Salmonella virulence factor SpiC is involved in expression of flagellin protein and mediates activation of the signal transduction pathways in macrophages

Kei-ichi Uchiya and Toshiaki Nikai

Correspondence

Kei-ichi Uchiya
kuchiya@ccmfs.meijo-u.ac.jp

Department of Microbiology, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan

SpiC is a virulence factor encoded within *Salmonella* pathogenicity island 2 (SPI-2). We have previously reported that infection of macrophages with *Salmonella enterica* serovar Typhimurium results in the SPI-2-dependent activation of the mitogen-activated protein kinase (MAPK) signalling pathways, leading to the expression of suppressor of cytokine signalling (SOCS)-3, which is involved in the inhibition of cytokine signalling. Here, we investigated the mechanism by which SpiC mediates the activation of signal transduction pathways in macrophages. Proteomic analysis showed that the level of FliC protein, a component of the flagellar filaments, was lower in the culture supernatant of a *spiC* mutant than in the supernatant from wild-type *Salmonella*. Furthermore, quantitative real-time RT-PCR showed that this mutant had a much lower level of *fliC* mRNA, indicating that SpiC regulates the transcription of FliC. We also found that the level of SOCS-3 in J774 macrophages was lower when they were infected with the *fliC* mutant than with wild-type *Salmonella*. FliC participated in the activation of MAPK pathways in *Salmonella*-infected macrophages, leading to the upregulation of SOCS-3 expression. Collectively, these results indicate that SpiC is involved in the expression of FliC, which plays a significant role in the SPI-2-dependent activation of MAPK pathways in *Salmonella*-infected macrophages.

Received 24 June 2008

Revised 6 August 2008

Accepted 7 August 2008

INTRODUCTION

Macrophages play a central role in host defence against many types of infection, and their function is affected by various mediators, including cytokines and eicosanoids. Gamma interferon (IFN- γ) is an essential contributor to macrophage activation and promotes the effective killing of pathogens that can survive within macrophages (Bhardwaj *et al.*, 1986; Green *et al.*, 1990; Suzuki *et al.*, 1988; van Dissel *et al.*, 1987). On the other hand, macrophage functions can be blocked by interleukin-4 (IL-4), IL-10 and transforming growth factor- β , as well as by prostaglandin E₂ (Bogdan & Nathan, 1993; Kunkel *et al.*, 1988; O'Farrell *et al.*, 1998; Strassmann *et al.*, 1994). Pathogen-associated molecular patterns (PAMPs), which are components of bacteria and viruses, are able to induce the expression of mediators by several types of cells, including macrophages, and influence the host immune system (Janeway & Medzhitov, 2002). PAMPs include cell wall components

derived from Gram-positive bacteria, LPS from Gram-negative bacteria, lipoteichoic acid, and flagellum, all of which are recognized by Toll-like receptors (TLRs). Specifically, bacterial cell wall components and lipoteichoic acid are recognized by TLR2 (Schwandner *et al.*, 1999), and LPS and flagellin bind TLR4 and TLR5, respectively (Hayashi *et al.*, 2001; Poltorak *et al.*, 1998). Ligand binding by TLRs leads to the induction of immune and inflammatory genes (Akira & Takeda, 2004).

Salmonellae are Gram-negative bacteria that cause a variety of disease syndromes in humans and animals. *Salmonellae* invade and destroy specialized epithelial cells in the host intestine, and migrate to the mesenteric lymph nodes, where they encounter, and subsequently survive and replicate within macrophages. Specific virulence factors encoded within the *Salmonella* pathogenicity island (SPI) are required at various stages of *Salmonella* infection (Groisman *et al.*, 1999). *Salmonella enterica* serovar Typhimurium, which causes gastroenteritis in humans and a systemic disease in mice similar to human typhoid fever, harbours two kinds of type III protein export systems: one for flagellar proteins as described above, and one for virulence factors. Several genes from the two systems share sequences in common (Aizawa, 2001;

Abbreviations: ERK, extracellular signal-regulated kinase; IFN, interferon; IL, interleukin; JNK, c-Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor κ B; PAMP, pathogen-associated molecular pattern; SOCS, suppressor of cytokine signalling; SPI-2, *Salmonella* pathogenicity island 2; TLR, Toll-like receptor; TTSS, type III secretion system.

Macnab, 2004). Some important virulence factors are directly delivered into the host environment by two different type III secretion systems (TTSSs) encoded on SPI-1 and SPI-2. The SPI-1 TTSS mediates bacterial entry into nonphagocytic cells (Galan, 2001), and the SPI-2 TTSS is required for survival and replication in the intracellular environment of host cells and for systemic infection in mice (Hensel *et al.*, 1998; Ochman *et al.*, 1996; Shea *et al.*, 1996).

SpiC is a virulence factor encoded within SPI-2. Previous studies have shown that a strain carrying a mutation in the *spiC* gene is unable to survive within macrophages and has greatly reduced virulence in mice. The SpiC protein is necessary for inhibiting the fusion of *Salmonella*-containing phagosomes with endosomal and lysosomal compartments (Uchiya *et al.*, 1999). Moreover, this protein is translocated by the SPI-2 TTSS to the cytosol of macrophages, where it interacts with host proteins such as TassC (Lee *et al.*, 2002) and Hook3 (Shotland *et al.*, 2003) to alter intracellular trafficking. On the other hand, several investigators have reported that SpiC is required for the translocation of SPI-2 effector proteins into target cells by interacting with SsaM, a SPI-2-encoded protein (Freeman *et al.*, 2002; Yu *et al.*, 2002, 2004). In addition, our recent studies have shown that SpiC is involved in *Salmonella*-induced activation of the signal transduction pathways in macrophages, leading to not only the production of IL-10 and prostaglandin E₂ but also the expression of suppressor of cytokine signalling (SOCS)-3, which is involved in the inhibition of IL-6 and IFN- γ signalling (Uchiya *et al.*, 2004; Uchiya & Nikai, 2004, 2005). As described above, the production of these mediators is thought to have an important role in the intracellular survival of *Salmonella* and its escape from the host defence system via modification of macrophage function.

In the present study, we investigated the mechanism by which SpiC mediates the activation of signal transduction pathways in macrophages following *Salmonella* infection. We found that SpiC promotes the expression of FliC at the transcriptional level. This expression of FliC activates mitogen-activated protein kinase (MAPK) signalling pathways, which play a significant role in the SPI-2-dependent induction of SOCS-3 expression by *Salmonella* infection.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains used in this study were derived from the wild-type *S. enterica* serovar Typhimurium strain 14028s. The *spiR::kan* derivative EG5799 and the *spiC::kan* derivative EG10128 have been described by Ochman *et al.* (1996) and Uchiya *et al.* (1999), respectively. The deletion mutants of *fliC* and *fljB* gene were constructed using the Red recombination system (Datsenko & Wanner, 2000). To delete the *fliC* gene, a kanamycin-resistance gene flanked by FLP recognition target sites from plasmid pKD4 was amplified by PCR using primers with regions homologous to the *fliC* gene (5'-GATCATGGCACAAGTCA-TTAATACAAACAGCCTGTCGCTGTTGACGTGTAGGCTGGAGC-

