The inflammatory cytokine tumor necrosis factor modulates the expression of *Salmonella* typhimurium effector proteins

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**Abstract**

Tumor necrosis factor α (TNF-α) is a host inflammatory factor. Bacteria increase TNF-α expression in a variety of human diseases including infectious diseases, inflammatory bowel diseases, and cancer. It is unknown, however, how TNF-α directly modulates bacterial protein expression during intestinal infection and chronic inflammation. In the current study, we hypothesize that *Salmonella* typhimurium senses TNF-α and show that TNF-α treatment modulates *Salmonella* virulent proteins (called effectors), thus changing the host-bacterial interaction in intestinal epithelial cells. We investigated the expression of 23 *Salmonella* effectors after TNF-α exposure. We found that TNF-α treatment led to differential effector expression: effector SipA was increased by TNF-α treatment, whereas the expression levels of other effectors, including gogB and spvB, decreased in the presence of TNF-α. We verified the protein expression of *Salmonella* effectors AvrA and SipA by Western blots. Furthermore, we used intestinal epithelial cells as our experimental model to explore the response of human intestinal cells to TNF-α pretreated *Salmonella*. More bacterial invasion was found in host cells colonized with *Salmonella* strains pretreated with TNF-α compared to *Salmonella* without TNF-α treatment. TNF-α pretreated *Salmonella* induced higher proinflammatory JNK signalling responses compared to the *Salmonella* strains without TNF-α exposure. Exposure to TNF-α made *Salmonella* to induce more inflammatory cytokine IL-8 in intestinal epithelial cells. JNK inhibitor treatment was able to suppress the effects of TNF-pretreated-*Salmonella* in enhancing expressions of phosphorylated-JNK and c-jun and secretion of IL-8. Overall, our study provides new insights into *Salmonella*-host interactions in intestinal inflammation.

**Background**

Tumor necrosis factor α (TNF-α) is a pleiotropic inflammatory cytokine with increased expression in many human diseases. These diseases include septic shock, cancer, AIDS, multiple sclerosis, diabetes, rheumatoid arthritis, and inflammatory bowel disease [1-6]. It is well documented that multiple factors from bacteria, viruses, and parasites stimulate production of TNF-α in the host [7-10]. Hence, in hosts with inflammatory diseases, enteric bacteria are potentially exposed to high levels of TNF-α.

Bacteria can sense signal molecules secreted by their hosts. This communication mechanism between bacterium is called “quorum sensing” (QS) [11,12]. QS utilizes hormone-like compounds referred to as autoinducers to regulate bacterial gene expression [13,14]. QS also applies to the communication between the host and bacteria [11]. However, it is unknown how TNF-α from host cells directly modulates bacterial protein expression during infection and chronic inflammation. *Salmonella* is a leading cause of gastrointestinal disease worldwide. *Salmonella* uses the type three secretion system (TTSS), a needle-like protein transport device to inject virulence proteins into eukaryotic host cells. These virulence factors, called effectors, paralyze or reprogram the eukaryotic cell to the benefit of the pathogen [15-17]. The activity of TTSS effectors allows bacteria to invade non-phagocytic cells or inhibit phagocytosis, regulate pro-inflammatory responses, prevent autophagy, or modulate intracellular trafficking [18]. *Salmonella* effectors display a large repertoire of...
biochemical activities and modulate the function of crucial host regulatory molecules [19-22].

Effectors are encoded via specific pathogenicity island 1 (SPI-1) and 2 (SPI-2). Over 30 Salmonella effectors, including AvrA, SipA, SipB, Gog B, and SpVB, have been shown to manipulate a succession of key signaling transduction pathways and physiological functions of host cells [19]. AvrA, SipA, SipB, SopB, SopD, SopE, SopE2 are SPI-1 effectors. SipA, SipB, SopB, SopD, SopE, SopE2 and other effectors are known to induce membrane deformation and ruffling that triggers bacterial internalization, promoting invasion [19,23,24]. The SPI-2 effectors, such as Gog B and SpVB, promote bacterial replication and systemic spread [19-22]. Recent studies indicate that there may be interplay between SPI-1 and SPI-2 effectors [19]. Although Salmonella is one of the best characterized pathogens, it remains unknown how virulence effector gene expression changes in response to host factors, such as TNF-α.

In Salmonella strains, AvrA is an acid-inducible effector that is strongly correlated with food hygiene and food-borne infection [25-27]. Our publications and others’ have demonstrated that AvrA is a multifunctional protein that plays a critical role in inhibiting inflammation, regulating epithelial apoptosis, and enhancing proliferation during bacterial infection [28-32]. Stimulation of inflammation by effectors is crucial for Salmonella to grow in the intestine [33]. Effectors, such as SipA, SopE, and SopB, are known to activate inflammation in host cells [24,34-41]. Un-controlled inflammation is harmful to the host, however, and eventually damages the niche occupied by Salmonella during infection. Salmonella secreted factor L (SseL) [42-44], SspH1 [45], StpP, and AvrA may reverse the activation of signaling pathways induced by other Salmonella effectors [19,46,47].

