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**Salmonella Virulence Factor SsrAB Regulated Factor Modulates Inflammatory Responses by Enhancing the Activation of NF-κB Signaling Pathway**

Lei Lei,* Wenbiao Wang,* Chuan Xia,* and Fenyong Liu*,†

Effectors proteins encoded by *Salmonella* pathogenicity islands play a key role in promoting bacterial intracellular survival, colonization, and pathogenesis. In this study, we investigated the function of the virulence-associated effector SrfA (SsrAB regulated factor) both in macrophages in vitro and in infected mice in vivo. SrfA was secreted into the cytoplasm during *S. Typhimurium* infection and disassociated IL-1R-associated kinase-1 (IRAK-1) from the IRAK-1–Toll interacting protein (Tollip) complex by interacting with Tollip. The released IRAK-1 was phosphorylated and subsequently activated the NF-κB signaling pathway, which enhanced the LPS-induced expression of inflammatory cytokines, such as IL-8, IL-1β, and TNF-α. The coupling of ubiquitin to endoplasmic reticulum degradation aa 183–219 domain of Tollip is the binding region for SrfA, and both the MDαaa239–226 and CTαaa357–377 regions of SrfA mediate binding to Tollip and NF-κB signaling activation. Deletion of SrfA in *S. Typhimurium* had no notable effects on its replication but impaired the induction of NF-κB activation in infected macrophages. The mice infected with srfA-deficient bacteria exhibited a decreased inflammatory response and an increased survival rate compared with those infected with wild-type *S. Typhimurium*. We conclude that SrfA is a novel *Salmonella* virulence effector that helps modulate host inflammatory responses by promoting NF-κB signaling activation. The Journal of Immunology, 2016, 196: 792–802.

*Salmonella enterica* serovar Typhimurium is a Gram-negative facultative intracellular pathogen. *S. Typhimurium* infections result in manifestations of diseases that range from gastroenteritis to enteric fever to life-threatening systemic disease in humans and a variety of other vertebrates (1). As one of the primary causes of foodborne disease in humans, *Salmonellosis* leads to tremendous morbidity and mortality, and is a severe public health threat and economic burden worldwide (2). Individuals carrying *S. typhi* with no symptoms also have an increased risk for development of hepatobiliary cancer (3).

Innate immune responses represent the first line of defense against *Salmonella* (4, 5). Central to the innate immune system, the NF-κB pathway controls inflammatory cytokine induction and cell death responses downstream of the recognition of pathogen-associated molecular patterns by the host pattern recognition receptors. TLR4, which is a founding member of the TLR family, was identified as the long-sought receptor that responds to bacterial LPS (6, 7). In the TLR4 signaling pathway, in response to LPS, MyD88 recruits IL-1R–associated kinase-1 (IRAK-1) by recognizing its death domain (8). IRAK-1 is subsequently auto-phosphorylated, dissociates from MyD88, and interacts with TNFR-associated factor-6 (9, 10), leading to NF-κB activation (11). Toll interacting protein (Tollip), a critical inhibitor of TLR-mediated innate immune responses, modulates the availability of IRAK-1, the critical upstream signaling molecule (12, 13) in the IL-1R and TLR signaling cascades (14–16). In resting cells, Tollip targets IRAK-1 in the cytosol and inhibits IRAK-1 phosphorylation to maintain the kinase in an inactive conformation. Over-expression of Tollip suppresses TLR4-mediated NF-κB activation, which suggests that Tollip impairs TLR4 signaling by blocking the phosphorylation of IRAK-1 (17).

The genomes of *Salmonella* species encode ~4500 genes, and >100 of these have been implicated in virulence (18, 19). Many *S. Typhimurium* virulence traits can be attributed to the presence of *Salmonella* pathogenicity islands (SPIs). Genes encoding a contact-dependent type III secretion system (T3SS) deliver virulence factors into host cells, which determine bacterial virulence, as well as intracellular survival and replication, during infection (20–23). A series of injected bacterial effectors found in SPIs have been reported to transport into the cytoplasm through T3SS and inhibit the NF-κB inflammatory signaling response (24–26). A recent study demonstrated that LegK1, a type IV effector, activates the NF-κB pathway by phosphorylating the IκB family of inhibitors and likely plays an important role in modulating macrophage defenses or inflammatory responses during *L. pneumophila* infection (27). Therefore, exploring the proinflammatory associated effector(s) correlated with virulence in *S. Typhimurium* is important for a more complete understanding of the pathogenesis of invasive *Salmonella*. 

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Abbreviations used in this article: BMM, bone marrow–derived macrophage; C2, conserved 2; co-IP, coimmunoprecipitation; CUE, coupling of ubiquitin to endoplasmic reticulum degradation; dpi, day postinfection; IL, immunoblot; IRAK-1, IL-1R–associated kinase-1; LB, Luria–Bertani; mAb, mouse Ab; QDO, synthetic dropout –Ttp/Leu/Adr/His; RT-PCR, real-time PCR; sirNA, small interfering RNA; SPI, *Salmonella* pathogenicity island; SrfA, SsrAB regulated factor; TBD, target of Myb1 (Tom1) binding domain; Tollip, toll interacting protein; T3SS, type III secretion system; WT, wild type; YTH, two-hybrid assay.

