Salmonella enterica Serovar Typhi Conceals the Invasion-Associated Type Three Secretion System from the Innate Immune System by Gene Regulation

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Abstract

Delivery of microbial products into the mammalian cell cytosol by bacterial secretion systems is a strong stimulus for triggering pro-inflammatory host responses. Here we show that Salmonella enterica serovar Typhi (S. Typhi), the causative agent of typhoid fever, tightly regulates expression of the invasion-associated type III secretion system (T3SS-1) and thus fails to activate these innate immune signaling pathways. The S. Typhi regulatory protein TviA rapidly repressed T3SS-1 expression, thereby preventing RAC1-dependent, RIP2-dependent activation of NF-kB in epithelial cells. Heterologous expression of TviA in S. enterica serovar Typhimurium (S. Typhimurium) suppressed T3SS-1-dependent inflammatory responses generated early after infection in animal models of gastroenteritis. These results suggest that S. Typhi reduces intestinal inflammation by limiting the induction of pathogen-induced processes through regulation of virulence gene expression.

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Introduction

One function of the innate immune system in the intestinal tract is to generate temporary inflammatory responses against invasive enteric pathogens while avoiding detrimental overreaction against harmless commensal bacteria under homeostatic conditions. In contrast to commensal microbes, pathogenic microbes express an array of virulence factors to manipulate host cell functions. Pathogen-induced processes, also known as patterns of pathogenesis [1], activate specific pathways of the innate immune system, enabling the host to distinguish virulent microbes from ones with lower disease-causing potential. By detecting pathogen-induced processes the host can escalate innate immune responses to levels that are appropriate to the threat [2].

Salmonella enterica serovar Typhimurium (S. Typhimurium), an invasive enteric pathogen associated with human gastroenteritis, triggers acute intestinal inflammation in the terminal ileum and colon, thereby producing symptoms of diarrhea and abdominal pain within less than one day after ingestion [3]. The inflammatory infiltrate in the affected intestinal tissue is dominated by neutrophils [4,5]. Similarly, neutrophils are the primary cell type in the stool during acute illness [6–8]. In contrast, individuals infected with serovar Typhi (S. Typhi) develop a febrile illness (typhoid fever) with systemic dissemination of the organism. In contrast to Salmonella-induced gastroenteritis, only a third of patients develop diarrhea that is characterized by a dominance of mononuclear cells in the stool [6]. The dominant cell type in intestinal infiltrates is mononuclear, while neutrophils are infrequent [9–11]. Unlike S. Typhi, interaction of S. Typhimurium with intestinal epithelial cells induces hepcidin A3-dependent transmigration of neutrophils [12]. Moreover, infection of human colonic tissue explants with S. Typhimurium results in the increased production of the neutrophil-attracting chemokine IL-8, while S. Typhi does not elicit this response [13]. These observations suggest that invasion of the intestinal mucosa by S. Typhimurium is accompanied by a rapid escalation of host responses leading to acute, purulent inflammation, while S. Typhi elicits little intestinal inflammation during early stages of infection, however the molecular mechanisms underlying these apparent differences are poorly defined.

One pathogen-induced processes that triggers pro-inflammatory immune responses is the transfer of bacterial molecules into the host cell cytosol by secretion systems. The invasion-associated type III secretion system (T3SS-1) expressed by all Salmonella serovars...
Author Summary

Bacterial pathogens translocate effector proteins into the cytoplasm of host cells to manipulate the mammalian host. These processes, e.g., the stimulation of small regulatory GTPases, activate the innate immune system and induce pro-inflammatory responses aimed at clearing invading microbes from the infected tissue. Here we show that strict regulation of virulence gene expression can be used as a strategy to limit the induction of inflammatory responses while retaining the ability to manipulate the host. Upon entry into host tissue, *Salmonella enterica* serovar Typhi, the causative agent of typhoid fever, rapidly represses expression of a virulence factor required for entering tissue to avoid detection by the host innate immune surveillance. This tight control of virulence gene expression enables the pathogen to deploy a virulence factor for epithelial invasion, while preventing the subsequent generation of pro-inflammatory responses in host cells. We conclude that regulation of virulence gene expression contributes to innate immune evasion during typhoid fever by concealing a pattern of pathogenesis.

and delivers effector proteins into the cytosol of epithelial cells [14]. A subset of these translocated effector proteins activate Rho-family GTPases [15–18], thereby triggering alterations in the host cell cytoskeleton that result in bacterial invasion of epithelial cells [19]. Excessive stimulation of Rho-family GTPases activates the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and promotes the subsequent release of proinflammatory cytokines and chemokines [15,20,21]. In a bovine model of *S. Typhimurium*-induced gastroenteritis, the rapid induction of intestinal inflammation and diarrhea requires the T3SS-1 apparatus as well as the effector proteins SipA, SopA, SopB, SopD, and SopE2 [22–24]. Similarly, in a murine model of *Salmonella enterica* induced colitis, SipA, SopE and SopE2 can independently induce intestinal inflammation [25] and mutants lacking a functional T3SS-1 are unable to initiate neutrophil recruitment to the intestinal mucosa during early infection [25,26]. These findings indicate that T3SS-1-mediated effector translocation induces innate immune responses during *S. Typhimurium*-induced colitis.

Similar to *S. Typhimurium*, invasion of cultured intestinal epithelial cells by *S. Typhi* is mediated by the T3SS-1 [27]. Replacement of *S. Typhimurium* T3SS-1 effector proteins with their *S. Typhi* orthologues does not attenuate inflammatory responses elicited by *S. Typhimurium* in the intestinal mucosa of calves [28], demonstrating that *S. Typhi* T3SS-1 effector proteins can exhibit intrinsic pro-inflammatory properties in vivo. Thus, the molecular basis for the absence of T3SS-1-dependent innate immune responses early during *S. Typhi* infection remains unclear.

Results

In contrast to *S. Typhimurium*, *S. Typhi* fails to activate the NF-kB signaling pathway in human epithelial cells

To study the induction of pro-inflammatory signaling pathways upon infection with *S. Typhimurium* and *S. Typhi*, we employed a human epithelial cell line permanently transected with a NF-kB-dependent luciferase reporter (HeLa 57A) [29]. Infection with the *S. Typhimurium* wild-type strain SL1344 resulted in a significant increase (7-fold; \( P < 0.01 \)) in luciferase activity compared to mock-infected cells (Fig. 1A), while a derivative of *S. Typhimurium* SL1344 carrying a mutation in the T3SS-1 apparatus gene *invA* (SW767) did not elicit NF-kB signaling [20,30]. In contrast to the *S. Typhimurium* wild-type, the *S. Typhi* wild-type strain Ty2 failed to trigger NF-kB activation (Fig. 1A), suggesting that *S. Typhi* is a poor activator of T3SS-1-dependent inflammatory processes in human epithelial cells.

Effect of the *viaB* operon on T3SS-1 mediated inflammatory responses in epithelial cells

The T3SS-1 mediates invasion of non-phagocytic cells. *S. Typhi* has been reported to differ from *S. Typhimurium* with regards to invasion of human epithelial cells [31–33], thus raising the possibility that the observed differential activation of the NF-kB signaling pathway could be due to varying degrees of invasiveness. To test this hypothesis, HeLa cells were infected with *S. Typhimurium* and *S. Typhi* strains and a gentamicin protection assay was performed (Fig. 1B). The *S. Typhimurium* wild type SL1344 and the *S. Typhi* wild type Ty2 were recovered in similar numbers, while the respective isogenic *invA*-deficient mutants displayed significantly reduced invasiveness. T3SS-1 activity has two functional consequences: manipulation of host signaling pathways and subsequent bacterial uptake. To discern between effects mediated directly by the T3SS-1 or indirectly by increasing the intracellular bacterial load, we next sought to reinstate invasiveness of the *S. Typhimurium* *invA* mutant without restoring T3SS-1 function. Expression of the *Erwinia pseudotoxigenica* invasin, encoded by the plasmid pRI203, raised invasiveness of the *S. Typhimurium* *invA* mutant comparable to the wild type strain (Fig. 1B), but failed to restore the ability to induce NF-kB activation in epithelial cells (Fig. 1C) [34]. Taken together, these observations indicate that immune evasion by *S. Typhi* did not directly correlate with the intracellular bacterial load or invasiveness.