Intestinal epithelial cells are physically linked by intercellular junctional complexes that regulate multiple functions including polarity, mechanical integrity, and signaling capacity [48]. Salmonella can invade and replicate within intestinal epithelial cells during the infection process [49]. Nontyphoidal Salmonella serotypes such as Salmonella typhimurium provoke an intense intestinal inflammatory response, consisting largely of neutrophil migration across the epithelial lining of the intestine [50,51]. Studies of S. typhimurium-infected laboratory animals and cultured epithelial cells have shown that bacteria rapidly enter epithelial cells after transient degeneration of the host cell surface microvilli and induce inflammatory responses [52-58]. Not surprisingly, the ability of S. typhimurium to enter epithelial cells constitutes a crucial step in pathogenesis. Salmonella invasion of the intestinal epithelium requires the virulence-associated TTSS [19,28,34,53,59]. Within the host intestine specialized antigen-sampling M cells, which reside in the epithelium overlying lymphoid tissues in the gut, are a preferred site of Salmonella invasion [60]. The factors involved in Salmonella-M cell interactions, however, are not well understood. Clearly, studying effectors can uncover important mechanisms of regulation in host-bacteria interaction.

A recent study demonstrated that Salmonella gastro-enteritis increases short- and long-term risk of inflammatory bowel disease [61]. Chronic intestinal inflammation enhances TNF-α levels in the host [62]. Therefore, enteric Salmonella is potentially exposed to TNF-α. In the current study, we hypothesize that Salmonella senses the host inflammatory factor TNF-α and that TNF-α treatment modulates Salmonella TTSS effectors, thus changing the host-bacteria interaction. We investigated the gene expression of Salmonella effectors changed by TNF-α and responses of the human intestinal cells to TNF-α treated Salmonella. We verified the expression levels of some effector proteins by Western blots. Furthermore, we used human intestinal epithelial cells as our experimental model to explore bacterial invasion and the proinflammatory NF-κB and c-Jun N-terminal kinase (JNK) signaling pathways in response to Salmonella strains with or without TNF-α pre-treatment. We found that TNF-α treatment modulated effector expression in a differentiated manner. Salmonella strains pre-treated with TNF-α induced more bacteria internalization and a more severe inflammatory response in intestinal epithelial cells than untreated Salmonella strains. Our study provides new insights into host factor regulation of bacterial effector expression through inflammatory responses.

Materials and methods

Bacterial strains and growth conditions

Salmonella strains (listed in Table 1) include wild-type (WT), S. typhimurium ATCC 14028s, S. typhimurium PhoR+ [63], Salmonella typhimurium 1344 (SL1344), and an AvrA mutant strain lacking the AvrA gene (SL1344AvrA- (provided by Dr. Jorge Galan from Yale University) [25]. Wild-type S. typhimurium 14028s AvrA - was generated in our laboratory based on previously published methods by Hamilton et al., and Miller et al. [64,65]. Briefly, the AvrA gene, flanked by upstream and downstream Salmonella chromosome sequences, was cloned into pMAK705 (chloramphenicol resistant). The construct plasmid was transformed into the Salmonella WT14028s strain by electroporation with a Gene Pulser apparatus (Bio-Rad, Munich, Germany) and grown at 30°C on chloramphenicol plates. Resulting colonies were then grown at 42°C to select for integrants. The integrants were subsequently grown at 30°C, the temperature at which the plasmid can leave
the chromosome and autonomously replicate. AvrA gene deletion was screened by PCR. AvrA deletion was also verified by Western blot using the anti-AvrA antibody. The resulting strain was named SL14028s AvrA-.

Bacteria were grown under the following conditions: non-agitated microaerophilic bacterial cultures were prepared by inoculation of 10 ml of Luria-Bertani broth with 0.01 ml of a stationary phase culture with or without TNF-α (10 ng/ml), followed by overnight incubation (~18 h) at 37°C, as previously described [53]. Overnight cultures of bacteria were concentrated 33-fold in Hank’s balanced salt solution (HBSS) supplemented with 10 mM HEPES, pH 7.4. The overnight cultures from the TNF-α pretreated Salmonella strains were washed thoroughly with HBSS 3 times to get rid of potential TNF-α residue in the media. The bacteria were then resuspended in fresh HBSS for cell lysis or colonization in the intestinal epithelial cells.

