Fish and Shellfish Immunology 87 (2019) 721–729

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Salmonella spv locus affects type I interferon response and the chemotaxis of neutrophils via suppressing autophagy

Lidan Wang, Yangli Li, Yuanhui Liu, Lingli Zuo, Yuanyuan Li, Shuyan Wu**, Rui Huang*

Department of Microbiology, Medical College of Soochow University. No. 199, Ren Ai Road, Suzhou, Jiangsu, 215123, PR China

ARTICLE INFO

Keywords:
S. Typhimurium
spv operon
Autophagy
Type I interferon response
Neutrophils chemotaxis

ABSTRACT

Salmonella is a facultative intracellular pathogen that can cause significant morbidity and mortality in humans and animals. *Salmonella* plasmid virulence (*spv*) gene sequence is a highly conserved 6.8 kb region which exists in the plasmid of most pathogenic *Salmonella*. Autophagy is a degradation process of unnecessary and dysfunctional cytoplasm components to maintain cellular homeostasis, which could affect host inflammatory responses, such as type I interferon response. Type I interferon response can promote the antibacterial activity of macrophage as well as the secretion of cytokines and neutrophil chemokines. We previously reported that *spv* locus could suppress autophagy and the aggregation of neutrophils in zebrafish larvae. To explore the influence of *spv* locus on *Salmonella* escaping from the innate immune responses and the underlying mechanism, the models of *Salmonella enterica* serovar Typhimurium infected macrophages *in vitro* and zebrafish larvae *in vivo* were used in this study. The interactions among *spv* locus, autophagy, type I interferon response and the chemotaxis of neutrophils were investigated. Western blot was used to detect the expression levels of autophagy related proteins and RT-qPCR was used to measure the mRNA levels of type I interferon response and the neutrophil chemokines. The chemotaxis of neutrophils were observed by Laser Scanning confocal microscopy. Autophagy agonist Torin 1 was also involved to interfere the autophagy influx. Results showed that *spv* locus could restrain type I interferon response and the chemotaxis of neutrophils via suppressing autophagy, which provided substantial foundation to study the mechanism of *Salmonella* escaping the innate immunity.

1. Introduction

According to the statistics from Centers for Disease Control and Prevention of USA, 1 million foodborne diseases were caused by *Salmonella* every year. To date, the *Salmonella enterica* species can be classified into more than 2,600 serotypes, among which *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium, STM) is one of the most important serovars causing infection with a broad range of hosts [1]. *S.* Typhimurium contains a plasmid with a highly conserved 6.8 kb region which is called *Salmonella* plasmid virulence (*spv*) operon. *spv* locus is one of the most critical virulence factors in pathogenic *Salmonella* spp. which can promote bacterial growth and reproduction in host cells and tissues, leading to severe infection and clinical disease [2,3]. *spv* operon is consist of positive transcriptional regulator *spvR* and structure genes *spvABCD*. *spvB*, with the ADP-ribosyltransferase activity, could inhibit the formation of autophagosome at the early stage of autophagy by depolymerization of F-actin filaments [4].

Macrophages and neutrophils are phagocytes in host innate immune system, which play crucial roles in the clearance of *Salmonellae*. In addition, it is well known that macrophages and neutrophils can also regulate inflammatory responses to protect the mucosa from pathogens that might breach the gastrointestinal barrier, playing an irreplaceable role in the early stage of infection [5]. The devoured *Salmonella* by macrophages would either be eliminated or spread to other parts of the body after inducing the death of macrophages. As the most abundant leukocytes, neutrophils can rapidly migrate to infection sites under the affection of chemoattractants to clear bacteria, and work as a "scavenger" to clear the infected macrophages that failed to control *Salmonellae* by producing a large number of oxidative stress reactions through virtue of NAPDH and peroxidase [6]. While neutrophil accumulation in the intestinal lumen is a prominent histological feature of gut inflammation and can mediate clearance of pathogens, whether they can efficiently navigate to pathogen colonization foci is not clear. Not only is autophagy a highly conserved eukaryotic cellular recycling process to affect cellular homeostasis, but also it is verified to combat infection with pathogens [7,8]. Autophagy can remove bacteria by

* Corresponding author.
** Corresponding author.
E-mail addresses: wushuyan@suda.edu.cn (S. Wu), hruisdm@163.com (R. Huang).

https://doi.org/10.1016/j.fsi.2019.02.009
Received 10 October 2018; Received in revised form 2 February 2019; Accepted 8 February 2019
Available online 10 February 2019

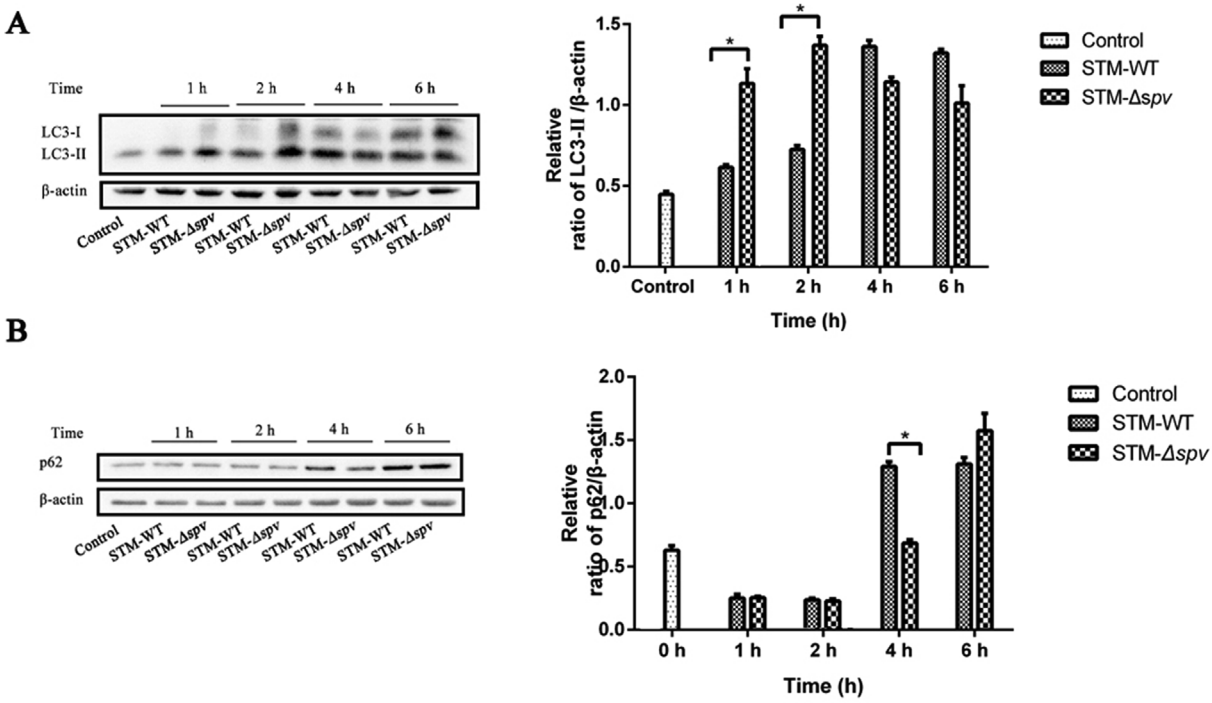

Fig. 1. The effect of *spv* locus on autophagy in macrophages. The expressions of autophagy related proteins LC3 and p62 were detected by western blot analysis. (A and B) The relative ratio of LC3-II/β-actin and p62/β-actin was measured by ImageJ. Student's *t*-test was used for statistical analysis. Data represented three independent experiments combined. Errors bars represented SEM. $*P < 0.05$.

lysosomal degradation directly and affect the inflammatory responses by regulating neutrophil degranulation, reactive oxygen species (ROS) production, as well as interferon responses [9–11]. In the process of autophagy, the autophagosome contained with invading bacteria would fuse with lysosomes or mature endosomes, on which exists the Toll-Like Receptor 3 (TLR3). TLR3 can activate TIR-domain-containing adaptor-inducing INF-β (TRIF) by recognizing double-stranded RNA (dsRNA) of degraded bacteria. The interaction between TRIF and TNF receptor-associated factor 3 (TRAF3) would facilitate type I interferon response to secret IFN-β [12,13]. IFN-β can stimulate natural killer cells (NKs) to release type II interferon IFN-γ, which would induce the activation of a variety of IFN-stimulated genes (ISGs) to promote the secretion of neutrophil chemokines resulting in efficient navigation of neutrophils to infection foci [14].