Despite causing disparate disease entities, the genomes of *S. Typhimurium* and *S. Typhi* display remarkable similarity. Chromosomal DNA sequences of both serovars are highly syntenic, with mostly minor inversions, deletions and insertions [35,36]. One DNA region that is present in *S. Typhi* but absent from *S. Typhimurium* is the *Salmonella* pathogenicity island 7 (SPI-7). Situated within SPI-7 is the *viaB* locus, an operon encoding regulatory (*viaA*), biosynthesis (*viaBCDE*), and export (*viaGBCDE*) genes involved in the production of the virulence (Vi) capsular polysaccharide of *S. Typhi* [37] (Fig. S1A). The *viaB* locus has been shown to suppress Toll-like receptor (TLR) signaling pathways [13,38,39]. We therefore explored the contribution of the *viaB* locus on diminishing NF-kB activation in epithelial cells (Fig. 1D and S1B). Deletion of the entire *viaB* locus in *S. Typhi* (*ΔviaB*) mutant; SW347) markedly increased the ability to activate NF-kB in epithelial HeLa cells (\( P < 0.001 \)). Akin to the findings with *S. Typhimurium*, NF-kB signaling induced by the *S. Typhi* *viaB* mutant was independent of invasiveness (Fig. S1C) but required a functional T3SS-1 since inactivation of *invA* in the *viaB* mutant background (Δ*viaB invA* mutant, STV4) completely abolished luciferase activity (\( P < 0.001 \)) (Fig. S1D). These results supported the idea that the *viaB* locus attenuates T3SS-1-induced, pro-inflammatory signaling pathways in human epithelial cells.

The *viaB* locus has been shown to alter interaction of *S. Typhi* with host cells through multiple distinct mechanisms (reviewed in [40]). The Vi capsular polysaccharide prevents complement deposition, phagocytosis, and TLR4 activation, while the regulatory protein TviA is known to dampen TLR5 signaling. We therefore wanted to discern whether the absence of NF-kB signaling in human epithelial cells is due to the production of the Vi capsule or due to altered gene expression mediated by TviA. To this end, the *tviA* gene cloned into a low copy number plasmid (pTVIA1) was introduced into a *S. Typhi* *viaB* mutant (STV2). Expression of *tviA* under control of the native promoter significantly lowered NF-kB activation (\( P < 0.01 \)) in...
comparison to cells infected with the S. Typhi *viaB* mutant carrying the empty vector control (pWSK29). Remarkably, expression of *tviA* reduced inflammatory responses to levels comparable to the *S. Typhi* wild-type strain (Fig. 1D and S1B), suggesting that the regulatory protein TviA is involved in dampening inflammatory responses in cultured human epithelial cells.

**TviA reduces T3SS-1-mediated inflammation in the bovine ligated ileal loop model**

We had recently demonstrated that a *S. Typhimurium* strain carrying the *S. Typhi* *viaB* locus on a plasmid elicits less mucosal inflammation in a bovine ligated ileal loop model than the isogenic *S. Typhimurium* wild type ATCC14028 [38], raising the possibility that TviA might be involved in suppressing inflammatory responses in *vivo*. To delineate the relative contribution of the Vi capsule and the regulator TviA to reducing inflammatory responses in the bovine ligated ileal loop model [23], we repeated these studies with derivatives of *S. Typhimurium* strain ATCC14028 in which the *phoN* gene in the chromosome had been replaced with the entire *S. Typhi* *viaB* locus (*phoN::viaB* mutant, TH170) or the *tviA* gene only (*phoN::tviA* mutant, SW474). In these strains, transcription of *tviA* and the downstream genes is solely

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**Figure 1. S. Typhi does not elicit inflammatory responses in epithelial cells.** Human epithelial cells permanently transfected with a NF-κB-luciferase reporter system (HeLa 57A) were infected with the indicated *Salmonella* strains at a multiplicity of infection of 5 or mock treated (bacterial growth media alone). (A) Cells were infected with the *S. Typhimurium* wild type SL1344, an isogenic *invA* mutant (SW767), the *S. Typhi* wild type Ty2, an isogenic *invA* mutant (SW222). Luciferase activity as a measure of NF-κB activation was determined after 5 h (N = 3). (B) Monolayers of cells were infected for 1 h with the indicated *Salmonella* strains. Bacterial numbers recovered after 90 min of Gentamicin treatment were standardized to the number of the bacteria in the inoculum (N = 4). Plasmid pRI203 encodes the *Y. pseudotuberculosis* invasin. (C) Luciferase activity exhibited by *Salmonella*-infected HeLa57A cells was determined as described above (N = 4). (D) Cells were infected the *S. Typhi* wild-type strain Ty2, a Δ*viaB* mutant (SW347), an *invA* Δ*viaB* mutant (STY4), a Δ*viaB* mutant (STY2) harboring the cloning plasmid pWSK29 (pWSK), a Δ*viaB* mutant (STY2) expressing the *tviA* gene (pTVIA1) and luciferase activity determined 5 h after infection (N = 5). Bars represent geometric means ± standard error. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not statistically significant.

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controlled by the native S. Typhi promoter [41,42]. This strategy was chosen to ensure that attenuation of intestinal inflammation in this model was not caused by introduction of the \textit{viaB} locus on a multi-copy plasmid [38].

We compared the \textit{phoN}::\textit{viaB} mutant and the \textit{phoN}::\textit{tviA} mutant to a strain carrying an antibiotic resistance gene inserted chromosomally in the \textit{phoN} gene (\textit{phoN} mutant, AJB715). The \textit{phoN}::\textit{viaB} mutant, the \textit{phoN}::\textit{tviA} mutant, and the isogenic \textit{phoN} mutant were recovered in equal numbers from gentamycin-treated tissue samples five hours after inoculation (Fig. 2A), suggesting that neither the \textit{tviA} gene nor the entire \textit{viaB} locus interfered with tissue invasion. Consistent with our previous observations [38], the \textit{phoN}::\textit{viaB} mutant elicited less fluid accumulation (Fig. 2B) and less pathological changes in the mucosa (Fig. 2C and D) than the isogenic \textit{phoN} mutant. Remarkably, expression of \textit{tviA} alone (\textit{phoN}::\textit{tviA} mutant) significantly reduced fluid accumulation and inflammation compared to the \textit{phoN} mutant (\textit{P}<0.01). The responses elicited by the \textit{phoN}::\textit{tviA} mutant and the \textit{phoN}::\textit{viaB} mutant were indistinguishable, suggesting that the \textit{viaB}-mediated attenuation of inflammatory responses five hours after inoculation of bovine ligated ileal loops with \textit{S. Typhimurium} was mostly attributable to the action of the \textit{tviA} regulatory protein. Taken together, these data suggested that gene regulation mediated by \textit{TviA} could dampen inflammatory processes \textit{in vivo}.

