Salmonella type III effector SpvC, a phosphothreonine lyase, contributes to reduction in inflammatory response during intestinal phase of infection

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Summary

Salmonella phosphothreonine lyase SpvC inactivates the dual-phosphorylated host mitogen-activated protein kinases (MAPK) through β-elimination. While SpvC can be secreted in vitro by both Salmonella pathogenicity island (SPI)-1 and SPI-2 type III secretion systems (T3SSs), translocation of this protein into the host cell cytosol has only been demonstrated by SPI-2 T3SS. In this study, we show that SpvC can be delivered into the host cell cytoplasm by both SPI-1 and SPI-2 T3SSs. Dephosphorylation of the extracellular signal-regulated protein kinases (ERK) was detected in an SPI-1 T3SS-dependent manner 2 h post infection. Using a mouse model for Salmonella enterocolitis, which was treated with streptomycin prior to infection, we observed that mice infected with Salmonella enterica serovar Typhimurium strains lacking the spvC gene showed pronounced colitis when compared with mice infected with the wild-type strain 1 day after infection. The effect of SpvC on the development of colitis was characterized by reduced mRNA levels of the pro-inflammatory cytokines and chemokines, and reduced inflammation with less infiltration of neutrophils. Furthermore, the reduction in inflammation by SpvC resulted in increased bacterial dissemination in spleen of mice infected with Salmonella. Collectively, our findings suggest that SpvC exerts as an anti-inflammatory effector and the attenuation of intestinal inflammatory response by SpvC is involved in systemic infection of Salmonella.

Introduction

Several Gram-negative bacteria deliver a repertoire of effector proteins, which act as anti-inflammatory effector molecules, into host cells through the type III secretion system (T3SS) to interfere with host inflammatory pathways (Alfano and Collmer, 2004; He et al., 2004; Mudgett, 2005; Navarro et al., 2005; Gaán and Wolff-Watz, 2006; Grant et al., 2006). These signalling cascades in the host cell include the NF-κB signalling pathway (Ribet and Cossart, 2010). For example, OspG from Shigella flexneri interacts with ubiquitin conjugating enzymes whose targets include IκBa, and interferes with IκBa degradation inhibiting the NF-κB activation (Kim et al., 2005). S. flexneri also secretes IpaH9.8, an effector with E3 ubiquitin ligase activity (Rohde et al., 2007; Ashida et al., 2010). Similarly, the acetylating activity of YopJ from Yersina enterocolitica has been implicated in the inhibition of the NF-κB pathway (Mittal et al., 2006; Mukherjee et al., 2006). Recently, a type III secretion effector, NleC, from enteropathogenic and enterohaemorrhagic Escherichia coli (EPEC and EHEC) was identified as a zinc protease that suppresses NF-κB activation by cleaving its subunit p65 (Yen et al., 2010).

In addition to the NF-κB pathway, the mitogen-activated protein kinase (MAPK) pathway is another central signalling cascade that is essential for host immune response. Therefore, several pathogens target the MAPK signalling pathway to facilitate their infection. Recently, several studies provided evidence that the Shigella type III effector OspF directly targets and inhibits MAPK activities, leading to bacterial survival and proliferation at the initial infection site (Arbibe et al., 2007; Kramer et al., 2007; Li et al., 2007). OspF specifically dephosphorylates extracellular signal-regulated protein kinases (ERK) 1/2, p38 kinases and probably c-Jun NH2-terminal kinases (JNK) through phosphothreonine lyase activity, thereby dampening the host immune response to bacterial infection. Particularly, inhibition of MAPK activities by OspF attenuates recruitment of polymorphonuclear leucocytes to...
infected tissues in mouse and rabbit animal models (Arbibe et al., 2007). Other virulence factors including SpvC from Salmonella enterica serovar Typhimurium (S. Typhimurium), VirA from Chromobacterium violaceum and HopAI1 from the plant pathogen Pseudomonas syringae possess the same phosphothreonine lyase activity (Li et al., 2007; Zhang et al., 2007; Mazurkiewicz et al., 2008).

Salmonella enterica serovar Typhimurium, a facultative intracellular bacterium that colonizes the intestinal tract and causes inflammatory diarrhoea, encodes two distinct type III secretion machineries within its pathogenicity islands 1 (SPI-1) and 2 (SPI-2). It was previously demonstrated that effector proteins translocated through both SPI-1 and SPI-2 T3SSs are important in eliciting intestinal inflammation (Coombes et al., 2005; Hapfelmeier et al., 2008). SPI-1 effectors are required to invade intestinal epithelial cells and trigger cytokine release during the early stages of infection even in the absence of SPI-2 T3SS, while SPI-2 effectors mediate the induction of mucosal inflammation in the intestine at later stages of infection. Salmonella strains lacking SPI-1 and SPI-2 cannot elicit intestinal inflammation (Coombes et al., 2005).

