African Journal of Microbiology Research Vol. 6(17), pp. 3963-3969, 9 May, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.623
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Salmonella enterica serovar *typhi* plasmid pR_{ST98} enhances caspase-3 mediated macrophage apoptosis by suppressing nitric oxide production

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Accepted 29 March, 2012

Plasmid pR_{ST98} is a hybrid antibiotic resistance-virulence plasmid isolated from *Salmonella enterica* serovar *typhi* (*S. typhi*). Previously, we transferred pR_{ST98} into attenuated *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) strain RIA to create a transconjugant pR_{ST98}/RIA and indicated that pR_{ST98} could enhance apoptosis of infected murine macrophages J774A.1. However, *S. typhimurium*-mice interaction cannot reflect all characteristics of *S. typhi*-human interaction. The present study 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 to gain more precise and further insight into the role of pR_{ST98} in interaction between *S. typhi* and human macrophage. Macrophage apoptosis, caspase-3 activity and nitric oxide (NO) production were assayed. The results demonstrated that macrophages infected with ST8 and ST8-c-pR_{ST98} displayed more extensive apoptosis than those infected with ST8-ΔpR_{ST98}. Further studies showed that pR_{ST98} could increase caspase-3 activity and suppress NO production. Pretreated macrophages with NO synthase inhibitor nitro-L-arginine methyl ester (L-NAME) enhanced *S. Typhi*-induced macrophage apoptosis. The research data indicate that the plasmid pR_{ST98} can enhance caspase-3 mediated macrophage apoptosis by suppressing NO production.

Key words: *Salmonella enterica* serovar *typhi*, plasmid pR_{ST98}, macrophage, apoptosis, nitric oxide, caspase-3 activity.

INTRODUCTION

Typhoid fever, also known as enteric fever, is caused by *Salmonella typhi*, a gram-negative bacterium classified as a *serovar* of the species *Salmonella enterica*. The symptoms of patients with typhoid fever usually present with a history of prolonged fever, headache, abdominal discomfort and general lethargy. Ingestion of polluted food or water is main transmission of this disease. With strict sanitary and hygienic measures, the incidence of typhoid fever has decreased in recent years. But it remains a serious public health problem in developing and even in developed countries (Crump and Mintz, 2010). According to the estimation of the World Health Organization (WHO), 22 million typhoid fever cases occur each year, 5% of which are fatal. What is worse, the treatment of infected patients has become complicated as *S. typhi* acquires a considerable variety of genes by plasmids moving between bacteria through conjugation, such as, antibiotic-resistance and virulence genes (Morita et al., 2010).

An outbreak of typhoid fever, affected 13 provinces and cities, took place in the mid to late 1980s in China. The patients of this outbreak had severe symptoms and exhibited complications with high mortality rates. Studies

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of our lab demonstrated the antibiotic-resistance *S. typhi* strains with a 98.6 mega-Dalton (159 kb) plasmid were the main causative agent for the epidemic. This plasmid mediated multi-drug resistance to chloramphenicol, streptomycin, trimethoprim, sulfonamide, gentamycin, neomycin, cephalosporin, ampicillin, carbenicillin and tetracycline and was named as pR_{ST98} (Huang and Mu, 1994). Further research indicated that pR_{ST98} is a hybrid resistance-virulence plasmid carrying both antibiotic resistance and virulence genes. Plasmid pR_{ST98} was associated with the virulence increasing in mice, such as lethality, infection of spleen, liver and mesenteric lymph nodes and serum resistance; pR_{ST98} could also cause its host bacteria resistance to the phagocytes and enhance the intracellular growth of *Salmonella* strains in phagocytes. In 2005, we identified *Salmonella* plasmid virulence (*spv*) homologous genes which were considered to be absent from *S. typhi* before identifying it on pR_{ST98} by Southern blot and DNA sequence analysis (Huang et al., 2005). *Spv* genes have been shown to be required for *Salmonella* intracellular growth and systemic infection (Libby et al., 2000).