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SrfA (SsrAB regulated factor) is encoded by srfA, a gene controlled by the two-component system SsrAB in SPI-2, and is associated with Salmonella virulence (28, 29), whereas the specific SrfA mechanism contributing to Salmonella pathogenesis is still unknown. In this study, we attempted to elucidate the intracellular target and intervention mechanism of SrfA, as well as examine the effects of srfA deficiency on host population dynamics both in macrophages in vitro and in infected mice in vivo (30, 31). Our findings demonstrated that SrfA is a secretory virulence effector and regulates inflammatory responses to Salmonella infection. S. Typhimurium is an ideal candidate for bacteria-mediated tumor therapy and is an intriguing chemotherapy agent (32, 33). Genetically modified attenuated S. Typhimurium was used for vaccine development and has also been used as an Ag carrier to deliver heterologous pathogenic Ags specified by multicity plasmids (34–36). Our research contributes to understanding Salmonella pathogenesis and to developing novel therapeutic approaches.

Materials and Methods
Bacteria strains and cell culture
The wild-type (WT) S. Typhimurium strain 14028s and its isogenic derivative srfA gene-deleted mutant (ΔsrfA) were used in this study. Bacterial cultures were grown overnight in Luria–Bertani (LB) medium. 293T, HepG2, and 293T/TLR4 cells (Invitrogen, Carlsbad, CA) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Macrophage RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. All of the mammalian cells were cultured at 37°C in 5% CO2.

Isolation and culture of murine bone marrow–derived macrophages
Bone marrow–derived macrophages (BMMs) were derived from the bone marrow cells as described previously (37, 38). Bone marrow cells were harvested from the femurs of 6- to 8-wk-old female BALB/c mice and cultured in RPMI 1640 containing 20% FBS (Gibco; Life Technologies), 1% penicillin/streptomycin, and 30% L929 cell–conditioned medium in a humidified incubator at 37°C and 5% CO2 to promote differentiation. The cells were washed twice with PBS every 2–3 d, and fresh BMMs medium was added. After 6 d, BMM differentiation was confirmed by monitoring the expression of the cell-surface marker F4/80, preferentially expressed in mature macrophages (39). BMMs in six-well plates (Corning Costar, Schiphol-Rijk, the Netherlands) were used for bacterial infection and the secretion assay.

Mice
Six- to eight-week-old female BALB/c mice were purchased from the Animal Experiment Center of Wuhan University/Animal Biosafety Level-III Laboratory. All of the mice were housed in specific pathogen-free conditions. The animal studies and the protocol for animal experiments were approved by the Institutional Animal Care and Use Committee at Wuhan University. All efforts were made to minimize suffering.

Plasmids
The plasmid constructs and primers used in this study are listed in Supplemental Table I. The pDNA encoding Tollip was obtained from ProteinTech (GenBank no. BC004420). The tollip gene was PCR-amplified and cloned into the pGADT7 (Clontech, Mountain View, CA) or pcDNA3.1 (+) vector (Invitrogen) to generate pAD-tollip and pDNA-tollip after enzymatic digestion, respectively. The truncated Tollip fragments such as the conserved 2 (C2) coupling of ubiquitin to endoplasmic reticulum degradation (CUE) (residues 55–273), Tollip delC2 (residues deleted 55–182), N-terminal target of Myb1 (Tom1) binding domain (TBD/C2) (residues 1–182), CUE (residues 183–273), CUE-ct (residues 220–273), TBDC2-CUE-nt (residues 1–219), C2CUE-nt (residues 55–219), C2 (residues 55–182), and Tollip delCUE-nt (residues deleted 183–219) were also obtained using a PCR-based assay. The PCR products were digested and cloned into pGADT7 or pRK11-Flag to generate recombinant plasmids of truncated tollip mutants. The srfA gene was amplified by PCR using the S. Typhimurium genomic DNA as template. The PCR product was cloned into pGBKTT7 (Clontech) or pcDNA3.1 (+) vectors to generate pBD-srfA and pcDNA-srfA plasmids after enzymatic digestion, respectively. Similarly, a series of truncated srfA mutants was amplified, digested, and cloned into a pGBKTT7 or pRK11-HA vector to generate recombinant plasmids. Plasmid-encoding full-length IRAK-1 was a kind gift from Han J (Xiamen University, Xiamen, Fujian, China). For expression in mammalian cells, the human irak-1 cDNA fragment was cloned into pcDNA3.1 (+). The tollip or srfA gene was also cloned into pGEX-6p-1 or pET-28a (+) (Novogen, Madison, WI) for prokaryotic expression, respectively. The accuracy of all of the constructs was confirmed by DNA sequencing.

