# Suppression of dendritic cell and T-cell activation by the pR<sub>ST98</sub> Salmonella plasmid

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Received February 12, 2014; Accepted October 24, 2014

DOI: 10.3892/mmr.2014.2919

Abstract. Salmonella evades host immune response via the expression of a variety of pathogenic factors. The ' $pR_{ST98}$ ' plasmid of Salmonella enterica serotype Typhi (S. Typhi) is involved in conferring the multidrug-resistance and virulence of S. typhi. However, its specific effect on host-cell function has remained elusive. Dendritic cells (DCs) are key regulators of immune responses. The present study therefore aimed to investigate whether  $pR_{ST98}$  may target DCs involved in mediating the adaptive immune response. In vivo experiments with Salmonella enterica serotype Typhimurium χ3337 and  $\chi$ 3337/pR<sub>ST98</sub> revealed that pR<sub>ST98</sub> may influence multiple important functions of murine DCs, including maturation, survival and cytokine production. In addition, pRST98 markedly contributed to decreasing T-cell activation. These data suggested that by targeting the aforementioned functions of DCs, pR<sub>ST98</sub> may partially overturn the adaptive immune defense mechanisms of the host, which are required for elimination of this pathogen from infected tissues. This may contribute to the evasion of host adaptive immune responses by S. Typhi and therefore provide a target for the prevention and treatment of typhoid fever.

## Introduction

Immune surveillance by dendritic cells (DCs) is important in the mediation of innate and acquired immunity. To activate T lymphocytes following infection, DCs internalize and eliminate invading microbes and exhibit pathogen-derived antigens on their surface in the form of major histocompatibility

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complex (MHC) molecules (1,2). Salmonella is a facultative intracellular pathogen and the successful engagement of T lymphocytes is required for the initiation of protective immunity (3). The activity of pathogen-specific T cells significantly reduces the levels of infection and colonization by virulent bacteria, via direct elimination of bacteria or by enhancing the innate immune response. However, certain microbial pathogens have evolved molecular mechanisms aimed at interfering with DC activity in order to evade the specific adaptive immune response. These mechanisms are termed 'immune evasion' and significantly influence pathogen virulence (4,5).

The action of multiple pathogenic factors of Salmonella, encoded by the chromosome and the plasmid, is required during infection (6). The significance of large plasmids in determining the virulence of Salmonella has been the subject of numerous studies (7-9). In a previous study by our group, the 98.6 mDa, 150 kb Salmonella enterica serotype Typhi (S. Typhi) plasmid, designated as pR<sub>ST98</sub>, was isolated during a survey of multidrug-resistant S. Typhi strains, and is known to mediate bacterial multidrug resistance to chloramphenicol, streptomycin, trimethoprin, sulphonamide, gentamicin, neomycin, kanamycin, cephalosporin, ampicillin, carbencillin and tetracycline. Patients infected with S. Typhi carrying  $pR_{ST98}$ exhibited more severe symptoms and complications with high rates of mortality (10). However, only one plasmid existed in the isolates. It was therefore hypothesized that pR<sub>ST98</sub> may be a mosaic-like structure responsible for not only drug resistance, but also increased bacterial virulence.

On the plasmids of all pathogenic *Salmonella* spp., except *S*. Typhi, there is a highly conserved 8 kb region which encodes virulence phenotypes, and this gene is designated as the *Salmonella* plasmid virulence (spv) gene (11). The virulence genes on  $pR_{ST98}$  were previously detected by spv-specific polymerase chain reaction and DNA analysis, and the results indicated that  $pR_{ST98}$  shared 99.8% homology with spv (10). This study was the first, to the best of our knowledge, to reveal that spv homologous genes existed on the  $pR_{ST98}$  plasmid. These early studies demonstrated that  $pR_{ST98}$  enhanced the virulence of host bacteria by increasing survival and mediating macrophage apoptosis via the mitochondrial pathway (12). However, the phenotype remained of

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*Key words: Salmonella enterica* serotype Typhi, plasmid, dendritic cell, T cell

interest, since the mechanism of  $pR_{ST98}$ -increased bacterial virulence has remained to be elucidated.

The virulence of *Salmonella* is frequently serovar-specific, for example, *S*. Typhi causes typhoid fever in humans, but no disease is associated with *S*. Typhi infection of mice. In the present study, in order to determine whether  $pR_{ST98}$  was involved in the specific immune response *in vivo*,  $pR_{ST98}$  was transferred into *S*. Typhimurium  $\chi 3337$  (a virulence plasmid-cured strain) and the conjugative strain  $\chi 3337/pR_{ST98}$  was used in a murine salmonellosis model. The effect of  $pR_{ST98}$  on important functions of murine DCs, including maturation as well as its effects on T cells. The results of the present study may aid the elucidation of the mechanism underlying *S*. Typhi immune evasion.