Reverse transcription polymerase chain reaction (RT-PCR)
Total RNA was extracted from bacteria using a Qiagen RNeasy mini kit (Cat: 74104. Qiagen, Valencia, CA) according to the manufacturer’s protocol. Total RNA was further digested with DNase I (Cat: 18068-015. Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by gel electrophoresis. Extracted RNA yield and purity was then determined by measuring absorbance in the 220 nm to 350 nm range. From the resulting spectra, the concentration of nucleic acids was estimated using the absorbance values at 260 nm, while the purity of each sample was determined by calculating the 260/280 and 260/230 ratios. RNA reverse transcription was performed using a SuperScript III kit (Invitrogen, Cat: 18080-051) according to the manufacturer’s directions. cDNA reaction products were then used in a quantitative PCR reaction. The reaction mixture was subjected to 29 cycles of PCR amplification using Taq polymerase (Fermentas, Glen Burnie, Maryland. Cat: EP0404). All PCR primers (Table 2) were designed using Lasergene software (DNASTar, Madison, WI). PCR products were separated on 2% agarose gels and densitometry readings of the DNA bands were taken using a Kodak IS2000R. The densitometry value of each PCR band was detected using KODAK MI 4.0.3. All expression levels were normalized to the bacterial reference gene, Mdh, of the same sample, using forward (5′-ATGAAAGTCG-CAGTCCTCGGCGCTGCTGGCGG-3′) and reverse (5′-ATATCTTTTTYTTCAGCGTATCCAGCAT-3′) primers for malate dehydrogenase (Mdh) [66]. All PCR reactions were performed in triplicate. The digital images are representative of the original data.

**Immunoblotting for bacterial SipA and AvrA**
Bacteria were lysed in buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and sonicated. Equal amounts of total proteins were loaded, separated by SDS-PAGE, and processed for immunoblotting with an anti-SipA antibody (generated by Dr. Ho-Young Kang, Pusan National University, Korea) or anti-AvrA antibody. For the anti-AvrA antibody, a 15-amino-acid peptide CGEEPFLPSDKA-DRY was designed based on AvrA amino acids 216-230. Two rabbits were injected with the peptide and a polyclonal antibody for AvrA was tested and purified, as previously described [30]. Immunoblotting was visualized by enhanced chemi-luminescence (ECL). Chemiluminescent signals were collected and scanned from ECL Hyperfilm (Amersham Pharmacia Biotech) with a Scanjet 7400c backlight flatbed scanner (Hewlett-Packard Co., Palo Alto, CA). Bands were quantified using Kodak MI software (v.4.0.3). The digital images are representative of the original data.

**Intestinal epithelial cell culture**
Human colonic epithelial HCT116 cells (American Type Culture Collection, Manassas, VA) were grown in DMEM (high glucose, 4.5 g/L) supplemented with 10% (vol/vol) fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin.

**S. typhimurium invasion of human epithelial monolayers**
Infection of HCT116 cells was performed by a previously described method [53]. Bacterial solution (~20 bacteria/epithelial cell) was added and bacterial invasion was assessed after 1 hour. Cell-associated bacteria, representing bacteria adhered to and/or internalized into the monolayers, were released by incubation with 100 μl of 1% Triton X-100 (Sigma). Internalized bacteria

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**Table 1 Salmonella strains used in this study**

| Name               | Description                                      | Reference or source |
|--------------------|--------------------------------------------------|---------------------|
| Salmonella SL14028s| Wild-type pathogenic Salmonella typhimurium       | ATCC                |
| SL14028s AvrA-     | SL14028s without AvrA                            | Constructed in our lab |
| SL1344             | Wild-type Salmonella SL1344 strain               | Hardt et al. 1997   |
| SL1344 AvrA-       | SL 1344 mutation without AvrA gene               | Hardt et al. 1997   |
| PhoP+              | Non-pathogenic complex regulator mutant derived from SL14028s | Miller et al. 1990  |

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were those obtained from lysis of the epithelial cells with 1% Triton X-100, 20 min after the addition of gentamicin (50 μg/ml). Gentamicin, an aminoglycoside antibiotic, does not permeate eukaryotic plasma membranes and is therefore cytolytic only to extracellular populations of bacteria while intracellular bacteria populations remain viable [67]. For both cell associated and internalized bacteria, 0.9 ml LB broth was then added and each sample was vigorously mixed and quantitated by plating for CFU on MacConkey agar medium.

**Immunoblotting for epithelial cell signaling**

Intestinal epithelial cells were incubated with equal numbers of the indicated *S. typhimurium* strain (about 20 bacteria per epithelial cell) for 30 minutes, washed, and incubated in fresh DMEM for 30 minutes as previously described [53,68,69]. Cells were rinsed twice in ice-cold HBSS, lysed in protein lysis buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and sonicated. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with one of the following primary antibodies: anti-p65 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-IκBα, anti-JNK, anti-phospho-IκBα, anti-phospho-c-JUN (Cell Signal, Beverly, MA), or anti-β-actin (Sigma-Aldrich, Milwaukee, WI, USA) antibodies and visualized by ECL.