Yeast two-hybrid screen
The main procedures for the two-hybrid assay (YTH) were followed as previously described (40). In brief, pBD-srfA, a bait plasmid containing the GAL4 DNA-binding domain, was transformed into Saccharomyces cerevisiae AH109 containing a cdNA library derived from human fetal brain (Clontech). Positive clones were selected on synthetic dropout medium lacking four nutrients, tryptophan, leucine, adenine, and histidine (synthetic dropout — Trp/Leu/His/Ade) (QDO), and were tested for β-galactosidase activity with a colony-lift filter assay according to the manufacturer’s instructions. DNA constructs that contained the sequence coding for the interaction partners of SrfA (designated as pACT2-cDNA) from positive colonies were extracted and determined with the sequencing primer 3′- ACT AGT ACA TGG GAT 3′. Coexpression of pAD-T with pBD-p53 was used as a positive control, and pBD-lam and pAD-T served as negative controls.

Coimmunoprecipitation and Western blot analysis
For immunoprecipitation assays, transfected cells were lysed in lysis buffer [150 mM NaCl; 1 mM EDTA; 20 mM Tris-HCl, pH 7.4; 1 mM EGTA; 1% Nonidet P-40; and 1% protease inhibitor mix (Roche)]. Clarified cell lysates were incubated with the indicated mouse Abs (mAbs), including anti-Tollip (Santa Cruz, Santa Cruz, CA), anti-SrfA, anti-HA (Sigma, St. Louis, MO), and anti-Flag (Sigma) at 4°C with mild shaking before protein G-agarose beads were added to capture the immunocomplexes. Precipitates were pelleted, washed with lysis buffer (500 mM NaCl), and boiled in Laemmli buffer. For Western blot analysis, the denatured polyepitopes or immunoprecipitated proteins from cell lysates or coimmunoprecipitation (co-IP) were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Protein levels were analyzed using indicated primary Abs, including the mAbs anti-HA, anti-Flag, anti–IRAK-1 (Sigma), anti-SrfA, anti-Tollip (Santa Cruz), and anti–β-actin (Origene, Rockville, MD) and the rabbit Ab anti–phospho-IRAK1 (pThr209). These Abs were incubated in an assay with anti-mouse or anti-rabbit IgG Abs conjugated to HRP (Vector Laboratories, Burlingame, CA). Bands were detected with ECL reagent and quantitated using a Gel Documentation Station (Bio-Rad, Hercules, CA). The mouse anti-SrfA mAb was self-prepared by modifying a previously described procedure (41).

Generation of GST-Tollip and His-SrfA and in vitro pull-down assay
GST-Tollip and His-SrfA fusion proteins were expressed in Escherichia coli BL21 (DE3) cells and purified by GST affinity chromatography or nickel column chromatography, respectively. For GST pull-down assays, protein G-agarose beads with anti-GST Ab (Proteintech) were incubated with purified GST or GST-Tollip before being washed with PBS and incubated with purified His-SrfA. The His pull-down assay procedure was only performed using protein G-agarose beads with anti-His Ab (Origene) and incubated primarily with purified His or His-SrfA and then with GST-Tollip. The proteins bound to the beads were then subjected to immunoblot (IB) analysis using the indicated Abs.

Immunofluorescence microscopy analysis
Transfected cells were washed three times with PBS (0.1% BSA), fixed in 4% (w/v) paraformaldehyde, and permeabilized using 0.2% Triton X-100. The cells were then blocked with 5% BSA and incubated with appropriate primary and secondary Abs. The primary Abs used were mouse anti-SrfA, goat anti-Tollip, and rabbit anti–IRAK-1. The secondary Abs used were Alexa Fluor 647–conjugated anti-mouse IgG (Invitrogen), FITC–conjugated anti-rabbit IgG (Proteintech), and Alexa Fluor 555–conjugated anti-goat IgG (Invitrogen). Nuclear staining was performed with DAPI (Invitrogen). Confocal images were collected using an Olympus confocal FV1000 microscope in separate channels and analyzed using the Olympus Fluoview software. The digital images were subsequently merged with Simple PCI software (Compix, Sewickley, PA), and representative images are shown.
**Transfection and reporter gene assays**

Cells were plated and transfected on the following day with the indicated plasmids or small interfering RNA (siRNA) using Lipofectamine (Invitrogen), following the protocols recommended by Invitrogen.

For reporter gene assays, 293/TLR4 cells were seeded in 24-well dishes and transfected with indicated plasmids with an NF-kB reporter (pNF-kB-luc) using Lipofectamine (Invitrogen). In the same experiment, an empty control plasmid was added to ensure that each transfection received the same amount of total DNA. To normalize for transfection efficiency, we added a pRL-TK Renilla luciferase reporter plasmid to each transfection. Approximately 24 h after transfection, cells were left untreated or were stimulated with 100 ng/ml LPS for 6 h before the dual-luciferase assay. The secretion analysis of inhibitor concentration of gentamicin (10 

**RNA interference and quantitative real-time PCR analysis**

Three siRNAs, including tollip-siRNA1, tollip-siRNA2, and tollip-siRNA3 (Ribobio, Guangzhou, China), that targeted different unique regions of the Tollip 3'UTR were designed and used for a Tollip-specific RNA interference assay (Supplemental Table II). For RNA interference, the cells were plated and transfected on the following day with the indicated tollip-siRNA molecules using Lipofectamine (Invitrogen) with NC-siRNA (nontargeting 20–25 nt siRNA; Ribobio) as a negative control. After 48 h of transfection, the cells were recovered for Western blotting or luciferase assays.