TGCTTC-3' and 5'-ATCAATCGCCGGATTAACGCAGTAAAGAG-AGGACGTTTTGCGGAACATATGAATATCCTCCTTAGT-3'). Kanamycin-resistant strains were obtained by transforming the PCR products into strain 14028s harbouring the λ Red recombinase on plasmid pKD46. Disruption of the *fliC* gene was confirmed by PCR using the *fliC*-specific primers. The kanamycin-resistance gene was then removed by transforming the strain with plasmid pCP20, which expresses the FLP recombinase, resulting in an in-frame deletion of the *fliC* gene. The *fljB*, *spiB* and *ssaV* genes were deleted in the same way but using the following primers: for *fljB*, 5'-TAA-CGTAACAGAGACAGCACGTTCTGCGGGACCTGGTTAGCCTG-CGTGTAGGCTGGAGCTGCTTC-3' and 5'-TTATGGCACAAGT-AATCAACACTAACAGTCTGTGCTGCTGACCCCATATGAATA-TCCTCCTTAGT-3'; for *spiB*, 5'-TTACAAGGCCGGGAAGTATGG-CTGAATGAAGGTAACCTGTCACCTGGTGTAGGCTGGAGCTGC-TTC-3' and 5'-GGATAGTTAATCAAAGTATCATAATGTTTAAT-CGTTACCACATCGCATATGAATATCCTCCTTAGT-3'; and for *ssaV*, 5'-AAATTTCTGGAGTCGCAATGCGTTCATGGTTAGGTG-AGGGAGTCAGTGTAGGCTGGAGCTGCTTC-3' and 5'-CATTG-TCCGCCAACTCCTCTTCGCTAAGGTCAATACTTTCTACCACA-TATGAATATCCTCCTTAGT-3'. Plasmid pEG9127 is a derivative of pBAC108L and contains the cloned *spiC* gene (Uchiya *et al.*, 1999). Bacteria were grown at 37 °C in Luria broth (LB). Kanamycin was used at 50 μ g ml⁻¹.

RNA preparation and quantitative real-time RT-PCR. Bacteria were grown in LB. When the OD₆₀₀ of the culture reached 1.8, total RNA was isolated using an RNeasy kit (Qiagen) in accordance with the manufacturer's protocol. The isolated RNA was treated with DNase I (Invitrogen) to remove contaminating DNA, and 2 μ g RNA was reverse-transcribed with SuperScript II reverse transcriptase using random primers. Real-time PCRs were performed in 50 μ l reaction mixtures containing 1 μ l cDNA, 0.9 μ M of each primer, 0.25 μ M of each fluorescent probe, and TaqMan Universal Master Mix (Applied Biosystems). Amplification was carried out in 96-well optical plates on a 7300 Real-Time PCR system (Applied Biosystems) with an initial incubation for 2 min at 50 °C, followed by 10 min at 95 °C and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The housekeeping gene 16S rRNA was used as an internal standard for quantification of the total RNA. The primer pairs and fluorescent probes were designed using Primer Express software version 3.0, and synthesized by Applied Biosystems. The specific fluorescent probes were labelled at the 5' end with the reporter dye 6-carboxyfluorescein (FAM). The sequences of the primer-probe combinations were as follows: for *fliC*, 5'-GGATACGCTGAATGTGCAACA-3', 5'-GAGTCACCTCACCGTT-CGTCTTA-3', and 6-FAM-CAGGATATGCCGATATA (fluorescent probe); and for 16S rRNA, 5'-AGATGGGATTAGCTTGTGTTGA-3', 5'-GTAACGTCATGCTGCGGTGA-3', and 6-FAM-CCACAA-CACCTTCCTC (fluorescent probe). Threshold cycle values were calculated from the amplification plots, and the amount of *fliC* gene expression was determined relative to the level of gene expression in wild-type *Salmonella* after both values were normalized to 16S rRNA levels. Each sample was analysed in triplicate.

Construction of *fliC-lacZ* fusion on a plasmid. To construct a transcriptional fusion of the *fliC* promoter region to the promoterless *lacZ* gene in the promoter-probe vector pRL124 (Malo & Loughlin, 1988), a 0.45 kb DNA fragment containing the *fliC* promoter region was amplified by PCR using two primers, 5'-CGGGGTACCGGCTATTTCGCCGCCTAAGA-3' and 5'-CCGGAATTCGCTGTTAGCA-GACTGAACCG-3'. The PCR product, digested with *KpnI* and *EcoRI*, was ligated into the corresponding sites of pRL124, producing pRL-*fliC*.

β -Galactosidase assay. Bacteria were grown overnight in LB at 37 °C, and diluted 1:100 into fresh LB and grown with aeration to OD₆₀₀ 1.8. β -Galactosidase activity was measured with the substrate

o-nitrophenyl β -D-galactoside, as described elsewhere (Miller, 1972). Each sample was assayed in triplicate.

Preparation of secreted and whole-cell proteins. An overnight culture in LB was inoculated into 15 ml fresh LB at a 1:100 dilution. The cultures were grown at 37 °C with mild aeration to OD₆₀₀ 1.8. A 1 ml sample of the culture was centrifuged at 18 500 *g* for 15 min to remove bacterial cells or supramolecules such as flagella or pili. After filtration through a 0.2 μ m pore-size filter (Advantec), filtrates were mixed with TCA (final concentration 6%), placed on ice for 30 min, and centrifuged at 14 000 *g* for 20 min. After drying, the pellets were dissolved in 20 μ l SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2.5 % SDS, 10 % glycerol, 0.01 % bromophenol blue, 25 mM DTT) and boiled for 5 min. For preparation of whole-cell proteins, the bacterial pellet from the centrifugation step was suspended in 1 ml cold water and processed as described above. The pellets were dissolved in 200 μ l SDS sample buffer and boiled for 5 min.

2D gel electrophoresis. Secreted proteins from bacteria grown to OD₆₀₀ 1.8 were extracted and solubilized in rehydration buffer [8 M urea, 2 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.4 % immobilized pH gradient buffer (pH 3–10), 0.001 % bromophenol blue and 50 mM DTT]. After quantification of protein by the Bradford assay (Bio-Rad) with BSA as a standard, a total of 125 μ l lysate containing 50 μ g protein was applied to 7 cm IPG ReadyStrips covering a pH range from 3 to 6 (Bio-Rad). IEF of proteins was carried out with the PROTEAN IEF cell (Bio-Rad) using a preset method that allowed a minimum of 10 000 V-h. Focused strips were subjected to SDS-PAGE using a 10 % acrylamide gel and then stained with Bio-Safe Coomassie (Bio-Rad).

Protein identification by MALDI-TOF MS. Protein spots were excised from the Coomassie-stained 2D gels, washed in 1:1 (v/v) 25 mM NH₄HCO₃/acetonitrile, and subjected to in-gel digestion with 50 ng trypsin μ l⁻¹ (Promega) overnight at 37 °C. The extracted peptides were desalted with a ZipTip column (Millipore) and then analysed by MALDI-TOF MS using a Voyager PK2 mass spectrometer (Applied Biosystems). Proteins were identified using the MASCOT program.

Cell culture and bacterial infection. J774 E, a mannose-receptor-positive murine macrophage cell line, was maintained at 37 °C in an incubator containing 5 % CO₂ in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10 % heat-inactivated fetal calf serum (HyClone), 100 U penicillin ml⁻¹, and 100 μ g streptomycin ml⁻¹. The day before infection, the macrophages were plated at a density of 1.0×10^6 per well in six-well tissue-culture plates (Falcon) or 0.4×10^6 per well in 24-well plates in medium without antibiotics. Bacteria (25 per macrophage) were added, and the plates were centrifuged at 500 *g* for 10 min at room temperature. The cells were then incubated for 20 min at 37 °C to permit phagocytosis, and free bacteria were removed by three washes with PBS (Sigma-Aldrich). After washing, Dulbecco's modified Eagle's medium containing 12 μ g gentamicin ml⁻¹ was added to kill extracellular bacteria, after which the cells were incubated at 37 °C for the indicated times.

pcDNA3.1-*fliC* plasmid construction and transfection. The *fliC* gene was amplified by PCR using the primers 5'-CGGGATC-CATGGCACAAGTCATTAATACA-3' and 5'-CCGGAATTTTAACG-CAGTAAAGAGAGGAC-3'. The PCR product was digested with *Bam*HI and *Eco*RI and then ligated into the corresponding sites of pcDNA3.1(+) (Invitrogen), producing pcDNA3.1-*fliC*. The resulting construct was verified by direct sequencing. For transfection experiments, macrophages were plated at a density of 1.0×10^6 per well in six-well plates and then transfected with 2 μ g pcDNA3.1-*fliC* plasmid using FuGENE HD transfection reagent (Roche) according to the manufacturer's protocol. After 30 h, cytosolic extracts were prepared from the macrophages and analysed by Western blotting.