**Real-time quantitative PCR analysis of the IL-8 mRNA**

Total RNA was extracted from epithelial cell monolayers using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA integrity was verified by gel electrophoresis. RNA reverse transcription was done using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer’s directions. The RT cDNA reaction products were subjected to quantitative real-time PCR using the MyiQ single-color real-time PCR detection system (Bio-Rad) and iQ SYBR green supermix (Bio-Rad) according to the manufacturer’s directions. IL-8 cDNA was amplified by using primers to the human IL-8 gene that are complementary to regions in exon 1(5’-TGCAGATAACTCTCCACACT-3’) and overlapping the

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**Table 2 PCR Primers for Salmonella effector proteins**

| Gene | Forward primers | Reverse primers | Access No. |
|------|-----------------|-----------------|------------|
| AvrA | 5’GAATGGACGGCTTTGAGAATGAC3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461786.1 |
| gogB | 5’TCCCCATATATACGCGCGGCGGCGGATGAC3’ | 5’CTGGTGCCCTTGTAGATTGGTGA3’ | NP_461519.1 |
| luxR | 5’GAATGGACGGCTTTGAGAATGAC3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461786.1 |
| luxS | 5’TCCCCATATATACGCGCGGCGGCGGATGAC3’ | 5’CTGGTGCCCTTGTAGATTGGTGA3’ | NP_461519.1 |
| pipB1 | 5’AGAGATGCCGGTCTTGGTAC3’ | 5’CTTGGAGATGGTACGGTGA3’ | NP_460061.1 |
| pipB2 | 5’ATCCATACGCGGCAACGCA3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461706.1 |
| sifA | 5’ATGCTCTAGGTTGCTTGGTAC3’ | 5’CTTGGAGATGGTACGGTGA3’ | NP_460061.1 |
| sifB | 5’TCCCCATATATACGCGCGGCGGCGGATGAC3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461706.1 |
| sipA | 5’TGCCTCTATCTATTGGTAC3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461706.1 |
| sipB | 5’ATCCATACGCGGCAACGCA3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461706.1 |
| sipC | 5’ATCCATACGCGGCAACGCA3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461706.1 |
| sipD | 5’TCCCCATATATACGCGCGGCGGCGGATGAC3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461706.1 |
| sipE | 5’ATCCATACGCGGCAACGCA3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461706.1 |
| sipF | 5’TCCCCATATATACGCGCGGCGGCGGATGAC3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461706.1 |
| sipG | 5’TCCCCATATATACGCGCGGCGGCGGATGAC3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461706.1 |
| sipH | 5’TCCCCATATATACGCGCGGCGGCGGATGAC3’ | 5’TGGTGCCCTTGTAGATTGGTGA3’ | NP_461706.1 |

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Salmonella
with
before
was added directly to the culture medium one hour. For Salmonella, HCT116 (with SP600125 pretreatment) were incubated above. For Salmonella, μHCT116 (with SP600125 pretreatment) were incubated with Salmonella three times in HBSS and incubated DMEM for 6 hours.

Salmonella-induced human IL-8 secretion
HCT116 cells were cultured in DMEM, followed by incubation in Salmonella-containing HBSS (1.6 x 10^10 bacteria/ml) for 30 min, washed 3 times in HBSS, and incubated at 37°C for 6 hours. Cell supernatants were removed and assayed for IL-8 by ELISA in 96-well plates as described previously [53].

Treatment with JNK inhibitor SP600125
To determine whether the effects of TNF is required for JNK, cells were treated with a JNK inhibitor SP600125 (EMD Biosciences, San Diego, CA). SP600125 (50 μM) was added directly to the culture medium one hour before Salmonella treatment. For Western blot assay, HCT116 (with SP600125 pretreatment) were incubated with Salmonella (SP600125 50 μM) 1 hour, washed three times in HBSS and incubated HBSS (SP600125 50 μM) for 1 hour, then harvested. Levels of indicated proteins were determined by Western blotting as described above. For Salmonella invasion and IL-8 ELISA: HCT116 (with SP600125 pretreatment) were incubated with Salmonella (SP600125 50 μM) 1 hour, washed three times in HBSS and incubated DMEM for 6 hours.

Statistical analysis
Data are expressed as means ± SD. All statistical tests were 2-sided. P values of less than .05 were considered to be statistically significant. Differences between two samples were analyzed by a Student’s t-test. Statistical analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC).

Results
The alteration of Salmonella effector gene expression after TNF-α treatment
We first tested whether TNF-α treatment changes the mRNA expression levels of Salmonella effectors. We used TNF-α at a concentration of 10 ng/ml, which is similar to the pathologic concentration in an inflamed intestine or patient serum [70]. Using RT-PCR, we investigated the mRNA expression of Salmonella effectors in the pathogenic Salmonella typhimurium SL1344 with or without TNF-α treatment. As shown in Fig. 1A, SipA was up-regulated by TNF-α, whereas gogB and spvB were down-regulated by TNF-α exposure (Fig. 1A). We tested 23 Salmonella effectors, those showing an upregulation of mRNA expression in response to TNF-α are shown in Fig. 1B and those that were down-regulated following TNF-α exposure are shown in Fig. 1C. TNF-α significantly upregulated the mRNA expression of SipA, whereas it down-regulated the mRNA expression of gogB and spvB. Overall, this PCR data suggests that certain effectors are responsive to the host inflammatory factor TNF-α.