As a crucial vertebrate model organism, zebrafish has been employed on researches for nearly 50 years. In last 10 years, it was widely used to study on human related illness, owing to its merits of simple structure, rapid growth, short life cycle, easy of breeding and so on [15,16]. In addition, zebrafish embryo develops its immune system since 1 day post fertilization (dfp) and the adaptive immunity does not mature until 4 week post fertilization (wpf), which provides an excellent animal model to investigate the interplay between infection and the innate immune responses [17]. Similar to mammals, type I interferon response also occurs in zebrafish model. With a highly homology with mammals, IRF3 and IRF7 are the key regulatory factors of type I interferon response in zebrafish [18]. Zebrafish IFN 1 is homologous to mammalian IFN-β and is a paramount product of type I interferon response. C-X-C motif ligand 8-L1 (CXCL8-L1) and CXCL8-L2 are of great importance in resisting *Salmonella* infection, which only exist in zebrafish and human, but not in mice [19,20]. Zebrafish larvae were used in this project to explore the functions of CXCL8-L1 and CXCL8-L2 on the chemotaxis of neutrophils properly. Especially, due to their transparency, transgenic zebrafish larvae labeled with specificity fluorescent proteins, such as Tg (*lyz*:GFP), make it convenient to track the interaction between bacteria and host immune cells directly.

In our previous studies, *spv* operon can aggravate *S.* Typhimurium infection by suppressing autophagy and the functions of phagocytes (macrophage and neutrophil) at the early period of infection as well as inducing Th1/Th2 shift to Th2 polarization during the adaptive immunity [21,22]. Nevertheless, the specific mechanism on how *spv* locus affect the chemotaxis of neutrophils remains unclear. In the present research, both macrophage and zebrafish were infected with STM-WT and STM–*Δspv* to investigate the influence of *spv* operon on autophagy, type I interferon response and their interaction. Furthermore, the effect and mechanism of *spv* locus on neutrophils chemotaxis were also explored in zebrafish larvae.

2. Materials and methods

2.1. Zebrafish maintenance and embryo collection

Wild-type AB and transgenic zebrafish Tg (*lyz*:GFP) were kindly provided by the Center for Circadian Clocks at Soochow University. Collecting from natural spawning, embryos were staged according to the established criteria and reared in Holtfreter buffer (60 mmol/L NaCl, 1.3 mmol/L $CaCl_2$, 0.67 mmol/L KCl, 0.3 mmol/L $NaHCO_3$, pH 7.0 and 10^{-5}% methylene blue) at 28.5 °C [23].

2.2. Bacterial strains

S. Typhimurium strain χ3306 (STM-WT) and *spv* locus deletion-mutant strain UF110 (STM-*Δspv*) were kindly supplied by Professor Paul A. Gulig [24]. Both strains were grown to mid-logarithmic phase in Luriae Bertani (LB) broth at 37 °C. STM-WT and STM-*Δspv* strains with red fluorescence protein (RFP-STM-WT and RFP- STM-*Δspv*) were constructed in our laboratory and cultured in the media with 100 μg ml^{-1} Ampicillin.

2.3. Cell culture

RAW 264.7 mouse macrophage cell lines were cultured in DMEM High Sugar medium (HyClone) supplemented with 10% fetal calf serum

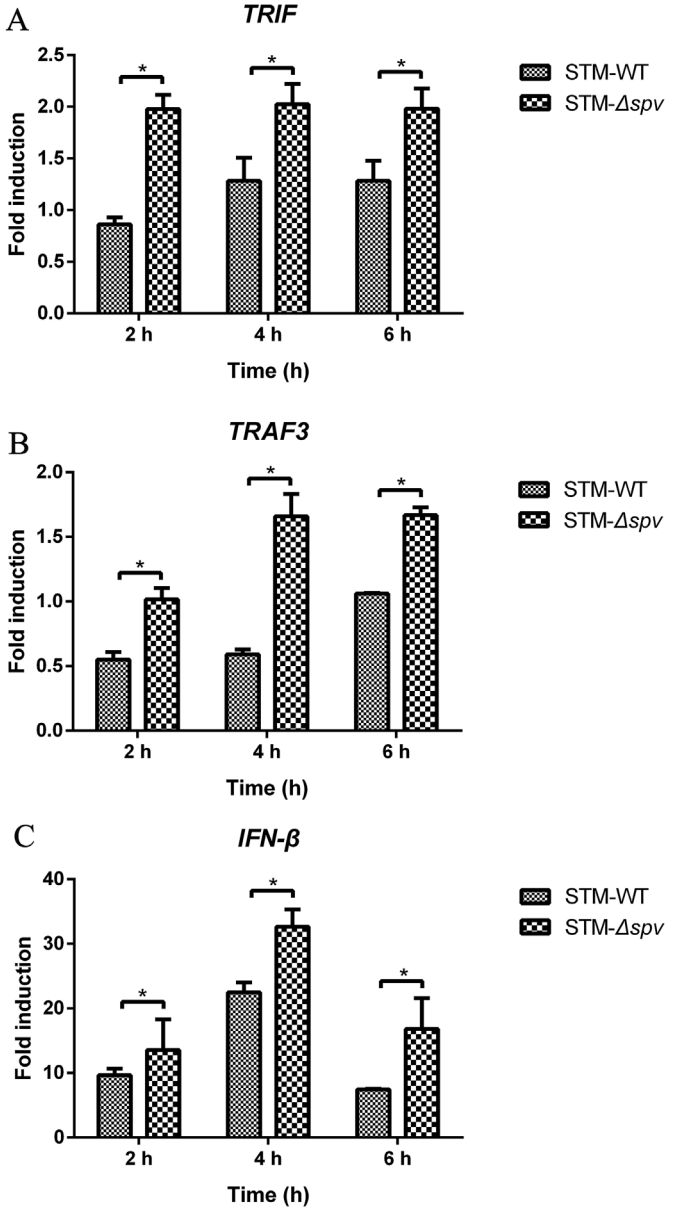

Fig. 2. The influence of *spv* locus on type I interferon response in macrophages. The mRNA levels of type I interferon response related genes *TRIF* (A), *TRAF3* (B) and *IFN-β* (C) were measured by RT-qPCR. Student's *t*-test was used for statistical analysis. Data represented three independent experiments combined. Errors bars represented SEM. $*P < 0.05$.

(Bovogen) and in a humidified incubator containing 5% CO_2 at 37 °C.

2.4. Cell in vitro infection assays

After cultured in Luriae Bertani (LB) medium overnight which containing 0.3 M NaCl, bacteria were diluted 1:33 with LB broth to subculture for 3 h. The optical density of bacteria was determined by spectrophotometry at 600 nm with viable plate counts before infection. Cells were infected with STM-WT and STM-*Δspv* at the multiplicity of infection (MOI) of 10 respectively. DMEM High Sugar medium (10% FBS) containing amikacin (100 μg ml^{-1}) was added to cells which were washed with PBS for 3 times to clear the extracellular bacteria at 1 h post infection (hpi). Cells were washed with PBS at 3 hpi and added with DMEM High Sugar medium (10% FBS) containing amikacin (10 μg ml^{-1}) to inhibit the growth of extracellular bacteria released from infected RAW264.7 cells. Cells were collected at 1, 2, 4 and 6 hpi.

2.5. Bacterial infection in zebrafish larvae

The process of bacterial culture and density determination of bacteria are the same with above. Zebrafish larvae at 5 dpf were divided randomly into the infection groups and the control group (each group containing 30 larvae). The infection groups of larvae were infected with 10^9 CFU/ml STM-WT and STM-*Δspv* by immersion, while the control group of larvae was treated with the same amount of Holtfreter buffer. Zebrafish larvae were collected at 6 hpi, 8 hpi and 10 hpi, respectively.