For quantitative real-time PCR (RT-PCR) analysis, total cellular RNA was extracted using TRizol (Invitrogen) according to the manufacturer’s protocol and reverse transcribed to synthesize first-strand cDNA using oligo (dT) as a primer. The CDNA was incubated and detected by SYBR Green Real-time PCR Master Mix (TOYOBO, Japan) with the addition of specific forward and reverse primers. Quantitative RT-PCR was performed with an ABI 7500 device (Applied Biosystems, Foster City, CA). Human-specific genes were amplified using the following primers: IL-8-RT-F: 5'-ACT GAG AGT GTA GAG TGG AC-3', IL-8-RT-R: 5'–AAC CTC CTG CAC CCA GTT TTC-3', TNF-α-RT-F: 5'-CTC CCT AAT CAG CCC TCT G-3', TNF-α-RT-R: 5'-GAG GAC CGT GGA GTA GAT GAG-3', Tollip-RT-F: 5'-GCG TCG ACA TGA CGA CCA CGG TCA G-3', Tollip-RT-R: 5'-CTG AAT TCC TCC CCC ATC TGC AGC A-3', β-actin-RT-F: 5'-ATG TGC CAG AGG GAT GAC AAG-3', β-actin-RT-R: 5'-TAG AGC CAC CAA TCC ACA GAC-3'. The PCR results were derived from three independent experiments.

**In vitro growth analysis of Salmonella, bacterial infection, and secretion assay**

The growth analysis of Salmonella strains in LB broth was performed as previously described (42). In brief, a single colony of each Salmonella strain was incubated in 2 ml LB broth overnight (~16 h) at 37°C with shaking. The culture was diluted and inoculated into fresh LB broth (50:1) and cultured at 37°C with shaking. At 0, 2, 4, 6, 8, 10, 12, 14, 16, and 24 h after inoculation, 100 μL bacterial culture was collected and used for analysis by limiting dilution before being plated on LB agar to determine the CFU. Each sample was analyzed in triplicate and repeated at least three times. The growth curve of WT S. Typhimurium 14028s and the derived ΔsrfA mutant was then generated.

For infection and secretion assays, Salmonella strains were cultured in LB broth overnight at 37°C without shaking. An overnight culture of bacteria was added to cells at multiplicities of infection of 50:1. After addition of the bacteria, the cells were briefly centrifuged to synchronize invasion and incubated for 30 min at 37°C with 5% CO2/air. The cells were then washed with PBS, and fresh medium containing 100 μg/mL gentamicin was added for 1 h to kill any remaining extracellular bacteria. The culture medium was then washed again and replaced with a minimum inhibitory concentration of gentamicin (10 μg/mL) for the remainder of the assay. The secretion assay of Salmonella effector SrfA was performed as previously described (43). Salmonella- incubated cells were harvested and lysed with 1% Triton X-100 for 10 min at 1, 6, 12, 24, 36, and 48 h postinfection to detect the secretion of SrfA in cytoplasm using DNAK (Abcam, New Territories, Hong Kong) as an internal control.

**Animal infection**

Groups of six mice were infected by oral gavage. For infection with Salmonella strains, mice were starved for food and water for 4 h and then infected orally with 5 × 107 CFU ΔsrfA mutant or WT Salmonella in 100 μL LB broth. Control mice were given 100 μL sterile LB broth. The mice were observed daily for signs of distress and mortality for 2 wk. Inoculation doses of infected Salmonella were verified by serial dilution and plating onto LB agar. To determine the in vivo distribution and bacterial load in organs, we sacrificed the mice at indicated time points by carbon dioxide inhalation. Blood was taken by cardiac puncture, and the livers and spleens were aseptically collected. Liver and spleen tissues were weighed and homogenized in sterile PBS. Tenfold serial dilutions of liver and spleen homogenates were plated onto agar plates and incubated overnight at 37°C. The number of bacteria per milligram of tissue was calculated.

For histopathological analysis, tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and analyzed by H&E staining, which was performed according to a standard protocol. The histological inflammatory lesions were quantified scored in a blinded manner as previously described (44). All of the animal experiments were conducted according to local animal care advisory committee guidelines.

**ELISA assay**

Serum was collected from mice at the indicated time points postinfection. IL-1β and TNF-α cytokine levels were measured with ELISA kits (Biolegend) according to the manufacturer’s protocol. Six mice were used per condition, and each sample was measured in duplicate. The expression results are the means ± SD.

**Statistical analysis**

Statistical analysis of the data was performed using Student t test (Microsoft Excel) or one-way ANOVA (GraphPad Prism 5.0). The values are reported as the means ± SD of data obtained from at least three independent experiments. A p value <0.05 was considered statistically significant.