SpvC, a Salmonella T3SS effector, shares 63% identical amino acids with Shigella OspF and inactivates ERK1/2, p38 and JNK in vitro as a phosphothreonine lyase (Li et al., 2007; Zhu et al., 2007; Mazurkiewicz et al., 2008). While SpvC is secreted into the culture supernatant by either SPI-1 or SPI-2 T3SS, translocation of SpvC into the cytoplasm of infected macrophages is only demonstrated by the SPI-2 T3SS (Mazurkiewicz et al., 2008). More importantly, SpvC is a virulence factor required for systemic infection in mice (Matsui et al., 2001). The Salmonella strain expressing SpvC from plasmids show significantly reduced cytokine production, including IL-8 and TNF-α, from infected host cells (Mazurkiewicz et al., 2008). Although the effect of SpvC on the host’s immune response in mouse infection is still unclear, it appears that SpvC downregulates localized cytokine production at infection sites, thereby facilitating bacterial growth and colonization.

In this study, we demonstrated that translocation of SpvC into the host cell cytosol is dependent on SPI-1 T3SS when bacteria are grown under SPI-1 inducing conditions. In addition, using the streptomycin-pretreated mouse model infected with S. Typhimurium that causes acute enterocolitis characterized by massive infiltration of neutrophils, we demonstrated that the Salmonella spvC mutant induces more pro-inflammatory cytokines in infected mouse caeca than the wild-type strain at an early stage of infection. We further found that by evasion of the immune response in the intestinal mucosa by SpvC in the early stages of infection facilitates bacterial dissemination at the later stages of infection. These findings suggest that SpvC can play a role in modulating the intensity of the inflammation as an anti-inflammatory effector and enhance Salmonella growth at the infection site, leading to spread of bacteria to systemic organs.

Results

SpvC is translocated into host cells by not only SPI-2 T3SS but also SPI-1 T3SS

As SpvC is rapidly degraded within host cells, it is difficult to detect the translocated protein by immunofluorescence microscopy in cells infected with Salmonella (Mazurkiewicz et al., 2008). Therefore, to examine whether SpvC is delivered to the host cell cytosol by SPI-1 T3SS, we constructed a gene fusion between spvC and cyaA, which encodes the activity domain of the calmodulin-dependent adenylate cyclase from Bordetella pertussis and used this as an enzymatic reporter for protein translocation (Sory and Cornelis, 1994). The SpvC–CyaA fusion protein was then produced in S. Typhimurium wild-type or in a mutant strain lacking invA (SPI-1 T3SS-deficient mutant), ssaV (SPI-2 T3SS-deficient mutant) or invA and ssaV genes (SPI-1 and SPI-2 T3SSs-deficient mutant). To confirm the SPI-1 T3SS function in the strains tested, we used SipC, a component of the SPI-1 T3SS translocon, fused to CyaA (SipC–CyaA) as a positive control. Each strain was cultivated under SPI-1 T3SS-inducing conditions and evaluated for production and secretion of SpvC–CyaA by Western blot analysis with rabbit anti-SpvC antiserum. As with the SipC–CyaA fusion protein, the SpvC–CyaA fusion protein was detected in culture supernatant collected from the wild-type and SPI-2 mutant strains with a functional SPI-1 T3SS, but not from the invA and invA ssaV double mutant strains, which are deficient in SPI-1 T3SS function (data not shown). Analysis of whole-cell fractions revealed that a similar level of SpvC–CyaA fusion protein was detected in the wild-type and in all mutant strains constructed (data not shown), indicating that the inability of the invA mutant to secrete the SpvC–CyaA is due to a defect in protein secretion.

To examine SPI-1 T3SS-dependent delivery of SpvC into host cells, HeLa cells were infected with the wild-type or mutant strain deficient invA, ssaV or invA ssaV, each carrying a plasmid expressing SpvC–CyaA fusion protein. Salmonella strains were grown under SPI-1 inducing conditions and infected with HeLa cells. After 2 h of infection, the HeLa cells were lysed and cytosolic levels of cAMP were measured. Infection with the wild-type strain expressing CyaA fusion to SpvC led to a dramatic increase in cytosolic levels of cAMP compared with cells infected with a strain harbouring the vector plasmid without an insert, indicating that SpvC–CyaA is translo-
SpvC can be translocated into host cells by SPI-1 and SPI-2. Translocation of SpvC–CyaA was assayed at 2 h after S. Typhimurium infection of HeLa cells (A) and at 18 h after infection of RAW264.7 cells (B). Each bar represents the average ± standard error values from three independent experiments. Asterisks indicate that differences are statistically significant (P < 0.05).

Fig. 1. SpvC can be translocated into host cells by SPI-1 and SPI-2. Translocation of SpvC–CyaA was assayed at 2 h after S. Typhimurium infection of HeLa cells (A) and at 18 h after infection of RAW264.7 cells (B). Each bar represents the average ± standard error values from three independent experiments. Asterisks indicate that differences are statistically significant (P < 0.05).