Although, we identified *spv* homologous genes on pR_{ST98}, the mechanism of pR_{ST98}-increased bacterial virulence was still not fully elucidated. In previous studies, we transferred pR_{ST98} into an attenuated *S. enterica* serovar *typhimurium* (*S. typhimurium*) strain RIA to create a trans-conjugant pR_{ST98}/RIA and then cultured with murine macrophage-like cell line J774A.1. The result indicated that pR_{ST98} could enhance the virulence of its host bacteria by increasing macrophage apoptosis (Wu et al., 2010). Because *S. typhi* only causes infection in humans, *S. typhimurium* that has a high degree of genome homology with *S. typhi* and a broad host range has been used extensively as an experimental model for typhoid fever. However, recent reports have shown that *S. typhimurium*-mice interaction cannot reflect all characteristics of *S. typhi*-human interaction. For example, *Salmonella* pathogenicity island (SPI)-2 is essential for intracellular survival of *S. typhimurium* in mice (Waterman and Holde, 2003), but not required for survival of *S. typhi* in human macrophages (Forest et al., 2010). In an effort to gain more precise and further insight into the role of pR_{ST98} in the interaction between *S. typhi* and human macrophage, in the present study, we examined apoptosis, caspase-3 activity and NO production in human-derived macrophage- like cell line THP-1 infected with wild-type (ST8) and pR_{ST98}- deletion (ST8-*Δ*pR_{ST98}) *S. typhi* strains.

MATERIALS AND METHODS

Bacterial strains and culture

Wild-type *S. typhi* strain ST8 harboring with pR_{ST98} 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 by our lab. 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 and infection

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. The next day, medium and non-adherent cells was removed and replaced with fresh complete medium. Cells were rested 2 days following chemical differentiation. Mid-logarithmic phase growth cultures of *S. typhi* strains ST8, ST8-*Δ*pR_{ST98} and ST8-c-pR_{ST98} were added to THP-1 macrophages at a multiplicity of infection (MOI) 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 extracellular growth of bacteria released from infected THP-1 cells. All assays were conducted in triplicate and repeated at least three times.

Preparation of samples for transmission electron microscopy

THP-1 cells were cultured and infected as described previously. Time point at 2 and 6 h post-infection was examined. Briefly, the adherent cells were scraped with a cell scraper and pelleted by centrifugation. The cells were then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide, and dehydrated through a series of graded acetone washes. Samples were embedded in epoxy resin, sectioned and placed onto 200-mesh copper grids. The grids were stained with uranyl acetate and lead citrate, and the samples were examined for the presence of apoptotic cells using a Hitachi TEM.

Flow cytometry analysis of apoptosis

Macrophage apoptosis was analyzed with flow cytometer after propidium iodide (PI) staining, apoptotic population appeared as a sub-G1 peak. THP-1 cells were harvested and washed with PBS. The cells were then fixed in 70% ethanol at 4°C for 24 h, treated with RNaseA at 37°C for 30 min and stained with PI in the dark for 30 min. The samples were analysed by a FACScan flow cytometer (FC500, Beckman Coulter, Brea, CA, USA).

Assay of caspase-3 activity

Caspase-3 activity was assayed using commercial colorimetric caspase assay kits (Beyotime, China). THP-1 cells were harvested and washed with PBS. The cells were then resuspended in lysis buffer and incubated on ice for 15 min. The cell debris was removed by centrifugation at 12000 rpm for 10 min at 4°C, and the supernatant was collected for the colorimetric assay of caspase-3 activity. Para-nitroanilide (pNA)-conjugated specific substrates for caspase-3 (DEVD-pNA) was used according to the manufacturer's instructions. Cleaved substrates were quantified by reading absorbance at 405 nm with a microplate spectrophotometer (uQuant, Bio-Tek Instruments Inc, Winooski, VT, USA).