\textbf{TviA represses expression of regulatory, structural, and effector proteins of the \textit{S. Typhi} T3SS-1}

A functional T3SS-1 is required for the induction of intestinal host responses in cattle [22,24,43]. A \textit{S. Typhimurium} strain carrying a mutation in the T3SS-1 apparatus gene \textit{invA} (\textit{invA} \textit{phoN} mutant, SW737) was significantly less invasive than a \textit{phoN} mutant (Fig. 2A) (\textit{P}<0.05). Interestingly, inactivation of \textit{invA} (\textit{invA} \textit{phoN} mutant) reduced fluid accumulation (Fig. 2B) and intestinal inflammation (Fig. 2C and D) by a magnitude that was similar to that observed for the \textit{phoN}::\textit{tviA} mutant. This finding was consistent with the idea that \textit{TviA} reduces T3SS-1-dependent host responses \textit{in vivo}, prompting us to further investigate the mechanism by which \textit{TviA} inhibits T3SS-1 gene expression. \textit{TviA} is a key activator of the \textit{tviBCDEvexABCDE} operon but can also control transcription of genes outside its own operon (Fig. S2A). Expression of \textit{TviA} results in diminished motility and flagellin secretion due to downregulation of the flagellar regulon by repressing transcription of the \textit{flhDC} genes [42,44]. \textit{FlhDC}, the master regulator of flagellar gene expression, activates transcript...
tion of class II flagellar genes, such as *fliA* and *βz* [45,46]. *FliA* is a positive regulator of class III flagellar genes, including flagellin [45,47]. To determine whether reduced motility or diminished flagellin production could account for the TviA-dependent reduction in NF-κB activation, we inactivated the *fliC* gene encoding the sole flagellin of the monophasic serovar Typhi, thereby rendering strains carrying these mutations flagellate and non-motile. Deletion of the entire *viaB* operon (∆*viaB ΔfliC* mutant, SW483) in the *fliC* background (∆*fliC* mutant, SW359) significantly increased NF-κB signaling in infected HeLa and HEK293 epithelial cells (Fig. S2B and S2C). Expression of TviA from a plasmid (pTVI1A) in a *viaB* *fliC* mutant reduced luciferase activity to levels comparable to the *fliC* mutant (Fig. S2B and S2C), demonstrating that TviA-dependent repression of NF-κB activation was flagellin-independent.

Gene expression profiling experiments suggest that TviA affects transcription of T3SS-1 genes through the following signaling cascade [42]: By repressing transcription of *flhDC*, TviA downstream-regulates expression of *Fliz*. The regulatory protein Fliz is an activator of *hilA* [48–50], the master regulator of T3SS-1 genes [51,52], thus placing T3SS-1 gene expression under negative regulation by TviA. *hilA* activates transcription of the activator, the regulatory protein FliZ is an activator of class III flagellar genes, including flagellin *fliC*. The bacteriophage-encoded TviA orthologues of these effectors to the induction of NF-κB signaling [45,47]. To determine whether reduced motility or diminished flagellin production could account for the TviA-dependent reduction in NF-κB activation, we inactivated the *fliC* gene encoding the sole flagellin of the monophasic serovar Typhi, thereby rendering strains carrying these mutations flagellate and non-motile. Deletion of the entire *viaB* operon (∆*viaB ΔfliC* mutant, SW483) in the *fliC* background (∆*fliC* mutant, SW359) significantly increased NF-κB signaling in infected HeLa and HEK293 epithelial cells (Fig. S2B and S2C). Expression of TviA from a plasmid (pTVI1A) in a *viaB* *fliC* mutant reduced luciferase activity to levels comparable to the *fliC* mutant (Fig. S2B and S2C), demonstrating that TviA-dependent repression of NF-κB activation was flagellin-independent.

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**In the absence of TviA, the *S. Typhi* T3SS-1 effector protein SopE is the major inducer of NF-κB activation in epithelial cells**

We next determined which T3SS-1 effector proteins contributed to pro-inflammatory responses elicited by *S. Typhimurium* and *S. Typhi*. Previous work has demonstrated that SopE, SopE2, SopB, and SipA contribute to NF-κB activation in epithelial cells [15–17,53]. The bacteriophage-encoded *sopE* gene is present in *S. Typhi* Ty2 but absent from *S. Typhimurium* strain ATCC 14028. To better model the contribution of TviA on attenuating T3SS-1-induced host responses, we chose to continue our studies using the *S. Typhimurium* strain SL1344, an isolate that carries the *sopE* gene while *SopB* and *SipA* are critical for the induction of inflammatory responses in epithelial cells upon infection with *S. Typhi*. A *S. Typhi* *invB* *invF* *sopE* *sopB* *sipA* mutant (SW1211) elicited pronounced NF-κB activation, but a more modest NF-κB activation was observed with the *S. Typhi* *invB* *invF* *sopE* *sopB* *sipA* mutant (SW1214) (Fig. 3). This indicated that, akin to the findings with the *S. Typhimurium* strain SL1344, SopE, SipA, and SopB are critical for the induction of inflammatory responses in epithelial cells upon infection with *S. Typhi*, while *invB* and *invF* are non-essential for the induction of inflammatory responses in epithelial cells upon infection with *S. Typhi*.

**Figure 3. Effect of the regulator TviA on NF-κB activation triggered by *S. Typhi* T3SS-1 effectors.** HeLa cells permanently transfected with a NF-κB-luciferase reporter system (HeLa 57A) were either mock treated (bacterial growth media alone) or infected with the *S. Typhi* wild type Ty2, a ∆*invB* mutant (SW347), a *sopB* *sipA* *sopE* ∆*invB* mutant (SW1217), an *invB* *viaB* mutant (STY4), a *sipA* *sopE* ∆*invB* mutant (SW1214), a *sipA* *sopE* ∆*invB-vexE* mutant (SW1215), a *sopB* *sopE* ∆*invB-vexE* mutant (SW1216), a *sopB* *sopE* ∆*invB-vexE* mutant (SW1213), a *sopB* *sopE* ∆*invB* mutant (SW1212), or a *sopB* *sipA* ∆*invB-vexE* mutant (SW1212). After 5 h, luciferase activity was measured (N=4). Bars represent geometric means ± standard error. *, P<0.05; **, P<0.01; ns, not statistically significant. doi:10.1371/journal.ppat.1004207.g003

TviA reduces activation of the Rac1 and NOD1/2 signaling pathway

Since SopE triggered the most pronounced host responses in the absence of *viaA*, we focused our further analysis on this signaling pathway. Mechanistic studies in cultured epithelial cells have revealed that the bacterial guanine nucleotide exchange factor (GEF) SopE activates the Rho-family GTPase Ras-related C3 botulinum toxin substrate 1 (RAC1) [15]. Excessive stimulation of RAC1 by bacterial effectors is sensed by the nucleotide-binding oligomerization domain-containing protein 1 (NOD1) [30]. Activation of NOD1 leads to phosphorylation of the receptor-
interacting serine/threonine-protein kinase 2 (RIP2) and activation of NF-κB signaling in epithelial cells [15,20,30,54]. The NOD1/2 signaling pathway in HeLa cells can also be triggered by SipA [34], although this pathway plays a lesser role in the SopE-encoding strain SL1344 (Fig. S4). Taken together, these findings raised the possibility that TviA-mediated downregulation of SopE allows S. Typhi to abate immune recognition by the RAC1-NOD1/2-RIP2 signaling pathway. To test this hypothesis, we abrogated RAC1 and RIP2 signaling by either ectopically expressing a dominant negative form of RAC1 (RAC1-DN) [30,55] or by treating cells with the RIP2 inhibitor (SB203580) (Fig. 4). Consistent with previous reports, ectopic expression of a GFP-SopE fusion protein alone was sufficient to induce NF-κB activation while no upregulation of this signaling pathway was observed with a GFP-SopE construct lacking GEF activity (GFP-SopE G168A) [30,56]. Simultaneous expression of the GFP-SopE fusion protein and a RAC1-DN construct abrogated NF-κB signaling (Fig. 4A).

Infection of HeLa cells with the S. Typhi wild type or the T3SS-1-deficient viaB invA mutant did not result in a statistically significant increase in NF-κB activation and abrogation of RAC1 or RIP2 signaling did not further impact signaling (Fig. 4A, B, and C). In marked contrast, infection with the S. Typhi viaB mutant led to a substantial upregulation of NF-κB-driven responses. Abrogation of RAC1 or RIP2 activity significantly blunted the induction of NF-κB responses in cells infected with the viaB mutant. Moreover, NF-κB activation in cells infected with a viaB sopB sipA mutant was inhibited when cells were transfected with a plasmid construct encoding RAC1-DN (Fig. 4C), suggesting that SopE, translocated into host cells in the absence of TviA, could activate NF-κB signaling in a RAC1-dependent manner. Treatment with the RIP2 inhibitor did not impact T3SS-1-mediated invasion of S. Typhi strains towards epithelial cells (Fig. S5), excluding the possibility that the RIP2 inhibitor inadvertently interfered with the function of the T3SS-1 machinery. Collectively, these data supported the idea that TviA restricts activation of the RAC1-NOD1/2-RIP2 signaling pathway in S. Typhi-infected epithelial cells.