For controls, macrophages were transfected with the same amount of empty vector.

Immunofluorescence microscopy. Macrophages were grown on glass coverslips in 24-well cell-culture plates and infected as described above. At 3 h after infection, samples were fixed in 3.7 % paraformaldehyde for 10 min and washed three times in PBS. Samples were then permeabilized in 0.2 % Triton X-100 for 10 min to allow entry of the primary antibodies, including rabbit polyclonal anti-*Salmonella* antibodies (Bioscience) and murine monoclonal anti-*FliC* antibodies (BioLegend). The secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 594-conjugated goat anti-mouse IgG. Samples were mounted onto glass slides using Vectashield (Vector laboratories) and viewed at $\times 63$ magnification on a confocal laser scanning microscope (LSM510 META, Zeiss).

Western blot analysis. Western blot analyses were performed essentially as described previously (Uchiya & Nikai, 2004). The peptide fragment of the *FliC* and *FljB* polypeptides with a common sequence VQSANSTNSQSDLDLSIQ was synthesized, and antiserum specific for the oligopeptide was obtained by immunization of rabbits with the peptide coupled to keyhole limpet haemocyanin using benzidine. The resulting anti-flagellin peptide antibody was used at a dilution of 1:300. Cell lysates from macrophages infected with the respective *Salmonella* strains were prepared, and detection of SOCS-3 protein and the three MAPKs, including extracellular signal-regulated kinase (ERK), p38 and c-Jun amino-terminal kinase (JNK), was performed as described previously (Uchiya & Nikai, 2005). I κ B- α was detected with a 1:500 dilution of anti-I κ B- α antibody (Santa Cruz Biotechnology).

Statistical analysis. Each experiment was performed at least three times. The results are expressed as means $\pm$ SDs. The data were analysed by analysis of variance with Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Identification of flagellin protein by MALDI-TOF MS

The *spiC* gene is adjacent to the *SpiR* (*SsrA*)/*SsrB* two-component regulatory genes, and is the initial gene of operons that encode the structural and secretory components of SPI-2. In the present study, to further examine the function of *SpiC*, we carried out a proteomic analysis of the culture supernatant proteins from bacteria grown to OD₆₀₀ 1.8 in LB, because the expression of the *spiC* gene has been reported to be induced at OD₆₀₀ >1.5 when the bacteria are grown in LB (K.-i. Uchiya and T. Nikai, unpublished results). We used 2D PAGE to compare the proteins in culture supernatants from the wild-type strain and a *spiC* mutant, which carries a nonpolar mutation in the *spiC* gene. The 2D gel profiles revealed a protein spot (Fig. 1, indicated by black arrowheads) that was more intense in the secreted proteins from the wild-type strain than in those from the *spiC* mutant. The corresponding spots were excised from the gel, digested with trypsin, and analysed by MALDI-TOF MS. The MASCOT program was employed for the identification of the protein spots. Search results using the NCBI nr protein database showed that protein scores

Fig. 1. 2D electrophoretic profiles of culture supernatant proteins from wild-type *Salmonella* (WT) and its *spiC* mutant. Bacteria were grown in LB, and the proteins secreted in the media were extracted when the OD₆₀₀ was 1.8. 2D gel electrophoresis was performed by using IEF gel strips (pH 3–6) followed by SDS-PAGE. The protein spots indicated by the black arrowheads were more intense for wild-type *Salmonella* than for the *spiC* mutant. The protein in these spots was identified as FliC by peptide mass fingerprinting using MALDI-TOF MS. The molecular masses and pH ranges are indicated.

greater than 60 were significant ($P < 0.05$), and there was only one protein with a protein score of 70. MASCOT identified both spots as containing the flagellin protein FliC, a component of flagellar filaments, suggesting that the absence of SpiC results in a reduction in the level of secreted FliC.

SpiC is involved in the expression of flagellin protein

S. enterica serovar Typhimurium expresses two antigenically distinct flagellins that are encoded by the *fliC* and *fljB* genes and coordinately expressed by a phase-variation

mechanism (Macnab, 1996). Because these flagellin proteins are exported into the medium from the cytoplasm via the flagellum-specific type III export system (Komoriya *et al.*, 1999; Macnab, 1996), we suspected that SpiC participates in either the transport or the expression of flagellin proteins. To examine these possibilities, we grew the bacteria to OD₆₀₀ 1.8 in LB to induce the expression of the *spiC* gene, and then analysed the proteins both in the medium and contained in the cells by Western blotting with a rabbit anti-flagellin antiserum that recognizes a peptide common to FliC and FljB. From their amino acid sequences, FliC and FljB are predicted to be 51.6 and 52.5 kDa, respectively. We observed bands corresponding to FliC and FljB in the wild-type strain. The identities of the two bands were also confirmed by comparison of the patterns from the wild-type, *fliC*, *fljB* and double-mutant strains (Fig. 2). Although a similar amount of FljB was detected in the wild-type strain and the *spiC* mutant, in both the culture supernatant and whole-cell lysates, the level of FliC was much lower in the *spiC* mutant than in the wild-type strain. Complementation of the *spiC* mutant with a plasmid carrying the wild-type *spiC* gene (pEG9127) restored FliC to the level found in the wild-type strain. These results demonstrated that SpiC participates in the expression but not the transport of FliC protein.

To determine whether the expression of FliC is regulated at the transcriptional level, we analysed the expression of the *fliC* gene by fusing the transcriptional regulatory region of *fliC* to *lacZ* in pRL124 (Fig. 3a) and by quantitative real-time RT-PCR (Fig. 3b). In both assays, the expression level of the *fliC* gene in the *spiC* mutant was greatly reduced compared with the wild-type strain, indicating that SpiC participated in the regulation of FliC transcription.

We further examined the involvement of other SPI-2-encoded virulence factors in the expression of the *fliC* gene. As expected, a mutation in the *spiR* gene (Ochman *et al.*,

Fig. 2. Detection of flagellin proteins in wild-type and mutant *Salmonella* strains. Bacteria were cultured in LB to OD₆₀₀ 1.8, and secreted proteins (a) and whole-cell proteins (b) were analysed by Western blotting using an anti-flagellin peptide antibody. Lanes: 1, wild-type *Salmonella*; 2, *spiC* mutant strain; 3, *spiC* mutant strain carrying the *spiC*-containing plasmid pEG9127; 4, *fliC* mutant strain; 5, *fljB* mutant strain; 6, *fliC/fljB* double-mutant strain. In both fractions, the amount of FliC in the *spiC* mutant was greatly reduced in comparison with wild-type *Salmonella*. Arrows a and b indicate FljB and FliC proteins, respectively. Molecular masses are indicated to the left of the blots.

