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Salmonella Pathogenicity Island 2-Dependent Expression of Suppressor of Cytokine Signaling 3 in Macrophages

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***Salmonella* pathogenicity island 2 (SPI-2), which is located at centisome 30.7 on the chromosome of *Salmonella enterica* serovar Typhimurium, is required for growth within macrophages and systemic infection in mice. We recently reported that the infection of macrophages with *Salmonella* induces the expression of cyclooxygenase-2 in a manner dependent on SPI-2 (K. Uchiya and T. Nikai, Infect. Immun. 72:6860–6869, 2004). In the present study, gene expression analysis using a cDNA array further showed the involvement of SPI-2 in the expression of suppressor of cytokine signaling 3 (SOCS-3), which is involved in the inhibition of cytokine signaling via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway. A high level of SOCS-3 expression was induced in J774 macrophages infected with wild-type *Salmonella* compared to that in macrophages infected with a strain carrying a mutation in the *spiC* gene within SPI-2. Other members of the SOCS family were not detected in *Salmonella*-infected macrophages. The SPI-2-induced up-regulation of SOCS-3 expression was dependent on activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. Furthermore, the inhibition of gamma-interferon-induced STAT-1 and interleukin-6-induced STAT-3 tyrosine phosphorylation correlated with the expression of SOCS-3. Taken together, these results indicate that *Salmonella* causes SPI-2-dependent activation of ERK1/2, leading to SOCS-3 expression, which in turn inhibits cytokine signaling via the JAK/STAT pathway.**

Cytokines and eicosanoids, such as prostaglandins (PGs) and leukotrienes, are known to affect the function of macrophages, which play a central role in the host defense against many types of infection. Gamma interferon (IFN-γ) is an essential contributor to macrophage activation and promotes the effective killing of pathogens that can survive within macrophages (3, 17, 38). On the other hand, macrophage functions can be blocked by interleukin-4 (IL-4) and IL-10 as well as by transforming growth factor beta and PGE_2 (6, 22, 25, 37, 43). In particular, IL-10 and PGE_2 can inhibit the production of reactive oxygen or nitrogen intermediates in activated macrophages (5, 16, 26, 27). Thus, the modulation of key cytokine and eicosanoid production can affect the host defense against many infections.

It is well known that cytokine signaling is inhibited by the suppressor of cytokine signaling (SOCS) family, which includes SOCS-1 to -7 and the cytokine-inducible Src homology 2 domain-containing protein (34, 46). These SOCS proteins act as negative regulators of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, which is activated in response to a variety of cytokines and hormones. The phosphorylation of STAT by JAK is required for its dimerization, nuclear translocation, DNA binding, and gene activation (44). Among the SOCS proteins, the functions and the regulation of expression are the most well studied for SOCS-1 and SOCS-3. Both SOCS proteins can inhibit JAK tyrosine kinase activity, preventing the phosphorylation of STAT factors. SOCS-1 does this by directly binding to the kinase domain of JAK, whereas SOCS-3 binds to the cytokine receptor. SOCS-3 can also interact with some target sequences present on JAK and STAT proteins (21, 28, 29, 30, 45). The expression of SOCS-1 and SOCS-3 is tightly regulated within immune cells, and their induction occurs not only through cytokines but also in response to lipopolysaccharide (LPS) and gram-positive bacteria (4, 35, 36).

Salmonella enterica is a facultative intracellular bacterium capable of surviving within macrophages, and this ability is important for the establishment of a systemic infection in vivo (13). Some important virulence factors required for this function are encoded within *Salmonella* pathogenicity island 2 (SPI-2) (9, 18, 19, 24, 31). Previous work has shown that a mutant with a mutation in the SPI-2 gene *spiC* is unable to survive within macrophages and has a highly attenuated virulence in mice. The SpiC protein is necessary for inhibiting the fusion of *Salmonella*-containing phagosomes with lysosomal compartments (39). Moreover, this protein is translocated into the cytosol of *Salmonella*-infected macrophages by the type III secretion system encoded within SPI-2, and it interacts with host proteins, such as TassC (23) and Hook3 (32), which are implicated in cellular trafficking. On the other hand, some studies have shown that SpiC is required for the translocation of SPI-2 effector proteins into target cells by interacting with SsaM, a SPI-2-encoded protein (14, 47, 48). Thus, more research is needed to clarify the molecular function of SpiC.

In addition to these reports, we have recently shown that SpiC is involved in *Salmonella*-induced activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway, which leads to the expression of cyclooxygenase 2 (COX-2). This results in an increase in PGE_2 and PGI_2 production in *Salmonella*-infected macrophages (41). Moreover, the receptor-mediated activation of cyclic AMP (cAMP)-dependent

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protein kinase by these prostanoids plays a significant role in the survival of *Salmonella* within macrophages. Thus, SpiC participates in the intracellular survival of *Salmonella* by regulating the signal transduction pathways in macrophages.

