA, rapamycin.

Discussion

Previous research by the authors demonstrated that pR_{ST98} was associated with increased virulence in mice, such as lethality; infection of spleen, liver, and mesenteric lymph nodes; and serum resistance; pR_{ST98} could also cause resistance of its host bacteria to phagocytes. However, the knowledge about pR_{ST98} is still limited. The present study provides evidence that pR_{ST98} could suppress autophagy in infected macrophages. Autophagy is a catabolic process by which a cell degrades its own components through the lysosomal machinery.^{6,7} It was initially reported as a process that eliminates damaged organelles, superfluous portions of cytoplasm, and long-lived, aberrant, or aggregated proteins. Recent studies have revealed that autophagy contributes to cellular defense against microbial invaders in innate and adaptive immunity.^{8,9} Birmingham et al. reported that autophagy could target *Salmonella* in the cytosol. Although the fate of *Salmonella* targeted by autophagy is still unclear, autophagy-deficient (*atg5*^{-/-}) cells were more permissive for intracellular growth by *Salmonella* than normal cells, allowing increased bacterial growth in the cytosol, demonstrating that autophagy restricted the intracellular growth of *Salmonella*.¹⁰ As pR_{ST98} could suppress autophagy in infected macrophages, the possibility that pR_{ST98} may affect the bacterial intracellular growth was considered. Measurement of intracellular bacterial growth confirmed the hypothesis that wide-type strain ST8 and pR_{ST98}^- complemented strain ST8-c- pR_{ST98} multiplied faster in infected macrophages than pR_{ST98} -deletion strain ST8- ΔpR_{ST98} . Pretreated infected macrophages with autophagy inducer RAPA could reduce the growth of ST8 and ST8-c- pR_{ST98} , but had no effect on the growth of ST8- ΔpR_{ST98} , thus suppression of autophagy contributed to an increased bacterial intracellular growth.

Recent studies have also revealed that autophagy plays an important role in modulating cell death.¹¹ Hernandez et al. reported that *S. typhimurium* could cause macrophage cell death by inducing autophagy.¹² However, in the present study it was found that pR_{ST98} not only suppressed autophagy in infected macrophages, but also enhanced cell death in infected macrophages, and pR_{ST98} -enhanced macrophage cell death could be modulated by autophagy inducer RAPA. The present data showed that pR_{ST98} may enhance macrophage cell death by suppressing autophagy. This discrepancy may be the result of the use of different strains. Although *S. typhimurium* and *S. typhi* have a high degree of genome homology and *S. typhimurium* has been used extensively as an experimental model for typhoid fever, the genetic differences between these two pathogens still cause several differences at the level of the host's response to infection.¹³ Thus, the autophagy level of infected macrophages induced by *S. typhi* and *S. typhimurium* may be different. The level of autophagy may impact its biological effects on host cells. Autophagy could restrict *Salmonella* growth in the host cells. The ability of *Salmonella* to induce host cell death is related to its growth in the host cell. One of the mechanisms *Salmonella* uses to induce host cell death is to deliver effector proteins through type III systems (T3SSs) to alter signal transduction pathways and affect the balance between

pro and anti-apoptotic factors in the host cells.¹⁴ Bacterial survival declines, and fewer effector proteins will be delivered into host cells. Thus, moderate autophagy may prevent the host cell from undergoing cell death induced by *Salmonella*. However, autophagy also involves reactive oxygen species (ROS) accumulation resulting from degradation of catalase, an important antioxidant.¹⁵ Excessive autophagy will destroy the balance between ROS production and degradation, and lead to ROS accumulation. ROS can oxidize cell constituents such as lipids, proteins, and DNA, thus damaging cell structures. Excessive autophagy may result in host cell death.