Responses to TNF-α treatment in Salmonella strains with or without AvrA
Our previous studies found that the Salmonella effector AvrA inhibits proinflammatory NF-κB pathway (Collier-Hyams et al., 2002) and stabilizes β-catenin and IκBα [71]. We reasoned that the expression levels of AvrA in the bacterial strains may alter their responses to TNF-α treatment. Therefore, we tested effector expression levels in pathogenic Salmonella strains and corresponding AvrA mutants with or without TNF-α treatment. SL14028s with AvrA gene expression is known to express the AvrA protein only at low pH [27]. As shown in Fig. 2, SipA expression was not changed by TNF-α in SL14028s, whereas SipA mRNA in SL1344 was significantly elevated by TNF-α. PhoP^C is a mutation derived from SL1344 [63]. Interestingly, the SipA mRNA was undetectable in PhoP^C.

To confirm that TNF-α pretreatment had no effects on bacterial growth, we measured the optical density (O.D.) of the bacteria in LB after TNF-α treatment for 18 hours. Over the still culture period, no significant difference was observed between the bacterial strain SL1344 with or without TNF-α pretreatment (Fig. 2B). Similar results were found in the SL1344 AvrA-strain with or without TNF-α treatment (Fig. 2C).

In Table 3, we summarize the changes of effector gene expression after TNF-α 18-hour treatment in Salmonella strains with or without AvrA expression.

Alteration of Salmonella effector proteins after TNF-α treatment
Effector protein expression may be different from mRNA levels. We therefore examined strains of Salmonella to determine whether SipA protein levels respond to TNF-α treatment. As shown in Fig. 3, SipA expression was elevated by TNF-α in SL14028s and SL1344. To make sure the difference we observed was not due to protein loading variation, we stained the membrane with Ponceau S Red that indicates total protein levels (Fig. 3B). Relatively equal amounts of proteins in each lane were visible. We also found that SipA and AvrA...
could not be detected in the AvrA deletion strain derived from SL14028s (Fig. 3A). Without AvrA, SL1344 AvrA-did not alter SipA expression after TNF-\(\alpha\) treatment. In addition, we generated an anti-AvrA antibody to detect the level of AvrA protein expression. SL14028s is known to express the AvrA protein only at low pH [26,27]. Therefore, we did not detect AvrA in the SL14028s group cultured in LB at pH 7.5. AvrA expression is high in the SL1344 strain and increased with TNF-\(\alpha\) exposure. Taken together, we found that TNF-\(\alpha\) significantly increased SipA protein expression in the pathogenic SL14028s and SL1344 strains (Fig. 3C).

**TNF-\(\alpha\) pretreatment of Salmonella enhances invasion of host cells**

We then examined whether pre-treating Salmonella with TNF-\(\alpha\) contributes to the physiological function of...
Salmonella, such as invasion. To determine whether TNF-α contributed to Salmonella invasion, we counted the number of Salmonella invading the human intestinal epithelial HCT116 cells. We found that TNF-α pretreatment of Salmonella increased the amount of internalized bacteria in epithelial cells versus untreated Salmonella SL1344 (Fig. 4A). In the Salmonella SL1344 AvrA-strain, we also found that TNF-α enhanced bacterial invasion of host cells (Fig. 4B). Moreover, we examined the number of cell-associated bacteria, including bacteria adhered to and/or internalized into the epithelial monolayers. Our data showed no significant difference of Salmonella associated with epithelial cells with or without TNF-α pretreatment (Fig. 4C SL1344 and Fig. 4D SL1344 AvrA-). Furthermore, we used a JNK inhibitor, SP600125, to treat cells in order to confirm the enhanced bacterial invasion is related to the JNK pathway. Significantly less number of invaded bacteria was found in SL14028S group with SP600125 compared to the no-inhibitor groups (P < 0.05 Fig. 4E). However, invaded bacterial numbers in the TNF pretreatment group and non-TNF treatment group were still significantly different (P < 0.05 Fig. 4D), suggesting that SP600125 could not block the effect of TNF-pretreated Salmonella in enhancing invasion. These in vitro data indicates that TNF-α pretreatment changes the ability of Salmonella to internalize into host cells.