2.6. Western blot analysis

The expressions of autophagy related proteins LC3 and p62 were detected to investigate the interaction between *spv* locus and autophagy in macrophages and zebrafish larvae. With the function of inhibiting mammalian target of rapamycin (mTOR), Torin 1 (Beyotime Biotechnology, China) was used as the autophagy agonist to explore the interplay among autophagy, type I interferon response and neutrophils chemotaxis [25]. Cells were pretreated with 5 nM Torin1 for 3 h. Zebrafish larvae at 3 dpf were primed with 0.4 μmol/L Torin 1 for 48 h. Proteins were fractionated on 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (PVDF). After blocking with 5% non-fat milk, the PVDF membrane was incubated with LC3 (Novusbio, USA) or p62 (MBL, Japan) antibody overnight at 4 °C and washed for 3 times with Tris-buffered saline containing 0.1% Tween-20 (TBST). Then the membrane was probed with the second antibody for 1 h at room temperature and washed for 3 times with TBST. Finally, the protein detection was performed by using chemiluminescence reagent (ECL, Biological Industries). Protein contents were measured by Image J software program.

2.7. Assessment of bacterial survival in macrophages and in zebrafish larvae

After washed three times with PBS, the infected macrophages were added with 200ul μl 0.3% Triton X-100 for 7 min. Cell lysates were collected and serially diluted 10-fold in PBS. A 100 μl aliquot in soft agar medium were plated on nutrient agar plates and grown overnight in 37 °C.The infected zebrafish larvae (n = 10/group) were collected at 8 hpi and washed three times with PBS. These larvae were grinded in 200 μl 0.3% Triton X-100 and the tissue homogenates were serially diluted 10-fold in PBS. A 500 μl aliquot in soft agar medium were plated on nutrient agar plates and grown overnight at 37 °C.

2.8. Real-time quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA of macrophages and zebrafish larvae infected with STM-WT and STM-*Δspv* were extracted by Trizol reagent (Invitrogen) and reverse transcribed by cDNA Reverse Transcription Kit (Transgene). EvaGreen 2X qPCR MasterMix (abm, Canada) was used as the real-time quantitative Polymerase Chain Reaction (RT-qPCR) reagent. Reactions were performed in a 20 μl volume which containing 10 μl EvaGreen 2X qPCR MasterMix, 7.8 μl double-distilled water, 0.6 μl forward primers, 0.6 μl reverse primers, and 1 μl cDNA template. All primers for RT-qPCR were shown in Table S1 (macrophage) and S2 (zebrafish). The *β-actin* primers were used in all as internal control. The qPCR conditions were as follows: 95 °C for 5 min, then 40 cycles of 95 °C for 3 s, 60 °C for 60 s.

2.9. Laser scanning confocal microscopy

Transgenic zebrafish larvae Tg (*lyz*:GFP) at 5 dpf were immersed in 10^9 CFU/ml RFP-STM-WT and RFP-STM-*Δspv*. Larvae were collected at different points and covered by 0.2% low melting agarose after anesthetized through tricaine to observe the dissemination of bacteria and the aggregation of neutrophils. The number of bacteria aggregation of neutrophils in zebrafish intestine were assessed by Image J.

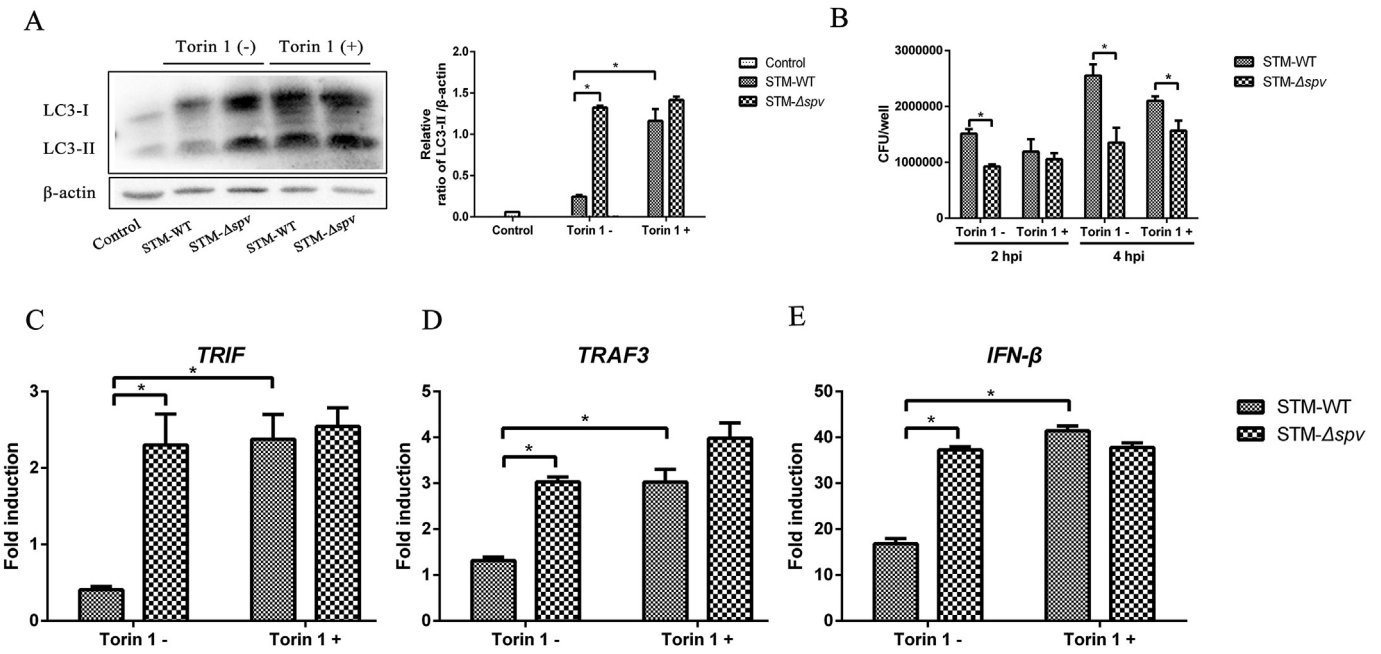

Fig. 3. The interaction between the inhibition of autophagy and suppression of type I interferon response by *spv* operon in macrophages. With or without the intervention of Torin 1, cells were incubated with STM-WT and STM-*Δspv*. (A) The expressions of autophagy related protein LC3 were detected by western blot analysis. The relative ratio of LC3-II/β-actin was measured by ImageJ. (B) The intracellular bacterial load was assessed by CFU counting. (C to E) The transcript levels of type I interferon response related genes *TRIF* (B), *TRAF3* (C) and *IFN-β* (D) were detected by RT-qPCR. One-way analysis of variance (ANOVA) and Student's *t*-test were used for statistical analysis. Data represented three independent experiments combined. Errors bars represented SEM.*$P < 0.05$.

2.10. Statistical analysis

Statistical analysis was performed with SPSS 17.0. All data were represented as the mean ± standard deviation (S.D.) from three replications. Comparisons of data were analyzed by using the Student's *t*-tests and the one-way analysis of Variance (ANOVA). It was considered as significant difference when *P* value was less than 0.05.

2.11. Ethics statement

All animal experiments described in the present study were conducted at the Center for Circadian Clocks at Soochow University and approved by the Animal Experimental Committee of Soochow University (Grant 2111270).

3. Results

3.1. The impact of spv locus on autophagy in macrophages

At 1 hpi, 2 hpi, 4 hpi and 6 hpi, cells were collected to detect the expressions of autophagy related proteins LC3 and p62 by immunoblot. The data showed that the expressions of LC3-II in STM-WT infection groups were significantly lower than those in STM-*Δspv* infection groups at 1 hpi and 2 hpi ($P < 0.05$) (Fig. 1A). At 4 hpi, the expression of p62 in STM-WT infection group was obviously higher compared with that in STM-*Δspv* infection group ($P < 0.05$) (Fig. 1B). Results demonstrated that *spv* operon could inhibit the autophagy in macrophages when infected with *Salmonella*.

3.2. The influence of spv locus on type I interferon response in macrophages

Cells were incubated with STM-WT and STM-*Δspv* separately to detect the mRNA levels of *TRIF*, *TRAF3* and *IFN-β* by using RT-qPCR at 2 hpi, 4 hpi and 6 hpi. According to the results, the mRNA levels of *TRIF*, *TRAF3* and *IFN-β* in STM-WT infection group were lower than those in STM-*Δspv* ($P < 0.05$) (Fig. 2A–C). These data suggested that *spv* operon could restrain type I interferon response in macrophages.