Heterologous expression of TviA in S. Typhimurium blunts T3SS-1-dependent responses in vivo

In addition to repressing T3SS-1 genes, TviA also suppresses flagella expression (Fig. S2A) [57]. Flagellin is known to induce pro-inflammatory responses by activating TLR5 [58] and the

Figure 4. In the absence of tviA, S. Typhi induces NF-κB activation in a RAC1- and RIP2-dependent manner. (A and B) HeLa cells permanently transfected with a NF-κB luciferase reporter system (HeLa 57A) were transfected with pCMV-myc (vector, black bars) or pRAC1-DN (RAC1-DN, white bars) (A) Cells were transfected with the indicated GFP-SopE constructs or infected with the indicated S. Typhi strains for 5 h (N = 3). (B) Cells were mock-treated, infected with the indicated S. Typhi strains, or treated with the NOD1 agonist C12-iE-DAP (an acylated derivative of γ-D-Glu-mDAP) for 5 h. (C) HeLa 57A cells were either treated with dimethyl sulfoxide (DMSO) or RIP2 inhibitor SB203580 dissolved in DMSO and subsequently infected with the indicated S. Typhi strains or were mock treated (bacterial growth media alone) (N = 3). Bars represent geometric means ± standard error. *, P<0.05; **, P<0.01; ns, not statistically significant.
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NLRC4- [nucleotide-binding oligomerization domain (NOD)-like receptor (NLR)] family caspase-associated recruitment domain (CARD)-containing protein 4) inflammasome, while our initial experiments in the bovine ligated ileal loop model suggest that TviA could mitigate mucosal inflammation (Fig. 2), it is conceivable that TviA-mediated gene regulation of flagellar biosynthesis could have affected flagellin-dependent innate immune pathways. To better study consequences of the expression of TviA on the RAC1-NOD1/2-RIP2 signaling pathway in an animal model, we therefore generated a \( \text{phoN::tviA} \) mutant in the \( S. \text{Typhimurium SL1344} \) background (SW760). Akin to the findings with \( S. \text{Typhi} \), expression of TviA in \( S. \text{Typhimurium} \) reduced transcription of T3SS-1 genes (Fig. S3), and the \( \text{phoN::tviA} \) mutant elicited significantly less \( P<0.05 \) NF-κB activation than the \( \text{phoN} \) control strain (Fig. 5A and S6). We next introduced the \( \text{tviA} \) gene into \( S. \text{Typhimurium SL1344} \) derivatives that only expressed the most potent inducers of the NF-κB pathway, SipA (\( \text{sopE2 sopE2 sopE2 sipA phoN::tviA} \) mutant; SW809) and SopE (\( \text{sopE2 sopE2 sipA phoN::tviA} \) mutant; SW807). Upon infection of HeLa cells (Fig. 5B), strains carrying the \( \text{phoN::tviA} \) insertion elicited significantly less luciferase activity than the respective \( \text{phoN} \) mutants \( P<0.01 \), indicating that TviA is able to reduce the NF-κB activation elicited by the \( S. \text{Typhimurium} \) orthologues of SopE and SipA. Inhibition of RIP2 significantly reduced NF-κB activation levels induced by the wild-type strain or the \( \text{phoN} \) mutant (Fig. 5C). The modest response induced by the \( \text{phoN::tviA} \) mutant was further blunted by inhibition of RIP2 signaling \( P<0.05 \) (Fig. 5C), suggesting that TviA-mediated regulation of T3SS-1 is partially able to avoid induction of the NOD1/2-RIP2 pathway in vitro.

To exclude any effects of TviA on flagellin-dependent pathways, we introduced the \( \text{phoN::tviA} \) mutation into a non-motile \( S. \text{Typhimurium} \) strain lacking phase 1 and 2 flagellins, FliC and FljB (\( \text{fliC fljB} \) mutant, SW762) (Fig. 6A). Both the \( \text{fliC fljB} \) mutant and the \( \text{fliC fljB phoN} \) mutant (SW793) elicited significant levels of NF-κB activation in cultured epithelial cells (Fig. 6A), while this response was greatly reduced in cells infected with the \( \text{fliC fljB phoN::tviA} \) mutant (SW764). Inactivation of the essential T3SS-1 gene \( \text{invA} \) completely abolished the ability to induce NF-κB signaling (Fig. 6A).

![Figure 5. Expression of TviA in S. Typhimurium SL1344 reduces T3SS-1-driven NF-κB activation in epithelial cells.](image-url)

HeLa 57A cells were infected with \( S. \text{Typhimurium} \) or mock treated with bacterial growth media (mock treatment) and luciferase activity determined 5 h after infection (N = 4). (A) The SL1344 wild type, an \( \text{invA} \) mutant (SW767), a \( \text{phoN} \) mutant (SW759), and a \( \text{phoN::tviA} \) mutant (SW760) were used to infect monolayers of HeLa 57A cells. (B) Cells were infected with the SL1344 wild type, an isogenic \( \text{sopB sopE2 sopE sipA phoN} \) mutant (SW868), a \( \text{sopB sopE2 sopE2} \) mutant (SW940), a \( \text{sopB sopE2 sipA} \) mutant (SW867), and derivatives thereof carrying a \( \text{phoN} \) (SW808; SW806) or a \( \text{phoN::tviA} \) (SW809; SW807) mutation, respectively. (C) Prior to infection with the indicated \( S. \text{Typhimurium} \) strains, cells were either treated with DMSO or the RIP2 inhibitor (SB203580) dissolved in DMSO. Bars represent geometric means ± standard error. *, \( P<0.05 \); **, \( P<0.01 \); ns, not statistically significant.

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Because introduction of a mutation in cecal mucosa at this early time point were T3SS-1-dependent, Peyer’s patches (Fig. S7). Inflammatory responses observed in the dependent, early inflammatory responses

Collectively, these data suggested that TviA represses T3SS-1-

independent mechanism.

Typhimurium to elicit pro-inflammatory gene expression.

derivatives carrying an additional mutation in 

12 h after infection, the relative abundance of

access by the

S.

treated mouse model [61]. In this model, detection of cytosolic

processes in the intestinal mucosa, we used the Streptomycin pre-

B activation was determined as described above. (B and C) Groups of streptomycin-pretreated C57BL/6 mice were intragastrically inoculated

with either a

TviA

mutant (SW793) (Fig. 6B and C).

P

0.05; **, P<0.01; ns, not statistically significant.

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To directly assess the ability of TviA to impede inflammatory processes in the intestinal mucosa, we used the Streptomycin pre-
treated mouse model [61]. In this model, detection of cytosolic access by the

S. Typhimurium T3SS-1 through the NOD1/2 signaling pathway contributes to intestinal inflammation early during infection [30,34,62]. Compared to mock infected mice, transcript levels of the pro-inflammatory genes Nos2, encoding inducible nitric oxide synthase (iNOS), and Tnfα, encoding tumor necrosis factor (TNF-α, were significantly (P<0.05) elevated in the cecal mucosa at 12 hours after infection with a non-flagellated S. Typhimurium phoN fliC fljB mutant (SW793) (Fig. 6B and C). Introduction of the S. Typhi tviA gene into a S. Typhimurium fliC fljB mutant (phoN::tviA fliC fljB mutant, SW764) significantly (P<0.05) reduced pro-inflammatory gene expression (Fig. 6B and 6C), but not bacterial numbers recovered from intestinal contents or Peyer’s patches (Fig. S7). Inflammatory responses observed in the cecal mucosa at this early time point were T3SS-1-dependent, because introduction of a mutation in invA abrogated the ability of S. Typhimurium to elicit pro-inflammatory gene expression. Collectively, these data suggested that TviA represses T3SS-1-dependent, early inflammatory responses in vivo through a flagellin-independent mechanism.