SpvC inactivates ERK1/2, p38 and JNK MAPKs through its phosphothreonine lyase activity (Li et al., 2007; Mazurkiewicz et al., 2008). Inactivation of ERK1/2 MAPK by SpvC is dependent on a functional SPI-2 T3SS (Mazurkiewicz et al., 2008). Our study demonstrated that SpvC is translocated into host cells by SPI-1 and SPI-2 T3SSs (Fig. 1). To determine whether SpvC translocated via SPI-1 T3SS inactivates MAPKs, we examined dephosphorylation of ERK1/2 using ΔB-Raf:ER cells. This cell line is a derivative of NIH 3T3 fibroblast cells in which the kinase domain of B-Raf is expressed as a fusion protein with the hormone-binding domain of the oestrogen receptor (ΔB-Raf:ER), and activation of the ERK1/2 signalling pathway can be induced by addition of 4-hydroxy-tamoxifen (4-HT), an antagonist of oestrogen and suppressed by the MEK inhibitor U0126 (Fig. 2A) (Pritchard et al., 1995). To confirm the availability of this cell line, we performed in vitro dephosphorylation assays with purified GST-SpvC or GST-SpvC K136A, an inactive mutant of SpvC (Li et al., 2007; Zhu et al., 2007). The lysates from ΔB-Raf:ER cells treated with 4-HT were incubated with GST-SpvC and subjected to immunoblotting with a rabbit monoclonal anti-phosphorylated ERK1/2 (phospho-ERK1/2) antibody. As expected, phospho-ERK1/2 was completely reduced when the cell lysate was incubated with GST-SpvC, but not GST-SpvC K136A or GST alone (Fig. 2B), indicating that ΔB-Raf:ER cells is available for dephosphorylation assays with SpvC.

Next, to examine whether the inactivation of MAPK in host cells is dependent on SPI-1 T3SS function, the amount of phospho-ERK1/2 in 4-HT-treated ΔB-Raf:ER cells infected with different S. Typhimurium strains was analysed by immunoblot analysis using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. Treatment with 4-HT induced accumulation of phospho-ERK1/2 in uninfected ΔB-Raf:ER cells (mock) and in cells infected with either ΔspvC or SPI-1 T3SS mutant strains (Fig. 2C). In contrast, infection with the wild-type or SPI-2 mutant showed significantly reduced activation of ERK1/2 in cells treated with 4-HT (Fig. 2C). Introduction of a plasmid expressing SpvC, but not SpvC K136A, into the ΔspvC mutant could decrease the amount of phospho-ERK1/2 in ΔB-Raf:ER cells treated with 4-HT at the wild-type level (Fig. 2D). However, the same levels of activated ERK1/2 were detected when 4-HT-treated ΔB-Raf:ER cells were infected with an SpvC overexpressing SPI-1 mutant (Fig. 2D), indicating that the translocation of SpvC into host cells and inactivation of ERK1/2 in host cells are required for a functional SPI-1 T3SS.
SpvC modulates intestinal inflammation reaction during early stages of S. Typhimurium infection

SPI-1 T3SS is required by Salmonella to invade and replicate within intestinal epithelial cells, and to stimulate innate immune response in the intestinal mucosa, resulting in an acute inflammation of intestines (Hapfelmeier and Hardt, 2005). This SPI-1-induced inflammation is triggered in the early stages of infection and exhibits many pathological changes including epithelial damage, submucosal oedema and infiltration of neutrophils into the lamina propria (Barthel et al., 2003). Therefore, we hypothesized that SpvC would influence early inflammatory responses in vivo. To demonstrate this, we induced colitis in streptomycin-pretreated mice and evaluated the pathological changes in these mice early after S. Typhimurium infection. We first determined that a mutation in spvC would not affect bacterial colonization in the caecum, colon contents, Peyer’s patches, mesenteric lymph nodes, spleen or liver of mice infected with S. Typhimurium. BALB/c mice were treated with streptomycin 24 h prior to infection with S. Typhimurium wild-type, ΔspvC, ΔSPI-1 or ΔSPI-2 strains (C), and trans-complemented wild-type, ΔspvC, ΔSPI-1 or ΔSPI-2 strains (D). Asterisks indicate that differences were statistically significant (P < 0.05).

Fig. 2. SpvC dephosphorylates ERK1/2 MAPK.
A. Immunoblots of phospho-ERK1/2 and ERK1/2 in ΔB-raf:ER cell lines were treated with 4-HT or U0126 to activate or inhibit the ERK1/2 signalling pathway.
B. Immunoblots of phospho-ERK1/2 and ERK1/2 in the activated ΔB-Raf:ER cells incubated with GST-SpvC proteins.
C and D. Dephosphorylation assays of the activated ΔB-Raf:ER cells infected with S. Typhimurium wild-type, ΔspvC, ΔSPI-1 or ΔSPI-2 strains (C), and trans-complemented wild-type, ΔspvC, ΔSPI-1 or ΔSPI-2 strains (D). Asterisks indicate that differences were statistically significant (P < 0.05).
large plasmid, or ΔspvC (spvC K136A'), a ΔspvC mutant replacing ΔspvC with an spvC K136A allele on the large plasmid. Infected mice were sacrificed 1 day after infection. No significant differences in the bacterial numbers were observed in these samples at this time (Fig. 3A and data not shown).