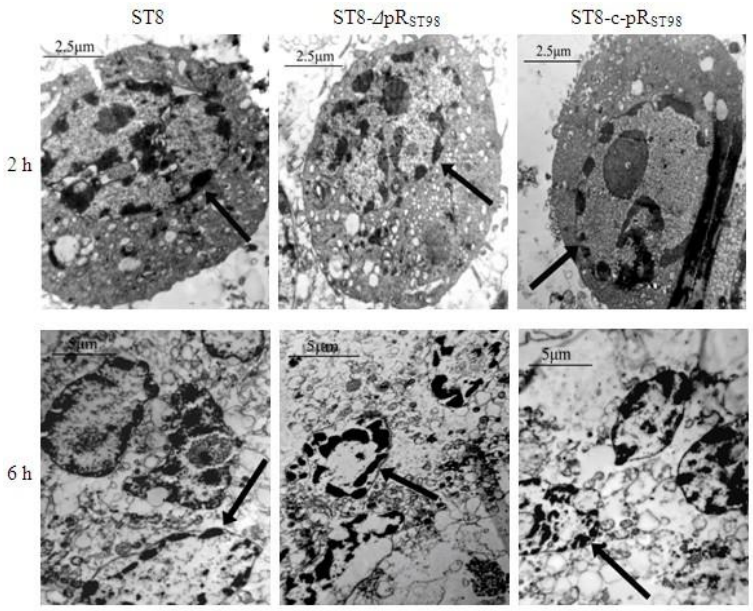

Figure 1. Transmission electron micrographs of THP-1 cells infected with *S. typhi* at 2 h and 6 h post-infection. Black arrows denoted condenses and marginated nuclear chromatin of THP-1 cells.

Measurement of nitric oxide (NO)

Nitric oxide (NO) released from macrophages infected with *S. typhi* was measured by means of the Griess reaction, which detects the amount of nitrite accumulated (Green et al., 1982). The supernatant was collected and an equal volume of Griess reagent was added. The mixture was incubated for 10 min at room temperature and absorbance was recorded at 540 nm. Blank and standards were also run in parallel. In some experiments, THP-1 cells were pretreated with NO synthase inhibitor L-NAME for 30 min.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Experimental results were analyzed for their significance by Student's *t*-test using a SPSS program. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Morphological features of macrophage apoptosis

As shown in Figure 1, at 2 h post-infection, THP-1 cells infected with *S. typhi* ST8, ST8-*Δ*pR$_{ST98}$ and ST8-c-pR$_{ST98}$ exhibited morphological features of apoptosis, such as condenses and marginated nuclear chromatin. But there was no significant difference between these three groups in amount of apoptotic cells at this point. At 6 h post-infection, these changes were more obvious and it was observed that more cells infected with ST8 and ST8-c-pR$_{ST98}$ exhibiting morphological features of apoptosis than cells infected with ST8-*Δ*pR$_{ST98}$.

Percentage of macrophage apoptosis

Though observation of cell ultra-structure by TEM is the gold standard to determine apoptosis, it is a qualitative standard and cannot reflect amount of apoptotic cells very accurately. The relationship between pR$_{ST98}$ and macrophage apoptosis was further confirmed by flow cytometer analysis after PI staining. As shown in Figure 2, the percentage of macrophage apoptosis induced by ST8, ST8-*Δ*pR$_{ST98}$ and ST8-c-pR$_{ST98}$ at 2 h post-infection was 23.05 ± 1.63, 22.10 ± 2.55 and 21.99 ± 1.89%, respectively, in contrast to 6.76 ± 1.09% in control. There was no significant difference between macrophage apoptosis induced by ST8, ST8-*Δ*pR$_{ST98}$ and ST8-c-pR$_{ST98}$ ($P > 0.05$). Amount of apoptotic cells displayed increasing tend over incubation time. The percentage of macrophage apoptosis induced by ST8, ST8-*Δ*pR$_{ST98}$ and ST8-c-pR$_{ST98}$ at 6 h post-infection was 70.31 ± 2.50%、53.02 ± 2.24% and 68.26 ± 2.32%, respectively, in contrast to 6.90 ± 1.12% in control. Significantly higher percentage of macrophage apoptosis induced by ST8 and ST8-c-pR$_{ST98}$ than ST8- *Δ*pR$_{ST98}$ was observed at this appoint ($P < 0.01$).

Caspase-3 activity

Whether caspase-3 activated by *S. typhi* infection was determined using colorimetric substrate assays of lysates from infected macrophages. As shows in Figure 3, caspase-3 activity was consistent with the percentage of macrophage apoptosis. Caspase-3 activity (OD_{405}) of