Fig. 3. Expression of the *fliC* gene in a *spiC* mutant. (a) β -Galactosidase activity from a *fliC-lacZ* transcriptional fusion expressed by wild-type *Salmonella* (WT) and *spiC* mutant strain grown in LB to OD₆₀₀ 1.8. β -Galactosidase activity is expressed as Miller units. WT (pRL124) carries the vector with the promoterless *lacZ*. (b, c) Quantitative analysis of *fliC* mRNA expression in mutants for the *spiC* gene (b) or other SPI-2-encoded genes (c). Bacteria were cultured in LB, and total RNA was extracted from wild-type *Salmonella*, *spiC* mutant strain, *spiC* mutant strain carrying pEG9127 (*spiC*⁺), *spiR* mutant strain, *spiB* mutant strain and *ssaV* mutant strain when the OD₆₀₀ was 1.8. Quantitative real-time RT-PCR was conducted using a TaqMan probe. Levels of *fliC* mRNA were normalized to those of 16S rRNA, and the results are shown relative to the expression in wild-type *Salmonella*.

1996), which is a two-component regulatory gene involved in the expression of SPI-2-encoded genes, resulted in defective expression of the *fliC* gene, similar to the *spiC* mutant (Fig. 3c); however, the defective phenotype was not seen in the *spiB* and *ssaV* mutants, which lack a putative component of the SPI-2 TTSS (Ochman *et al.*, 1996; Hensel *et al.*, 1997). These results suggested the specific involvement of SpiC in the expression of the *fliC* gene.

Involvement of FliC in the induction of SOCS-3 expression in *Salmonella*-infected macrophages

SOCS-3 is known to inhibit cytokine signalling via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway (Kile & Alexander, 2001; Suzuki *et al.*, 2001). Our previous studies showed that SpiC participates in the induction of SOCS-3 expression by affecting the signal transduction pathways in *Salmonella*-infected macrophages (Uchiya & Nikai, 2005); however, the mechanism by which SpiC mediates activation of the signal transduction pathways in macrophages after *Salmonella* infection remains unknown. Because we found that SpiC contributes to the expression of FliC, we first examined the involvement of FliC in the induction of SOCS-3 expression in macrophages using two methods. In one set of experiments, flagellin (FliC) isolated from *S. enterica* serovar Typhimurium strain 14028 (Alexis) was added to cell cultures, and in the other set of experiments, macrophages were transfected with the pcDNA3.1-fliC plasmid, which carries the wild-type *Salmonella fliC* gene. After treatment, cell lysates were prepared and the expression of SOCS-3 was assessed by Western blotting. Treatment of macrophages with purified

FliC induced the dose-dependent expression of SOCS-3 when measured 1.5 h post-treatment (Fig. 4a). The extent of induction was lower 3 h post-treatment. Furthermore, transfection of macrophages with plasmid pcDNA3.1-fliC resulted in an approximately 1.8-fold increase in SOCS-3 expression compared with the vector control (Fig. 4b), indicating that FliC can induce SOCS-3 expression.

We next examined whether FliC is involved in the induction of SOCS-3 caused by infection of macrophages with *Salmonella*. Almost no difference was observed between the uptake of bacteria by macrophages infected with the wild-type and that of macrophages infected with mutant *Salmonella* strains (data not shown). Fig. 5 shows that, at 2.5 h post-infection, the levels of SOCS-3 in *fliC* mutant-infected macrophages were reduced by up to 50% compared with the levels in wild-type *Salmonella*-infected macrophages, and that the level of SOCS-3 induced by the *fliC/fliB* double mutant was twofold lower than that induced by the *fliC* mutant. Similar patterns of SOCS-3 expression were also observed at 8 h post-infection, although there were no significant differences at 1 h post-infection. A mutation of the *fliB* gene, however, had little effect on the expression of SOCS-3. Taken together with the results from Figs 2 and 3, these findings demonstrate the involvement of FliC in SPI-2-dependent induction of SOCS-3 expression. Furthermore, the expression of SOCS-3 in macrophages infected with the *fliC* mutant did not decrease to the level seen for the *spiC* mutant-infected macrophages. This result indicates that FliC contributes to SPI-2-dependent induction of SOCS-3 expression but that there must also be FliC-independent mechanisms.

Fig. 4. Involvement of FliC in the induction of SOCS-3 expression in macrophages. Cytosolic extracts from macrophages treated with purified FliC (a) or transfected with empty vector (pcDNA3.1) or pcDNA3.1-fliC (b) were prepared at the indicated times after treatment and analysed by Western blotting using an anti-SOCS-3 antibody. (c) Levels of SOCS-3 normalized to actin levels. The values are shown relative to untreated or pcDNA3.1-transfected cells. Expression of SOCS-3 by macrophages was enhanced by treatment with purified FliC or by transfection with pcDNA3.1-fliC.

FliC participates in SOCS-3 expression via activation of MAPK pathways

We next focused on the role of FliC in the signal transduction pathways that govern *Salmonella*-induced expression of SOCS-3. In a previous study, we showed that both ERK and p38 MAPK signal transduction pathways play important roles in the induction of SOCS-3 expression by *Salmonella* infection (Uchiya & Nikai, 2005). Therefore, we examined the involvement of FliC in the activation of MAPK pathways by measuring the levels

of phosphorylated MAPK in *Salmonella*-infected macrophages. Western blotting using phospho-specific antibodies (Fig. 6) revealed that the levels of MAPK phosphorylation in *Salmonella*-infected macrophages corresponded with the levels of SOCS-3 expression. Infection of macrophages with the wild-type strain resulted in higher levels of phosphorylated ERK, p38 and JNK than did infection with the *spiC* mutant, indicating that SpiC participates in the activation of the MAPK pathways in *Salmonella*-infected macrophages. The levels of MAPK phosphorylation in the *fliC*-

Fig. 5. Western blot analysis of SOCS-3 expression in macrophages infected with *Salmonella*. Cytosolic extracts from macrophages infected with wild-type (WT), *spiC*, *fliC*, *fliB* or *fliC/fliB* double-mutant *Salmonella* were prepared at the indicated times post-infection and were analysed by Western blotting using an anti-SOCS-3 antibody. (a) Images of the original blots. (b) Levels of SOCS-3 normalized to actin levels. The graphs show the percentages compared to uninfected macrophages. The levels of SOCS-3 in the macrophages infected with the *fliC* or *fliC/fliB* double mutant were decreased compared with that of wild-type *Salmonella*-infected cells. * $P < 0.05$; ** $P < 0.005$; # $P < 0.001$, significantly different from macrophages infected with *spiC* *Salmonella*. UI, uninfected.

Fig. 6. Western blot analysis of phospho-ERK (p-p44/42), phospho-p38 (p-p38) and phospho-JNK (p-p54/46) in macrophages infected with *Salmonella*. Cytosolic extracts prepared 2.5 h after infection with wild-type (WT), *spiC*, *fliC*, *fliB* or *fliC/fliB* double-mutant *Salmonella* were analysed with the indicated antibodies. (a) Image of the original blots. After analysis with an anti-phospho-ERK, -phospho-p38 or -phospho-JNK antibody (top), the membranes were stripped and reprobed with an antibody to ERK, p38 or JNK (bottom). (b) Densitometric analysis of the amounts of phospho-ERK (p-p44), phospho-p38 and phospho-JNK (p-p54) normalized to the amounts of ERK (p44), p38 and JNK (p54) in the same samples. The graphs show the percentages compared with uninfected macrophages. The extent of MAPK pathway activation was lower in the *fliC* mutant-infected cells than in the wild-type *Salmonella*-infected cells. * $P < 0.05$; ** $P < 0.005$; # $P < 0.001$, significantly different from macrophages infected with *spiC* *Salmonella*. UI, uninfected.

mutant-infected macrophages were lower than in the wild-type strain-infected macrophages, but the levels were not as low as those in the *spiC* mutant-infected macrophages, indicating that the *fliC* mutant is weaker in the activation of the MAPK pathways than the *spiC* mutant. Taken together, these results indicate that FliC partially participates in the SPI-2-dependent expression of SOCS-3 through the activation of the MAPK pathways.