**TNF-α pretreated Salmonella changes the host response**

We further hypothesized that TNF-α treatment changes Salmonella effector protein expression, thus altering the host’s inflammatory responses. The c-Jun N-terminal kinase (JNK) pathway is known to be regulated by the Salmonella effector AvrA [29,71]. Salmonella increases JNK phosphorylation [29]. We tested for the alteration of these two pathways as read-outs of inflammatory responses from host cells. We found that TNF-α pretreated Salmonella SL1344 could enhance c-JUN, p-c-JUN, and p-JNK expression in HCT116 cells (Fig. 5A). Statistical data further showed a significant difference in expression of p-c-JUN and p-JNK induced by Salmonella with or without TNF-α treatment (Fig. 5B and 5C). Moreover, we confirm the role of JNK pathway with a JNK inhibitor, SP600125. Inhibitor treatment blocked the enhancement of both p-c-JUN and p-JNK induced by Salmonella with or without TNF-α (Fig. 5D). In addition, we tested the activity of AP-1, a transcription factor which is a heterodimeric protein associated with c-Jun [72]. However, we did not find the difference in induction of AP-1 activity by Salmonella without TNF or with TNF-pretreatment (data not shown).
IL-8 mRNA and protein levels in intestinal epithelial cells induced by *Salmonella* with or without TNF-α treatment

Cytokine IL-8 expression and secretion are common readouts for inflammatory responses in the host cells [73]. It is known that pathogenic *Salmonella* increases IL-8 through both transcriptional regulation and protein expression levels [58,71,73,74]. We reasoned that exposure to TNF-α makes pathogenic *Salmonella* more aggressive, inducing more severe inflammatory responses as compared to *Salmonella* without TNF-α treatment. We assessed the effect of TNF-α exposed *Salmonella* on IL-8 mRNA expression in human intestinal HCT116 cells. IL-8 mRNA real-time PCR showed that HCT116 cells significantly increased the level of IL-8 mRNA expression after TNF-α pretreated *Salmonella* colonization (Fig. 6A). In contrast, cells colonized with untreated *Salmonella* expressed less inflammatory IL-8 mRNA (Fig. 6A). Both pathogenic SL14028s and SL1344 had similar trends: TNF-α pretreated *Salmonella* induced significantly higher amounts of IL-8 mRNA, over 2.5 folds as compared to untreated *Salmonella* (Fig. 6A). Furthermore, we examined IL-8 protein secretion into the cell media caused by bacterial infection. As shown in Fig. 6B, an increase in IL-8 protein secretion was detected in the cell media after TNF-α pretreated *Salmonella* SL14028s colonization for 6 hours. In contrast, less IL-8 protein secretion was induced by untreated *Salmonella* SL14028s colonization (Fig. 6B). SL1344 had similar trends: TNF-α pretreatment induced significantly higher amounts of IL-8 secretion compared to untreated *Salmonella* (Fig. 6A). Overall, there is a significant difference of IL-8 secretion in cells colonized with *Salmonella* strains with or without TNF-α pretreatment. A possibility of the increased IL-8 could be due to the enhanced internalized bacteria after TNF pretreatment. We further tested the relationship between the bacterial loading, intercellular bacterial number and IL-8 secretion. However, we did not find that IL-8 secretion linearly related to the invaded bacterial numbers in the cells (data not shown). The enhanced bacterial invasion by TNF treatment and the increased IL-8 could be two different physiological effects in the host cells. Increased bacterial invasion is not necessary to induce increased IL-8 secretion.

### Table 3 Bacteria effector gene expression after 18-hour treatment with TNF-α in *Salmonella* strains with or without AvrA

| Gene | SB1117 (AvrA-) | SB300 (with AvrA) | phop^c | SL14028s (AvrA-) | SL14028s (with AvrA) |
|------|----------------|-------------------|--------|------------------|----------------------|
| sipA | ↓              | ↑                 | ND     | ↑                | ↓                    |
| sipB | ↑              | ↑                 | ↓      | ↓                | ↑                    |
| sipC | ↑              | ↑                 | ND     | ↓                | ↓                    |
| sipA | ↓              | ↑                 | ↓      | ↓                | ↓                    |
| sipB | ↓              | ↓                 | ↓      | ↑                | ↑                    |
| sopO | ↑              | ↑                 | ↓      | ↓                | ↓                    |
| sopE2| ↑              | ↑                 | ND     | ↓                | ↓                    |
| sipP | ↑              | ↑                 | ND     | ↓                | ↑                    |
| sipG | ↓              | ↑                 | ↓      | ↑                | ↑                    |
| sopB1| ↑              | ↑                 | ND     | ↓                | ↓                    |
| sopB2| ↑              | ↑                 | ↑      | ↑                | ↑                    |
| sipA | ↓              | ↓                 | ↓      | ↑                | ↑                    |
| sipB | ↓              | ↓                 | ↓      | ↑                | ↑                    |
| sopC | ↑              | ↑                 | ND     | ↓                | ↓                    |
| ssaB | ↓              | ND                | ↑      | ↑                | ↓                    |
| spvB | ↓              | ↑                 | ↑      | ND               | ↓                    |
| sseF | ↑              | ↑                 | ↓      | ↑                | ↑                    |
| sseG | ↑              | ↑                 | ↓      | ↓                | ↑                    |
| sseI | ↑              | ↑                 | ↓      | ↑                | ↑                    |
| sseJ | ↓              | ↑                 | ↑      | ↑                | ↑                    |
| sseL | ↑              | ↓                 | ↑      | ↑                | ↑                    |
| sspH2| ND             | ↓                 | ↓      | ↑                | ↓                    |
| sbpF | ↓              | ↑                 | ND     | ↓                | ↑                    |
| luxS | ↑              | ↑                 | ↑      | ↓                | ↑                    |