**Results**

**SrfA is a secreted cellular signaling effector of Salmonella**

Among a series of Salmonella pathogenicity-associated proteins, SrfA, encoded by srfA, is thought to be associated with Salmonella virulence within a host (29, 45). To understand the function of the SrfA protein in S. Typhimurium, we generated an srfA mutant Salmonella strain ΔsrfA. Growth curve analysis showed that WT S. Typhimurium strain and the ΔsrfA mutant had similar growth characteristic, suggesting that deletion of the srfA gene did not affect bacterial growth in vitro (Fig. 1A). In RAW264.7 macrophage cells infected with WT Salmonella, SrfA was expressed and secreted into the cytoplasm with DnaK, a classic structural Salmonella protein, which was used as an internal control. By contrast, no SrfA protein was observed in cells infected with the ΔsrfA mutant (Fig. 1B). Similar results were observed in cultured primary macrophage cells, the BMMs (Fig. 1C). To explore the effect of SrfA on the inflammatory response, activation of the NF-kB promoter was investigated in cells infected with Salmonella. In comparison with the ΔsrfA mutant, NF-kB promoter-driven luciferase activity was significantly upregulated in cells infected with WT Salmonella (Fig. 1D). These data demonstrated that SrfA is a secreted cellular signaling activator of Salmonella and is nonessential for bacterial growth.

**SrfA interacts with Tollip**

To investigate how SrfA functions in bacterial pathogenicity, we attempted to identify SrfA-interacting proteins with a YTH screen analysis. Using full-length srfA as bait, we screened nearly 1.5 × 107 independent clones from a human fetal brain-derived cDNA library and obtained 24 β-galactosidase–positive clones. Sequencing analysis and BLAST searches of GenBank indicated that one of these clones encoded Tollip. The cytoplasmic protein Tollip, as the most frequently represented binding partner of SrfA, was further investigated using YTH, pull-down, and co-IP assays. According to the YTH assay, SrfA interacted with full-length Tollip (Fig. 2A). The interaction of SrfA and Tollip was con-
firmed by the pull-down of purified His-SrfA with GST-Tollip (Fig. 2B) and by co-IP (Fig. 2C). In 293T/TLR4 cells cotransfected with the eukaryotic expression plasmids pCMV-srfA and pCMV-tollip, both Tollip and SrfA proteins were colocalized in the cytoplasm (Fig. 3B).

Tollip, a ubiquitin-binding protein that shepherds target proteins to the Tom1 complex in early endosomes, functions by modulating the availability of IRAK-1 in the IL-1R and TLR4 signaling cascades, thus serving as a critical regulator of innate immune responses (14–17). To investigate the effect of SrfA-Tollip inter-

**FIGURE 1.** Characterization of WT S. Typhimurium ST14028 compared with the ΔsrfA mutant and the identification of secretory SrfA correlated with NF-κB activation in infected cells. (A) Growth curve analysis of WT S. Typhimurium ST14028 (circles) and ΔsrfA S. Typhimurium (triangles) in LB broth. Bacterial cultures were collected at the indicated time points and used to determine CFU/ml. Data represent the mean ± SD from three independent experiments (n = 3). (B and C) Western blot analysis of the synthesis (cell lysates) and secretion (supernatant) of SrfA in WT Salmonella or ΔsrfA mutant-infected macrophages. Monolayers of RAW264.7 cells (B) or BMMs (C) were infected at a multiplicity of infection of 50 for WT or ΔsrfA S. Typhimurium. Cell lysates were harvested at 6, 12, 24, 36, and 48 h postinfection. The expression of SrfA was analyzed by Western blot using an anti-SrfA mAb. The expression of DnaK in bacterial cells was used as an internal control. (D) 293/TLR4 cells infected with WT S. Typhimurium ST14028 exhibited enhanced activity of the NF-κB promoter compared with cells infected with the ΔsrfA mutant strain. Twenty-four hours after transfection with a pNF-κB luciferase reporter plasmid and a pRL-TK Renilla luciferase plasmid, 293/TLR4 cells were infected with WT or ΔsrfA S. Typhimurium for an additional 8 h before the luciferase assays were performed (mean ± SD; n = 3). **p < 0.01.