Next, macroscopic analysis revealed that mice infected with ΔspvC and ΔspvC (spvC K136A') mutants showed similar acute caecal inflammation 1 day after infection (Fig. 3B). In contrast, caeca from mice infected with the wild-type and ΔspvC (spvC') mutant strain were the same as those from mock-infected mice, except for a slightly reduced caecum size (Fig. 3B). Another quantitative hallmark of intestinal inflammation is reduced caecum weight. We found that mice infected with ΔspvC and ΔspvC (spvC K136A') mutants had markedly decreased caecum weight compared with those infected with the wild-type and ΔspvC (spvC') mutant strain (Fig. 3C). Histopathological examination of the caeca of these mice supported the above results. As shown in Fig. 3D, mice infected with S. Typhimurium wild-type and ΔspvC (spvC') at day 1 after infection showed weak pathological changes in their caeca including a slight increased infiltration of neutrophils and macrophages into the lamina propria compared with mock infection. In contrast, much more severe pathological changes, such as submucosal oedema, a reduced number of goblet cells and epithelial erosions were observed in mice infected with ΔspvC as well as ΔspvC (spvC K136A') mutant strains. These histopathological changes were quantitatively confirmed by measuring the thickness of the caecal mucosa and the number of neutrophils infiltrating the lamina propria of the infected caeca (Fig. 3E and F). In contrast, any significant difference in the intestinal mucosa, where severe inflammatory responses are exhibited, were not detected in streptomycin-pretreated mice infected with either the wild-type or ΔspvC mutant strain 3 days after infection (data not shown). These results clearly indicate the possibility that SpvC is able to modulate the intestinal inflammatory reaction at infection sites during early stages of infection.

**SpvC modulates intestinal inflammation**

mRNA was extracted from the caeca of streptomycin-pretreated mice mock-infected or infected with *Salmonella* strains. As shown in Fig. 4, significantly higher mRNA levels of *Ifng*, *Tnfa*, *Kc*, *Mcp1* and *Mip2* were observed in the caeca of mice infected with ΔspvC or ΔspvC (spvC K136A') compared with the wild-type or ΔspvC (spvC') mutant strain. Collectively, these results suggest that SpvC and its enzymatic activity are responsible for the reduced production of inflammatory cytokines in the intestinal mucosa during early stages of *Salmonella* infection.

In addition, to address which T3SS is related to reduction of inflammatory responses, mice were infected with ΔSPI-1 or ΔSPI-2 and ΔspvC mutant strain, and pro-inflammatory cytokines and chemokines were measured. Lower expression levels of *Ifng*, *Kc* and *Mip2* genes were observed 24 h after infection with ΔSPI-1, or ΔSPI-1 and ΔspvC compared with the wild-type and the expression levels were similar between ΔSPI-1, and ΔSPI-1 and ΔspvC infection. (Fig. S1). In contrast, the mRNA levels in the caeca of mice infected with ΔSPI-2, and ΔSPI-2 and ΔspvC are the same as that of the wild-type (Fig. S1). Unexpectedly, no significant differences in expression levels were detected in caeca from mice infected with between ΔSPI-2, and ΔSPI-2 and ΔspvC strains (Fig. S1). These data suggest that not only SPI-1 but SPI-2 T3SSs contributes to attenuation of intestinal inflammatory responses during *S. Typhimurium* infection of streptomycin pretreated mice.

**SpvC inactivates ERK1/2 MAPK in mouse intestinal mucosa after *Salmonella* infection**

To assess the direct role of SpvC during early stages of infection, phospho-MAPKs levels in caeca infected with *Salmonella* strains were examined by immunohistochemistry using rabbit monoclonal anti-phospho-ERK1/2, anti-phospho-p38 or anti-phospho-JNK antibody 1 day after infection. Consistent with the results described above, a significant increase in phospho-ERK1/2 MAPK levels was detected in the villus epithelial cells and lamina propria of caeca infected with ΔspvC or ΔspvC (spvC K136A') mutant strains compared with caeca infected with the wild-type or ΔspvC (spvC') strain at similar levels to the mock infection (Fig. 5). However, no significant differences in phospho-p38 or phospho-JNK levels were observed in the caeca infected with all strains (Fig. S2). MAPKs signalling pathways are stimulated by cytokines, stress or growth factors and influence apoptosis, cell growth differentiation as well as inflammation.

To further analyse the role of SpvC in *Salmonella* pathogenesis, we demonstrated whether SpvC affects apoptosis or cell proliferation *in vitro*. To clarify the possibility that SpvC suppresses apoptosis, we examined the activity of
Fig. 3. SpvC reduces intestinal inflammation 1 day after S. Typhimurium infection of streptomycin-pretreated mice.
A. Bacterial numbers recovered from the caeca of mice after infection.
B. Photographs of caeca removed from the mice after infection; scale bars: 1 cm.
C. Caecum weights of the mice after infection.
D. Histopathological appearance of the murine caeca; scale bars: 100 µm.
E. Submucosa thickness of the murine caeca.
F. The number of neutrophils infiltrating the submucosa of the murine caeca.
Asterisks indicate that differences are statistically significant ($P < 0.05$). ns, not statistically significant.
caspase-3 and -7 in HeLa cells transfected with SpvC. No suppression of these caspases was observed in the SpvC-transfected cells (Fig. S3A). Next, to study whether SpvC is involved in cell proliferation, we determined the number of viable cells in culture based on quantification of the ATP present, which signals the presence of metabolically active cells. No difference in cell number transfected with pEGFP-C1 and pEGFP-SpvC was detected (Fig. S3B). Finally, we verified that SpvC blocks NF-κB signalling pathway, which trigger intestinal inflammatory responses as well as MAPK. No blocking was detected in the HeLa cells transfected with SpvC (Fig. S3C).

