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spv locus aggravates *Salmonella* infection of zebrafish adult by inducing Th1/Th2 shift to Th2 polarization

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ABSTRACT

Salmonella enterica serovar typhimurium (*S.* typhimurium) are facultative intracellular enteric pathogens causing disease with a broad range of hosts. It was known that Th1-type cytokines such as IFN-γ, IL-12, and TNF-α *etc.* could induce protective immunity against intracellular pathogens, while Th2-type cytokines such as IL-4, IL-10, and IL-13 *etc.* are proved to help pathogens survive inside hosts and cause severe infection. One of the critical virulence factor attributes to the pathogenesis of *S.* typhimurium is *Salmonella* plasmid virulence genes (*spv*). Until now, the interaction between *spv* locus and the predictable generation of Th1 or Th2 immune responses to *Salmonella* has not been identified. In this study, zebrafish adults were employed to explore the effect of *spv* locus on *Salmonella* pathogenesis as well as host adaptive immune responses especially shift of Th1/Th2 balance. The pathological changes of intestines and livers in zebrafish were observed by hematoxylin-eosin (HE) staining and electron microscopy. Levels of the transcription factors of Th1 (Tbx21) and Th2 (GATA3) were measured by real-time quantitative PCR (RT-qPCR). Expression of cytokines were determined by using RT-qPCR and ELISA, respectively. Results showed that *spv* operon aggravates damage of zebrafish. Furthermore, it demonstrated that *spv* locus could inhibit the transcription of *tbx21* gene and suppress the expression of cytokines IFN-γ, IL-12 and TNF-α. On the contrary, the transcription of *gata3* gene could be promoted and the expression of cytokines IL-4, IL-10 and IL-13 were enhanced by *spv* locus. Taken together, our data revealed that *spv* locus could aggravate *Salmonella* infection of zebrafish adult by inducing an imbalance of Th1/Th2 immune response and resulting in a detrimental Th2 bias of host.

1. Introduction

Salmonella spp. resulted in the greatest foodborne bacterial disease burden all over the world. Poultry, beef, turkey, dairy products, and vegetables are all reported to historically associate with outbreaks of *Salmonella enterica* serovar typhimurium (*S.* typhimurium) infection, and raises a globally important health problem [1]. *S.* typhimurium causes salmonellosis, with different symptoms varying from self-limiting gastroenteritis to life-threatening systemic infection. As gram-negative facultative intracellular bacteria, there are many factors contributed to *Salmonella* pathogenesis including flagellum, endotoxin, effectors of different types secretion system *etc.* In addition, a highly conserved 8-kb region identified on plasmid of pathogenic *Salmonella* spp. is defined as *Salmonella* plasmid virulence genes (*spv*), which is responsible for severe infection and clinical disease [2].

The adaptive immune system consists of humoral immunity mediated by B lymphocytes and cellular immunity mediated by T lymphocytes. Since the antibody in humoral immunity cannot enter the cell containing intracellular bacteria, the host mainly relies on cellular immunity against intracellular bacteria. T lymphocytes can be classified to $CD4^+$ T cells (the T helper cells, Th) and $CD8^+$ T cells (the cytotoxic T cells, CTL) according to their function. Upon antigenic stimulation, Th0 cells can differentiate into Th1 or Th2 cells and mainly elicit cell-mediated immunity responding to intracellular pathogens and humoral immunity responding to extracellular pathogens, respectively [3]. And additional subsets of Th cells have been recognized such as Treg, Th9, Th17, and Th22 *etc.* with distinct cytokine expression profiles and transcription factors (TFs) which enriched and specialize in mounting the immune

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responses to particular pathogens [4,5]. Th1 cells are closely related to the activation of CTLs, NK cells (nature killer cells) and macrophages, which are of great significance to resist intracellular pathogen infection. In contrast, Th2 cells are mainly involved in immune response to extracellular bacteria. As facultative intracellular bacteria, it is reasonable that the host mostly relies on Th1 immune response to *Salmonella* infection that leading to shift of Th1/Th2 balance to Th1 dominance. The differentiation of Th1 is regulated by the transcription factor T-bet (Tbx21) while transcription factor GATA-3 has been identified to play a relevant role in differentiation of Th2. T-bet is a Th1 specific T-box transcription factor which controls the expression of Th1 cytokines such as IL-12, IFN-γ, and TNF-α *etc*. GATA-3 is known to be a key regulator of Th2 development and influences the expression of Th2 cytokines such as IL-4, IL-10, and IL-13 *etc* [6,7]. Accordingly, the expression of T-bet and GATA-3 is able to represent the balance between Th1 and Th2 function.

Zebrafish as an excellent vertebrate model takes more advantages to study on bacterial infection and host immunity. Zebrafish are easier to maintain and handle than other mammalian animal models with a low-cost. Zebrafish larvae with only innate immunity have been widely used for the study of *S.* typhimurium [8–10]. *Salmonella* can be found in fish and shellfish naturally, therefore, zebrafish adults with well-developed immune systems similar to humans also have been regarded as good model for *Salmonella* infection [11,12].