## Materials and methods

Salmonella strains and growth conditions. In the present study, S. Typhimurium SR11 x3337 (a nalidixic acid-resistant, virulence plasmid-cured derivative of  $\chi 3306$ ) was used as a negative control. This strain was provided by Dr Roy Curtiss III (Arizona State University, Phoenix, AZ, USA). The multidrug-resistant S. Typhi strains harboring pR<sub>ST98</sub> were obtained from patients' blood during a typhoid fever outbreak in Suzhou, China between 1987 and 1992. Plasmid-free Escherichia coli K12W1485 Rifr F-Lac(+) (E. coli K12W1485) with a rifampicin resistance gene on the chromosome and S. Typhimurium SR11 x3337 were used as recipients to create the conjugant x3337/pR<sub>ST98</sub>. E. coli V517 (54.4, 7.3, 5.6, 5.2, 4.0, 3.0, 2.7 and 2.1 Kb) and Shigella flexneri 24570 (159.6, 4.0 and 3.0 Kb) harboring standard plasmids were used as size markers. All strains were grown to mid-logarithmic phase at 37°C in Luria-Bertani (LB) broth (Shanghai Kemin Biotechnology Co. Ltd., Shanghai, China) and quantified spectrophotometrically by determining optical density (OD) at 600 nm. Strains were subsequently centrifuged at 2,300 xg for 5 min and resuspended in RPMI-1640 medium (Gibco-BRL) without antibiotics prior to their addition to cells.

Conjugal transfer of  $pR_{ST98}$  and electrophoretic analysis. The conjugal test was performed as follows:  $pR_{ST98}$  was transferred from the clinical isolated multi-drug-resistant S. Typhi to a plasmid-free laboratory strain of E. coli K12W1485, and Shigella and Salmonella (SS) agar plates containing rifampicin (100  $\mu$ g/ml) and chloramphenicol (20  $\mu$ g/ml) (Beijing Biodee Biotechnology Co., Ltd., Beijing, China) were used. E. coli is able to ferment lactose, so that it can therefore be easily identified on an SS agar plate. pR<sub>ST98</sub> was transferred from *E. coli* K12W1485 to *S.* Typhimurium χ3337. *E. coli* K12W1485 receiving pR<sub>ST98</sub> (conjugant pR<sub>ST98</sub>/E. coli K12W1485) was used as the donor, while S. Typhimurium  $\chi$ 3337 was the recipient. The two strains of bacteria were grown for 16 h at 37°C in LB broth and mixed well by transferring 0.1 ml of each into 3 ml fresh LB broth for 4 h at 37°C. The mixture was centrifuged at 2,300 xg for 5 min and resuspended in normal saline. 0.1 ml of the suspension was transferred to a casein hydrolysate agar plate and grown for 16 h at 37°C. The lawn was collected and the serial dilution tube test was conducted. 0.1 ml of the suspension was transferred to an SS agar plate containing rifampicin (100  $\mu$ g/ml), chloramphenicol (20  $\mu$ g/ml) and ampicillin (25  $\mu$ g/ml) (Beijing Biodee Biotechnology Co., Ltd., Beijing, China). The colonies producing hydrogen sulfide were selected to be cultured a second time on the same selective agar, reactivated on LB agar plates and labeled as conjugant  $\chi$ 3337/pR<sub>ST98</sub>. Plasmid DNA extraction and electrophoretic analysis were applied using routine methods (13).

*Mice*. BALB/c mice were purchased from the Experimental Animal Center (Chinese Academy of Science, Shanghai Laboratory Animal Center, Shanghai, China). Mice were housed in a pathogen-free animal facility and maintained under standard environmental conditions (room temperature  $24\pm1^{\circ}$ C; relative air humidity  $50\pm2\%$ ; a 12-h light/dark cycle; fed standard rodent diet and water *ad libitum*). Mice were used aged 8-10 weeks (weight 30–40g) and animal experiments were performed following protocols approved by the institutional animal care and use committee of Bengbu Medical College.

Antibodies. Antibodies were obtained from eBioscience, Inc. (San Diego, CA, USA) unless otherwise stated. The fluorochrome-conjugated anti-mouse monoclonal antibodies (mAbs) used in the present study were as follows: anti-CD11c fluorescein isothiocyanate (FITC; N418), anti-MHC Class II(I-A) phycoerythrin (PE)-Cy5 (NIMR-4), anti-CD40 (PE; 1C10), anti-CD80 PE (16-10A1), anti-CD86 PE (PO3.1), anti-CD62L FITC (MEL-14), anti-CD44 PE (IM7), anti-CD3e peridinin chlorophyll (PerCP)-Cy5.5 (145-2C11), anti-CD8a allophycocyanin (APC) (53-6.7), anti-interleukin (IL)-12 PE (C 17.8), anti-interferon (IFN)- $\gamma$  PE (XMG1.2), anti-tumor necrosis factor (TNF)- $\alpha$  PE (TN3-19) and all isotype-matched control mAbs were from Caltag Laboratories (Invitrogen Life Technologies, Carlsbad, CA, USA).

*Cell culture and infection*. For the generation of bone marrow-derived DCs, bone marrow was flushed from the tibias and femurs of 8 to 10-week-old mice. Following red cell lysis with lysing solution (BD Biosciences) and washing, the progenitor cells were cultured in six-well plates at  $1\times10^6$ /well in RPMI containing 10% fetal calf serum supplemented with 10 ng/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml IL-4 (Biomics Biotechnology Co., Ltd., Nantong, China). These cells were cultured for six days at 37°C in 5% CO<sub>2</sub>, and fresh medium and cytokines were added on days two and four. On day six, the DCs were collected for use in the infection experiments.