* P < 0.05; *P < 0.01. ND: not detectable by PCR.
To confirm the effect of TNF-pretreated *Salmonella* on IL-8 secretion is through the JNK pathway, we further used the inhibitor SP600125 to treat cells, significant less IL-8 was found in the SL14028S *Salmonella* with SP600125 group compared to the non-inhibitor group (Fig. 6C P < 0.03). SP600125 treatment was able to decrease the IL-8 secretion significantly in the SL14028 + TNF group v.s. the SP600125 + SL14028 + TNF group (Fig. 6C P = 0.017). There was significant difference in SL14028S with or without TNF pretreatment (Fig. 6C P < 0.05). However, the difference

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**Figure 3** SipA and AvrA protein expression. (A) Western blot assay for the expression of SipA and AvrA. (B) Relative protein band intensity in Ponceau S Red staining. Data are reported as representative results from three independent experiments. (C) The relative intensity of the Western blot bands. The data are reported as the mean ± SD of three independent experiments. *P < 0.05 was considered significant.

**Figure 4** TNF-α pretreatment of *Salmonella* contributes to enhanced bacterial invasion in human intestinal epithelial HCT116 cells. (A) and (B) Number of internalized *Salmonella* (A: SL1344, B: SL1344 AvrA-) in the HCT116 cells. (C) and (D) Number of *Salmonella* associated with HCT116 cells. C: SL1344; D: SL1344 AvrA-. (E) Number of internalized *Salmonella* in the HCT116 cells with a JNK inhibitor SP600125 (50 μM) pretreatment. HCT116 cells were stimulated with *Salmonella* with or without TNF-α pretreatment for 30 min, washed, and incubated in fresh DMEM for 30 min. For both cell associated and internalized bacteria, 0.9 ml LB broth was then added and each sample was vigorously mixed and quantitated by plating for CFU on MacConkey agar medium. The mean ± SD is from three replicate experiments.
between TNF pretreatment or no-TNF treatment was abolished after SP600125 pretreatment (Fig. 6C). Taken together, these IL-8 data indicate that TNF-α pretreated Salmonella stimulated more inflammatory responses in the intestinal epithelial cells through the JNK pathway.

**Discussion**

The aim of this study was to determine the effect of TNF-α on Salmonella effector expression and the ability of TNF-α pretreated Salmonella to induce inflammatory responses in host epithelial cells. We investigated the regulation of Salmonella effectors in a variety of contexts, including mRNA expression, protein expression, and host-bacteria interaction. Furthermore, we explored the response of human intestinal cells to TNF-α pretreated Salmonella. Bacterial invasion was enhanced in cells colonized with TNF-α pretreated Salmonella. Salmonella strains with TNF-α pretreatment induced higher proinflammatory responses compared to untreated Salmonella. Overall, our data show that TNF-α exposure makes Salmonella more virulent and enhances inflammation in host intestinal cells. This study provides a new insight into the Salmonella-host interaction in intestinal inflammation and infection.

Our study demonstrates that Salmonella senses the host inflammatory factor TNF-α and responds by changing its effector protein expression and enhancing its virulence, such as invasion. However, it is unknown how Salmonella senses TNF-α in the environment and whether Salmonella has a receptor for TNF-α. Recent findings have begun to reveal the molecular mechanisms by which bacteria can sense small innate immune molecules and modulate virulence gene expression. Wu et al. demonstrated that Pseudomonas aeruginosa recognizes host immune activation and responds by enhancing their virulence phenotype [75].
Zaborina et al. showed dynorphin regulation of bacterial pathogenesis and cross-signaling between quorum sensing and quinolone signaling [76]. Norepinephrine modulates interactions between enterohemorrhagic Escherichia coli (EHEC) and the colonic epithelium by increasing bacterial adherence to the colonic mucosa [77]. EHEC uses a QS regulatory system to “sense” that it is within the intestine and then activates genes essential for intestinal colonization [13]. The QS system used by EHEC is known as the LuxS/autoinducer 2 (AI-2) system extensively involved in interspecies communication [13]. Given that eukaryotic cell-to-cell signaling typically occurs through hormones, and bacterial cell-to-cell signaling occurs through QS, QS may be used as a “language” by which bacteria and host cells communicate [13]. In S. typhimurium, the PhoQ sensor kinase is activated by host antimicrobial peptides. PhoQ then promotes the expression of virulence genes through a phosphorelay cascade [78]. However, it is still unknown how pathogenic Salmonella senses TNF-α, thus changing the expression of the bacterial effectors. Studies in Pseudomonas raise the possibility that TNF-α sensors or receptors for TNF-α are encoded for in the prokaryotic genome. Further studies on the Salmonella quorum sensing system and effector regulation will provide insights into this powerful and effective bacteria-host interaction.