3.3. The interaction between autophagy and type I interferon response affected by spv locus

With the intervention of Torin 1, cells were incubated with bacteria for 2 h to detect the expression of autophagy related protein LC3 by western blot. It showed that the expression of LC3-II in STM-WT infection group was lower than that in STM-*Δspv* group without the interference of Torin 1. After the pretreatment of Torin 1, the expression of LC3-II in STM-WT infection group was improved significantly and there was no significant difference in the comparison with STM-*Δspv* infection group (Fig. 3A).

In order to explore whether the autophagic response is required for *Salmonella* clearance and *spv* locus could inhibit it, Torin 1-mediated induction of autophagy in bacterial clearance by macrophages was investigated. With or without the pretreatment of Torin 1, the bacterial survival was assessed at 2 hpi and 4 hpi. The results showed that the CFU (Colony-Forming Units) counting in STM-WT infection group were significantly higher than STM-*Δspv* infection group without the assistance of Torin 1 at 2 hpi and 4 hpi ($P < 0.05$)(Fig. 3B). After the pretreatment of Torin 1, the survival of bacteria was reduced in STM-WT infection group and the difference between the two groups was lower ($P > 0.05$) (Fig. 3B), at 2 hpi. However, at 4 hpi, the bacterial load with the treatment of Torin 1 in STM-WT group was still significantly higher than that in STM-*Δspv* group. These findings suggested that the augmentation of autophagy would help cell to eliminate bacteria and autophagy is an early response to maintain the homeostasis in cells.

Cells were incubated with bacteria for 4 h to detect the transcript levels of gene *TRIF*, *TRAF3* and *IFN-β* by RT-qPCR with or without the pretreatment of Torin 1. The results showed that the transcript levels of *TRIF*, *TRAF3* and *IFN-β* in STM-WT infection group were lower than that in STM-*Δspv* infection group without the assistance of Torin 1 ($P < 0.05$). But with the pretreatment of Torin 1, the mRNA levels of type I interferon response related genes were evidently increased in

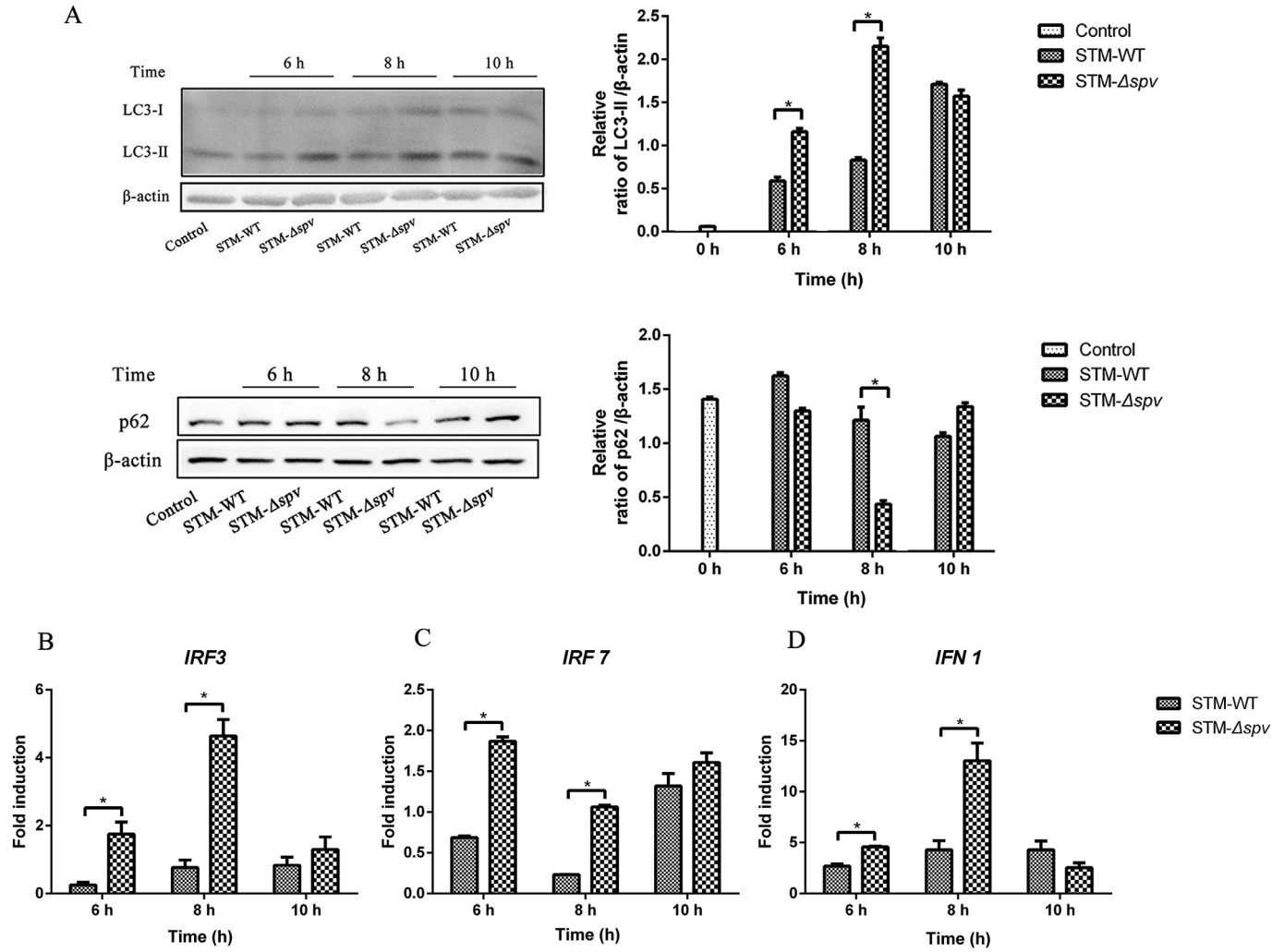

Fig. 4. The influence of *spv* locus on autophagy and type I interferon response in zebrafish larvae. (A) The expressions of autophagy related proteins LC3 and p62 were detected by western blot analysis. The relative ratio of LC3-II/β-actin and p62/β-actin were detected by ImageJ. (B to D) The mRNA levels of *IRF3* (B), *IRF7* (C) and *IFN 1* (D) were measured by RT-qPCR. Student's *t*-test were used for statistical analysis. Data represented three independent experiments combined. Errors bars represented SEM. $^{*}P < 0.05$.

STM-WT infection groups ($P < 0.05$) and was comparable to STM-*Δspv* infection groups ($P > 0.05$) (Fig. 3C–E). These data demonstrated that *spv* locus could inhibit type I interferon response through affecting autophagy *in vitro*.

3.4. Effect of spv locus on autophagy and type I interferon response in zebrafish larvae

The expressions of LC3 and p62 were detected in zebrafish larvae at 6 hpi, 8 hpi and 10 hpi. It showed that the expressions of LC3-II in STM-WT infection groups were lower than those in STM-*Δspv* groups at 6 hpi and 8 hpi ($P < 0.05$), the expressions of p62 in STM-WT infection groups were higher than those in STM-*Δspv* infection groups at 8 hpi ($P < 0.05$). But there were no significant differences in the expressions of LC3-II at 10 hpi between the two infection groups ($P > 0.05$) (Fig. 4A). The data demonstrated that *spv* operon could inhibit autophagy in zebrafish larvae.

After infected with 10^9 CFU/ml STM-WT and STM-*Δspv*, zebrafish larvae were collected to detect the mRNA levels of *IRF3*, *IRF7* and *IFN 1*. Compared with STM-*Δspv* infection groups, the transcript levels of *IRF3*, *IRF7* and *IFN 1* in STM-WT infection groups were significantly lower at 6 hpi and 8 hpi ($P < 0.05$) (Fig. 4B–D). There were no statistical differences in transcript levels of *IRF3*, *IRF7* and *IFN 1* between STM-WT and STM-*Δspv* infection groups at 10 hpi ($P > 0.05$). These findings indicated that *spv* locus could suppress the type I interferon response in zebrafish larvae infection model.