Collectively, we clearly demonstrated that SpvC modulates intestinal inflammation during the early stages of Salmonella infection through the downregulation of the ERK1/2 MAPK signalling pathway.

SpvC allows systemic infection by S. Typhimurium by evading intestinal immune response

Reduced inflammatory response in the intestinal mucosa due to infection by S. enterica serovar Typhi (S. Typhi) is required for the dissemination of bacteria to systemic organs (Tsolis et al., 2008). In the case of S. Typhi, the viaB locus which is absent from the S. Typhimurium genome is implicated in attenuating intestinal inflammatory responses by evading the host immune systems (Pickard et al., 2003; Wilson et al., 2008). Therefore, to determine the role of SpvC in systemic Salmonella infection, the streptomycin-pretreated mice were infected with the S. Typhimurium wild-type and ΔspvC mutant strains, and bacterial translocation to the spleen was monitored 2 day after infection. While no difference was found between the number of S. Typhimurium strains recovered from the colon contents (Fig. 6A), a decreased number of bacteria from spleens infected with S. Typhimurium strains lacking a functional spvC gene were found when compared with those infected with the wild-type and the complemented strain ΔspvC (spvC+) (Fig. 6B). However, the wild-type strain and the spvC-deficient mutant strains were recovered in comparable numbers from spleens of orally infected mice without pretreatment with streptomycin, which showed no inflammatory response in the intestinal mucosa, at the same stage of infection (data not shown). These data indicate that the reduced intestinal inflammation by SpvC allows bacterial dissemination in systemic organs.

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**Fig. 5.** SpvC inactivation of ERK1/2 by phosphothreonine lyase activity.

A. Immunohistochemical analysis of anti-phospho-ERK1/2 immunostaining of sections of caecum from streptomycin-pretreated mice after 24 h of infection with *S.* Typhimurium wild-type, ΔspvC, ΔspvC (spvC+) and ΔspvC (spvC K136A†). Boxes indicate the area shown at the higher magnification; scale bars: 100 μm. Data are representative of three experiments.

B. The number of phosphorylated ERK1/2-positive cells of the caeca from *Salmonella*-infected mice. Asterisks indicate that differences were statistically significant (*P* < 0.05).

**Fig. 6.** SpvC promotes bacterial dissemination in the systemic stage of infection. The streptomycin-pretreated mice were inoculated intragastrically with *S.* Typhimurium strains. Bacterial numbers recovered from colon contents (A) and spleen (B) at 2 days after infection. Asterisks indicate that differences were statistically significant in wild-type infection (*P* < 0.05). ns, not statistically significant.

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Discussion

The recognition of conserved bacterial antigens by specific pattern-recognition receptors activates defence responses that are essential against non-pathogenic and pathogenic microorganisms. The early immune response triggered by bacterial antigens include the activation of MAPK signalling cascades and transcriptional reprogramming in host animals and plants (Ausubel, 2005; Akira et al., 2006; He et al., 2006). To prevent early elimination by host defence systems, Salmonella modulates host immune response by delivering anti-inflammatory effectors into the host cells. To control the host immune response, Salmonella uses a T3SS effector, SpvC, which is a phosphothreonine lyase, and its enzymatic activity inactivates MAPKs including ERK1/2, p38 and JNK (Mazurkiewicz et al., 2006; Zhu et al., 2007; Mazurkiewicz et al., 2008). Although overexpression of SpvC in Salmonella strains significantly reduces cytokine production (Mazurkiewicz et al., 2008), the role of SpvC in Salmonella systemic infection has yet to be clarified. In this study, we found that Salmonella SpvC modulates host immune response by reducing inflammatory cytokines during the early stages of infection. It suggests that this reduction is due to the dephosphorylation of MAPK, at least ERK1/2, by the direct effect of SpvC as phosphothreonine lyase. Nevertheless, some previous reports have been indicated that SpvC dephosphorylates ERK1/2, p38 and JNK in vitro; however, we showed the inactivation in only ERK1/2 in vivo. The differences may be explained by the antibody specificity or variations in assay conditions. Interestingly, it has been published that IL-8 chemokine production induced by Salmonella infection is inhibited by the MEK inhibitor U0126 (Murli et al., 2001). This suggests that activation of ERK1/2 is required for Salmonella-induced inflammation. In addition, ERK1/2 phosphorylation induces the expression of nucleoporins to regulate nucleocytoplasmic transport (Kosako et al., 2009). Therefore, dephosphorylation of ERK1/2 by SpvC may be blocked export of some transcriptional factors from the cytoplasm to nucleus, resulting in reduction of intestinal inflammation.

Recent studies have examined the in vivo role of SpvC using mouse models and found no evidence that SpvC is required for bacterial colonization and reduced immune response in the intestinal mucosa (Kappeli et al., 2011). This discrepancy is probably due to different Salmonella strains used for these studies. In the Salmonella-induced enterocolitis model, the intensity of the inflammatory reaction is affected both by virulent phenotypes of Salmonella strains and the host background genetics, including the Nramp1 genotype (Valdez et al., 2009). Infection of streptomycin-pretreated mice with S. Typhimurium strain SL1344, a highly virulent strain in mice, results in severe inflammatory responses accompanied by histopathological changes such as submucosal oedema, a reduced number of goblet cells and epithelial erosion 8 h after infection (Barthel et al., 2003). In contrast, infection by ATCC 14028 exhibits a delayed inflammatory response and takes longer (~ 48 h after infection) to reach the same levels of inflammatory reaction as by strain SL1344 (Haneda et al., 2009), indicating that infection by strain SL1344 elicits a faster host inflammatory response than ATCC 14028 strain in S. Typhimurium-induced colitis. Therefore, the effect of SpvC may not be detected by strong inflammatory responses triggered by strain SL1344 in the intestinal mucosa early after infection.