Discussion

S. Typhi invades the intestinal mucosa without triggering the massive neutrophil influx observed during gastroenteritis caused by non-typhoidal serovars. Here we show that one mechanism for attenuating host responses is a TviA-mediated repression of T3SS-

1, a virulence factor known to induce potent inflammatory host responses. Effector molecules translated by the T3SS-1 into the host cell cytosol activate Rho-family GTPases [15–17]. The activation of Rho-family GTPases is a pathogen-induced process that is sensed by NOD1 [21,30], which ultimately results in the activation of pro-inflammatory responses in vitro [15,20,34] and in vivo [30,62]. However, S. Typhi requires a functional T3SS-1 to invade the intestinal epithelium during infection [27]. Our data suggest that S. Typhi might have evolved to invade the intestinal epithelium without inducing a potent antibacterial inflammatory response by regulating T3SS-1 expression in a TviA-dependent manner. Osmoregulation prevents expression of TviA in the intestinal lumen, which renders S Typhi invasive [42] (Fig. 2A). However, TviA expression is rapidly upregulated upon entry into tissue [63], resulting in repression of T3SS-1 and flagella expression while biosynthesis of the Vi capsule is induced [42,44]. Here we show that TviA prevented NF-κB activation in epithelial cells by reducing T3SS-1-dependent expression of RAC1. Furthermore, the TviA-mediated reduction of T3SS-1-dependent inflammatory responses elicited at early time points in animal models was independent of flagella and the Vi capsule. These data support the hypothesis that TviA attenuates inflammation because it rapidly turns off T3SS-1 expression upon entry into tissue, thereby concealing a pathogen-induced process from the host.

Bovine ligated ileal loops are suited to model the initial 12 hours of host pathogen interaction, a time period during which inflammatory responses are largely T3SS-1-dependent [22,23,64]. Similarly, in the mouse colitis model, inflammatory responses elicited in the cecum at early time points (i.e. during the first 2 days) after infection are largely T3SS-1-dependent [61,65,66]. However, mechanisms independent of T3SS-1 are responsible for cecal inflammation observed at later time points (i.e. at days 4 and 5 after infection) in the mouse colitis model [65]. Expression of the S. Typhi Vi capsular polysaccharide in S. Typhimurium leads to an attenuation of these T3SS-1-independent inflammatory responses in the mouse colitis model [41], by reducing complement activation and TLR4 signaling [67,68]. Thus the viaB locus reduces intestinal inflammation by multiple different mechanisms (Fig. S2A). A TviA-mediated repression of T3SS-1 reduces early inflammatory responses while the Vi capsular polysaccharide attenuates responses generated through T3SS-1-independent mechanisms at later time points. It is tempting to speculate that the result of these immune evasion mechanisms is a reduction in the intestinal inflammatory response that could contribute to differences in disease symptoms caused by typhoidal and non-typhoidal serotypes.

Materials and Methods

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol on mouse experiments was approved by the Institutional Animal Care and Use Committee of the University of California, Davis ( Permit Number: 16179). The protocol on calf experiments was approved by the Institutional Committee at the Universidade Federal de Minas Gerais, Brazil ( Permit Number: CETEA 197/ 2006).

Bacterial strains and culture conditions

The bacterial strains, including relevant properties, are listed in table 1. Unless noted otherwise, bacteria were aerobically grown at 37°C in Luria-Bertani (LB) broth (10 g/l tryptone, 5 g/l yeast
## Table 1. Bacterial strains and plasmids used in this study.

| Strain designation | Relevant characteristics/Genotype | Source/Reference |
|--------------------|-----------------------------------|------------------|
| S. Typhi            |                                   |                  |
| Ty2                | Wild-type strain, Vi⁺             | ATCC 700931b     |
| STY2               | Ty2 ΔviaB::KanR                    | [13]             |
| STY4               | Ty2 ΔviaB::KanR invA::pINV5 (Cm³) | [13]             |
| SW74               | Ty2 Δtvb⁻::E-Cm³ Vi⁻              | [57]             |
| SW242              | Ty2 invA::pINV5 (Cm³)             | [57]             |
| SW347              | Ty2 ΔviaB, Vi⁻                     | [42]             |
| SW559              | Ty2 Δlic                       | [42]             |
| SW398              | Ty2 ΔviaB Δlic invA::pSW127        | This study       |
| SW483              | Ty2 ΔviaB Δlic                   | This study       |
| SW611              | Ty2 Δtvb⁻::E-Cm³ invA::pSW127      | This study       |
| SW904              | Ty2 Δtvb⁻::E, Vi⁻                | This study       |
| SW1207             | Ty2 ΔviaB sopB::MudJ              | This study       |
| SW1208             | Ty2 tvb⁻::E sopB::MudJ            | This study       |
| SW1209             | Ty2 ΔviaB ΔsopE                    | This study       |
| SW1210             | Ty2 tvb⁻::E ΔsopE                  | This study       |
| SW1211             | Ty2 ΔviaB sopB::MudJ ΔsipA         | This study       |
| SW1212             | Ty2 Δtvb⁻::E sopB::MudJ ΔsipA      | This study       |
| SW1213             | Ty2 Δtvb⁻::E sopB::MudJ ΔsopE      | This study       |
| SW1214             | Ty2 ΔviaB ΔsopE ΔsipA             | This study       |
| SW1215             | Ty2 Δtvb⁻::E ΔsopE ΔsipA           | This study       |
| SW1216             | Ty2 ΔviaB sopB::MudJ ΔsopE         | This study       |
| SW1217             | Ty2 ΔviaB sopB::MudJ ΔsopE ΔsipA  | This study       |
| S. Typhimurium      |                                   |                  |
| AJB715             | IR715 phoN::KanR                   | [73]             |
| C5019              | ATCC14028 phoN::Tn10dCm           | [74]             |
| IR715              | ATCC14028 Nal⁺                     | [75]             |
| SL1344             | Strp⁺                             | [76]             |
| SPN305             | IR715 Δlic::pSPN29                  | [42]             |
| SW284              | IR715 phoN: Cm³                   | [42]             |
| SW399              | IR715 invA::pSW127                 | [71]             |
| SW474              | IR715 phoN::tvA-Cm³               | [42]             |
| SW562              | IR715 ΔinvA::TetR                  | [77]             |
| SW737              | IR715 phoN::KanR ΔinvA::TetR      | This study       |
| SW751              | IR715 phoN::pSW208                  | This study       |
| SW759              | SL1344 phoN::Cm³                   | This study       |
| SW760              | SL1344 phoN::tvA-Cm³               | This study       |
| SW761              | SL1344 Δlic                       | This study       |
| SW762              | SL1344 Δlic fjb5001::MudJ           | This study       |
| SW764              | SL1344 Δlic fjb5001::MudJ phoN::tvA-Cm³ | This study     |
| SW766              | SL1344 Δlic fjb5001::MudJ phoN::tvA-Cm³ ΔinvA::TetR | This study     |
| SW767              | SL1344 ΔinvA::TetR                | This study       |
| SW793              | SL1344 Δlic fjb5001::MudJ phoN::pSW208 | This study     |
| SW794              | SL1344 Δlic fjb5001::MudJ phoN::pSW208 ΔinvA::TetR | This study     |
| SW798              | SL1344 sopB::MudJ                  | [30]             |
| SW800              | SL1344 sopB::pSB1039               | [30]             |
| SW806              | SL1344 ΔsipA sopB::MudJ sopE2::pSB1039 phoN::Tn10dCm | This study     |
| SW807              | SL1344 ΔsipA sopB::MudJ sopE2::pSB1039 phoN::tvA-Cm³ | This study     |
| SW808              | SL1344 ΔsopE sopB::MudJ sopE2::pSB1039 phoN::Tn10dCm | This study     |
| SW809              | SL1344 ΔsopE sopB::MudJ sopE2::pSB1039 phoN::tvA-Cm³ | This study     |
extract, 10 g/l NaCl) or LB agar (15 g/l agar). To induce expression of \textit{tviA} and Vi capsule biosynthesis genes, an overnight culture in LB broth was diluted 1:50 in tryptone yeast extract (TYE) broth (10 g/l tryptone, 5 g/l yeast extract) or Dulbecco’s modified Eagle’s medium (DMEM) as indicated and incubated aerobically at 37°C for 3 h. When appropriate, antibiotics were added to LB broth cultures or LB agar plates at the following concentrations: carbenicillin (0.1 mg/ml), chloramphenicol (0.03 mg/ml), kanamycin (0.05 mg/ml), nalidixic acid (0.05 mg/ml), and tetracycline (0.01 mg/ml).