Detection of FliC in *Salmonella*-infected macrophages

As FliC was found to be required for the induction of SOCS-3 expression through the activation of MAPK pathways in *Salmonella*-infected macrophages, we subsequently investigated whether FliC can be detected in *Salmonella*-infected macrophages by confocal laser scanning microscopy using anti-FliC antibodies. Three hours after bacterial internalization, flagellar filaments composed of FliC were present around bacteria, and detected in about 70 % of macrophages infected with the wild-type strain; however, almost none were observed in macrophages infected by the *spiC* mutant (Fig. 7). In addition, the defective expression in the *spiC* mutant could be rescued by introducing pEG9127, which carries the *spiC* gene. These results suggest that the presence of FliC-derivative filaments inside *Salmonella*-infected macrophages is important for the induction of SOCS-3 expression.

The NF- κ B pathway participates in SpiC-dependent expression of SOCS-3

Because FliC is reported to activate the NF- κ B pathway in epithelial cells (Ogushi *et al.*, 2001; Reed *et al.*, 2002; Takahashi *et al.*, 2001) and because the NF- κ B pathway participates in the induction of SOCS-3 expression (Hayashi *et al.*, 2002), we investigated the participation of the NF- κ B signalling pathway in SPI-2-dependent expression of SOCS-3. As shown in Fig. 8(a), MG-132, a NF- κ B inhibitor, dose-dependently reduced the expression of SOCS-3 in wild-type *Salmonella*-infected macrophages, suggesting the importance of the NF- κ B pathway in *Salmonella*-induced SOCS-3 expression. To further confirm the participation of the NF- κ B pathway, we measured the level of NF- κ B activation in *Salmonella*-infected macrophages. In resting cells, NF- κ B makes a complex with the inhibitory protein I κ B in the cytoplasm (Baldwin, 1996). Upon appropriate cell stimulation, I κ B is rapidly phosphorylated, ubiquitinated and then degraded by the 26S proteasome (Brown *et al.*, 1995; May & Ghosh, 1998). This degradation of I κ B frees NF- κ B to enter the nucleus and stimulate the transcription of target genes. Therefore, we followed the activation of NF- κ B by measuring the level of I κ B- α degradation. The experiments were performed in the presence of cycloheximide ($0.1 \mu\text{g ml}^{-1}$) to block the synthesis of new I κ B- α . LPS, an activator of NF- κ B, was used as a positive control. As shown in Fig. 8(b), the levels of I κ B- α protein in the wild-type strain-infected macro-

Fig. 7. Confocal immunofluorescence analysis of FliC in macrophages infected with wild-type *Salmonella* (WT) or *spiC* mutant strain. Cells were fixed 3 h after bacterial internalization, and were labelled for *Salmonella* (green) and FliC (red). (a) Fluorescent images. (b) Percentages of *Salmonella*-containing macrophages that were stained with an antibody to FliC were enumerated by fluorescence microscopy. FliC was detected in about 70 % of macrophages infected with the wild-type strain, but in almost none infected with the *spiC* mutant. The defective detection of the *spiC* mutant could be rescued by introducing pEG9127, which carries the *spiC* gene. Bars, 10 μm .

phages at 2.5 and 5 h post-infection were decreased up to 60 and 45 % compared with those of uninfected macrophages, respectively, whereas infection with the *spiC* mutant had little effect on the level of I κ B- α protein.

Taken together, these results demonstrate that the NF- κ B pathway plays a significant role in SPI-2-dependent expression of SOCS-3. In contrast, the *fliC* mutant and *fliC/fliB* double mutant caused a similar amount of I κ B- α

Fig. 8. Involvement of the NF- κ B signalling pathway in *Salmonella*-induced expression of SOCS-3. (a) Effect of the NF- κ B inhibitor MG-132 on *Salmonella*-induced expression of SOCS-3. Macrophages were infected with wild-type *Salmonella* in the presence of the indicated concentrations of inhibitor or 0.1 % DMSO (solvent control). At 5 h post-infection, cytosolic extracts were prepared, and SOCS-3 expression was analysed by Western blotting with an anti-SOCS-3 antibody. MG-132 dose-dependently suppressed the expression of SOCS-3. (b) Degradation of I κ B- α in *Salmonella*-infected macrophages. Macrophages were infected with wild-type or mutant strains of *Salmonella* in the presence of cycloheximide (0.1 $\mu\text{g ml}^{-1}$). Macrophages were also treated with LPS (10 $\mu\text{g ml}^{-1}$) as a positive control. At 2.5 and 5 h post-infection, cytosolic extracts were prepared, and I κ B- α degradation was analysed using an anti-I κ B- α antibody. (c) Levels of SOCS-3 or I κ B- α normalized to actin levels. The graphs show percentages of the value for untreated or uninfected cells. The NF- κ B pathway participated in the *Spic*-dependent but not the *FliC*-dependent expression of SOCS-3. # $P < 0.001$, significantly different from macrophages infected with the wild-type strain. UI, uninfected.

degradation to that of the wild-type strain, indicating that FliC does not participate in the activation of NF- κ B in *Salmonella*-infected macrophages.

DISCUSSION

In the current studies, we demonstrated that SpiC, a protein encoded within SPI-2, is involved in the expression of FliC at the transcriptional level. We also showed that the SpiC-dependent expression of FliC plays a significant role in the activation of the signalling pathways that lead to SOCS-3 induction in *Salmonella*-infected macrophages.

SOCS-3 is a member of the SOCS family, which includes SOCS-1 to -7 and Shc (Starr *et al.*, 1997; Yoshimura *et al.*, 1995). These SOCS proteins act as negative regulators of the JAK/STAT signalling pathway. Ligand binding by cytokine receptors activates JAK, which then phosphorylates STAT proteins. This phosphorylation by JAK is required for the dimerization of STAT and its nuclear translocation, binding of DNA, and activation of gene transcription (Kile & Alexander, 2001; Yasukawa *et al.*, 1999). In addition to inhibiting the response to IL-6 by blocking the phosphorylation of STAT-3, SOCS-3, like SOCS-1, has been reported to inhibit IFN- γ signalling by blocking STAT-1 phosphorylation (Song & Shuai, 1998; Stoiber *et al.*, 1999). Cytokines, especially IFN- γ , are essential contributors to macrophage activation, promoting the effective killing of certain intracellular pathogens (Bhardwaj *et al.*, 1986; Green *et al.*, 1990; Suzuki *et al.*, 1988; van Dissel *et al.*, 1987). This suggests that the expression of SOCS-3 affects host defences against intracellular pathogens by inhibiting IFN- γ signalling. In our previous studies, cDNA array analysis revealed that the expression of SOCS-3 in *Salmonella*-infected macrophages is upregulated in a SPI-2-dependent manner, leading to the inhibition of IL-6 and IFN- γ signalling via the JAK/STAT signalling pathway (Uchiya & Nikai, 2005). This suggested that the expression of SOCS-3 is important for the virulence of *Salmonella*; however, the mechanism by which SpiC mediates the induction of SOCS-3 expression in macrophages after *Salmonella* infection remains unknown. It has been reported that SOCS-3 expression is stimulated not only by cytokines but also by PAMPs, such as LPS, bacterial CpG-DNA, and Gram-positive bacteria (Bode *et al.*, 1999; Dalpke *et al.*, 2001; Stoiber *et al.*, 1999, 2001). In the present study, we focused on the role of flagella, which are abundant on the bacterial surface, in SPI-2-dependent expression of SOCS-3.