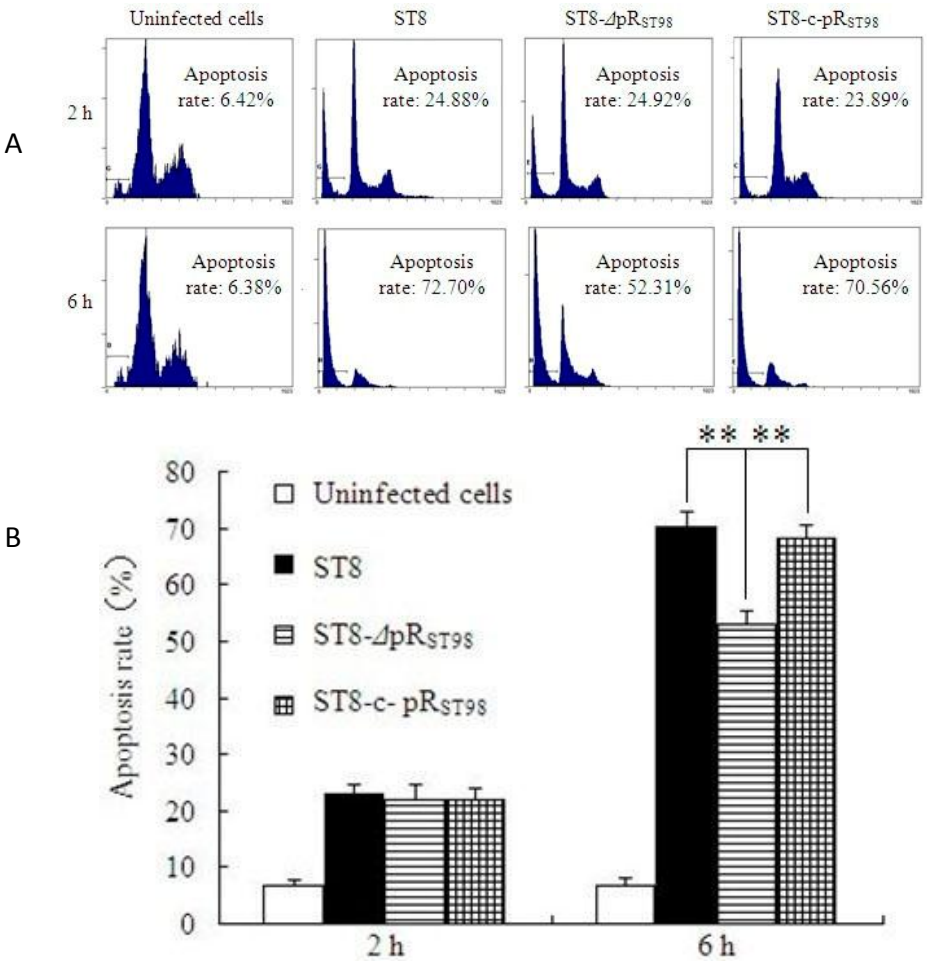

Figure 2. Percentage of macrophage apoptosis in THP-1 cells infected with *S. typhi* at 2 h and 6 h post-infection. (A) Representative results of percentage of macrophage apoptosis analyzed with flow cytometer after PI staining; (B) the mean percentage of macrophage apoptosis analyszd with flow cytometer after PI staining. Statistical analysis was performed by student *t*-test (**P < 0.01).

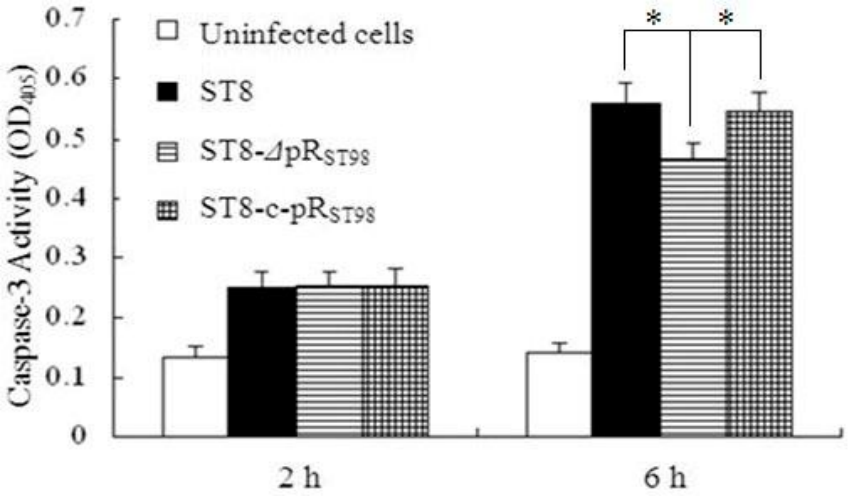

Figure 3. Caspase-3 activity in THP-1 cells infected with *S. typhi* at 2 h and 6 h post-infection. Statistical analysis was performed by student *t*-test (*P < 0.05).