3.5. Effect of spv locus on bacterial survival and the chemotactic function of neutrophils in zebrafish larvae

Tg (*lyz*:GFP) larvae at 5 dpf were infected with STM-WT and STM-*Δspv*.The dissemination of bacteria and the chemotaxis of neutrophils in intestine were observed by laser confocal microscopy (Fig. 5A). There were much more RFP-STM-WT in zebrafish intestinal tract than RFP-STM-*Δspv* at 6 and 8 hpi ($P < 0.05$) (Fig. 5B). At 6hpi and 8 hpi, less infiltrated neutrophils were observed in the infection sites of RFP-STM-WT infection groups than that in RFP-STM-*Δspv* infection groups ($P > 0.05$) (Fig. 5C). It demonstrated that *spv* locus may inhibit the chemotaxis of neutrophil and augment the burden of bacteria.

The chemotaxis of neutrophils was regulated by CXCL8-L1 and CXCL8-L2, which were released by macrophages under the modulation of IFN-γ. RT-qPCR was employed to determine the transcript levels of gene *IFN-γ*, *CXCL8-L1* and *CXCL8-L2* of zebrafish larvae. The results showed that the transcript levels of *IFN-γ*, *CXCL8-L1* and *CXCL8-L2* in STM-WT infection groups were significantly lower than those in STM-*Δspv* infection groups at 6 hpi and 8 hpi ($P < 0.05$) (Fig. 5D–F). These

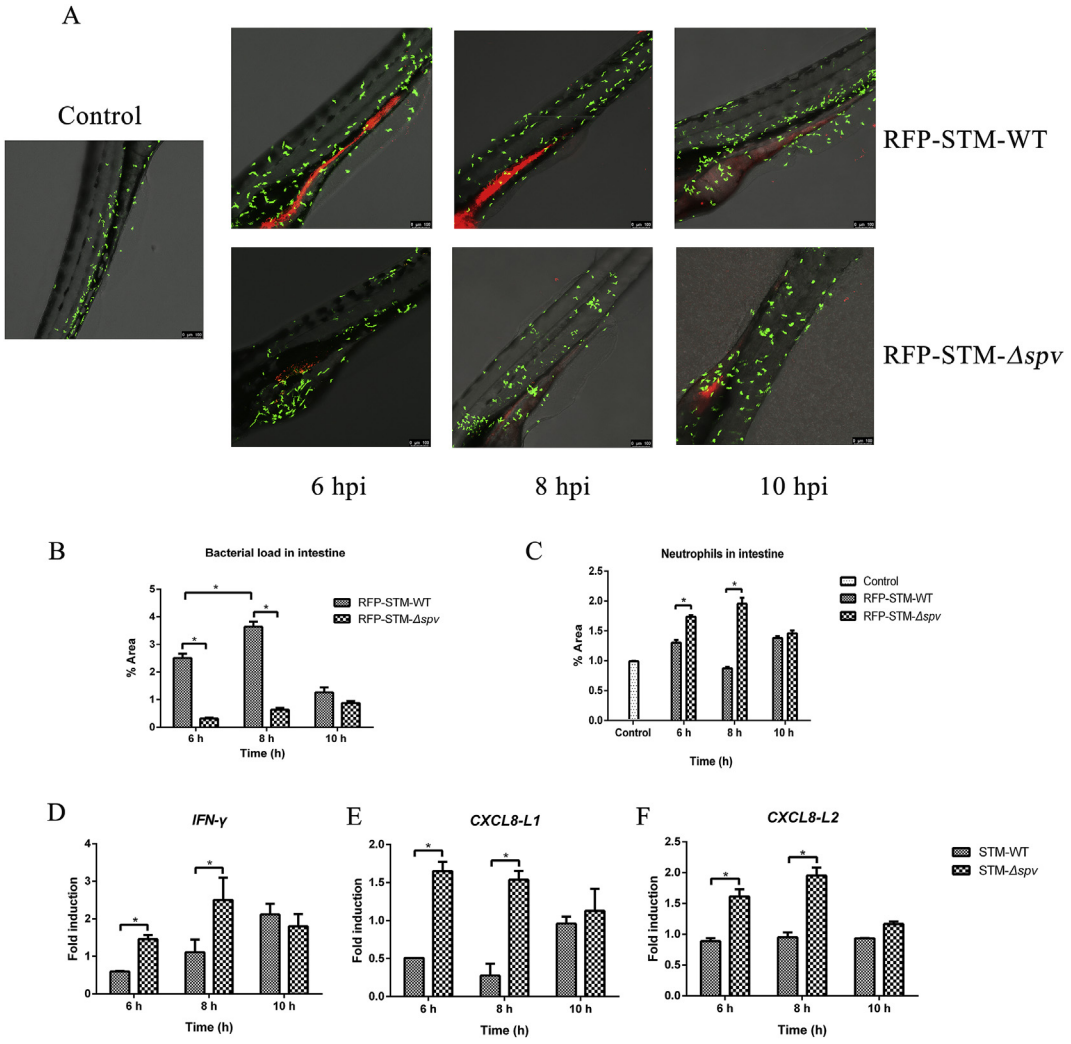

Fig. 5. The effect of *spv* operon on the chemotaxis of neutrophils in zebrafish larvae. (A) Bacteria dissemination and neutrophil chemotaxis were observed by laser confocal microscopy at 6 hpi, 8 hpi and 10 hpi. (B to C) The bacterial load and aggregation of neutrophils were measured by Image J. The green indicated neutrophils, while the red indicated STM-WT or STM-*Δspv*. Scale bar, 100 μm. (D to F) The transcript levels of *IFN*-γ (D), *CXCL8-L1* (E) and *CXCL8-L2* (F) were measured by RT-qPCR. Student's *t*-test were used for statistical analysis. Data represented three independent experiments combined. Error bars represented SEM. *$P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

data suggested that *spv* locus could decrease the release of IFN-γ and chemokines of neutrophils.

3.6. The interplay among autophagy, type I interferon response and neutrophil chemotaxis influenced by spv locus

Zebrafish larvae were primed with or without Torin 1 for 48 h and collected to detect the expressions of LC3 by western blot at 8 hpi. Results showed that the expression of LC3-II in STM-WT infection group was lower than that in STM-*Δspv* group without Torin 1 treatment ($P < 0.05$). After the treatment with Torin 1, the expression of LC3-II in STM-WT infection group was increased markedly and there was no significant difference in comparison with STM-*Δspv* infection group ($P > 0.05$) (Fig. 6A).

With or without the pretreatment of Torin 1, zebrafish larvae were immersed in bacteria for 8 h to assess the bacterial survival. The results showed that the survival bacteria of STM-WT infection group were more than STM-*Δspv* infection group without the assistance of Torin 1 (Fig. 6B) ($P < 0.05$). After the pretreatment of Torin 1, the survival bacteria was evidently declined in STM-WT infection group ($P < 0.05$) and was comparable to that in STM-*Δspv* infection group ($P > 0.05$). These findings demonstrated that the depressed autophagy process by *spv* locus could affect the elimination of bacteria.

Zebrafish larvae were pretreated with or without Torin 1 for 48 h and collected at 8 hpi. The transcript levels of type I interferon-related gene *IRF3*, *IRF7* and *IFN 1* were detected in zebrafish. Without the intervention of Torin 1, the transcript levels of *IRF3*, *IRF7* and *IFN 1* were significantly higher in the STM-*Δspv* infection group than those in the STM-WT infection group ($P < 0.05$) (Fig. 6C–E). After the pretreatment of Torin 1, the mRNA levels of *IRF3*, *IRF7* and *IFN 1* in STM-WT infection group were increased, and there were no significant differences in comparison with STM-*Δspv* infection group ($P > 0.05$) (Fig. 6C–E). The transcript levels of *IFN*-γ, *CXCL8-L1* and *CXCL8-L2*, that generating in the downstream of type I interferon response, were also determined by RT-qPCR. Without the pretreatment of Torin 1, the transcript levels of *IFN*-γ, *CXCL8-L1* and *CXCL8-L2* were significantly higher in STM-*Δspv* infection group than in STM-WT infection group ($P < 0.05$). However, the mRNA levels of *IFN*-γ, *CXCL8-L1* and *CXCL8-L2* were increased in STM-WT infection groups after the treatment of Torin 1, and were comparable with STM-*Δspv* infection group ($P > 0.05$) (Fig. 6 F-H). These findings implicated that *spv* operon could decrease the secretion of chemokines through inhibiting autophagy and type I interferon response in zebrafish larvae infection model.