**FIGURE 2.** Identification of the interaction between the S. Typhimurium effector SrfA and the cytoplasmic anti-inflammatory protein Tollip. (A) Interaction of SrfA with the anti-inflammatory protein Tollip was confirmed using a yeast two-hybrid system. Transformed yeast cells were plated onto QDO and subjected to a β-galactosidase activity test (β-gal). All of the samples (QDO, β-gal) were prepared from the same experiments at the same time and in parallel. (B) GST pull-down assays and His pull-down assays using purified GST-Tollip and His-SrfA. His-SrfA and GST-Tollip were induced to express in E. coli BL21 (DE3) strains and purified using affinity chromatography. The interaction between SrfA and Tollip in vitro was determined by reciprocal His- and GST-pull-down assays. (C) Co-IP analysis of SrfA and Tollip expressed in 293T cells. 293T cells were cotransfected with two plasmids pCMV-srfA and pCMV-tollip. Twenty-four hours after transfection, cell lysates were immunoprecipitated with either anti-SrfA, anti-Tollip mAbs, or control IgG. The immunoprecipitants were denatured and separated on SDS-polyacrylamide gels and detected using anti-Tollip or anti-SrfA mAbs.
action on IRAK-1 activation, we analyzed the interaction of IRAK-1, Tollip, and SrfA with a co-IP assay. In cells cotransfected with pCMV-tollip and pCMV-irak-1 or pCMV-srfA plasmids, Tollip coprecipitated with IRAK-1 or SrfA separately. However, the interaction between Tollip and IRAK-1 was impaired by the coexpression of SrfA. Confocal analysis revealed that Tollip colocalized predominantly with SrfA in the cytoplasm and not with IRAK-1 in HepG2 cell cultures transfected with pCMV-tollip and pCMV-irak-1 (upper panels), pCMV-tollip and pCMV-srfA (middle panels), or all three plasmids (bottom panels). At 24 h posttransfection, cells were fixed and incubated with mouse anti-SrfA, goat anti-Tollip, or rabbit anti-IRAK-1 primary Abs. Alexa Fluor 647-conjugated anti-mouse IgG, FITC-conjugated anti-goat IgG, and Alexa Fluor 555-conjugated anti-goat IgG were incubated as fluorescent secondary Abs. The subcellular localization of Tollip (green) with SrfA (yellow) and IRAK-1 (red) was visualized using fluorescent microscopy (original magnification ×100). The cell nuclei were stained with DAPI (blue). When all three proteins were coexpressed, Tollip colocalizes with SrfA in the cytoplasm and without IRAK-1. Construction of truncated tollip mutants based on its linear structure and identification of the binding domain of Tollip to SrfA using YTH and co-IP assays. White box represents the TBD domain (aa 1–54), black box represents the C2 domain (aa 55–182), and gray box represents the CUE domain (aa 183–273) of Tollip, respectively. Dark gray box represents CUE-nt domain (aa 183–219) of Tollip that mediate the interaction with SrfA. Plus signs (+) represent positive interaction with SrfA, and dashes (−) indicate no interaction with SrfA. Numbers in brackets represent the numbers of amino acid residues in Tollip. The interaction region of Tollip with SrfA was located in the CUE domain of Tollip.

SrfA attenuated the interaction of Tollip with IRAK-1 by binding the CUE domain of Tollip. SrfA inhibited the binding of Tollip to IRAK-1 by co-IP analysis. 293T cells that were monotonically transfected or cotransfected with pCMV-srfA, pCMV-tollip, and pCMV-irak-1 were immunoprecipitated with anti-Tollip mAb or control IgG and immunoblotted with anti-SrfA and anti–IRAK-1 mAbs. The affinity between Tollip and IRAK-1 was attenuated by the coexpression of SrfA.

SrfA-induced phosphorylation of IRAK-1 and LPS-triggered NF-κB activation is dependent on the interaction with Tollip To explore the mechanism of SrfA regulation of IRAK-1 signaling, we tested the effect of SrfA on the phosphorylation of IRAK-1 induced by LPS. Our data revealed that LPS-induced IRAK-1 phosphorylation was inhibited by Tollip overexpression, and the inhibition was partially eliminated by SrfA expression. We subsequently investigated the effect of SrfA on LPS-stimulated, NF-κB promoter-driven luciferase expression. Our results revealed that SrfA increased LPS-stimulated, NF-κB promoter-driven luciferase expression in a dose-dependent manner. By contrast, Tollip inhibited LPS-stimulated, NF-κB promoter-
driven luciferase expression in a dose-dependent manner, and the inhibition was partially relieved by coexpression with SrfA (Fig. 4B). The LPS-induced transcription of cytokines downstream of NF-kB signaling, such as IL-8, IL-1β, and TNF-α, was also significantly increased when SrfA was coexpressed (Fig. 4C).

Three tollip-siRNAs that targeted different unique regions of the Tollip mRNA were designed (Supplemental Table II) and transfected into 293/TLR4 cells to knock down endogenous tollip levels and proved to be efficient both by RT-PCR and by Western blot detection (Fig. 5A). When endogenous tollip expression was effectively knocked down using tollip-siRNA 123, LPS-stimulated NF-κB promoter-driven luciferase expression was not regulated by SrfA (Fig. 5B). SrfA truncation analysis revealed that both the C4(aa207–226) and the M4(aa357–377) regions of SrfA are essential for binding to Tollip. SrfA mutants with deleted C4(aa207–226) and M4(aa357–377) regions (SrfA-del/m4c4) lost the ability to interact with Tollip (Fig. 6A). The mutant SrfA-del/m4c4 failed to stimulate LPS-promoted NF-κB activation compared with full-length SrfA (Fig. 6B). Furthermore, Tollip inhibition of LPS-stimulated, NF-κB promoter-driven luciferase expression was not upregulated by the mutant SrfA-del/m4c4 (Fig. 6B). These results suggested that SrfA activation of LPS-stimulated NF-κB activation was dependent on the interaction between SrfA and Tollip.

To investigate the contribution of SrfA to bacterial pathogenesis, we infected groups of BALB/c mice with WT or ΔsrfA S. Typhimurium, and bacterial colonization of the liver and spleen was measured at 5 and 9 d postinfection (dpi). Mice infected with WT S. Typhimurium showed clinical signs of malaise, lost weight, and all died within 9 dpi, whereas 70% of mice infected with ΔsrfA S. Typhimurium survived (Fig. 7A, 7B). The data showed that, based on the CFU counts from the organ lysates, both WT and ΔsrfA S. Typhimurium colonized the livers and spleens of mice, and ΔsrfA mutant exhibited significantly higher bacterial loads than WT S. Typhimurium at 5 dpi (p < 0.05; Fig. 7C). However, there was no significant difference between the CFU number of WT and ΔsrfA S. Typhimurium in the spleens or livers at 9 dpi (p > 0.05; Fig. 7C). Analysis of apoptosis based on TUNEL assays (46, 47) identified that, compared with WT S. Typhimurium, the macrophages infected with the ΔsrfA mutant showed significantly fewer apoptotic cells (p < 0.01; Supplemental Fig. 1), which might affect bacterial spread between host cells.