Previous data have shown that SpvC is translocated through SPI-2 T3SS into the host cytoplasm relatively late in the process of infection by intracellular bacteria (Mazurkiewicz et al., 2008). In this study, we found that SpvC functions as not only SPI-2 but also SPI-1 effectors and is produced by extracellular bacteria after bacterial contact with host cells. Although it was initially suggested that SPI-1 and SPI-2 T3SSs induce different environmental conditions and function independently, recent observations have provided evidence of considerable functional overlap between SPI-1 and SPI-2 during the infection processes. For example, SPI-1 effector SopB persists in Salmonella-infected macrophages many hours after invasion, and regulates macrophage-inducible nitric oxide synthase levels (Drecktrah et al., 2005). Likewise, SPI-2 is typically expressed intracellularly, but its expression also occurs during the initial stage of infection in the intestinal lumen (Brown et al., 2005). Furthermore, SPI-1 regulator HilD differentially regulates both SPI-1 and SPI-2 regulons in a growth phase-dependent manner during growth in LB medium (Bustamante et al., 2008). Therefore, it is likely that the dual roles of SPI-1 and SPI-2 are important for Salmonella pathogenesis during the intestinal and/or systemic phases of infection. Indeed, in addition to the importance of SpvC translocation through SPI-2 T3SS for bacterial growth during systemic infection (Matsui et al., 2001; Mazurkiewicz et al., 2008), translocation of SpvC through SPI-1 T3SS is a key mechanism that facilitates successful dissemination of bacteria to systemic organs from the initial sites of infection.

Salmonella enterica cause severe intestinal inflammation at the entry site of infection. This inflammatory response is triggered by SPI-1 effectors, SopE, SopE2 and SopB that activate Rho GTPases Rac-1 and Cdc42. Activation of these GTPases leads to the stimulation of MAPK and NF-kB pathways, which trigger the intestinal inflammatory response (Bruno et al., 2009). In addition, SopE induces caspase-1 activation to elicit intestinal inflammation (Müller et al., 2009). The triggering of an
inflammatory response is a common virulence strategy of enteropathogenic bacteria that allows them to adhere and colonize host tissues by competing with the microbial flora (microbiota), which provide an efficient barrier against non-resident bacteria, termed colonizationresistant flora (microbiota), which provide an efficient barrier that allows them to adhere and colonize host tissues by competing with the microbial flora. Thus, the manipulation of cell signalling pathways by Salmonella SPI-1 effectors modulates the activated MAPK pathway by its phosphothreonine lyase activity and thus modulates transcriptional reprogramming to pro-inflammatory cytokines (see Fig. 7). Importantly, the effectors SpvC and SspH1 are translocated to host cells by both SPI-1 and SPI-2 T3SSs, indicating that both T3SSs participate in the process of repressing the activated inflammation during the intestinal phase of infection.

A murine model of enteric salmonellosis provides a tool for elucidating the molecular details of host–pathogen interactions and facilitates our understanding of the roles of SPI-1 and SPI-2 in the generation of intestinal inflammation during Salmonella infection. In this study, we showed that an SPI-1 and SPI-2 effector SpvC targets phosphorylated ERK in intestinal epithelial cells and negatively modulates the MAPK signalling pathway by its phosphothreonine lyase activity. We also demonstrated that SpvC is responsible for the anti-inflammatory effect of Salmonella during the early intestinal phases of infection and that the reduced inflammatory response in the intestinal mucosa, is required to spread the bacteria to systemic sites. Overall, the presence of multiple anti-inflammatory effectors modulating MAPK and NF-κB pathways in Salmonella indicate that the manipulation of cell signalling is essential for successful systemic infection by this bacteria.

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Experimental procedures

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table S1. The S. Typhimurium wild-type strain used in this study was ATCC 14028 and its spontaneous nalidixic acid-resistant derivative strain SH100 (Gotth et al., 2003). E. coli DH5α and S17.1::pir were used as hosts for the construction of plasmids and for the conjugation of the pir-dependent suicide plasmid pWM91 (Metcalf et al., 1996) into S. Typhimurium respectively. Unless otherwise indicated, bacteria were grown in Luria–Bertani (LB) broth or on LB agar. Antibiotics were added to the media at the following concentrations: ampicillin, 100 µg ml⁻¹; chloramphenicol, 25 µg ml⁻¹; nalidixic acid, 50 µg ml⁻¹; or kanamycin, 25 µg ml⁻¹.