For infection of DCs,  $1.5 \times 10^5$  cells (invasion assays),  $4 \times 10^5$  cells (apoptosis assays) or  $10^6$  cells (co-stimulatory molecule expression analysis) were seeded in 24- or six-well plates in medium without antibiotics and infected with the corresponding *S*. Typhimurium strains at a multiplicity of infection (MOI) of 20 bacteria per DC. Bacteria were centrifuged onto DC at 400 xg for 5 min. Following 1 h of infection,  $100 \ \mu g/ml$  amikacin (AMK; Beijing Biodee Biotechnology Co.) was added to exterminate extracellular bacteria. Then, the cells were washed and further incubated with medium containing  $10 \ \mu g/ml$  AMK to prevent extracellular growth of bacteria released from the infected DC for an additional, indicated length of time. Bacterial invasion and DC viability assays. S. Typhimurium strains were grown to the mid-log growth phase, at which the bacteria are most invasive. DCs were infected in triplicate as aforementioned. To evaluate bacterial infectivity, AMK-treated DCs were permeabilized for 30 min with 0.1% Triton X-100 (Shanghai Fushen Biotechnology Co., Ltd., Shanghai, China) in phosphate-buffered saline (PBS; Hefei Bomei Biotechnology Co., Ltd., Hefei, China). Lysates of 2 h were plated on agar plates for overnight incubation at 37°C. At the same time-point, DC viability was analyzed using a FACSCalibur flow cytometer (BD Biosciences), using staining of fragmented DNA with propidium iodide and labeling of cells with fluorescently tagged Annexin V (BD Biosciences), which binds to phosphatidylserine present in the outer membrane of cells during early-stage apoptosis.

Detection of cell-surface markers. The infected cells were harvested at 24 h post-infection and incubated with mouse anti-FcRII/III mAb for 10 min at 4°C. Fluorochrome-conjugated antibodies were added to cells, which were incubated on ice for 30 min and then washed twice with wash buffer. Cells were kept on ice and analyzed by flow cytometry and CellQuest<sup>™</sup> version 3.3 software (BD Biosciences, Franklin Lakes, NJ, USA) and WinMDI 2.8 (winmdi.software.informer.com/2.8/).

*Bacterial infection of mice. S.* Typhimurium strains were grown overnight in LB medium, harvested in the logarithmic phase (OD600=0.8-1.0), washed once in PBS and resuspended in PBS with 15% glycerol (Hefei Bomei Biotechnology Co.). The cultures were stored in aliquots at -80°C. The bacterial titers were determined by plating serial dilutions on LB agar plates. For infection, aliquots were thawed and appropriately diluted in PBS. In experiments in which DC and T-cell activation were analyzed, mice were infected intravenously with 500 organisms and sacrificed by cervical dislocation on the fifth or tenth day following infection. In experiments in which the intracellular cytokines of splenic DCs were studied, mice were administered a single intravenous injection of 10<sup>7</sup> bacteria and sacrificed 24 h subsequently. Total splenocytes were subjected to flow-cytometric analysis.

*Flow-cytometric analysis of spleen cells*. Single-cell suspensions were obtained by passing spleens through stainless steel meshes followed by erythrocyte lysis. Cells (10<sup>6</sup>) were incubated with anti-FcRII/III mAb to block unspecific antibody binding. Following 10 min of incubation, cells were stained with FITC-conjugated mouse anti-CD11c, PE-conjugated mouse anti-CD40, PE-conjugated mouse anti-CD80, PE-conjugated mouse anti-CD3e, APC-conjugated mouse anti-CD8a, FITC-conjugated mouse anti-CD62L and PE-conjugated mouse anti-CD44. Following 30 min on ice, cells were washed and analyzed.

Intracellular cytokine expression was analyzed in a splenocyte preparation that had been incubated for 5 h in the presence of GolgiPlug<sup>TM</sup> (brefeldin A solution, 10 µg/ml; BD Biosciences). Cells were then washed with PBS supplemented with 0.1% bovine serum albumin (Beijing Leigen Biotechnology Co., Ltd., Beijing, China) and were surface stained for CD11c to identify splenic DCs and for co-stimulatory molecules. Cells were subsequently fixed, permeabilized and stained



Figure 1. Identification of conjugal transfer of pR<sub>ST98</sub>. Plasmids were extracted and analyzed by electrophoresis. Lanes: 1, *Salmonella enteric* serovar Typhimurium (*S*. Typhimurium) carrying a 150 kb plasmid (pR<sub>ST98</sub>); 2, *Escherichia coli* (*E. coli*) K12W1485 containing pR<sub>ST98</sub> plasmid donor in the second conjugal transfer step; 3, *S*. Typhimurium  $\chi$ 3337; 4, conjugant  $\chi$ 3337/pR<sub>ST98</sub>; 5, plasmid size marker *Shagella flexneri* 24570; 6, plasmid size marker *E. coli* V517.

with anti-IL-12 PE, anti-IFN- $\gamma$  PE or anti-TNF- $\alpha$  PE using the cytofix/cytoperm kit (eBioscience, Inc.) according to the manufacturer's instructions. Flow cytometry was performed on splenocytes from individual mice stimulated separately.

Assessment of bacterial burden. Single-cell suspensions were obtained from the spleens of infected mice in RPMI-1640. An aliquot of the suspension was lysed and evaluated to determine the number of viable bacteria. Colony-forming units (CFU) were determined by plating serial dilutions of tissue homogenates on LB agar plates.

Statistical analysis. Values are presented as the mean  $\pm$  standard deviation. Data obtained from independent experiments were analyzed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference between values. The statistical software package SPSS 15.0 (SPSS, Inc., Chicago, IL, USA) was used for all data analyses.