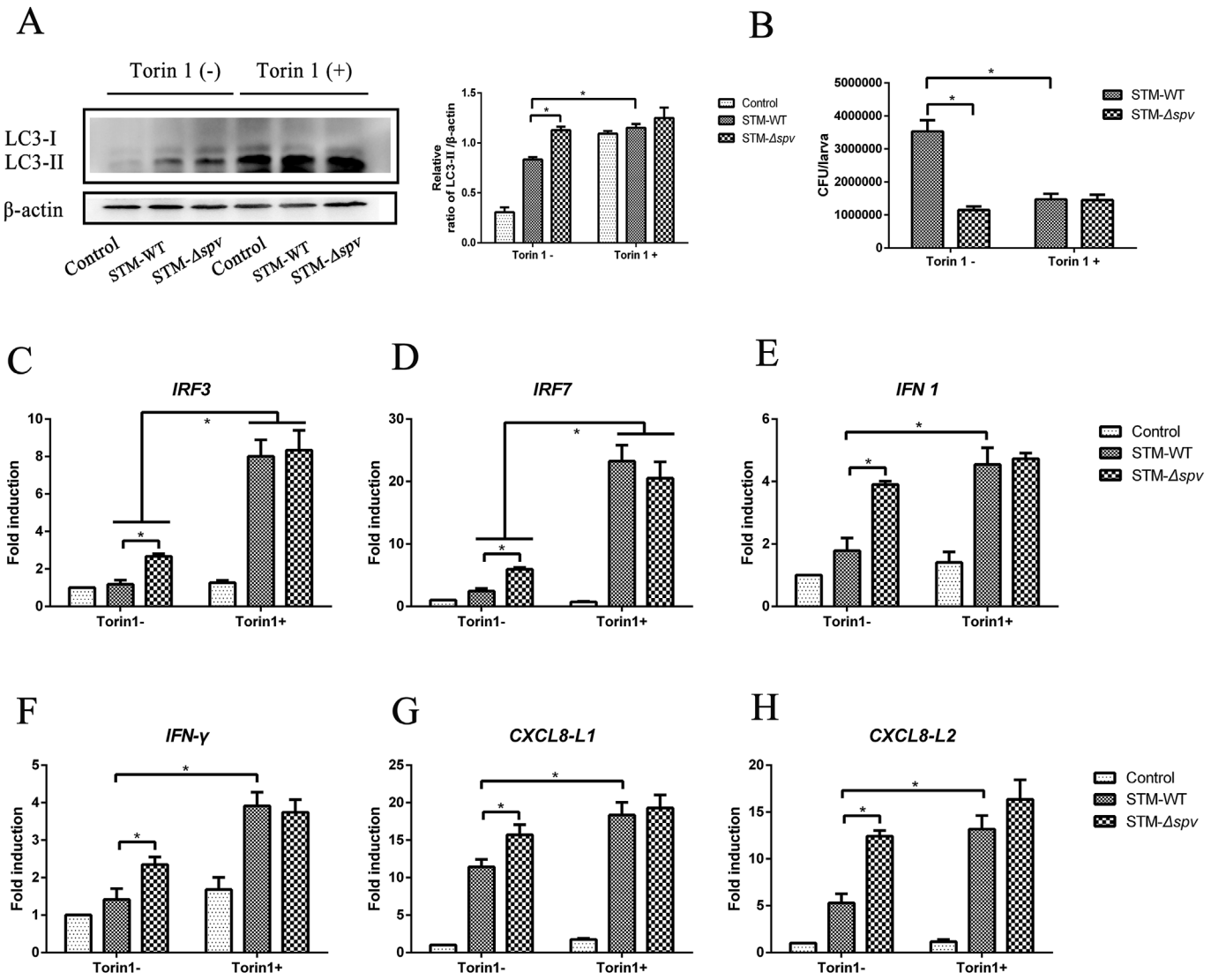

Fig. 6. The impact of *spv* operon on the mechanism for neutrophils chemotaxis in zebrafish larvae. With or without the pretreatment of Torin 1, the larvae at 5 dpf were immersed in STM-WT and STM-Δ*spv*. (A) The expression of autophagy related protein LC3 was detected by western blot analysis. The relative ratio of LC3-II/β-actin was measured by ImageJ. (B) The number of viable bacteria was measured by CFU counting. (C to H) The mRNA levels of *IRF3* (C), *IRF7* (D), *IFN 1* (E), *IFN*-γ (F), *CXCL8-L1* (G) and *CXCL-L2* (H) were detected by RT-qPCR. One-way analysis of variance (ANOVA) and Student's *t*-test were used for statistical analysis. Data represented three independent experiments combined. Errors bars represented SEM.*$P < 0.05$.

4. Discussion

Salmonellae are enteric pathogens and responsible for about 115 million human infections per year all around the world [26]. The interaction between *Salmonella* and host defense has been explored for many years. Our previous studies demonstrated that *spv* locus could affect autophagy and the aggregation of neutrophils. But the specific mechanism of *spv* locus on the chemotaxis of neutrophils are still not fully illuminated.

Exiting on the membrane of lysozymes or mature endosomes, TLR3 could recognize degraded pathogen components residing in the autophagosome when it fused with lysozymes or mature endosomes, stimulating TRIF, TRAF3, IRF3 and the downstream signaling pathway. Therefore, we hypothesized that the process of antimicrobial autophagy may initiate the TRIF-dependent type I interferon response. In this study, the expressions of autophagy associated proteins LC3 and p62 were detected. At the same time, transcript levels of type I interferon response related gene *TRIF*, *TRAF3* and *IFN-β* were measured in macrophages. Results indicated that *spv* locus could inhibit autophagy as well as type I interferon response *in vitro*. As an ATP competitive inhibitor, Torin 1 is commonly used as the autophagy agonist by combining with the autophagy inhibitor target of Rapamycin (TOR). In order to investigate the interplay between the inhibition of autophagy and type I interferon response by *spv* locus, cells were pretreated with Torin 1 and the expression of LC3 as well as the transcript levels of macrophage type I interferon response related genes were detected. With the intervention of Torin 1, the expression of LC3- II in cells infected with STM-WT was significantly increased to a comparable level to those incubated with STM-Δ*spv* at 2 hpi. At 4 hpi, the transcript levels of type I interferon response related genes in STM-WT infection group were improved and showed no difference with STM-Δ*spv* infection group by the interference of Torin 1. The bacterial load in STM-WT infection group is higher than that in STM-Δ*spv* infection group at 2 hpi and 4 hpi. After being treated with Torin 1, the differences of bacterial load between two groups are lower at 2 hpi. At 4 hpi, the viable bacteria in STM-WT group are more than STM-Δ*spv* group with treatment of Torin1, which can be explained by the phase of autophagy. These data suggested that the type I interferon response inhibited by *spv* locus could be relieved by the activation of autophagy. In addition, the interplay between suppression of autophagy and type I interferon

response by *spv* operon were also verified in zebrafish larvae.