We next examined histological spleen and liver sections because these are the major organs where Salmonella replication occurs.
Morphologically, hepatosplenomegaly was observed in mice infected with WT *Salmonella* Typhimurium compared with the ΔsrfA-infected mice. Similarly, histopathological analysis revealed that microabscesses and necrotic areas existed in the spleen and liver of mice infected with WT bacteria, whereas significantly fewer histological lesions were observed in the spleen or liver of mice infected with the ΔsrfA mutant (*p* < 0.01; Fig. 7D, 7E).

We further tested IL-1β and TNF-α concentrations in the sera of mice infected with WT and ΔsrfA *Salmonella* Typhimurium. From 1 to 7 dpi, IL-1β and TNF-α levels in the sera increased over time (Fig. 7F). Both the IL-1β and the TNF-α levels in the sera of mice infected with WT *Salmonella* were significantly higher than from those infected with the ΔsrfA mutant (*p* < 0.05; Fig. 7F). These results suggested that *Salmonella* virulence factor SrfA plays a critical role in inducing an inflammatory response and pathological damage in infected mice (Fig. 8).

**Discussion**

The genes encoding SPI-2 T3SS and the cognate effector proteins form a complex virulon controlled by the SsrAB two-component system (48). SrfA, one of the gene products controlled by SsrAB, is associated with *Salmonella* virulence. However, the exact function and mechanism of SrfA for *Salmonella* pathogenesis is still unclear. In this study, we investigated the role of *Salmonella* SrfA in bacterial pathogenesis. SrfA is secreted into the cytoplasm of macrophages and induces the activation of NF-κB signaling during *Salmonella* infection, leading to an increase in the inflammatory responses. SrfA interacts specifically with the cytoplasmic protein Tollip, an endogenous modulator of TLR signaling, and released IRAK-1 from the Tollip–IRAK-1 complex. Tollip is a modular protein with an N-terminal target of Myb1 (Tom1) binding domain, a central conserved 2 (C2) domain, and a C-terminal coupling of ubiquitin to CUE domain (49). In resting cells, Tollip binds to IRAK-1, forming the Tollip-IRAK-1 complex that inhibits the phosphorylation and activation of IRAK-1 (17). In *Salmonella*-invaded macrophages, SrfA is secreted into the cytoplasm and competitively binds to Tollip, removing it from the Tollip–IRAK-1 complex through the CUE domain and leading to the release and phosphorylation of IRAK-1. Phosphorylated IRAK-1 mediates NF-κB activation and induces downstream inflammatory responses (Fig. 8). Down syndrome candidate region 1-1S isofrom, also known as RCAN1, also causes the dissociation of the IRAK-1–Tollip complex, leading to the activation of TAK1 and enhancement of the IL-1β–stimulated inflammatory response (50). SrfA, as a bacterial effector, mimics the function of endogenous Down syndrome candidate region 1-1S and interacts with Tollip, promoting the dissociation of the Tollip–IRAK-1 complex and leading to the activation of both IRAK-1 and subsequent NF-κB signaling (51).

The NF-κB/REL family of transcription factors is central to both the innate and the adaptive immune responses and plays critical roles in host defense against invading pathogens (52). The activation of NF-κB signaling is also postulated to be important for cell death protection (53, 54). Bacterial pathogens have evolved diverse pathological associated effectors, such as YopJ (*Yersinia* outer protein J) in *Yersinia* (21, 55, 56) and IpaH0722, OspB and OspF in *Shigella* (20, 57, 58), to manipulate host NF-κB signaling. *Salmonella* also uses multiple effectors to dampen the host’s inflammatory response. In *Salmonella*, the bacterial effector AvrA, as a member of the YopJ/Avr family of proteins (59), debiquitinates IκB, inhibiting NF-κB activation (24, 60), and is considered the first bacterial effector from a mammalian pathogen that limits virulence in vertebrates by inhibiting the activation of the key proinflammatory NF-κB transcription factor and augmenting apoptosis in human epithelial cells. *Salmonella* secreted factor L, which was demonstrated to exhibit debiquitinating ac-
Salmonella translocated effector protein SspH1, a member of the bacterial leucine-rich repeat motif repeat protein family (62), is translocated to the mammalian nucleus by intracellular S. Typhimurium and inhibits NF-kB–dependent gene expression, such as IL-8, to attenuate the host’s inflammatory response (22, 25). All of these NF-kB–modulating effectors are inhibitory in nature and manipulate host NF-kB signaling at the level of IκB.