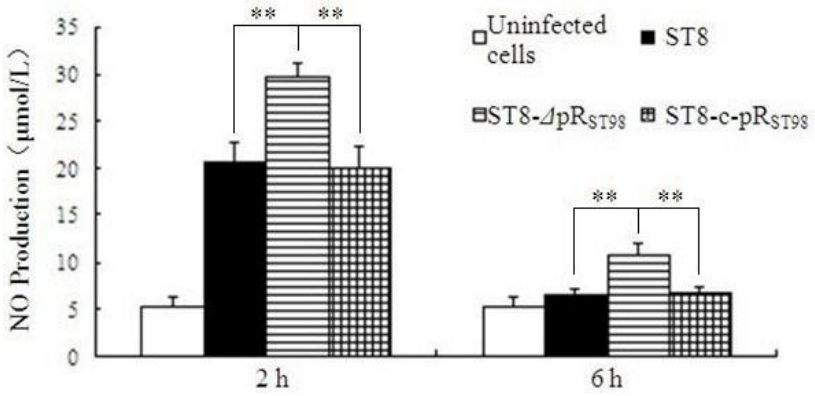

Figure 4. Level of nitrite in the culture supernatants of THP-1 cells infected with *S. typhi* at 2 h and 6 h post-infection. Statistical analysis was performed by student *t*-test (***P* < 0.01).

control macrophages infected with ST8, ST8- *Δ*pR$_{ST98}$ and ST8-c-pR$_{ST98}$ at 2 h post-infection was 0.248 ± 0.030, 0.251 ± 0.027 and 0.252 ± 0.029, respectively. However, no significant difference between macrophages infected with ST8, ST8-*Δ*pR$_{ST98}$ and ST8-c-pR$_{ST98}$ was observed at this appoint (*P* > 0.05). Significant difference was observed at 6 h post-infection, caspase-3 activity of macrophages infected with ST8, ST8-*Δ*pR$_{ST98}$ and ST8-c-pR$_{ST98}$ at 6 h post-infection was 0.559 ± 0.035, 0.467 ± 0.027 and 0.546 ± 0.033, respectively (*P* < 0.05). Caspase-3 activity of macrophages infected with ST8 and ST8-c-pR$_{ST98}$ was significantly higher than ST8-*Δ*pR$_{ST98}$.

NO production

The amount of nitrite in supernatant was assayed. As shown in Figure 4, macrophages infected with ST8-*Δ*pR$_{ST98}$ showed a significant higher nitrite level compared to macrophages infected with ST8 and ST8-c-pR$_{ST98}$ at both 2 h and 6 h post-infection (29.58 ± 1.52 versus 20.69 ± 2.00 μmol/L and 19.97 ± 2.19 μmol/L at 2 h post-infection; 10.77 ± 1.17 versus 6.48 ± 0.63 μmol/L and 6.64 ± 0.67 μmol/L at 6 h; *P* < 0.01).

Effect of NO suppression on *S. typhi* induced macrophage apoptosis

In order to investigate whether NO plays a role in apoptosis after *S. typhi* infection, THP-1 cells were pretreated with NO synthase inhibitor L-NAME. As shown in Figure 5, compared with untreated macrophages, L-NAME enhanced significantly induced macrophage apoptosis (44.25 ± 2.40% versus 53.90 ± 1.89%; *P* < 0.01). But its effect on ST8 and ST8-c-pR$_{ST98}$ induced macrophage apoptosis was limited (65.66±2.99 and 63.39±2.80% versus 66.34±2.67 and 63.65±2.39%; *P* > 0.05).