IFN-β, the production of type I interferon response, could induce the secretion of type II interferon IFN-γ and stimulate the secretion of chemokine CXCL9 and CXCL10 in mice, which was critical for the recruitment of neutrophils to infection foci [13]. However, in zebrafish, it was demonstrated that the specific neutrophil chemokine CXCL8-L1 and CXCL8-L2 were required for resisting *Salmonella* infection [19]. To explore the potential mechanism of *spv* operon on aggravating *Salmonella* infection, the mRNA levels of cytokines *IFN-γ*, *CXCL8-L1* and *CXCL8-L2* were measured, transgenic zebrafish larvae were also involved to observe the dissemination of bacteria and neutrophils accumulation visually. Data showed that *spv* locus could restrain the transcript levels of these genes and the recruitment of neutrophils. Torin 1 was used to further confirm the interaction among autophagy, type I interferon response and chemotaxis of neutrophils in zebrafish. With the intervention of Torin 1, the levels of autophagy, the transcription of type I interferon response related genes as well as the neutrophils chemokines in STM-WT infection group were improved. With the intervention of Torin 1, the difference of bacterial load between two groups is lower, suggesting that the assessment of bacterial survival was affected by autophagy rather than type I interferon response. These results demonstrated that *spv* locus may restrain the secretion of neutrophil chemokines via inhibiting autophagy and type I interferon response, and ultimately affect the neutrophil chemotactic function to promote bacterial survival and proliferation. It is perplexing that the transcript levels of *IRF3* and *IRF7* in both STM-WT and STM-*Δspv* infection groups were enhanced significantly while the transcript levels of *IFN 1*, *IFN-γ*, *CXCL8-L1* and *CXCL8-L2* were improved only in STM-WT infection group with the intervention of autophagy agonist (Fig. 6). The latest research may be helpful for explaining our results, which demonstrated that selective autophagy could degrade the adaptor TRIF thus modulating innate inflammatory signaling [27]. In detail, the activation of autophagy in STM-*Δspv* infection group with Torin 1 spurs a mass of secretion of IRF3 and IRF7, however, the undue autophagy may clear the adaptor TRIF to some extent resulting in a slight increase in the transcript levels of *IFN 1*, *IFN-γ*, *CXCL8-L1* and *CXCL8-L2*.

It is widely known that neutrophils play a paramount role in clearing extracellular bacteria by phagocytosis, but various researches focused on their functions of eliminating intracellular bacteria in recent years. Gasdermin D-dependent neutrophils death were induced by activated noncanonical inflammasome signaling pathway, which could mediate the host defense against intracellular *Salmonella ΔsifA* [28]. The extracellular vesicles that released from *Mycobacterium tuberculosis*-infected neutrophils can promote the clearance of intracellular *Mycobacterium* by early superoxide anion production and autophagy induction [29]. Interestingly, neutrophils from mice that is whether susceptible or resistant to infection can eliminate facultative intracellular bacterium *Listeria monocytogenes* effectively. These findings arrest our great attention to figure out the potent and specific ability of neutrophil in defending against intracellular bacteria [30].

Since the essential role that neutrophils serve in determining the outcome of infection as mentioned above, researches on neutrophil chemotaxis shed novel light on host defense mechanisms against facultative intercellular pathogens. And the investigation on the specific intracellular signaling pathway that determining the neutrophil chemotaxis *in vivo* was of great significance for the development of anti-inflammatory therapies [31]. After the primary chemoattractants such as formyl-peptides were produced by bacteria or necrotic cells in inflammatory sites, the activated secondary chemoattractants were released from the surrounding tissue and resident immune cells, which would activate the nearby endothelia and recruit neutrophils in a distance [32].

To date, it was confirmed that CXCL8 served as the most important secondary chemoattractants among CXCL2, CXCL8 and leukotriene B_4 (LTB_4) [33]. Acting on C-X-C chemokine receptor 1 and 2 (CXCR1 and CXCR2) which expressing in a variety of cells, CXCL8 could participate in the clearance of pathogens and delay disease progressions [34]. Our data suggested that *spv* locus might suppress IFN-γ to impact CXCL8-L1 and CXCL8-L2. Similarly, IFN-γ could influence CXCR3 of CXCL9, CXCL10, and CXCL11 to the defense against *Salmonella* infection in mice, which implied that IFN-γ could facilitate macrophages to secret chemokines and promote the chemotaxis of neutrophil [35]. However, some researches revealed that IFN-γ may mediate the suppression of neutrophil by Staphylococcal Enterotoxin Type A (SEA) or increasing SOCS3 expression in neutrophils [36,37]. It is possible that the modulation of IFN-γ on the aggregation of neutrophil is dependent on different pathogens.

Recently, more and more investigations focused on motility and migration of neutrophils in response to infection and the related mechanisms were not restricted to the secretion of chemokines. The activation of transcription factor FOXO1 by bacterial challenge is demanded not only to mobilize neutrophils in response to infection, but also facilitate the functions of neutrophil including chemotaxis, phagocytosis, and bacterial killing *in vivo* [38,39]. By inducing autocrine ATP signaling and subsequently promoting myosin light chain phosphorylation, lipopolysaccharide (LPS) was identified as a potent signal for inhibiting neutrophil migration [40]. Moreover, endothelial IL-6 paracrine signaling serves as an essential role in neutrophil migration and survival during infection [41]. The fact that transendothelial migration (TEM) of neutrophils is limited to submucosal vessels when responding to *Salmonella*/endotoxin-induced inflammation may regard as the regulator of recruitment of neutrophils to the inflamed intestines [40]. In addition, the infiltration of neutrophils is a double-edged sword, which would cause severe inflammation when there is excessive accumulation of neutrophils. The infiltration of neutrophils in intestine could lead to damage of tissues, such as disorganization of intestinal barrier [5]. Accordingly, the balance of neutrophils aggregation is necessary for the host to stay in good condition, among which neutrophil reverse migration was proposed recently [42].

In conclusion, this study demonstrated that *spv* locus could inhibit type I interferon response and the chemotaxis of neutrophil via restraining autophagy. These findings provide substantial information for studying the mechanism of *Salmonella* escaping the host innate immune response. In order to further illustrate the pathogenesis of *spv* locus on *Salmonella* infection, more attention would be paid to both forward and reverse migration of neutrophils in the following investigations.

Acknowledgements

We thank Professor Paul A. Gulig, University of Florida, USA, for kindly supplying *S.* Typhimurium wide type strain χ3306 and *spv* operon deletion-mutant strain UF110 and Dr. Wilbert Bitter (Vrije Universiteit, Amsterdam, the Netherlands) for the RFP pGMDs3 plasmid. We are also grateful for Professor Wang Han (Center for Circadian Clocks and School of Biology & Basic Medical Sciences, Soochow University) who provided help in zebrafish experiments.

This work was supported by the Natural Science Foundation of China (No. 81671976, No.31670140) and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.009.

References

[1] J. Mambu, I. Virlogeux-Payant, S. Holbert, O. Grepinet, P. Velge, A. Wiedemann, An updated view on the rck invasin of Salmonella: still much to discover, Front. Cell. Infect. Microbiol. 7 (2017) 500.

[2] D.G. Guiney, J. Fierer, The role of the spv genes in Salmonella pathogenesis, Front.