### Results

Conjugal transfer of plasmid  $pR_{ST98}$ . S. Typhi causes typhoid fever in humans only; therefore, S. Typhimurium was employed to infect mice in order to study the pathogenesis of  $pR_{ST98}$  in vivo.  $pR_{ST98}$  was initially transferred from the multi-drug-resistant S. Typhi to E. coli K12W1485 and then transferred from E. coli K12W1485 to S. Typhimurium  $\chi$ 3337 to create the conjugant  $\chi$ 3337/p $R_{ST98}$ . Electrophoretic analysis indicated that an additional 150-Kb p $R_{ST98}$  plasmid was found in  $\chi$ 3337/p $R_{ST98}$ , compared to the recipient  $\chi$ 3337, which confirmed that p $R_{ST98}$  was transferred into  $\chi$ 3337 (Fig. 1).

 $pR_{ST98}$  does not influence bacterial invasion. To date the role of  $pR_{ST98}$  in bacterial invasion has remained elusive. The invasion ability of *S*. Typhimurium strains with or without  $pR_{ST98}$ was compared. The results demonstrated that *S*. Typhimurium  $\chi$ 3337 and conjugant  $\chi$ 3337/p $R_{ST98}$  were equally invasive (Fig. 2A). This therefore indicated that the expression of  $pR_{ST98}$ did not increase the ability of the bacteria to invade DCs.

DC viability is decreased following infection with  $pR_{ST98}$ S. Typhimurium. A previous study demonstrated that the infection of macrophages or DCs with S. Typhimurium expressing



Figure 2. Entry of *Salmonella* to murine DCs and DC viability. DCs were infected with *Salmonella enteric* serovar Typhimurium ( $\chi$ 3337 or the conjugant  $\chi$ 3337/pR<sub>ST98</sub>; multiplicity of infection 20) or left untreated. Following 1 h of infection, bacteria were washed away and cells were cultured for 2 h in the presence of amikacin to eliminate extracellular bacteria. (A) Cells were lysed and intracellular bacteria were plated on Luria-Bertani agar plates to determine intracellular CFU. Values are expressed as the mean of four independent experiments ± standard deviation. (B) Viability of DCs at the same time-point was assessed by flow cytometry following staining with fluorescence labeled Annexin V and PI. The results of one representative experiment are shown. \*\*P<0.01 vs. control, \*P<0.05 vs.  $\chi$ 3337 strain. DC, dendritic cell. CFU, colony-forming units; PI, propidium iodide; ns, no significant difference.

the type III secretion system resulted in early apoptosis (14). To assess whether  $pR_{ST98}$  of *S*. Typhimurium induced mortality of murine DCs and whether this may be involved in mediating the ability of DCs to stimulate T cells, an apoptosis assay was performed using *S*. Typhimurium-infected murine DCs. As illustrated in Fig. 2B, *S*. Typhimurium induced apoptosis in murine DCs. Two hours after infection, *S*. Typhimurium  $\chi 3337/pR_{ST98}$ -infected DCs exhibited a higher rate of apoptosis than that of  $\chi 3337$ -infected cells. Therefore,  $pR_{ST98}$  may be an important factor involved in *Salmonella*-induced cell death.

S. Typhimurium *induces DC maturation*. To study the effects of *Salmonella*  $pR_{ST98}$  on DC maturation, cells were infected with the corresponding bacterial strains, and the expression of co-stimulatory molecules involved in T-cell activation was measured by flow cytometric analysis following 24 h of further incubation to facilitate the biosynthesis of novel surface molecules. Unstimulated DCs are physiologically immature; however, following infection with *S*. Typhimurium

 $\chi$ 3337 or  $\chi$ 3337/pR<sub>ST98</sub>, DCs exhibited a mature phenotype, manifested as an increase in the expression of co-stimulatory molecules. Phenotypic changes following the infection of DCs with corresponding strains are indicated in Fig. 3. Compared to *S*. Typhimurium  $\chi$ 3337/pR<sub>ST98</sub>- and  $\chi$ 3337-infected DCs exhibited increased expression levels of all three surface molecules (CD80, CD86 and MHC II), indicated by increased fluorescence intensity.

 $pR_{ST98}$  enhances bacterial burden following S. Typhimurium infection. BALB/c mice were intravenously infected with 500 bacteria of corresponding S. Typhimurium strains. Infection resulted in massive expansion of bacteria in the spleen and liver. The two bacterial strains exhibited the same course. The number of bacteria recovered from  $\chi$ 3337/pR<sub>ST98</sub>-infected mice was significantly higher than that of  $\chi$ 3337-infected mice ten days post-infection (Fig. 4). As expected, the  $\chi$ 3337/pR<sub>ST98</sub> strain was virulence-enhanced compared to the  $\chi$ 3337 strain, and the titer in the spleen and liver was 10<sup>7</sup> bacteria/organ.

Suboptimal activation of DCs results from infection with the conjugant,  $\chi 3337/pR_{ST98}$ . In vivo, Salmonella is usually contained within splenic DCs, which are the most efficient antigen-presenting cell (APC) types capable of stimulating naïve T cells (15). The present study therefore aimed to determine how the numbers and activation states of splenic DCs were altered during infection with S. Typhimurium. It was demonstrated that the overall number of splenic DCs increased significantly five days following infection with either strain, compared to that of mock-infected animals. A significant difference in the number of DCs in the spleen was observed between  $\chi$ 3337- and  $\chi$ 3337/pR<sub>ST98</sub>-inoculated mice (Fig. 5A and B). To determine whether DC activation was distinct following wild-type  $\chi$ 3337 or the conjugant  $\chi$ 3337/pR<sub>ST98</sub> infection, cell surface expression of co-stimulatory molecules (CD40, CD80 and CD86) on CD11c(+) splenic DCs, as well as their ability to secrete the pro-inflammatory cytokines, was analyzed. Fig. 5C indicates that the extent of CD40, CD80 and CD86 upregulation is distinct following  $\chi$ 3337 infection, compared to that following  $\chi$ 3337/pR<sub>ST98</sub> immunization. S. Typhimurium  $\chi$ 3337 infection induced greater co-stimulatory molecule expression than  $\chi$ 3337/pR<sub>ST98</sub> immunization five days post-infection.