However, recent research has exhibited that bacterial spread and distribution in the tissues occurs in parallel with the onset and escalation of the host immune response (63), and inflammatory host cell death leads to the extrusion of invasive Salmonella and might also contribute to cell-to-cell transmission and dissemination in systemic tissues (64). Our data demonstrated that NF-kB signaling was activated by the S. Typhimurium effector SrfA through the regulation of IRAK-1 activation. This activation contributes to Salmonella-promoted inflammation, which might explain the subsequent release and dissemination of the invasion-primed Salmonella.

Although essential to disease, it is still unclear how Salmonella spreads from one focus of infection to another. Apoptosis of macrophages in the liver is observed during systemic Salmonella infection (65–67). This observation raises a hypothesis that apoptotic host cell death might constitute a mechanism allowing bacterial spread to new cells that occurs in the context of a far more active necrotic cell death process, ensuring an ongoing protective intracellular environment in which a subset of bacteria can avoid detection and targeting by the humoral branch of the immune system (68–70). In cell culture, Salmonella-induced apoptosis requires the activation of TLR4 by bacterial LPS (65, 71). Previous reports demonstrated that phosphorylated activated IRAK-1 also promotes downstream apoptosis (12, 13, 68). Therefore, we further investigated the effect of srfA deletion mutation on cell apoptosis both quantitatively and qualitatively (46, 47). Our data indicated that the apoptosis of cells infected with DsrfA mutant was significantly decreased compared with WT S. Typhimurium using a flow cytometry assay (p < 0.01; Supplemental Fig. 1A) and a fluorescent microscopic assay (Supplemental Fig. 1B). This finding suggests that SrfA promotes the phosphorylation of IRAK-1 and LPS-induced NF-kB activity and, subsequently, apoptosis downstream of IRAK-1 (12, 13, 72). A reasonable explanation for the intracellular dissemination of invasive Salmonella based on this study is that SrfA induces apoptosis among host cells via the activation of...
IRAK-1 and facilitates cell-to-cell spread during *Salmonella* infection.

Recent studies of enteropathogenic *E. coli* indicated that enteropathogenic *E. coli*-induced inflammation involves a balance between proinflammatory proteins including flagellin and an unidentified T3SS-independent 50-kDa protein, as well as anti-inflammatory proteins such as intracellular T3SS-dependent factors (73). A *Salmonella*-induced inflammatory response might also be advantageous as a consequence of the balance between proinflammatory and anti-inflammatory proteins. The

**FIGURE 7.** Inactivation of srfA decreases the virulence and inflammatory response to *Salmonella* in infected-mice. (A) Body weight loss (%) of BALB/c female mice infected intragastrically with 10⁴ WT or ΔsrfA mutant bacteria (six animals per group). Data represent the mean ± SD from three independent experiments. (B) Survival (%) of mice orally infected with 10⁴ WT or ΔsrfA mutant bacteria (six animals per group). Data represent the mean ± SD from three independent experiments. (C) Bacterial enumeration in the livers and spleens of infected mice. Mice (six animals per group) were infected orally with 10⁴ WT or the ΔsrfA mutant S. Typhimurium strain. At 5 and 9 dpi, the organs were harvested aseptically for colony counts, and organ lysates were diluted before plating on selective LB plates. Data represent the mean ± SD from three independent experiments. **p < 0.01. (D)** Histopathological analysis of the liver and spleen tissues from infected mice by H&E staining. The mice were sacrificed at 7 dpi, and liver and spleen tissues were collected for histopathological analysis. Representative micrographs from each group are shown. The arrows indicate microabscesses/necrotic areas in the liver and spleen (original magnification ×20). (E) Histological scores of *S. Typhimurium*-infected liver and spleen at day 7. The maximum total liver inflammation score was 16, whereas the spleen was 12. (F) The expression levels of IL-1β and TNF-α in the sera of infected mice (six animals per group) were determined by ELISA. Error bars indicate the SDs (mean ± SD; n = 3). WT compared with ΔsrfA. *p < 0.05, **p < 0.01, ns, not significant.

**FIGURE 8.** Schematic diagram displaying the role of SrfA during *S. Typhimurium* infection. Bacterial LPS activates TLR4, leading to the recruitment of MyD88, TIRAP, and IRAK-1 for complex formation in the myddosome. LPS promotes IRAK-1 autophosphorylation in the myddosome. Phosphorylated IRAK-1 is released from the complex to signal to downstream effectors for proinflammatory responses. Tollip and IRAK-1 form complexes to inhibit the IRAK-1 signaling pathway. During *S. Typhimurium* infection, SrfA was secreted into the cytoplasm, where it bound competitively to Tollip with IRAK-1, leading to the release of IRAK-1 from IRAK-1–Tollip complexes and to activation of the immune response by NF-κB activation.
Salmonella proinflammatory effector SrfA, combined with other anti-inflammatory proteins including AvrA and Salmonella secreted factor L, might regulate the immune response of host cells at different phases during Salmonella infection and control the intracellular survival and replication of invasive Salmonella. In conclusion, SrfA is a secreted virulence effector of Salmonella. SrfA stimulated the activation of IRAK-1 and LPS-induced NF-κB signaling by binding to Tollip and subsequently inducing inflammatory responses and pathogenesis. This discovery provides us with a different perspective for investigating the interaction of pathogens and the host during infection.

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Disclosures

The authors have no financial conflicts of interest.

References


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