Microbiol. 2 (2011) 129.
[3] L. Wei, S. Wu, Y. Li, Y. Chu, R. Huang, Salmonella enterica serovar Typhi plasmid impairs dendritic cell responses to infection, Curr. Microbiol. 65 (2012) 133–140.
[4] Y. Chu, S. Gao, T. Wang, J. Yan, G. Xu, Y. Li, et al., A novel contribution of spvB to pathogenesis of Salmonella Typhimurium by inhibiting autophagy in host cells, Oncotarget 7 (2016) 8295–8309.
[5] P. Broz, M.B. Ohlson, D.M. Monack, Innate immune response to Salmonella typhimurium, a model enteric pathogen, Gut Microb. 3 (2012) 62–70.
[6] N.A. Burton, N. Schurmann, O. Casse, A.K. Steeb, B. Claudi, J. Zankl, et al., Disparate impact of oxidative host defenses determines the fate of Salmonella during systemic infection in mice, Cell Host Microbe 15 (2014) 72–83.
[7] K.R. Parzych, D.J. Klionsky, An overview of autophagy: morphology, mechanism, and regulation, Antioxidants Redox Signal. 20 (2014) 460–473.
[8] J. Huang, J.H. Brumell, Bacteria-autophagy interplay: a battle for survival, Nat. Rev. Microbiol. 12 (2014) 101–114.
[9] T. Riffelmacher, F.C. Richter, A.K. Simon, Autophagy dictates metabolism and differentiation of inflammatory immune cells, Autophagy 14 (2018) 199–206.
[10] A. Bhattacharya, Q. Wei, J.N. Shin, E. Abdel Fattah, D.L. Bonilla, Q. Xiang, et al., Autophagy is required for neutrophil-mediated inflammation, Cell Rep. 12 (2015) 1731–1739.
[11] T. Riffelmacher, A. Clarke, F.C. Richter, A. Stranks, S. Pandey, S. Danielli, et al., Autophagy-dependent generation of free fatty acids is critical for normal neutrophil differentiation, Immunity 47 (2017) 466–480 e5.
[12] B. Cai, J. Wu, X. Yu, X.Z. Su, R.F. Wang, FOSL1 inhibits type I interferon responses to malaria and viral infections by blocking TBK1 and TRAF3/TRIF interactions, mBio 8 (2017).
[13] K.A. Owen, C.J. Anderson, J.E. Casanova, Salmonella suppresses the TRIF-dependent type I interferon response in macrophages, mBio 7 (2016) e02051-15.
[14] K.I. Kim, O.A. Malakhova, K. Hoebe, M. Yan, B. Beutler, D.E. Zhang, Enhanced antibacterial potential in UBP43-deficient mice against Salmonella typhimurium infection by up-regulating type I IFN signaling, J. Immunol. 175 (2005) 847–854.
[15] D.J. Grunwald, J.S. Eisen, Headwaters of the zebrafish – emergence of a new model vertebrate, Nat. Rev. Genet. 3 (2002) 717–724.
[16] P.R. Saraceni, A. Romero, A. Figueras, B. Novoa, Establishment of infection models in zebrafish larvae (Danio rerio) to study the pathogenesis of aeromonas hydrophila, Front. Microbiol. 7 (2016) 1219.
[17] V. Torraca, S. Mostowy, Zebrafish infection: from pathogenesis to cell Biology, Trends Cell Biol. 28 (2018) 143–156.
[18] H. Feng, Q.M. Zhang, Y.B. Zhang, Z. Li, J. Zhang, Y.W. Xiong, et al., Zebrafish IRF1, IRF3, and IRF7 differentially regulate IFNPhi1 and IFNPhi3 expression through assembly of homo- or heteroprotein complexes, J. Immunol. 197 (2016) 1893–1904.
[19] S. de Oliveira, A. Lopez-Munoz, F.J. Martinez-Navarro, J. Galindo-Villegas, V. Mulero, A. Calado, Cxcl8-l1 and Cxcl8-l2 are required in the zebrafish defense against Salmonella Typhimurium, Dev. Comp. Immunol. 49 (2015) 44–48.
[20] B. Yan, P. Han, L. Pan, W. Lu, J. Xiong, M. Zhang, et al., IL-1beta and reactive oxygen species differentially regulate neutrophil directional migration and Basal random motility in a zebrafish injury-induced inflammation model, J. Immunol. 192 (2014) 5998–6008.
[21] S.Y. Wu, L.D. Wang, J.L. Li, G.M. Xu, M.L. He, Y.Y. Li, et al., Salmonella spv locus suppresses host innate immune responses to bacterial infection, Fish Shellfish Immunol. 58 (2016) 387–396.
[22] S.Y. Wu, L.D. Wang, G.M. Xu, S.D. Yang, Q.F. Deng, Y.Y. Li, et al., Spv locus aggravates Salmonella infection of zebrafish adult by inducing Th1/Th2 shift to Th2 polarization, Fish Shellfish Immunol. 67 (2017) 684–691.
[23] C.B. Kimmel, W.W. Ballard, S.R. Kimmel, B. Ullmann, T.F. Schilling, Stages of Embryonic development of the zebrafish, Dev. Dynam. Official Pub. Am. Assoc. Anat. 203 (1995) 253–310.
[24] H. Matsui, C.M. Bacot, W.A. Garlington, T.J. Doyle, S. Roberts, P.A. Gulig, Virulence plasmid-borne spvB and spvC genes can replace the 90-kilobase plasmid in conferring virulence to Salmonella enterica serovar Typhimurium in subcutaneously inoculated mice, J. Bacteriol. 183 (2001) 4652–4658.
[25] J. Zhao, B. Zhai, S.P. Gygi, A.L. Goldberg, mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy, Proc. Natl. Acad. Sci. U. S. A 112 (2015) 15790–15797.
[26] Y. Seif, E. Kavvas, J.C. Lachance, J.T. Yurkovich, S.P. Nuccio, X. Fang, et al., Genome-scale metabolic reconstructions of multiple Salmonella strains reveal serovar-specific metabolic traits, Nat. Commun. 9 (2018) 3771.
[27] M. Samie, J. Lim, E. Verschueren, J.M. Baughman, I. Peng, A. Wong, et al., Selective autophagy of the adaptor TRIF regulates innate inflammatory signaling, Nat. Immunol. 19 (2018) 246–254.
[28] K.W. Chen, M. Monteleone, D. Boucher, G. Sollberger, D. Ramnath, N.D. Condon, et al., Noncanonical inflammasome signaling elicits gasdermin D-dependent neutrophil extracellular traps, Sci. Immunol. 3 (2018).
[29] V.D. Alvarez-Jimenez, K. Leyva-Paredes, M. Garcia-Martinez, L. Vazquez-Flores, V.G. Garcia-Paredes, M. Campillo-Navarro, et al., Extracellular vesicles released from Mycobacterium tuberculosis-infected neutrophils promote macrophage autophagy and decrease intracellular mycobacterial survival, Front. Immunol. 9 (2018) 272.
[30] M.G. Pitts, T.A. Combs, S.E.F. D'Orazio, Neutrophils from both susceptible and resistant mice efficiently kill opsonized Listeria monocytogenes, Infect. Immun. 86 (2018).
[31] S. de Oliveira, E.E. Rosowski, A. Huttenlocher, Neutrophil migration in infection and wound repair: going forward in reverse, Nat. Rev. Immunol. 16 (2016) 378–391.
[32] P.V. Afonso, M. Janka-Junttila, Y.J. Lee, C.P. McCann, C.M. Oliver, K.A. Aamer, et al., LTB4 is a signal-relay molecule during neutrophil chemotaxis, Dev. Cell 22 (2012) 1079–1091.
[33] D. Kim, C.L. Haynes, Neutrophil chemotaxis within a competing gradient of chemoattractants, Anal. Chem. 84 (2012) 6070–6078.
[34] R.C. Russo, C.C. Garcia, M.M. Teixeira, F.A. Amaral, The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases, Expert Rev. Clin. Immunol. 10 (2014) 593–619.
[35] B. Chami, A. Yeung, M. Buckland, H. Liu, M.F. G, K. Tao, et al., CXCR3 plays a critical role for host protection against Salmonellosis, Sci. Rep. 7 (2017) 10181.
[36] A.P. Ferreira-Duarte, A.S. Pinheiro-Torres, G.F. Anhe, A. Condino-Neto, E. Antunes, I.A. DeSouza, MHC class II activation and interferon-gamma mediate the inhibition of neutrophils and eosinophils by staphylococcal Enterotoxin type A (SEA), Front. Cellular Infect. Microbiol. 7 (2017) 518.
[37] C. Ramakrishna, E.M. Cantin, IFNgamma inhibits G-CSF induced neutrophil expansion and invasion of the CNS to prevent viral encephalitis, PLoS Pathog. 14 (2018) e1006822.
[38] G. Dong, L. Song, C. Tian, Y. Wang, F. Miao, J. Zheng, et al., FOXO1 regulates bacteria-induced neutrophil activity, Front. Immunol. 8 (2017) 1088.
[39] D.P. Sullivan, T. Bui, W.A. Muller, V. Butin-Israeli, R. Sumagin, In vivo imaging reveals unique neutrophil transendothelial migration patterns in inflamed intestines, Mucosal Immunol 11 (2018) 1571–1581.
[40] X. Wang, W. Qin, X. Xu, Y. Xiong, Y. Zhang, H. Zhang, et al., Endotoxin-induced autocrine ATP signaling inhibits neutrophil chemotaxis through enhancing myosin light chain phosphorylation, Proc. Natl. Acad. Sci. U. S. A 114 (2017) 4483–4488.
[41] L.E. Hind, P.N. Ingram, D.J. Beebe, A. Huttenlocher, Interaction with an endothelial lumen increases neutrophil lifetime and motility in response to P aeruginosa, Blood 132 (2018) 1818–1828.
[42] S. Nourshargh, S.A. Renshaw, B.A. Imhof, Reverse migration of neutrophils: where, when, how, and why? Trends Immunol. 37 (2016) 273–286.