**Construction of plasmids**

Standard cloning techniques were performed to generate the plasmids listed in table 2. Cloning vectors and \(\text{ori}(\text{R6K})\)-based 

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### Table 1. Cont.

| Strain designation | Relevant characteristics\(*/\)Genotype | Source/Reference |
|--------------------|----------------------------------------|------------------|
| SW839              | SL1344 \(\Delta\text{sipA}\)::pSW244    | This study       |
| SW867              | SL1344 \(\Delta\text{sipA} \text{sopB-Mu}\)\(\text{sopE2-}::\text{pSB1039}\) | [30]             |
| SW868              | SL1344 \(\Delta\text{sipA} \text{sopE-}\)\(\text{sopB-Mu}\)\(\text{sopE2-}::\text{pSB1039}\) | [30]             |
| SW940              | SL1344 \(\Delta\text{sopE}\)\(\text{sopB-Mu}\)\(\text{sopE2-}::\text{pSB1039}\) | This study       |
| SW972              | SL1344\(\Delta\text{sipA}\)\(\Delta\text{sopE}\)\(\text{sopB-Mu}\)\(\text{sopE2-}::\text{pSB1039}\) | This study       |
| SW973              | SL1344 \(\Delta\text{sipA}\)\(\Delta\text{sopE}\)\(\text{sopB-Mu}\) \(\text{sopE2-}::\text{pSB1039}\) | [30]             |
| SW974              | IR715 \(\Delta\text{sipA}\)::pSW244     | [30]             |
| SW976              | SL1344 \(\Delta\text{sopE}\)           | [78]             |
| SW977              | SL1344 \(\Delta\text{sopE}\)::pSW245    | This study       |
| SW1009             | SL1344 \(\Delta\text{sopE}\)\(\text{sopE2-}::\text{pSB1039}\) | This study       |
| TH170              | IR715 \(\text{phoN-viaB}\)             | [41]             |

**E. coli**

| Strain designation | Relevant characteristics\(*/\)Genotype | Source/Reference |
|--------------------|----------------------------------------|------------------|
| TOP10              | \(\text{F}^{-}\) \(\text{mcrA}\) \(\Delta\text{mcr-hsd}(-\text{RMS-} \text{mcrBC})\) \(\Phi\)\text{80lacZ}-\text{M15}\) \(\Delta\text{lacX74}\) \(\text{araD139}\) \(\text{araE1}\) \(\text{mrr-}\text{hsdRMS-mcrBC}\) \(\text{lacZYA-argF}\) \(\text{U169}\) \(\text{W80lacZ}\) | Life Technologies |
| OH5\(\text{a}\) \(\text{piR}\) | \(\text{F}^{-}\) \(\text{endA1}\) \(\text{hsdR17}\) (\(\text{r}\)\(^2\)\(\text{m}\)\(^+\)) \(\text{zxx}\)\(::\text{RP4}2\) \(\text{KanR}\) \(\text{araE1}\) \(\text{mrr-}\text{hsdRMS-mcrBC}\)| Laboratory strain collection |
| S17\(\text{I}\) \(\text{a}\) \(\text{piR}\) | \(\text{F}^{-}\) \(\text{endA1}\) \(\text{hsdR17}\) (\(\text{r}\)\(^2\)\(\text{m}\)\(^+\)) \(\text{zxx}\)\(::\text{RP4}2\) \(\text{KanR}\) \(\text{araE1}\) \(\text{mrr-}\text{hsdRMS-mcrBC}\)| Laboratory strain collection |

\(^*\text{Cm}^\text{\textregistered} \): Chloramphenicol resistance; \(\text{Kan}^\text{\textregistered} \): Kanamycin resistance; \(\text{Nal}^\text{\textregistered} \): Nalidixic acid resistance; \(\text{Strep}^\text{\textregistered} \): Streptomycin resistance; \(\text{Tet}^\text{\textregistered} \): Tetracycline resistance (tetRA).

\(^{\text{a}}\text{American Type Culture Collection, Manassas, VA.}\)

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### Table 2. Plasmids used in this study.

| Plasmid designation | Relevant characteristics\(*/\)Genotype | Source/Reference |
|---------------------|----------------------------------------|------------------|
| pCMV-myc            | ori\(\text{(pMB1)}\) \(\text{bla}\) \(\text{PCMVIE}\) \(\text{myc-tag}\) | Clontech         |
| pCR2.1              | Cloning vector                         | Life Technologies |
| pEP185.2            | ori\(\text{(R6K)}\) \(\text{mobRP4}\) \(\text{cat}\) | [80]             |
| pEGFP-C1            | ori\(\text{(pMB1)}\) \(\text{Kan}^\text{\textregistered}\) \(\text{PCMVIE}\) \(\text{EGFP}\) | Clontech         |
| pGFP-SopE           | \(\text{sopE}\) cloned into \(\text{pEGFP-C1}\); \(\text{N}\)-terminal \(\text{GFP}\) tag | [30]             |
| pGFP-SopE-G168A     | \(\text{G168A}\) amino acid substitution in \(\text{sopE}\) in \(\text{pEGFP-C1}\); \(\text{N}\)-terminal \(\text{GFP}\) tag | [30]             |
| pNFkB-luc           | \(\text{NF}^\text{\textregistered}\)-responsive \(\text{luciferase reporter plasmid}\) | [81]             |
| pRAC1-DN            | Dominant-negative form of \(\text{hrAC1}\) cloned into \(\text{pCMV-myc}\); \(\text{T17N}\) \(\text{amino acid substitution substitution; N-terminal myc tag}\) | [30]             |
| pRDH10              | ori\(\text{(R6K)}\) \(\text{mobRP4}\) \(\text{cat}\) \(\text{tetC}\) \(\text{SacB}\) | [82]             |
| pR203               | \(\text{Y. pseudotuberculosis}\) \(\text{invasin gene in pREG153}\) | [83]             |
| pSW28               | Upstream and downstream regions of the \(\text{tviBCDEvexABCDE}\) region of \(\text{S. Typhi Ty2}\) in \(\text{pGP704}\) | [57]             |
| pSW208              | Internal fragment of the \(\text{S. Typhimurium phenN}\) gene cloned into \(\text{pEP185.2}\) | This study       |
| pSW233              | Upstream and downstream regions of the \(\text{tviBCDEvexABCDE}\) region of \(\text{S. Typhi Ty2}\) in \(\text{pRDH10}\) | This study       |
| pSW245              | Upstream and downstream region of the \(\text{S. Typhimurium sopE}\) gene in \(\text{pRDH10}\) | [78]             |
| pTK-LacZ            | Normalization of transfection efficiency | [84]             |
| pTVIA1              | \(\text{tvIA}\) under control of its native promoter in \(\text{pWSK29}\) | [38]             |
| pWSK10              | ori\(\text{(pSC101)}\) \(\text{bla}\) | [85]             |

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suicide plasmids were routinely maintained in E. coli TOP10 and DH3α λpir, respectively.

An internal fragment of the phoN coding sequence was PCR amplified from the S. Typhimurium IR715 chromosome using the primers listed in table 3, subcloned into pCR2.1 (TOPO TA cloning kit, Life Technologies), and cloned into pEP185.2 utilizing the unique XbaI and SacI restriction sites to give rise to pSW208. To generate pSW233, pSW28 was digested with EcoRI and the unique XbaI and SacI restriction sites to give rise to pSW208.