Furthermore, it was investigated whether the level of T-cell function induced by splenic DCs was correlated with the relative cytokine levels produced by these cells. As shown in Fig. 6, CD11c(+) splenic DCs from mice injected with *S*. Typhimurium produced IL-12, IFN- $\gamma$  or TNF- $\alpha$  24 h following infection without *in vitro* restimulation. The percentage of IL-12- or IFN- $\gamma$ -producing splenic DCs in the mice infected with  $\chi$ 3337/pR<sub>ST98</sub> was significantly lower than that of those infected with  $\chi$ 3337/pR<sub>ST98</sub> compared to that of  $\chi$ 3337-infected mice.

S. Typhimurium infection decreases T-cell activation. S. Typhimurium infection induced massive splenomegaly. On the tenth day post-infection, there was an almost three-fold increase in spleen cellularity. Total splenocytes were isolated



Figure 3. Expression of surface molecules on DCs 24 h following exposure to *Salmonella enteric* serovar Typhimurium. DCs were infected with  $\chi$ 3337 or conjugant  $\chi$ 3337/pR<sub>ST98</sub> for 1 h, washed and resuspended in amikacin-containing medium for 24 h. Cells were stained with specific anti-CD80, anti-CD86 or anti-MHC II antibodies. The cells were also stained with an isotype-matched control antibody. Results were calculated as: (geometric mean fluorescence intensity of stained cells) - (isotype control stained cells). Data are representative of three independent experiments.



Figure 4. Organ loads of  $\chi 3337$  and  $\chi 3337/pR_{ST98}$ . Mice were infected intravenously with a dose of 500 organisms/mouse. Mice infected with conjugant  $\chi 3337/pR_{ST98}$  showed increased organ colonization at 10 days post-infection, as compared to that of mice infected with  $\chi 3337$ . To determine organ colonization capacity, mice were sacrificed and their livers and spleens were lysed and plated for intracellular bacterial count. Organ colonization experiments were repeated >three times for each strain. Four mice per group were included in each experiment, \*P<0.05. CFU, colony-forming units.

from the naïve mice and mice infected with  $\chi 3337$  or the conjugant  $\chi 3337/pR_{ST98}$  at the fifth and tenth day post-infection and stained with specific antibodies. The effect of  $pR_{ST98}$  on the status of the CD4(+) and CD8(+) T-lymphocyte populations was determined. The mice infected with either strain of *S*. Typhimurium did not demonstrate a significant change in T-cell population compared to that in the control mice. T lymphocytes were subsequently further analyzed for the expression of the immune activators CD44 and CD62L. In the immune system, CD62L is expressed on naïve T cells, but its

expression declines upon activation. By contrast, the expression of CD44 is upregulated following activation of naïve T lymphocytes during their response against invading microbes (16). The mice were kept under pathogen-free conditions; however, it was observed that in naïve mice ~20% of splenic T cells exhibited a CD44(high) and CD62L(low) (activated) phenotype. Following infection with S. Typhimurium, expression of CD44 and CD62L on T cells was markedly altered (Fig. 7). ~30-40% of T cells acquired a CD44(high) and CD62L(low) phenotype at ten days post-infection. Although no significant difference was observed in the CD44(high) and CD62L(low) T-lymphocyte population in the  $\chi$ 3337-infected mice at five days post-infection, there was a <10% increase in this population at ten days post-infection compared to that in the  $\chi$ 3337/pR<sub>ST98</sub>-infected mice. These results therefore suggested that  $pR_{\rm ST98}$  did not affect the status of the T-lymphocyte population, but prevented T-cell activation.

## Discussion

The role of DCs in triggering anti-microbial immunity, particularly *in vivo*, has been well defined. *S*. Typhimurium is a bacterial pathogen that systemically disseminates from the site of infection (17). Studies have demonstrated that *S*. Typhimurium interferes with the activation of host adaptive immunity at multiple levels (18-20). *Salmonella* virulence factors perturb antigen processing and presentation by DCs, as well as the activation of *Salmonella*-specific T cells (21). These observations led to the hypothesis that this feature of virulent *Salmonella* is required to promote systemic dissemination in the host.