Construction of plasmids and mutant strains

The bacterial plasmids and primers used in this study are listed in Tables S1 and S2 respectively. To construct the complementing plasmids pYS2, spvC gene was amplified from the virulence plasmid of S. Typhimurium ATCC 14028 with primers, spvC-C-F1 and spvC-C-R1 and the PCR product was cloned into BamHI and SalI sites of pMW118 (Nippon Gene). Plasmid pYS5 was constructed by cloning the point-mutated spvC gene (spvC K136A) on pMW118. spvC K136A mutation was constructed with overlapping primers spvC (K136A)-F1 and spvC (K136A)-R1 using GeneTailor™ Site-Directed Mutagenesis System (Invitrogen). The mutation was verified by DNA sequencing.

A CyaA reporter plasmid, pMW-CyaA-2HA encoding the cyaA-2HA fusion gene, was used for the translocation assay of SpvC into host cells. To construct pMW-CyaA-2HA, the cyaA-2HA fragment was amplified from plasmid pMS109 carrying the cyaA gene of B. pertussis (Sory and Cornelis, 1994) with primers BP-cyaA-F and HA-Sph and the PCR product was cloned into pMW118. spvC or sipC (for control as an SPI-1 effector) genes were amplified from S. Typhimurium ATCC 14028 genomic DNA using primers spvC-CyaA-F1 and spvC-CyaA-R1 (for spvC) or sipC-CyaA-F1 and sipC-CyaA-R1 (for sipC). The amplified PCR products were cloned into the EcoRI and SacI sites of pMW-CyaA-2HA, generating plasmids pKO34 and pMY1 respectively. Similarly, to construct pACPJ-spvC-CyaA-2HA, spvC gene was amplified by PCR with spvC-CYA-A-F2 and spvC-Cya-A-R2 primers and cloned into pACPJ-CyaA-2HA (Miki et al., 2009) using In-Fusion HD cloning kit (Clonetech).

To construct plasmids expressing GST-SpvC and GST-SpvC K136A fusion proteins, spvC and spvC K136A genes were amplified using the primers SpvC-BamHI-FW2 and SpvC-SalI-RV2, and PCR products were cloned into the BamHI and SalI sites of pGEX-6P-1 (GH Healthcare).

To construct the spvC-deleted mutant, ΔspvC (HG204), the suicide vector pWM91 (Metcalf et al., 1996) was used. Two DNA fragments, spvC-A and spvC-B were amplified by PCR with primers, spvC-F1 and spvC-R2 for (spvC-A) and spvC-F2 and spvC-R2 (for spvC-B) using S. Typhimurium ATCC 14028 genomic DNA as a template. PCR fragments spvC-A and spvC-B were digested with HindIII and XbaI and HindIII respectively. Both digested spvC-A and spvC-B fragments were cloned into the HindIII and XbaI sites on pBluescript SKII(+), generating pHG1, which contains a region of deleted amino acids from 75 to 228 in spvC. A XhoI–NotI fragment of pHG1 was cloned into the same sites on pWM91. The resulting plasmid was transferred from E. coli S17.1::pir to S. Typhimurium SH100 by conjugation.

The PCR-based λ Red recombination system (Datsenko and Wanner, 2000) using pKD46 and pKD4 was performed to knock-in the wild-type spvC gene or the mutant type spvC gene (spvC K136A). In order to make SpvC knock-in mutants, PCR was performed using spvC-KI-F1 and spvC-KI-R1 primers. pYS2 or pYS5 plasmids (see above), containing the wild-type spvC gene or the mutant-type gene (spvC K136A), respectively, were used as templates. The DNA fragment containing the kanaR cassette was amplified by PCR with the primer set spvC-KI-F2-P1 and spvC-KI-R2-P2 from pKD4. Then, a second PCR was performed using the amplified spvC gene and kanaR cassette to construct spvC::kan fragments. Finally, spvC-kan and spvC K136A-kan were amplified with primers, spvC-KI-F3 and spvC-KI-R3, from the constructed spvC-kan or spvC K136A-kan fragment, then introduced into S. Typhimurium strain SH100 carrying plasmid pKD46 to give rise to strain TH1244 and TH1245 respectively. The insertions and the mutations were verified using PCR and DNA sequencing.

To construct S. Typhimurium TM937 (ΔssaV), a ΔssaV::cat mutation was transduced from SL1344 (Miki et al., 2009) into SH100 using a P22 phage. A catR cassette was eliminated by pCP20 (Datsenko and Wanner, 2000). S. Typhimurium TM1180 (invA::kan) and TH1267 (invA::kan ΔssaV) were constructed by P22 phage transduction of invA::kan mutation from a derivative of strain SL1344 (Galán et al., 1992) to strain SH100 and the ΔssaV mutant strain TM937.

Cell lines

HeLa cells were routinely cultured in minimal essential medium (MEM, Sigma) supplemented with 10% fetal calf serum (FCS). RAW264.7 and ΔB-Raf/ER cells (Pritchard et al., 1995) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 10% FCS.