The flagellum is composed of a basal body, a hook and a filament. Flagellin protein, a component of the filament, is transported from the cytoplasm by the flagellum-specific type III export system (Komoriya *et al.*, 1999) and is polymerized with the help of the cap protein FliD, producing long helical flagellar filaments (Ikeda *et al.*, 1996). *S. enterica* serovar Typhimurium expresses two antigenically distinct flagellins encoded by the *fliC* and *fliB*

genes. The alternative expression of these two genes is known as phase variation, and it occurs on a timescale of the order of 10^3 – 10^5 generations (Silverman, 1979). In the present study, proteomic analysis revealed that the level of FliC protein is much lower in the culture supernatant from the *spiC* mutant than in that from the wild-type strain. We further investigated the mechanism by which SpiC alters the levels of secreted FliC. SpiC is required for secretion of some virulence factors from the cytoplasm by the SPI-2 TTSS (Freeman *et al.*, 2002; Yu *et al.*, 2002), although the molecular mechanism is not known. Several genes that encode the SPI-2 TTSS and the flagellum-specific type III export system have sequences in common (Aizawa, 2001; Macnab, 2004). These findings suggest that, in addition to its role in SPI-2 TTSS, SpiC might participate in the export of flagellin proteins from the cytoplasm via the type III flagellar protein export system. Our results showed that the levels of FliC were reduced not only in the culture supernatant but also in whole-cell lysates of the *spiC* mutant, indicating that the reduced level of FliC was due to altered expression rather than an inability to transport FliC via the type III flagellar protein export system. Furthermore, quantitative real-time RT-PCR revealed that this mutant had a much lower level of *fliC* mRNA. These results indicate that SpiC controls the expression of FliC at the transcriptional level. A gel-shift assay did not provide evidence that SpiC binds to the promoter region of the *fliC* gene (data not shown), which suggests that SpiC participates indirectly in the induction of *fliC* mRNA expression. We further examined the possibility that SpiC alters flagellar phase variation by using the *fliB/fliA* mutant in which phase variation is eliminated. The *fliB* gene constitutes an operon with the *fliA* gene, which encodes a negative regulator of *fliC* expression (Yamamoto & Kutsukake, 2006). The expression level of FliC in the *fliB/fliA* mutant was decreased by a mutation of the *spiC* gene (data not shown). This suggests that SpiC is not involved in phase variation, although we cannot completely rule out this possibility. Taken together, these results suggest that SpiC affects the expression of the class 1 gene *fliHDC* or the class 2 gene *fliA* (Macnab, 1996), which, respectively, function as a master regulator of flagellar gene expression and a regulator of class 3 gene expression. Further studies are needed to clarify how SpiC contributes to the expression of the *fliC* gene.

The flagellar filament protein FliC is a potent stimulator of immune and proinflammatory responses (Gewirtz *et al.*, 2001b; Hayashi *et al.*, 2001). There have been many reports that it activates signal transduction pathways via TLR5 in cultured cells such as epithelial cells, leading to the induction of immune and proinflammatory genes (Eaves-Pyles *et al.*, 2001; Gewirtz *et al.*, 2001a; Sierro *et al.*, 2001; Zeng *et al.*, 2003). Okugawa *et al.* (2006) reported the induction of SOCS-1 by flagellin stimulation in Jurkat T cells. As described above, SpiC contributes to the expression of FliC, suggesting that FliC participates in SpiC-dependent expression of SOCS-3. As expected, we

found that the level of SOCS-3 expression in macrophages infected with the *fliC* mutant was lower than that of the wild-type strain-infected macrophages, whereas a mutation of the *fljB* gene had little effect on SOCS-3 expression. These results indicate that FliC plays a significant role in the SpiC-dependent expression of SOCS-3. The level of SOCS-3 expression in the *fliC* mutant-infected macrophages, however, did not drop to that in *spiC* mutant-infected macrophages, suggesting the existence of additional FliC-independent mechanisms in the SPI-2-dependent expression of SOCS-3.

In subsequent studies, we focused on the role of FliC in the signal transduction pathways governing the SPI-2-induced expression of SOCS-3. SPI-2-dependent SOCS-3 expression is reported to occur downstream of the ERK and p38 MAPK signalling pathways (Uchiya & Nikai, 2005). In the present study, we showed that, in addition to ERK and p38 MAPK, the NF- κ B pathway contributes to the SPI-2-dependent activation of SOCS-3 expression. In contrast, although FliC was involved in the activation of MAPK pathways, we could not find evidence for the involvement of FliC in NF- κ B activation, indicating that FliC is not required for all aspects of SPI-2-dependent activation of the signal transduction pathways. Flagellin is recognized by TLR5, and ligand binding by TLR5 leads to NF- κ B activation (Hayashi *et al.*, 2001; Zeng *et al.*, 2003). Therefore, our results suggest that FliC activates TLR5-independent signal transduction pathways in macrophages. In addition to TLR5, flagellin was recently shown to be recognized in the cytosol by two different nucleotide-binding oligomerization domain (Nod)-like receptors, Ipaf and Naip5 (also known as Birc1e) (Molofsky *et al.*, 2006; Ren *et al.*, 2006). Miao *et al.* (2006) and Franchi *et al.* (2006) have reported that flagellin activates a signalling pathway independent of TLR5, which leads to caspase-1 cleavage and the secretion of IL-1 β via Ipaf in the cytosol of *Salmonella*-infected macrophages. These findings indicate that Ipaf plays an important role in the induction of SOCS-3 expression by FliC in *Salmonella*-infected macrophages. This possibility is supported by our finding that SOCS-3 expression is elevated in pcDNA3.1-*fliC*-transfected macrophages.

There are several reports that the expression of FliC is downregulated in *Salmonella*-infected macrophages or during *Salmonella* infection in mice (Alaniz *et al.*, 2006; Cummings *et al.*, 2006; Eriksson *et al.*, 2003). However, our data showed that FliC was detected in about 70 % of macrophages infected with the wild-type strain but almost not at all in macrophages infected with the *spiC* mutant. Lyons *et al.* (2004) reported that infection of polarized epithelial cells by *Salmonella* leads to IL-8 expression by causing the SPI-2-dependent translocation of flagellin to a basolateral membrane domain expressing TLR5. Together with our results, this suggests that SPI-2 mediates not only the transcytosis of flagellin in infected cells but also its expression.

In conclusion, we showed that SpiC is required for the transcriptional expression of flagellin in *S. enterica* serovar Typhimurium. We also demonstrated that the SpiC-dependent expression of FliC activates MAPK signalling pathways in macrophages after *Salmonella* infection, leading to the induction of SOCS-3 expression. It is likely that these events play important roles in the pathogenesis of *Salmonella* infection.

ACKNOWLEDGEMENTS

We are grateful to Sunao Iyoda for helpful discussions. We also wish to thank Asami Sugita, Haruka Amano and Kayo Sobue for technical assistance. This work was supported by Grants-in-Aid for the 'Academic Frontier' Project for Private Universities (2007–2011) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and for Scientific Research (C) 17590403 from the Japan Society for the Promotion of Science (to K.-i. U.).