DISCUSSION

Interaction between pathogens and macrophages is essential for the pathogenesis of many infections. Macrophages have double roles in pathogenic infection (Gordon, 2007). On one hand, macrophages are key mediators in eliciting both innate and adaptive immune responses. As *the* scavengers of host, macrophages can engulf and digest off invading pathogens, present antigen to T lymphocytes and secret cytokines that regulate immune response. On the other hand, many pathogens reside and replicate in macrophages, even exploit macrophages as a crucial tool in the initiation of the disease. In order to colonize host successfully, pathogens evolve a variety of strategies to manipulate the fate of macrophages towards their benefits. Regarding *Salmonella*, bacteria mainly evolve two strategies: (i) Following invasion of macrophages, *Salmonella* resides in a membrane-bound compartment, known as the *Salmonella*-containing vacuole (SCV) that superficially resembles a phagosome. Unlike conventional phagosome, SCV is redirected away from the phagosomal maturation pathway and does not fuse with lysosome (Brumell and Grinstein, 2004), (ii) when replicating within macrophages, *Salmonella* also produces cytotoxins to stimulate macrophage apoptosis (Chanana et al., 2007), thus eludes host innate immune responses and spreads easily. The ability of *Salmonella* to initiate death of infected macrophages contributes to the severity of disease caused by *Salmonella* strains.

Our previous study demonstrated that pR$_{ST98}$ could increase apoptosis level of murine macrophage J774A.1. However, pR$_{ST98}$ was originally isolated from *S. typhi*. *S. typhimurium*-mice interaction and *S. typhi*-human interaction may differ in a number of respects. In the present study, we use *S. typhi* infected human macrophage THP-1, it was observed that pR$_{ST98}$ could increase the percentage of macrophage apoptosis

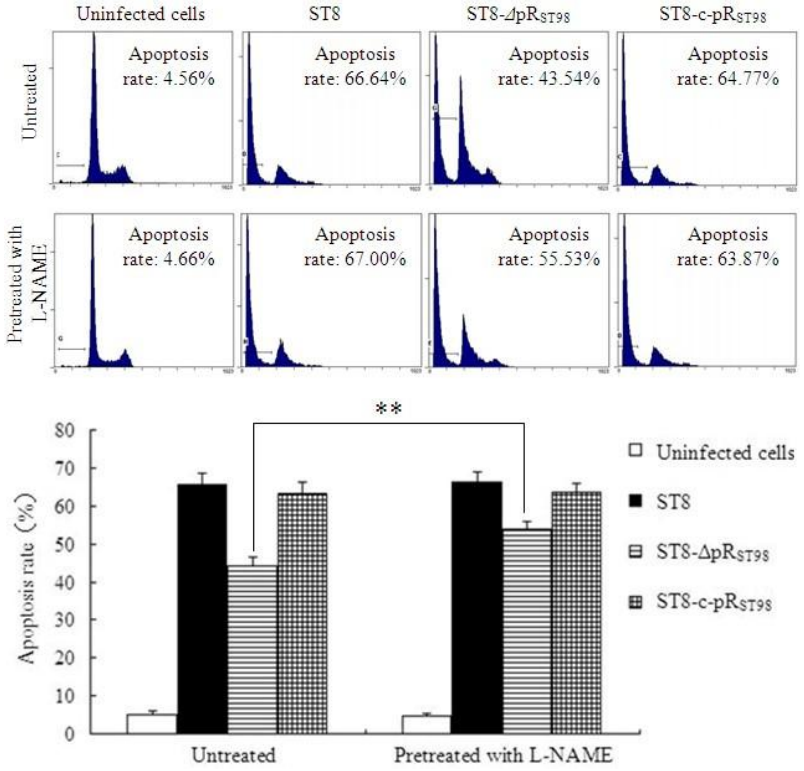

Figure 5. Effect of NO synthase inhibitor L-NAME on apoptosis induced by *S. typhi* at 6 h post-infection. (A) Representative results of percentage of macrophage apoptosis analysed with flow cytometer after PI staining; (B) the mean percentage of macrophage apoptosis analysed with flow cytometer after PI staining.Statistical analysis was performed by student *t*-test (**P < 0.01).

induced by *S. typhi*, as demonstrated by the results of cell ultra-structure observation using transmission electron microscopy and flow cytometer analysis. This is consistent with our previous study. Additionally, we find an interesting phenomenon that the percentage of apoptosis between macrophages infected with ST8, ST8-*Δ*pR$_{ST98}$ and ST8-c-pR$_{ST98}$ was not significantly difference at early phase, and that the cytotoxicity of pR$_{ST98}$ occurred later. Similar phenomenon of delayed cytotoxicity of virulent effectors was observed by Libby et al. (2000) in assaying the *spv* phenotype in macrophages and Paesold et al. (2002) in intestinal epithelial cells. Perhaps, the delayed cytotoxicity of pR$_{ST98}$ caused *S. typhi* infection. It would allow sufficient time for *S. typhi* to adapt to the host intracellular environment, replicate extensively and express genes for deep infection.