In previous studies by our group, it was demonstrated that plasmid  $pR_{ST98}$  enhanced the virulence of host bacteria by increasing survival and mediating macrophage apoptosis via the mitochondrial pathway (12). Consistent with these results,  $pR_{ST98}$  was found to influence multiple important functions of murine



Figure 5. Flow-cytometric analysis of splenic DCs from *Salmonella enteric* serovar Typhimurium (*S*. Typhimurium)-infected mice. Mice were given 500 *S*. Typhimurium  $\chi$ 3337 or conjugant  $\chi$ 3337/pR<sub>ST98</sub> intravenously. At five days post-infection, splenocytes from individual mice were stained and analyzed by flow cytometry. (A) Cells in the live lymphocyte gate were analyzed for CD11c(+) (surface staining to identify splenic DCs) cell populations. Representative dot plots indiacting the expression of CD11c on cells from the spleen of mice. The numbers represent the percentage of splenic DCs. (B) *Salmonella* infection generates an increase in splenic DCs. The y-axis represents the total number of splenocytes and the absolute number of splenic DCs. (C) Splenic DCs express lower levels of co-stimulatory molecules following  $\chi$ 3337/pRST98 infection than those following  $\chi$ 3337 infection. The histograms indicate CD40, CD80 and CD86 expression on gated CD11c(+) splenocytes from mice infected with  $\chi$ 3337 or  $\chi$ 3337/pR<sub>ST98</sub>. Four individual mice in each group were analyzed in  $\geq$ three independent experiments. \*P<0.05 vs. control, \*\*P<0.01 vs. control, #P<0.05 vs.  $\chi$ 3337 strain, ##P<0.01 vs.  $\chi$ 3337 strain. DC, dendritic cell; FITC, fluorescein isothiocyanate.

DCs, including maturation, survival and cytokine production. In addition,  $pR_{ST98}$  was also demonstrated to decrease T-cell activation. These results suggested that by targeting the aforementioned functions of DCs,  $pR_{ST98}$  may at least partially abrogate the adaptive immune defense mechanisms of the host, which are required for the elimination of the pathogen from infected tissues. However, further studies are required in order to determine the impact of  $pR_{ST98}$  on subsets of splenic DCs.

Previous studies by our group suggested that  $pR_{ST98}$  was not required for the invasion of non-phagocytic cells, including HeLa and Hep-2 cells (unpublished observations), but instead, its function was required to prevent significant entrance of *Salmonella* to phagocytic DCs. In the present study,  $pR_{ST98}$ activity was demonstrated not to differentially modulate the entry of *Salmonella* to DCs. Sundquist and Wick (22) reported that the death of DCs induced by *Salmonella* following infection may have a negative impact on the initiation of antibacterial immunity. However, besides macrophagy, DCs may also undergo cell death following interaction with pathogenic *Salmonella*,  $pR_{ST98}$  may potentially be one of the factors mediating the induction of DC death. Furthermore, it was revealed that *Salmonella*  APCs, characterized by enhanced expression of cell surface antigens, including CD80, CD86 and MHC II. This phenotypic alteration was similar to the response observed when DCs were treated with lipopolysaccharides, a response which is associated with increased levels of co-stimulatory surface molecules (23). Furthermore, the conjugant  $\chi$ 3337/pR<sub>ST98</sub>-treated DCs exhibited lower levels of activation and maturation than those of  $\chi$ 3337-treated DCs. These results provided evidence that pR<sub>ST98</sub>, one of the pathogenic factors of *Salmonella*, impaired the functions of DCs, predominantly via the induction of apoptosis and concurrent suppression of maturation.

Following investigation of the virulence of  $pR_{ST98}$  in DCs as well as macrophage cell lines, the virulence of the conjugant strain was evaluated in a murine model. To analyze the bacterial colonization in various organs, groups of BALB/c mice were intravenously infected with 500 CFU  $\chi$ 3337 or the conjugant  $\chi$ 3337/pR<sub>ST98</sub>. The organ load of the  $\chi$ 3337-infected mice was significantly lower than that of the  $\chi$ 3337/pR<sub>ST98</sub>-infected mice in spleen and liver on the tenth day post-infection. This suggested that pR<sub>ST98</sub> was essential for conferring *Salmonella* virulence. Furthermore, recent studies have shown that following intravenous infection of



Figure 6. Cytokines are produced by DCs following *Salmonella enteric* servar Typhimurium injection. BALB/c mice were injected with phosphate-buffered saline or  $10^7 S$ . *typhimurium*. The spleen cells were removed 4 h subsequently and incubated in complete medium for 5 h with brefeldin A ( $10 \mu g$ /ml) and further monitored for surface marker expression and intracellular cytokine production. Dot plots show intracellular cytokine production by CD11c(+) DCs. The percentages of cytokine-producing cells among gated CD11c(+) DCs are indicated. The results are from individual mice and are representative of three independent experiments, with a total of four mice per group. Values are expressed as the mean  $\pm$  standard deviation. \*P<0.05 vs. control, \*\*P<0.01 vs. control, #P<0.05 vs.  $\chi$ 3337 strain. DC, dendritic cell; IFN- $\gamma$ , interferon  $\gamma$ ; IL-12, interleukin 12; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .



Figure 7. Flow-cytometric analysis of T cells from *Salmonella enteric* serovar Typhimurium-infected mice. Groups of mice were infected intravenously with a dose of 500 organisms/mouse. Uninfected mice were used as a control. At various time-points, the population of CD4(+) or CD8(+) T cells and the expression of the activation markers CD44 and CD62L on the splenic T cells of  $\chi$ 3337- (open symbols) or conjugant  $\chi$ 3337/pR<sub>ST98</sub> (closed symbols)-infected mice was analyzed. Numbers indicate percentages of cells and values are expressed as the mean  $\pm$  standard deviation for three mice per group and time-point, \*P<0.05 vs.  $\chi$ 3337 strain. The results are representative of two independent experiments.