CyaA translocation assay

Translocation of SpvC via T3SS into host cells was measured using cAMP Biotrak Enzyme immunoassay (EIA) system (GE Healthcare). To demonstrate the translocation of the SpvC–CyaA fusion protein, HeLa or RAW264.7 cells were seeded into 24-well plates at a density of 2 x 10⁵ cells per well. S. Typhimurium strains expressing CyaA fusion proteins were grown for 16 h at 37°C in LB broth with shaking, diluted to 1:50 with LB broth containing 0.3 M NaCl and inoculated for 3 h without shaking until an A₆₀₀ of 0.6–0.8 was reached to induce functional SPI-1 T3SS (Bajaj et al., 1995). To induce SPI-2 T3SS, these bacteria were grown with shaking for 16 h prior to infection. Cells were infected with S. Typhimurium strains expressing CyaA fusion proteins at a multiplicity of infection (moi) of 100. Plates were centrifuged at 250 g for 1 min at room temperature to synchronize infection. After incubation for 60 min at 37°C in 5% CO₂, free bacteria were removed from the cells by three washes with PBS. An aliquot of 0.5 ml of DMEM supplemented with 10% heat-inactivated fetal bovine serum and 100 µg ml⁻¹ gentamicin were added to each well, and plates were incubated at 37°C in 5% CO₂. After more than 60 min, cells were washed three times with
PBS and lysed using the standard protocol recommended by the manufacturer. cAMP EIA was performed using an Ultraspec Visible Plate Reader II 96 (Amersham Biosciences). The data from at least three independent experiments is represented as means ± standard errors.

Dephosphorylation assay

ΔB-Raf:ER cells were seeded in 24-well plates at a density of 2 × 10⁴ cells per well. To activate ERK1/2 pathway, 4-hydroxytamoxifen (4-HT, Sigma) was added to cells at a final concentration of 1 μM before 30 min of infection by S. Typhimurium strains. ΔB-Raf:ER cells were treated with 20 μM U0126; a MEK1/2 inhibitor (Promega) was used as a negative control. Cells were infected with S. Typhimurium strains under the same conditions as the CyaA translocation assay. After 2 h of infection, cells were washed three times with PBS containing 1 mM Na₃VO₄ and 10 mM NaF, and solubilized with SDS sample buffer. The resulting samples were boiled for 5 min, separated by SDS-PAGE on a 10% gel and transferred to polyvinylidene fluoride membranes (Millipore). Proteins were analysed by immunoblotting with rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), anti-phospho-p38 MAPK (Thr180/Tyr182) or anti-phospho-SAPK/JNK (Thr183/Tyr185) rabbit antibody respectively (Cell Signalling Technology).

RNA extraction and quantitative real-time PCR

RNA was extracted from the mouse caecum using Tri reagent (Molecular Research Center) as described previously (Raffatellu et al., 2007). cDNA was synthesized using a QuantTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed with the primer pairs listed in Table S2 using QuantTect SYBR Green and the 7900HT Sequence Detection System (Applied Biosystems). The data were analysed using the comparative Ct method (Applied Biosystems).

Statistical analysis

Student’s t-test was used to analyse protein translocation, phosphothreonine lyase activity, caecum weight, bacterial number and cytokine expression. Values of P < 0.05 were considered statistically significant.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Supplemental experimental procedures.

Table S1. Bacterial strains and plasmids used in this study.

Table S2. Primers used for constructions of strains.

Fig. S1. Both SPI-1 and SPI-2 T3SSs are required for reduction in inflammatory responses by SpvC. Transcript levels of Sixf (A), Mip2 (B) and Kc (C) at 24 h after infection were measured by quantitative real-time PCR. Bars represent fold changes (geometric means) in mRNA levels compared with a group of mock-infected mice from the same point of time. Variability between samples is indicated by standard error error bars. ns indicates that differences were not statistically significant.

Fig. S2. SpvC is not involved in dephosphorylation of phospho-p38 and phospho-JNK. Immunohistochemical analysis of anti-phospho-p38 or phospho-JNK immunostaining of sections of caecum from streptomycin-pretreated mice after 24 h of infection with S. Typhimurium wild-type, ΔspvC, ΔspvC (spvC+) and ΔspvC

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(spvC K136A<sup>+</sup>). Scale bars: 100 μm. Data are representative of three experiments.

**Fig. S3.** SpvC does not affect other MAPK phenotypes, apoptosis and cell proliferation, and NF-κB activation.

A. Caspase-Glo 3/7 assay in HeLa cells transfected pEGFP-C1 or pEGFP-SpvC. Cells treated with 1 μM staurosporine (STS), an inducer of apoptosis and/or 1 μM of the broad-spectrum caspase inhibitor, Z-VAD-FMK, were used as control.

B. CellTiter-Glo ATP-based proliferation assay in HeLa cells transfected with pEGFP-C1 or pEGFP-SpvC. Cells treated with 5 μg ml<sup>−1</sup> actinomycin D, an inhibitor of gene transcription, were used as control. Asterisks indicate the difference was statistically significant compared with pEGFP-C1-transfected cells.

C. Fold increase in NF-κB-dependent luciferase activity in pEGFP-C1-, pEGFP-NleC- or pEGFP-SpvC-transfected HeLa cells unstimulated (white bar) or stimulated with TNF-α (black bar). Asterisk indicates the difference was statistically significant compared with pEGFP-C1-transfected cells stimulated with TNF-α.

**Fig. S4.** Other *Salmonella* type III effectors, AvrA and SspH1, reduce intestinal inflammation as well as SpvC. Transcript levels of *Ifng* (A), *Mip2* (B) and *Kc* (C) at 24 h after infection were measured by quantitative real-time PCR. Asterisks indicate that differences were statistically significant (*P* < 0.05).

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