Our data demonstrate that TNF-α exposure increases the expression of the Salmonella effector SipA. SipA contributes significantly to Salmonella host cell invasion in vitro and to Salmonella enterocolitis in vivo [35,41,79]. SipA also plays key role in maximizing pro-inflammatory responses [41]. Our bacterial invasion study further showed that cells colonized with TNF-α pretreated Salmonella had more internalized Salmonella. Moreover, Salmonella strains with TNF-α pretreatment induced higher proinflammatory responses, such as the activation of JNK and elevation of IL-8, compared to the Salmonella strains without TNF-α exposure. This observation is correlated with the enhanced expression of SipA in Salmonella exposed to TNF-α.

At the mRNA level SL1344 expressing AvrA has a significant ability to modify SipA in response to TNF-α, whereas SL14028s is less responsive (Fig.2A). We also found that an AvrA knockout strain derived from SL14028s had significantly decreased levels of SipA mRNA and protein. Salmonella SL14028s is known to be deficient in AvrA expression. AvrA protein expression was only detectable when SL14028s was cultured in low pH media [25-27]. The status of the effector AvrA may alter the expression of other effectors and the capacity of bacteria to induce host inflammation. Other factors in the environment may also contribute to the expression changes of Salmonella effectors. Although AvrA is known to regulate diverse bacteria-host

**Figure 6** TNF pretreatment of Salmonella contributes to enhanced IL-8 mRNA and proteins in human intestinal epithelial cells. Cells were cultured in DMEM, followed by Salmonella-containing HBSS for 30 min, washed 3 times in HBSS, and incubated at 37°C for 6 hours. Total RNA was extracted for real-time PCR. Cell supernatants were removed and assayed for IL-8 by ELISA. (A) IL-8 mRNA levels in the HCT116 cells after colonization with TNF-pretreated Salmonella. (B) IL-8 protein secreted into the cell culture media of the HCT116 cells after Salmonella infection. (C) IL-8 protein secreted into the cell culture media of the HCT116 cells after Salmonella infection. HCT116 cells were pretreated with a JNK inhibitor SP600125 (50 μM). In a single experiment, samples were assayed in triplicate. The data are reported as mean ± SD of three independent experiments. *P < 0.05 was considered significant.
interactions [28,29,71,80], the synergistic regulation of AvrA and other Salmonella effectors in response to the inflammatory status of the host cells remains unknown. Further investigations on the interaction of AvrA and its fellow effectors will help us to understand the network of Salmonella effectors in epithelial cell-bacteria cross-talk.

TNF-α exposure decreased the mRNA expression of SPI-2 effectors Gog B and SpvB, which are known to promote bacterial replication and systemic spread [19,20,22]. We did not examine the protein expression of these two proteins, while the cell culture system limited investigation into the physiologic relevance of the reduction of Gog B and SpvB. Long-term bacterial replication and systematic spread need to be examined in an in vivo model.

In summary, our current study answers the fundamental question of whether TNF-α expressed from host cells can change the expression level of Salmonella effectors, such as SipA, gogB, and spvB. Salmonella exposed to TNF-α induced more bacterial internalization, higher activity of JNK pathway with enhanced p-JNK and p-c-Jun in the host cells. As a consequence of the activation of the JNK pathway, the expression of inflammatory cytokines, such as IL-8, is higher in cells colonized with TNF-α pretreated Salmonella. Overall, Salmonella exposed to TNF-α caused enhanced inflammation in intestinal epithelial cells. We postulate that chronic inflammation with elevated TNF-α in host cells may change the behavior of pathogenic bacterial effectors and may make pathogens more virulent.

Conclusions
We found that TNF-α treatment modulated effector expression in a differential manner. The expression of effector SipA was increased after TNF-α exposure in pathogenic Salmonella. Enhanced bacteria internalization and more severe inflammatory responses of intestinal epithelial cells were found after Salmonella strains were exposed to TNF-α. Activation of the JNK pathway significantly elevates and enhances inflammation in intestinal epithelial cells. As a consequence, the expression of inflammatory cytokines, such as IL-8, is high in cells colonized with TNF-α pretreated Salmonella. Our studies provide new insights into host factor TNF-α regulation of Salmonella effector expression in bacterial invasion and inflammatory responses.

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All authors read and approved the final manuscript.

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