Apoptosis is an active process of programmed cell death (PCD), which leads to cell destruction and death. Classical apoptosis is triggered by two separate pathways: one is extrinsic pathway initiated by cell surface death receptors, and the other is intrinsic pathway which involves the disruption of mitochondrial membrane integrity (Burz et al., 2009). Both pathways activate executioner caspases (members of cysteine protease family) to cleave cellular substrates to produce the features associated with apoptosis. Caspase-3 is a central effector of caspase, activated caspase-3 is a protease with many cellular targets. Certain pathogens, such as *Salmonella*, can deliver effector proteins through type III protein secretion systems (TTSSs) to alter important signal transduction pathways and affect the balance between pro- and anti-apoptotic factors in the host cell, with caspase-3 as the major executioner of characteristic apoptotic changes (Guiney, 2005). The results demonstrate that pR$_{ST98}$ raised caspase-3 activity in infected cells, suggesting that macrophage apoptosis induced by pR$_{ST98}$ is related to caspase-3 activation.

Generation of NO is a host defense mechanism during pathogenic infection. NO has potent host defense functions, including antimicrobial actions via its cytotoxic or cytostatic effects (Nathan and Shiloh, 2000) and anti-apoptotic activity through inhibition of caspases activity via S-nitrosylation of the active cysteine in caspases (Slomiany and Slomiany, 2010). Alam et al. (2006, 2008) reported earlier that *Salmonella* infection caused higher bacterial burden and more extensive apoptosis in Peyer's patch tissue, liver tissue and peritoneal macrophages of inducible NO synthase (iNOS)- deficient mice than wild-

type mice. It indicated that a decrease or eventual absence of NO production may result in a deleterious outcome for host during pathogenic infection. Our present study gave similar results that pR_{ST98} suppressed NO production and resulted in higher percentage of infected macrophage apoptosis. Blocking of NO production with NO synthase inhibitor L-NAME enhanced significantly ST8-*Δ*pR_{ST98} induced macrophage apoptosis, but its effect on ST8 and ST8-c-pR_{ST98} induced macrophage apoptosis was limited. However, Chanana et al. (2007) reported a contrary result that, NO induced macrophage apoptosis following infection with *S. typhi*. Appearance of this opposite results may not affect the validity of our results. NO is a multifunctional molecule, its biological effects depend on the concentration of intracellular NO in combination with the intracellular environment and its interaction with other biological molecules (Wang et al., 2010).

In conclusion, our present study demonstrated that the pR_{ST98} enhances caspase-3 mediated macrophage apoptosis by suppressing NO production. However, our knowledge of pR_{ST98} obtained from present study is still limited. The mechanisms of pR_{ST98} mediating NO production are unclear. *Spv* locus is a highly conserved region consisting of five genes, one transcriptional regulator *spvR* and four structural genes *spvABCD*. It was reported that *spv*C had phosphothreonine lyase activity that can remove phosphate groups from host cells mitogen-activated protein kinases (MAPKs) thereby inactivating them (Mazurkiewicz et al., 2008). MAPKs have been demonstrated to be key players in regulating production of NO (Gao et al., 2012). Though, *spv* homologous genes have been found on pR_{ST98}, pR_{ST98} is a large plasmid containing complex sequences of unknown functions. However, It is not clear whether pR_{ST98} mediating NO production is directly related to *spv* genes or a result of interplay of several genes. Further exploration will be focused on the details of pR_{ST98} regulatory pathways.

ACKNOWLEDGEMENTS

We are grateful to Prof. Xueguang Zhang, Medical College of Soochow University, P. R. China, for providing human macrophage-like cell line THP-1. This study was supported by Natural Science Foundation of China (No. 30972768), Special Research Fund for the Doctoral Program of High Education (No. 20103201110009), Natural Science Foundation of Jiangsu province (No. BK2011286) and Qinglan Project of Jiangsu Province (No. SR13400211).

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