S. Typhimurium, >50% of bacteria that reach the spleen are located inside DCs (15). Therefore, the virulence capacity of S. Typhimurium to spread systemically into the host may be directly correlated with the bacterium's ability to avoid antigen presentation by DCs, without the activation of specific T-cell immunity. Quantitative changes in DCs were also apparent in response to Salmonella infection and significantly increased DC numbers were observed five days after infection. This increase may be due to DC migration to the spleen during infection, which occurs as a result of the functions of the spleen in initiating specific immunity and as a site of Salmonella replication (15). A marked maturation response of the splenic DCs was observed following infection and supported the hypothesis that pR<sub>ST98</sub> hindered DC maturation without influencing the total splenic DC number during infection.

Local inflammatory responses by innate cells within infected tissues may also influence antigen presentation and T-cell activation (17). The capacity of DCs to produce TNF- $\alpha$ , IL-12 and IFN- $\gamma$  during *Salmonella* infection was therefore examined. An increase in DCs producing TNF- $\alpha$  was detected among the splenic DCs of *Salmonella*-infected mice. However, the absolute number of splenic DCs producing TNF- $\alpha$  during the first day of infection was relatively low (~10<sup>4</sup>). The relative lack of DCs producing TNF- $\alpha$  suggested that DCs may not make a significant contribution to the control of initial bacterial replication. However, TNF- $\alpha$  produced by DCs may induce local effects during *Salmonella* infection in order to coordinate the maturation and migration of DCs. In this way, DC-derived TNF- $\alpha$  may facilitate a link between the innate and adaptive immune responses.

The results of the present study also revealed pR<sub>ST98</sub>-associated changes in DC cytokine secretion during Salmonella infection. In vivo production of IL-12 by DCs in response to intravenous administration of microbial stimuli is rapid and transient (24-26). Following 24 h of S. Typhimurium infection, a portion of DCs produced IL-12 without restimulation in vitro, which indicated that these DCs were likely involved in IL-12 production in vivo. These results were consistent with studies in which IL-12 production of liver CD11c(+) cells was analyzed by flow cytometry following stimulation with S. Typhimurium (27). IL-12 is an essential cytokine, which may provide protective immunity against Salmonella, as it is a key cytokine produced by APC to stimulate a Th1-directed response (28,29). Salmonella-specific T cells developing under the influence of IL-12 produce cytokines, including IFN-y, that enhance the bactericidal capacity of phagocytes and facilitate microbial elimination (30,31). These observations suggested that pR<sub>ST98</sub> interference with DC function may prevent the activation of T-cell-mediated immunity against antigens derived from this pathogen.

The activation and differentiation status of T cells during the course of infection was subsequently evaluated. T cells had acquired an activated phenotype after one week of infection. An almost 10% decrease in the CD44(high) and CD62L(low) T-lymphocyte population was observed in the  $\chi$ 3337/pR<sub>ST98</sub> infected mice ten days post-infection when compared to that in the  $\chi$ 3337 infected mice. It was therefore suggested that underlying the ability of pR<sub>ST98</sub> to impair the functions of DCs, is the inhibition of protective T-cell responses *in vivo*. However, the genetic basis of  $pR_{ST98}$  modulation of DCs and T-cell activation remains to be elucidated.

In conclusion, the characterization of the chimeric plasmid  $pR_{ST98}$ , isolated from *S*. Typhi, may be potentially important for elucidating the virulence mechanism of this pathogen. The results of the present study indicated that  $pR_{ST98}$  was a potent pathogenic factor of *Salmonella* and prevented DC-mediated activation of T cells, predominantly by inhibiting DC maturation. These results also potentially have significance in the treatment and prevention of typhoid fever.

## Acknowledgements

The present study was supported by the Natural Science Foundation of China (no. 30972768), the Natural Science Foundation of Jiangsu province (no. BK2011286), the Special Research Fund for the Doctoral Program of High Education (no. 20103201110009), the Qinglan Project of Jiangsu Province (no. SR13400211) the Provincial Project of Natural Science Research for Colleges and Universities of Anhui Province of China (no. KJ2013B142) and the Program for Scientific Innovation Research of College Graduates in Jiangsu Province (no. CX10B\_042Z).

#### References

- Steinman RM: Dendritic cells and the control of immunity: enhancing the efficiency of antigen presentation. Mt Sinai J Med 68: 160-166, 2001.
- Walseng E, Bakke O and Roche PA: Major histocompatibility complex class II-peptide complexes internalize using a clathrin- and dynamin-independent endocytosis pathway. J Biol Chem 283: 14717-14727, 2008.
- Mittrücker HW and Kaufmann SH. Immune response to infection with *Salmonella* typhimurium in mice. J Leukoc Biol 67: 457-463,2000.
- Bosio CM, Bielefeldt-Ohmann H and Belisle JT: Active suppression of the pulmonary immune response by *Francisella tularensis* Schu4. J Immunol 178: 4538-4547, 2007.
- 5. Lecours MP, Gottschalk M, Houde M, Lemire P, Fittipaldi N and Segura M: Critical role for *Streptococcus suis* cell wall modifications and suilysin in resistance to complement-dependent killing by dendritic cells. J Infect Dis 204: 919-929, 2011.
- Madajczak G and Binek M: Influence of spv plasmid genes group in *Salmonella Enteritidis* virulence for chickens. I. Occurrence of spv plasmid genes group in *Salmonella Enteritidis* large virulence plasmid. Med Dosw Mikrobiol 57:163-174, 2005 (In Polish).
- García-Quintanilla M and Casadesús J: Virulence plasmid interchange between strains ATCC 14028, LT2, and SL1344 of *Salmonella enterica* serovar Typhimurium. Plasmid 65: 169-175, 2011.
- García-Quintanilla M, Ramos-Morales F and Casadesús J: Conjugal transfer of the *Salmonella enterica* virulence plasmid in the mouse intestine. J Bacteriol 190: 1922-1927, 2008.
- Sajid SU and Schwarz S: Plasmid fingerprinting and virulence gene detection among indigenous strains of *Salmonella enterica* serovar enteritidis. J Ayub Med Coll Abbottabad 21: 83-86, 2009.
- Huang R, Wu S, Zhang X and Zhang Y: Molecular analysis and identification of virulence gene on pR(ST98) from multi-drug resistant *Salmonella* Typhi. Cell Mol Immunol 2: 136-140, 2005.
- Kurita A, Gotoh H, Eguchi M, *et al*: Intracellular expression of the *Salmonella* plasmid virulence protein, SpvB, causes apoptotic cell death in eukaryotic cells. Microb Pathog 35: 43-48, 2003.
- Wu S, Li Y, Xu Y, Li Q, Chu Y, Huang R and Qin Z: A Salmonella enterica serovar Typhi plasmid induces rapid and massive apoptosis in infected macrophages. Cell Mol Immunol 7: 271-278, 2010.
- Takahashi S and Nagano Y: Rapid procedure for isolation of plasmid DNA and application to epidemiological analysis. J Clin Microbiol 20: 608-613, 1984.