Table 3. Primers used in this study.

| Target                  | Sequence* | Reference |
|-------------------------|-----------|-----------|
| Mutagenesis             |           |           |
| S. Typhimurium phoN     | 5′-TCTAGACGATGGAACAGAAGCTG-3′<br>5′-GAGCTCTAATGCGAAGATGT-3′ | This study |
| Real time PCR           |           |           |
| Salmonella gmk          | 5′-TTGGAGGAGGCAGGTTT-3′ | [86] |
| Salmonella flhD         | 5′-ACAGGTGGTTGATCGGCCG-3′ | [63] |
| Salmonella hiA          | 5′-ATCAAGGCAAGAGATCGGTA-3′<br>5′-GAATAGGAACTCTCCGACGA-3′ | [42] |
| Salmonella invF         | 5′-GGTGCTCAAGATTCAAG-3′ | This study |
| Salmonella prgH         | 5′-CACTGAAAGCTGTTGAAGTT-3′<br>5′-CGCCAGTTATACGAGGTAG-3′ | This study |
| Salmonella sipA         | 5′-TCAAATTAGTGCCTGCG-3′<br>5′-TTCATCAGTAGCGTCTG-3′ | This study |
| Salmonella sopE         | 5′-CAARAMACTTCTACGGAGGA-3′<br>5′-ACATGAGCGGCGATTTG-3′ | This study |
| Murine Gapdh            | 5′-TGGAACAGTTGGTGGAGTCA-3′<br>5′-AGTGTCCTGAGTTGTTGG-3′ | [87] |
| Murine Tnfa             | 5′-AGCCAGGAGGAGAACAGAACAAAC-3′ | [68] |
| Murine Nos2             | 5′-TTCGGTGTGGTTGAACTTTC-3′<br>5′-CGCTTCCAGGTCCTTGGTAG-3′ | [88] |

*restriction endonuclease cleavage sites are underlined.
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Generation of mutants by allelic exchange

Plasmids were introduced into S17-1 λpir and conjugation performed as described previously [57]. The unmarked S. Typhi ΔviaBΔviaC invA mutant SW904 was constructed by inserting the plasmid pSW233 into the STY2 mutant chromosome, selecting for single crossover events (creating merodiploids) on LB agar plates containing Cm and Kan. Sucrose selection was performed as described previously [69] to select for a second crossover event, thus effecting deletion of the tviBCDExeABCDE genes, yielding SW904. The deletion was confirmed by PCR. To facilitate transduction of the unmarked ΔsopE mutation, pSW245 was introduced into this locus in the SW976 chromosome by conjugation with S17-1 λpir as the donor strain, creating SW977 as an intermediate.

Construction of mutants by P22-mediated generalized phage transduction

Phage P22 HT int-105 was utilized for generalized phage transduction in S. Typhimurium as described previously [70]. For S. Typhi recipients, a similar protocol was followed except the multiplicity of infection (MOI) was increased to 100.

A phage lysate of SW399 was used to transduce the invC::pSW127 mutation into SW483 and SW74, thus generating the S. Typhi ΔviaBΔviaC invA mutant (SW398) and the ΔviaBΔviaE invA mutant (SW611). SW1207 and SW1208 were created by transducing the sopB::MuD mutation from SW798 into the ΔsopE mutant (SW347) and the ΔviaBΔviaE mutant (SW904), respectively. The S. Typhi ΔviaBΔsopE (SW1209), ΔviaBΔviaEΔsopE (SW1210), ΔviaBΔsopB::MuDΔsopE (SW1216), and ΔviaBΔviaEΔsopB::MuDΔsopE (SW1213) mutants were constructed by transducing the ΔsopE::pSW243 mutation from SW977 into SW347, SW904, SW1207, and SW1208, respectively. Subsequent sucrose selection allowed selecting for mutants that had lost the plasmid by allelic exchange and generated a clean ΔsopE mutation, thus creating SW1209, SW1211, SW1216, and SW1213, respectively. Similarly, a P22 lysate of SW389 was used to transduce the ΔsopE::pSW244 mutation (SW839) into SW1207, SW1208, SW1209, and SW1210. The intermediates were subjected to sucrose selection, thus creating the clean ΔsopE mutation of strains SW1211, SW1212, SW1214, and SW1215, respectively. The ΔviaBΔsopB::MuDΔsopAΔsopE mutant (SW1217) was generated through
transduction of the ΔphoE::pSW245 mutation from SW977 into the SW1211 chromosome and sucrose selection. The S. Typhimurium SL1344 derivatives SW759 and SW760 were established by transducing the phoN::CamR and phoN::tviA-CmR mutations from SW284 and SW747 into the SL1344 wild type. Transduction of the ΔfljC::SPN29 from SPN305 into the SL1344 wild type and subsequent sucrose selection gave rise to the SL1344 fljC deletion mutant SW761. Subsequent introduction of the βIβ501::Muβ into this strain led to the SL1344 ΔfljC::βIβ501::Muβ6 mutant (SW762). To construct SW764 and SW793, the phoN::tviA-CmR (SW474) and phoN::pSW208 (SW751) mutations were transduced separately into SW762. Invasion-deficient derivatives of these strains were generated by transducing the invA::TetR mutation from SW562 into SW764, SW793, and SL1344, thus creating strains SW766, SW794, and SW767, respectively. SW806, SW807, SW808, and SW809 were generated by transducing the phoN::Tn10dCm (CS019) or phoN::tviA-CmR (SW474) into SW867 or SW940. The ΔinfC::pSW244 mutation (SW974) was moved into the SL1344 wild type to create SW839. SW940 was established by transduction of the sof::Muβ mutation (SW798) into SW796 and subsequent introduction of the sofE2::pSB1039 mutation (SW800). A P22 phage lysate of SW800 was used to create SW792 using SW1009 as the recipient strain. The phoN::kanR mutation from AJ715 was transduced into SW562 to give rise to the phoN::kanR invA::TetR mutant (SW737).

Tissue culture experiments
HeLa 57A cells [29,34] were generously provided by R. T. Hay (the Wellcome Trust Centre for Gene Regulation and Expression, College of Life Sciences, University of Dundee, United Kingdom). HEK-293 cells were obtained from ATCC (ATCC CRL-1573). Both cell lines were routinely cultured at 37°C in a 5% CO₂ atmosphere in DMEM containing 10% fetal bovine serum (FBS) (Life Technologies). For NF-kB activation and invasion experiments, cells were seeded in 24-well plates and 48-well plates (Corning) at densities of 1×10⁵ cells/well and 2×10⁵ cells/well, respectively, and incubated for 24 h prior to subsequent experiments.

Measurement of NF-kB activation in epithelial cells

S. Typhi and S. Typhimurium strains were pre-cultured in TYE broth as described above. HeLa 57A cells or HEK-293 cells transfected with a NF-kB-luciferase reporter construct were infected with the indicated strains at a final concentration of approximately 10⁶ colony forming units (CFU)/ml. To synchronize the infection, plates were centrifuged for 5 min at 500 g at room temperature. After 3 h, cells were washed with DPBS and incubated at 37°C for an additional 2 h in the presence of DMEM containing 10% fetal bovine serum (FBS) (Life Technologies). For NF-kB activation and invasion experiments, cells were seeded in 24-well plates and 48-well plates (Corning) at densities of 1×10⁵ cells/well and 2×10⁵ cells/well, respectively, and incubated for 24 h prior to subsequent experiments.

Bovine ligated ileal loop model
Salmonella Typhimurium was cultured in LB broth at 37°C under agitation, followed by subculture in fresh LB (without antibiotics) for 3 hours, at 37°C under agitation. Four–5 week-old male healthy Salmonella-free Holstein calves were used in this study. Ligated ileal loops were surgically prepared as previously described [23]. Ligated loops were mock treated with intraluminal injection of sterile LB broth or inoculated with 3 ml of suspensions containing 1×10⁸ CFU of the S. Typhimurium ATCC14028 phoN mutant (AJ715), a phoN::cyaB mutant (TH170), a phoN::tviA mutant (SW474), or a phoN::invA mutant (SW737). Ligated loops were surgically removed at 5 h after infection for tissue sampling and measurement of intraluminal fluid accumulation. Samples containing the intestinal mucosa and the associated lymphoid tissue were collected with a 6 mm biopsy punch. Each intestinal biopsy was kept in sterile PBS with 50 μg/ml of gentamicin for 1 h, homogenized in 2 ml of PBS, serially diluted, and plated on LB agar plates containing nalidixic acid. Additional biopsies were fixed by immersion in 10% buffered formalin, processed for paraffin embedding, cut and stained with hematoxylin and eosin. Histopathologic changes including hemorrhage, neutrophilic infiltration, edema, and necrosis and/or apoptosis were scored from 0 to 3 (0 for absence of lesions, and 1, 2, or 3 for mild, moderate, or severe lesion, respectively) for a combined total score ranging from 0 to 12.