In a latest research, we utilized the zebrafish larva model to study the effect of *spv* locus on host defense. Results showed that *spv* operon could restrain the host innate immune responses leading to an increase in bacterial survival in early stage of infection. Data revealed that *spv* operon could inhibit the function of neutrophils and macrophages, restrain the formation of autophagosomes in the earlier stage of autophagy [13]. However, the interplay between *spv* locus and the host adaptive immunity remains undefined. In the present study, zebrafish adult infection model was successfully established. The effect of *spv* locus on the damage of zebrafish was observed. The interaction between *spv* operon and the balance of Th1/Th2 in intestine and liver was explored firstly to further elucidate the host immune responses to *Salmonella* infection.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. typhimurium strain UF009 (STM-WT), a wild type strain with virulent *spv* genes, and *spv* locus deletion-mutant strain UF110 (STM-*Δspv*) were kindly supplied by Professor Paul A. Gulig [14]. Both strains were harvested by centrifugation at 3000 × g for 10 min and washed with the sterile Holtfreter buffer after grown to mid-logarithmic phase at 37 °C in Luriae Bertani (LB) broth. They were quantified spectrophotometrically by determining the optical density at 600 nm along with agar plating.

2.2. Zebrafish adults maintenance

The zebrafish wild type AB adults were bought from the zebrafish technology platform of Shanghai Institute of Biochemistry and Cell Biology (SIBCB). They were taken care to acclimatize under standard conditions at 28.5 °C and 14:10 h (light: dark) every day for a week in the loop filter water. To ensure their health, their feeding and swimming activities were observed and the healthy fish were selected to perform the study.

2.3. Construction of zebrafish adult infection model

Zebrafish adults at eight months post fertilization were randomly divided into the infection group and the control group. Suspensions of STM-WT were diluted to 1×10^7 CFU/ml, 1×10^6 CFU/ml, 1×10^5 CFU/ml, 1×10^4 CFU/ml and 1×10^3 CFU/ml gradually. The infection group of adults were oral infected with the indicated concentrations of STM-WT in volume of 5 μl, while the control group of adults were treated with an equivalent amount of Holtfreter buffer, each treatment group including ten adults. The gavage method was carried out according to relevant reference with slightly modification [15]. The adults were monitored carefully to ensure that they were treated successfully without rumination. After 21 days post infection (dpi), the survival curve of zebrafish was drawn and the median lethal dose (LD_{50}) was calculated with Reed-Muench method to determine the optimal administration of bacteria [16].

2.4. Assessment of bacterial survival

Zebrafish adults were oral infected with 5 μl suspension of STM-WT and STM-*Δspv* at indicated concentration. At 1 dpi, 7 dpi, and 14 dpi, the infected zebrafish were anesthetized in 0.2% Tricaine and then euthanized by incubation on ice for 15 min. Then zebrafish adults were pined to a dissecting sponge and intestines were separated. The details for anatomy of zebrafish adult were the same as the established protocol [17]. Intestines were collected into 1.5 ml microfuge tubes, homogenized with 100 μl PBS, and 50 μl aliquot was diluted and incubated overnight for CFU counting.

2.5. Histopathologic analysis

Zebrafish adults were infected with concentration of 2×10^5 CFU/ml STM-WT and STM-*Δspv* in volume of 5 μl. The intestines of 5 zebrafish adults were separated after dissection at 1dpi, 7 dpi and 14 dpi [17]. The tissue samples were fixed in 4% paraformaldehyde overnight at 4 °C, and then washed and dehydrated by a series of graded ethanol. Samples were embedded with paraffin after xylene. Subsequently, they were observed under the light-microscope after Hematoxylin-eosin (HE) staining, respectively (Olympus, Japan).

2.6. Preparation of samples for transmission electron microscopy

Zebrafish adults at 1 dpi and 7 dpi were dissected and then the intestines were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer. Samples were post-fixed in 1% osmium tetroxide, and dehydrated through a series of graded acetone washes. Following this, samples were embedded in epoxy resin, sectioned, and stained with uranyl acetate and lead citrate. The sections were subsequently examined under the transmission electron microscope (HT7700, Hitachi Co., Japan).

2.7. Real-time quantitative Polymerase Chain Reaction

Total RNA of intestine from zebrafish adult infected with STM-WT and STM-*Δspv* at 7 dpi and 14 dpi was extracted by Trizol reagent (Invitrogen) and reverse transcribed by cDNA Reverse Transcription Kit (Transgene). The real-time quantitative Polymerase Chain Reaction (RT-qPCR) reagent used was EvaGreen 2X qPCR MasterMix (abm, Canada). The qPCR conditions were as follows: 95 °C for 5 min, then 34 cycles of 95 °C for 15 s, 60 °C for 60 s. Reactions were performed in a 20 μl volume containing 10 μl EvaGreen 2X qPCR MasterMix, 7.8 μl double-distilled water, 0.6 μl of each forward and reverse primers, and 1 μl cDNA template. All