- 14. Cirillo DM, Valdivia RH, Monack DM and Falkow S: Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol Microbiol 30: 175-188, 1998.
- Yrlid U, Svensson M, Håkansson A, Chambers BJ, Ljunggren HG and Wick MJ: In vivo activation of dendritic cells and T cells during *Salmonella enterica* serovar Typhimurium infection. Infect Immun 69: 5726-5735, 2001.
- Baaten BJ, Li CR and Bradley LM: Multifaceted regulation of T cells by CD44. Commun Integr Biol 3: 508-512, 2010.
- 17. Hurley D, McCusker MP, Fanning S and Martins M: *Salmonella*-host interactions modulation of the host innate immune system. Front Immunol 5: 481, 2014.
- Alaniz RC, Cummings LA, Bergman MA, Rassoulian-Barrett SL and Cookson BT: *Salmonella* Typhimurium coordinately regulates FliC location and reduces dendritic cell activation and antigen presentation to CD4+ T cells. J Immunol 177: 3983-3993, 2006.
- Halici S, Zenk SF, Jantsch J and Hensel M: Functional analysis of the *Salmonella* pathogenicity island 2-mediated inhibition of antigen presentation in dendritic cells. Infect Immun 76: 4924-4933, 2008.
- Srinivasan A, Nanton M, Griffin A and McSorley SJ: Culling of activated CD4 T cells during typhoid is driven by *Salmonella* virulence genes. J Immunol 182: 7838-7845, 2009.
- Bueno SM, Riedel CA, Carreño LJ and Kalergis AM: Virulence mechanisms displayed by *Salmonella* to impair dendritic cell function. Curr Med Chem 17: 1156-1166, 2010.
- 22. Sundquist M and Wick MJ: *Salmonella* induces death of CD8alpha(+) dendritic cells but not CD11c(int) CD11b(+) inflammatory cells in vivo via MyD88 and TNFR1. J Leukoc Biol 85: 225-234, 2009.
- 23. Jiang HR, Muckersie E, Robertson M, Xu H, Liversidge J and Forrester JV: Secretion of interleukin-10 or interleukin-12 by LPS-activated dendritic cells is critically dependent on time of stimulus relative to initiation of purified DC culture. J Leukoc Biol 72: 978-985, 2002.

- 24. Huang LY, Reis e Sousa C, Itoh Y, Inman J and Scott DE: IL-12 induction by a Th1-inducing adjuvant in vivo: dendritic cell subsets and regulation by IL-10. J Immunol 167: 1423-1430, 2001.
- 25. Jiao X, Lo-Man R, Guermonprez P, *et al*: Dendritic cells are host cells for mycobacteria in vivo that trigger innate and acquired immunity. J Immunol 168: 1294-1301, 2002.
- 26. Schulz O, Edwards AD, Schito M, Aliberti J, Manickasingham S, Sher A and Reis e Sousa C: CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. Immunity 13: 453-462, 2000.
- Johansson C and Wick MJ: Liver dendritic cells present bacterial antigens and produce cytokines upon *Salmonella* encounter. J Immunol 172: 2496-2503, 2004.
- Lehmann J, Springer S, Werner CE, et al: Immunity induced with a Salmonella enterica serovar Enteritidis live vaccine is regulated by Th1-cell-dependent cellular and humoral effector mechanisms in susceptible BALB/c mice. Vaccine 24: 4779-4793, 2006.
- 29. Yoon WS, Choi HJ and Park YK: *Salmonella* Typhimurium harboring plasmid expressing interleukin-12 induced attenuation of infection and protective immune responses. J Gen Appl Microbiol 57: 115-122, 2011.
- 30. John B, Rajagopal D, Pashine A, Rath S, George A and Bal V: Role of IL-12-independent and IL-12-dependent pathways in regulating generation of the IFN-gamma component of T cell responses to *Salmonella* Typhimurium. J Immunol 169: 2545-2552, 2002.
- MacLennan C, Fieschi C, Lammas DA, et al: Interleukin (IL)-12 and IL-23 are key cytokines for immunity against Salmonella in humans. J Infect Dis 190: 1755-1757, 2004.