Together, the present results demonstrate that pR_{ST98} could suppress autophagy in infected macrophages. Suppression of autophagy may result in rapid bacterial replication as well as enhance macrophage cell death induced by *S. typhi*. However, the mechanism of pR_{ST98} suppressing macrophage autophagy is still relatively poorly understood. The *spv* locus is a highly conserved region consisting of five genes, one transcriptional regulator *spvR* and four structural genes *spvABCD*. It has been reported that the *spvC* gene could downregulate cytokines, such as TNF- α , released from infected cells.¹⁶ It is known that several cytokines, including TNF- α , can activate autophagy pathways.¹⁷ Thus, downregulating TNF- α production may suppress autophagy of infected cells. The authors found a gene sequence that is homologous to *spvC* on the pR_{ST98} plasmid. However, pR_{ST98} is a large hybrid plasmid containing complex sequences of unknown functions. It is not clear whether pR_{ST98} suppressing autophagy is directly related to *spvC* gene or a result of interplay of several genes; further exploration will be focused on the regulatory pathways of pR_{ST98} suppressing autophagy.

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Conflict of interest

All authors declare to have no conflict of interest.

REFERENCES

1. Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. *Bull World Health Organ.* 2004;82(5):346-53.
2. Crump JA, Mintz ED. Global trends in typhoid and paratyphoid fever. *Clin Infect Dis.* 2010;50(2):241-6.
3. Huang R, Wu S, Zhang X, Zhang Y. Molecular analysis and identification of virulence gene on $pR_{(ST98)}$ from multi-drug resistant *Salmonella typhi*. *Cell Mol Immunol.* 2005;2(2):136-40.

4. Fierer J. Extra-intestinal *Salmonella* infections: the significance of *spv* genes. *Clin Infect Dis*. 2001;32(3):519-20.
5. Kaminsky V, Abdi A, Zhivotovsky B. A quantitative assay for the monitoring of autophagosome accumulation in different phases of the cell cycle. *Autophagy*. 2011;7(1):83-90.
6. Kroemer G, Levine B. Autophagic cell death: the story of a misnomer. *Nat Rev Mol Cell Biol*. 2008;9(12):1004-10.
7. Baehrecke EH. Autophagy: dual roles in life and death? *Nat Rev Mol Cell Biol*. 2005;6(6):505-10.
8. Deretic V. Autophagy in immunity and cell-autonomous defense against intracellular microbes. *Immunol Rev*. 2011;240(1):92-104.
9. Huang J, Brumell JH. Autophagy in immunity against intracellular bacteria. *Curr Top Microbiol Immunol*. 2009;335:189-215.
10. Birmingham CL, Smith AC, Bakowski MA, Yoshimori T, Brumell JH. Autophagy controls *Salmonella* infection in response to damage to the *Salmonella*-containing vacuole. *J Biol Chem*. 2006;281(16):11374-83.
11. Amelio I, Melino G, Knight RA. Cell death pathology: cross-talk with autophagy and its clinical implications. *Biochem Biophys Res Commun*. 2011;414(2):277-81.
12. Hernandez LD, Pypaert M, Flavell RA, Galán JE. A *Salmonella* protein causes macrophage cell death by inducing autophagy. *J Cell Biol*. 2003;163(5):1123-31.
13. Sabbagh SC, Forest CG, Lepage C, Leclerc JM, Daigle F. So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. *FEMS Microbiol Lett*. 2010;305(1):1-13.
14. Guiney DG. The role of host cell death in *Salmonella* infections. *Curr Top Microbiol Immunol*. 2005;289:131-50.
15. Yu L, Wan F, Dutta S, et al. Autophagic programmed cell death by selective catalase degradation. *Proc Natl Acad Sci USA*. 2006;103(13):4952-7.
16. Mazurkiewicz P, Thomas J, Thompson JA, et al. SpvC is a *Salmonella* effector with phosphothreonine lyase activity on host mitogen-activated protein kinases. *Mol Microbiol*. 2008;67(6):1371-83.
17. Deretic V, Levine B. Autophagy, immunity, and microbial adaptations. *Cell Host Microbe*. 2009;5(6):527-49.