REFERENCES

- Aizawa, S. I. (2001). Bacterial flagella and type III secretion systems. *FEMS Microbiol Lett* **202**, 157–164.
- Akira, S. & Takeda, K. (2004). Toll-like receptor signalling. *Nat Immunol* **4**, 499–511.
- Alaniz, R. C., Cummings, L. A., Bergman, M. A., Rassoul-Barrett, S. L. & Cookson, B. T. (2006). *Salmonella typhimurium* coordinately regulates FliC location and reduces dendritic cell activation and antigen presentation to CD4⁺ T cells. *J Immunol* **177**, 3983–3993.
- Baldwin, A. S., Jr (1996). The NF- κ B and I- κ B proteins: new discoveries and insights. *Annu Rev Immunol* **14**, 649–681.
- Bhardwaj, N., Nash, T. W. & Horwitz, M. A. (1986). Interferon- γ -activated human monocytes inhibit the intracellular multiplication of *Legionella pneumophila*. *J Immunol* **137**, 2662–2669.
- Bode, J. G., Nimmesgern, A., Schmitz, J., Schaper, F., Schmitt, M., Frisch, W., Häussinger, D., Heinrich, P. C. & Graeve, L. (1999). LPS and TNF α induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. *FEBS Lett* **463**, 365–370.
- Bogdan, C. & Nathan, C. (1993). Modulation of macrophage function by transforming growth factor β , interleukin-4, and interleukin-10. *Ann N Y Acad Sci* **685**, 713–739.
- Brown, K., Gerstberger, S., Carlson, L., Franzoso, G. & Siebenlist, U. (1995). Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science* **267**, 1485–1488.
- Cummings, L. A., Wilkerson, W. D., Bergsbaken, T. & Cookson, B. T. (2006). *In vivo*, *fliC* expression by *Salmonella enterica* serovar Typhimurium is heterogeneous, regulated by ClpX, and anatomically restricted. *Mol Microbiol* **61**, 795–809.
- Dalpe, A. H., Oppen, S., Zimmermann, S. & Heeg, K. (2001). Suppressors of cytokine signaling (SOCS)-1 and SOCS-3 are induced by CpG-DNA and modulate cytokine responses in APCs. *J Immunol* **166**, 7082–7089.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Eaves-Pyles, T., Murthy, K., Liaudet, L., Virag, L., Ross, G., Soriano, F. G., Szabo, C. & Salzman, A. L. (2001). Flagellin, a novel mediator of *Salmonella*-induced epithelial activation and systemic inflammation: IkB α degradation, induction of nitric oxide synthase, induction of

- proinflammatory mediators, and cardiovascular dysfunction. *J Immunol* **166**, 1248–1260.
- Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. & Hinton, J. C. D. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol Microbiol* **47**, 103–118.
- Franchi, L., Am, A., Body-Malapel, M., Kanneganti, T. D., Ozören, N., Jagirdar, R., Inohara, N., Vandenabeele, P., Bertin, J. A. & other authors (2006). Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1 β in *Salmonella*-infected macrophages. *Nat Immunol* **7**, 576–582.
- Freeman, J. A., Rappale, C., Kuhle, V., Hensel, M. & Miller, S. I. (2002). SpiC is required for translocation of *Salmonella* pathogenicity island 2 effectors and secretion of translocon proteins SseB and SseC. *J Bacteriol* **184**, 4971–4980.
- Galan, J. E. (2001). *Salmonella* interactions with host cells: type III secretion at work. *Annu Rev Cell Dev Biol* **17**, 53–86.
- Gewirtz, A. T., Navas, T. A., Lyons, S., Godowski, P. J. & Madara, J. L. (2001a). Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* **167**, 1882–1885.
- Gewirtz, A. T., Jr, Simon, P. O., Schmitt, C. K., Taylor, L. J., Hagedorn, C. H., O'Brien, A. D., Neish, A. S. & Madara, J. L. (2001b). *Salmonella typhimurium* translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J Clin Invest* **107**, 99–109.
- Green, S. J., Crawford, R. M., Hockmeyer, J. T., Meltzer, M. S. & Nacy, C. A. (1990). *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN- γ -stimulated macrophages by induction of tumor necrosis factor- α . *J Immunol* **145**, 4290–4297.
- Groisman, E. A., Blanc-Portard, A.-B. & Uchiya, K. (1999). Pathogenicity islands and the evolution of *Salmonella* virulence. In *Pathogenicity Islands and Other Mobile Virulence Elements*, pp. 127–150. Edited by J. B. Kaper & J. Hacker. Washington, DC: American Society for Microbiology.
- Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M. & other authors (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**, 1099–1103.
- Hayashi, T., Kaneda, T., Toyama, Y., Kumegawa, M. & Hakeda, Y. (2002). Regulation of receptor activator of NF- κ B ligand-induced osteoclastogenesis by endogenous interferon- β (IFN- β) and suppressors of cytokine signaling (SOCS). *J Biol Chem* **277**, 27880–27886.
- Hensel, M., Shea, J. E., Raupach, B., Monack, D., Falkow, S., Gleeson, C., Kubo, T. & Holden, D. W. (1997). Functional analysis of *ssaJ* and the *ssaK/U* operon, 13 genes encoding components of the type III secretion apparatus of *Salmonella* pathogenicity island 2. *Mol Microbiol* **24**, 155–167.
- Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., Vazquez-Torres, A., Gleeson, C., Fang, F. C. & other authors (1998). Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Mol Microbiol* **30**, 163–174.
- Ikeda, T., Oosawa, K. & Hotani, H. (1996). Self-assembly of the filament capping protein, FliD, of bacterial flagella into an annular structure. *J Mol Biol* **259**, 679–686.
- Janeway, C. A., Jr & Medzhitov, R. (2002). Innate immune recognition. *Annu Rev Immunol* **20**, 197–216.
- Kile, B. T. & Alexander, W. S. (2001). The suppressors of cytokine signaling (SOCS). *Cell Mol Life Sci* **58**, 1627–1635.
- Komoriya, K., Shibano, N., Higano, T., Azuma, N., Yamaguchi, S. & Aizawa, S. (1999). Flagellar proteins and type III-exported virulence factors are the predominant proteins secreted into the culture media of *Salmonella typhimurium*. *Mol Microbiol* **34**, 767–779.
- Kunkel, S. L., Spengler, M., May, M. A., Spengler, R., Larrick, J. & Remick, D. (1988). Prostaglandin E₂ regulates macrophage-derived tumor necrosis factor gene expression. *J Biol Chem* **263**, 5380–5384.
- Lee, A. H., Zareei, M. P. & Daefler, S. (2002). Identification of a NIPSNAP homologue as host cell target for *Salmonella* virulence protein SpiC. *Cell Microbiol* **4**, 739–750.
- Lyons, S., Wang, L., Casanova, J. E., Sitaraman, S. V., Merlin, D. & Gewirtz, A. T. (2004). *Salmonella typhimurium* transcytoses flagellin via an SPI-2-mediated vesicular transport pathway. *J Cell Sci* **117**, 5771–5780.
- Macnab, R. M. (1996). Flagella and motility. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 123–145. Edited by F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & other authors. Washington, DC: American Society for Microbiology.
- Macnab, R. M. (2004). Type III flagellar protein export and flagellar assembly. *Biochim Biophys Acta* **1694**, 207–217.
- Malo, M. S. & Loughlin, R. E. (1988). Promoter-detection vectors for *Escherichia coli* with multiple useful features. *Gene* **64**, 207–215.
- May, M. J. & Ghosh, S. (1998). Signal transduction through NF- κ B. *Immunol Today* **19**, 80–88.
- Miao, E. A., Alpuche-Aranda, C. M., Dors, M., Clark, A. E., Bader, M. W., Miller, S. I. & Aderem, A. (2006). Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1 β via Ipaf. *Nat Immunol* **7**, 569–575.
- Miller, J. (1972). *Experiments in Molecular Genetics*, pp. 352–355. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Molofsky, A. B., Byrne, B. G., Whitfield, N. N., Madigan, C. A., Fuse, E. T., Tateda, K. & Swanson, M. S. (2006). Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *J Exp Med* **203**, 1093–1104.
- Ochman, H., Soncini, F. C., Solomon, F. & Groisman, E. A. (1996). Identification of a pathogenicity island required for *Salmonella* survival in host cells. *Proc Natl Acad Sci U S A* **93**, 7800–7804.
- O'Farrell, A. M., Liu, Y., Moore, K. W. & Mui, A. L.-F. (1998). IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat 3-dependent and -independent pathways. *EMBO J* **17**, 1006–1018.
- Ogushi, K., Wada, A., Niidome, T., Mori, N., Oishi, K., Nagatake, T., Takahashi, A., Asakura, H., Makino, S. & other authors (2001). *Salmonella enteritidis* FliC (flagella filament protein) induces human β -defensin-2 mRNA production by Caco-2 cells. *J Biol Chem* **276**, 30521–30526.
- Okugawa, S., Yanagimoto, S., Tsukada, K., Kitazawa, T., Koike, K., Kimura, S., Nagase, H., Hirai, K. & Ota, Y. (2006). Bacterial flagellin inhibits T cell receptor-mediated activation of T cells by inducing suppressor of cytokine signalling-1 (SOCS-1). *Cell Microbiol* **8**, 1571–1580.
- Poltorak, A., He, X., Smirnova, L., Liu, M. Y., Van Huffel, C. X., Birdwell, D., Alejos, E., Silva, M., Galanos, C. & other authors (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* **282**, 2085–2088.
- Reed, K. A., Hobert, M. E., Kolenda, C. E., Sands, K. A., Rathman, M., O'Connor, M., Lyons, S., Gewirtz, A. T., Sansonetti, P. J. & other authors (2002). The *Salmonella typhimurium* flagellar basal body protein FliE is required for flagellin production and to induce a proinflammatory response in epithelial cells. *J Biol Chem* **277**, 13346–13353.
- Ren, T., Zamboni, D. S., Roy, C. R., Dietrich, W. F. & Vance, R. E. (2006). Flagellin-deficient *Legionella* mutants evade caspase-1- and *Naip5*-mediated macrophage immunity. *PLoS Pathog* **2**, e18.