Invasion assays
Invasiveness of the indicated Salmonella strains was determined using a Gentamicin protection assay as described previously [71]. Briefly, HeLa 57A cells were infected at a MOI of 5 with Salmonella strains pre-cultured in TYE broth. After 1 h, cells were washed and media containing 0.1 mg/ml Gentamicin was added for 90 min. Diluted cell lysates (0.5% Triton-X-100) were spread on LB agar plates to determine the number of CFU per well. Invasiveness was calculated as percentage of recovered bacteria compared to the inoculum.

Bacterial gene expression analysis
Overnight cultures of the indicated S. Typhi and S. Typhimurium strains were diluted 1:50 in TYE broth and incubated at 37°C for 3 h. Total RNA was extracted from approximately 2×10⁸ CFU using the Aurum Total RNA Mini Kit (Biorad). 1 μg of total RNA was subjected to a separate DNase treatment (DNA-free kit, Life Technologies) and converted to cDNA using MvUL reverse transcriptase (Life Technologies) in a 25 μl volume as described previously [71]. 4 μl of this cDNA was used as the template for real time PCR analysis with the primers listed in table 3. Data was acquired on a ViiA 7 real-time PCR instrument (Life Technologies). Relative target gene expression was normalized to mRNA levels of the house keeping gene gmk, encoding guanylate kinase (ΔΔCt method). DNA contamination was less than 1% for all amplicons as determined by a separate RT-PCR mock reaction lacking reverse transcriptase.
Mouse colitis model

Animals were obtained from The Jackson Laboratory (Bar Harbor), housed under specific-pathogen-free conditions and provided with water and food ad libitum. Groups of female, 9–12 week old C57BL/6 mice were orally treated with 20 mg Streptomycin. After 24 h, these mice were inoculated as described previously [61] with either 0.1 ml LB broth (mock treatment) or 1 × 10⁹ CFU of the S. Typhimurium SL1344 fliC fliB phoN mutant (SW793), the fliC fliB phoN viaB4A mutant (SW764), the fliC fliB phoN invA mutant (SW794), or the fliC fliB phoN viaB4A invA mutant (SW766) suspended in 0.1 ml LB broth. 12 h after infection, animals were euthanized and tissues were collected. The bacterial load was determined by spreading serial 10-fold dilutions of homogenates on LB agar plates containing the appropriate antibiotics. Flash-frozen colonic tissue was homogenized in a Mini-beadbeater (Biospec Products) and RNA was extracted by the TRI reagent method (Molecular Research Center). cDNA was generated using MuLV reverse transcriptase and reverse transcription reagents (Life Technologies). SYBR Green (Life Technologies)-based real-time PCR was performed as described previously [72] using the primers listed in table 3. Data was acquired by a ViaA 7 real-time PCR system (Life Technologies) and analyzed using the comparative Ct method (ΔΔCt method). Murine target gene transcription within each sample was normalized to the respective levels of Gapdh mRNA.

Statistical analysis

Data obtained from tissue culture experiments, bacterial gene transcription experiments, and the bovine ligated ileal loop model was log-transformed prior to analysis with a paired Student’s t-test. Data obtained from tissue culture experiments, bacterial gene transcription experiments, and the bovine ligated ileal loop model was log-transformed prior to analysis with a paired Student’s t-test. Recovered bacterial inoculum. (C) Cells were infected at a multiplicity of infection (MOI) of 10 for 1 h and extracellular bacteria killed by treatment with Gentamicin for 90 min. Recovered bacterial numbers were standardized to the number of the bacteria in the inoculum. (D) To determine NF-κB activation, luciferase activity measured 5 h after infection (N = 3). Bars represent geometric means ± standard error. **, P<0.01; ns, not statistically significant. (TIF)

Figure S2 TviA reduces T3SS-1-induced NF-κB activation independent of flagellin expression. (A) Schematic representation of the TviA regulatory network in S. Typhi and effect on host signaling pathways. (B and C) HeLa 57A cells (B) or HEK-293 cells transiently transfected with a NF-κB-dependent reporter plasmid (pNFkB-luc) (C) were infected with a S. Typhi ΔfliC mutant (SW359), a ΔfliC ΔviaB mutant (SW483), derivatives carrying the cloning plasmids pWSK29 (pWSK) or the plasmid pTV1A1, and a ΔfliC ΔviaB invA mutant (SW398). Luciferase activity was quantified 5 h after infection to determine NF-κB activation levels (N = 3). Bars represent geometric means ± standard error. *, P<0.05; **, P<0.01; ***, P<0.001. (TIF)

Figure S3 Effect of TviA on bacterial gene expression in vitro. The S. Typhi wild type Ty2 (WT), a ΔviaB mutant (SW347), a ΔviaB invA mutant (SW74), the S. Typhimurium wild-type SL1344, a SL1344 phoN mutant (SW759), a SL1344 phoN::tetA mutant (SW760), the S. Typhimurium 14028 Na⁺ wild type (IR715), a 14028 Na⁺ phoA mutant (AJ715), and a 14028 Na⁺ phoA::tetA (SW474) were cultured in TYE broth for 3 h. RNA was extracted and qRT-PCR performed to determine the relative abundance of fliD (A), hilA (B), invF (C), pgpH (D), sopE (E), and sopE (F) mRNA. Data presented is fold change over the abundance of mRNA recovered from the respective wild-type strain after standardization to the housekeeping gene gmk. The dotted line indicates no change in gene expression. Bars represent geometric means from 3 (S. Typhimurium) or 4 (S. Typhi) independent experiments ± standard error. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not statistically significant. (TIF)

Figure S4 Contribution of SopE, SipA, SopB, and SopE2 to NF-κB activation in human epithelial cells. HeLa 57A cells were treated with media only (mock treatment) or infected with the indicated S. Typhimurium SL1344 derivatives. Certain Salmonella strains lacked defined T3SS-1 effector proteins to analyze the responses induced by SopB (light grey bar), SipA (white bar), and SopE (dark grey bar). NF-κB activation was assessed 5 h after infection based on a NF-κB-driven luciferase reporter system (N = 4). Bars represent geometric means ± standard error. **, P<0.01; ns, not statistically significant. (TIF)

Figure S5 Inhibition of RIP2 signaling does not affect invasiveness of S. Typhi strains. HeLa 57A cells pretreated with DMSO or RIP2 inhibitor (SB203580; dissolved in DMSO) were infected with the indicated S. Typhi strains at a MOI of 10 and invasion determined by a Gentamicin protection assay. Bars represent geometric means ± standard error. ns, not statistically significant. (TIF)

Figure S6 Expression of TviA in S. Typhimurium 14028 Na⁺ reduces T3SS-1-driven NF-κB activation in epithelial cells. HeLa 57A cells were infected with the S. Typhimurium 14028 Na⁺ derivatives or treated with bacterial growth media (mock treatment). Luciferase activity determined 5 h after infection (N = 3). Bars represent geometric means ± standard error. **, P<0.01; ns, not statistically significant. (TIF)

Figure S7 Bacterial colonization in the mouse colitis model. (A and B) Streptomycin-pretreated mice were infected with the indicated S. Typhimurium SL1344 derivatives as described in Figure 6. The bacterial load in the colon content (A) and the Peyer’s patches (B) was determined 12 h after infection. Bars represent geometric means ± standard error. ns, not statistically significant. (TIF)

Author Contributions

Conceived and designed the experiments: SEW MGW VP AMK TS FF RLS AJB. Performed the experiments: SEW MGW VP AMK TS FF LCF FC EAC GESA TAP RLS AJB. Analyzed the data: SEW MGW VP AMK TS FF LCF FC EAC GESA TAP RLS AJB. Wrote the paper: SEW RLS AJB.

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