- Schwandner, R., Dziarski, R., Wesche, H., Rothe, M. & Kirschning, C. J. (1999). Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J Biol Chem* **274**, 17406–17409.
- Shea, J. E., Hensel, M., Gleeson, C. & Holden, D. W. (1996). Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc Natl Acad Sci U S A* **93**, 2593–2597.
- Shotland, Y., Krämer, H. & Groisman, E. A. (2003). The *Salmonella* SpiC protein targets the mammalian Hook3 protein function to alter cellular trafficking. *Mol Microbiol* **49**, 1565–1576.
- Sierro, F., Dubois, B., Coste, A., Kaiserlian, D., Kraehenbuhl, J. P. & Sirard, J. C. (2001). Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. *Proc Natl Acad Sci U S A* **98**, 13722–13727.
- Silverman, M. (1979). Phase variation in *Salmonella*: genetic analysis of a recombinational switch. *Proc Natl Acad Sci U S A* **76**, 391–395.
- Song, M. M. & Shuai, K. (1998). The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. *J Biol Chem* **273**, 35056–35062.
- Starr, R., Willson, T. A., Viney, E. M., Murray, L. J., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D. & other authors (1997). A family of cytokine-inducible inhibitors of signaling. *Nature* **387**, 917–921.
- Stoiber, D., Kovarik, P., Cohney, S., Johnston, J. A., Steinlein, P. & Decker, T. (1999). Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN- γ . *J Immunol* **163**, 2640–2647.
- Stoiber, D., Stockinger, S., Steinlein, P., Kovarik, J. & Decker, T. (2001). *Listeria monocytogenes* modulates macrophage cytokine responses through STAT serine phosphorylation and the induction of suppressor of cytokine signaling 3. *J Immunol* **166**, 466–472.
- Strassmann, G., Patil-koota, V., Finkelman, F., Fong, M. & Kambayashi, T. (1994). Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E₂. *J Exp Med* **180**, 2365–2370.
- Suzuki, Y., Orellana, M. A., Schreiber, R. D. & Remington, J. S. (1988). Interferon- γ : the major mediator of resistance against *Toxoplasma gondii*. *Science* **240**, 516–518.
- Suzuki, A., Hanada, T., Mitsuyama, K., Yoshida, T., Kamizono, S., Hoshino, T., Kubo, M., Yamashita, A., Okabe, M. & other authors (2001). CIS/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. *J Exp Med* **193**, 471–481.
- Takahashi, A., Wada, A., Ogushi, K., Maeda, K., Kawahara, T., Mawatari, K., Kurazono, H., Moss, J., Hirayama, T. & other authors (2001). Production of β -defensin-2 by human colonic epithelial cells induced by *Salmonella enteritidis* flagella filament structural protein. *FEBS Lett* **508**, 484–488.
- Uchiya, K. & Nikai, T. (2004). *Salmonella enterica* serovar Typhimurium infection induces cyclooxygenase 2 expression in macrophages: involvement of *Salmonella* pathogenicity island 2. *Infect Immun* **72**, 6860–6869.
- Uchiya, K. & Nikai, T. (2005). *Salmonella* pathogenicity island 2-dependent expression of suppressor of cytokine signaling 3 in macrophages. *Infect Immun* **73**, 5587–5594.
- Uchiya, K., Barbieri, M. A., Funato, K., Shah, A. H., Stahl, P. D. & Groisman, E. A. (1999). A *Salmonella* virulence protein that inhibits cellular trafficking. *EMBO J* **18**, 3924–3933.
- Uchiya, K., Groisman, E. A. & Nikai, T. (2004). Involvement of *Salmonella* pathogenicity island 2 in the up-regulation of interleukin-10 expression in macrophages: role of protein kinase A signal pathway. *Infect Immun* **72**, 1964–1973.
- van Dissel, J. T., Stikkelbroeck, J. J., Michel, B. B., van den Barselaar, M. T., Leijh, P. C. & van Furth, R. (1987). Inability of recombinant interferon-gamma to activate the antibacterial activity of mouse peritoneal macrophages against *Listeria monocytogenes* and *Salmonella typhimurium*. *J Immunol* **139**, 1673–1678.
- Yamamoto, S. & Kutsukake, K. (2006). FljA-mediated posttranscriptional control of phase 1 flagellin expression in flagellar phase variation of *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **188**, 958–967.
- Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T. & other authors (1999). The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. *EMBO J* **18**, 1309–1320.
- Yoshimura, A., Ohkubo, T., Kiguchi, T., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Hara, T. & Miyajima, A. (1995). A novel cytokine-inducible gene CIS encodes an SH-2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO J* **14**, 2816–2826.
- Yu, X.-J., Ruiz-Albert, J., Unsworth, K. E., Garvis, S., Liu, M. & Holden, D. W. (2002). SpiC is required for secretion of *Salmonella* pathogenicity island 2 type III secretion system proteins. *Cell Microbiol* **4**, 531–540.
- Yu, X.-J., Liu, M. & Holden, D. W. (2004). SsaM and SpiC interact and regulate secretion of *Salmonella* pathogenicity island 2 type III secretion system effectors and translocators. *Mol Microbiol* **54**, 604–619.
- Zeng, H., Carlson, A. Q., Guo, Y., Yu, Y., Collier-Hyams, L. S., Madara, J. L., Gewirtz, A. T. & Neish, A. S. (2003). Flagellin is the major proinflammatory determinant of enteropathogenic *Salmonella*. *J Immunol* **171**, 3668–3674.

Edited by: J. G. Shaw