For the present study, we carried out a cDNA array analysis to identify additional genes that are up-regulated in a SPI-2-dependent manner in *Salmonella*-infected macrophages. We show that *Salmonella* infection causes SPI-2-dependent SOCS-3 expression, resulting in the inhibition of cytokine signaling via the JAK/STAT pathway.

MATERIALS AND METHODS

Reagents. Reagents for cell culture were purchased from Sigma-Aldrich (St. Louis, MO), and other reagents were purchased from the following sources: PD98052 and SB203580 were from Calbiochem (La Jolla, CA), SP600125 and NS-398 were from Biomol (Plymouth Meeting, PA), PGE_2 was from Cayman Chemicals (Ann Arbor, MI), recombinant murine IFN-γ was from Pharmingen (San Diego, CA), and recombinant murine IL-6 and a monoclonal anti-mouse IL-10 antibody were from R&D Systems (Minneapolis, MN). PD98052, SB203580, SP600125, and NS-398 were dissolved in dimethyl sulfoxide (DMSO). When these drugs were used, the final concentration of DMSO in the culture medium was 0.1%; this concentration of solvent did not affect the cellular responses.

Bacterial strains, plasmid, and growth conditions. The strains used for this study were derived from the wild-type *S. enterica* serovar Typhimurium strain 14028s. The *spiC*::*kan* derivative EG10128 and the *purB*::Tn*10* strain EG9652 were described by Uchiya et al. (39). Plasmid pEG9127 is a derivative of pBAC108L containing the cloned *spiC* gene (39). Bacteria were grown at 37°C in Luria broth (LB). Kanamycin and tetracycline were used at 50 μg/ml and 15 μg/ml, respectively.

Cell culture and bacterial infection. The J774 E clone, a mannose receptor-positive murine macrophage cell line, was maintained in a 37°C incubator with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. The day before infection, the macrophages were plated at a density of 1.0×10^6/well in 6-well tissue culture plates (Falcon; DB Biosciences, Franklin Lakes, NJ) or 0.4×10^6/well in 24-well plates in medium without antibiotics. Bacterial infections of macrophages were conducted as described previously (39), using a multiplicity of infection of 25 bacteria per macrophage.

Analysis of gene expression by cDNA array. Macrophages seeded in six-well plates were infected with bacteria. After incubation for 5 h, the total RNA was prepared as described previously (40). RNAs were treated with DNase I (Takara Biomedicals, Tokyo, Japan) to remove contaminating genomic DNA, and the RNA integrity was confirmed in a denaturing agarose gel. An Atlas pure total RNA labeling kit (Clontech Laboratories, Palo Alto, CA) was utilized according to the manufacturer's instructions for mRNA purification, cDNA synthesis, and cDNA labeling. Purified cDNAs were labeled with [α-^{32}P]dATP (3,000 Ci/mmol; Amersham Pharmacia Biotech, Tokyo, Japan), and the labeled probes were purified by spin column centrifugation as described in the manufacturer's protocol.

An analysis of mRNA expression was performed using an Atlas Mouse 1.2 array (Clontech Laboratories), which consists of 1,176 mouse cDNA fragments organized into broad functional groups. Following the manufacturer's instructions, the Atlas membranes were hybridized overnight with 1×10^6 cpm/ml of radioactive probe at 68°C in a rotation-hybridization oven. After being washed, the membranes were exposed to X-ray film using an intensifying screen for 1 to 3 days at −80°C. The X-ray films were scanned with a Phosphor Imager SI, and the level of gene expression was analyzed using Array Vision 6.0 software (Amersham Pharmacia Biotech). All spots on membranes were normalized with the ubiquitin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene signals. Data are expressed as relative changes in mRNA levels between wild-type *Salmonella*-infected and *spiC* or *purB* mutant-infected macrophages, and a 1.5-fold difference in wild-type *Salmonella*-infected samples relative to *spiC* mutant-infected samples was taken as the cutoff for scoring up-regulated expression.

Semiquantitative reverse transcription-PCR (RT-PCR). Total RNA (2 μg) from macrophages was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) using an oligo(dT) primer. PCRs were conducted in 20-μl reaction mixtures consisting of reaction buffer (Perkin-Elmer, Foster City, CA), a 0.5 μM concentration of each deoxynucleoside triphosphate, a 1 μM concentration of each primer, 1 μl cDNA, and 1 U *Taq* DNA polymerase (Perkin-Elmer) for cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplification was carried out for 22 cycles for SOCS-3, 25 cycles for interleukin-1 receptor antagonist (IL-1Ra), 24 cycles for monocyte chemoattractant protein 3 (MCP-3), 35 cycles for cornichon-like protein (CNIL) and cytotoxic cell protease 1 (CCP-1), 26 cycles for leukocyte adhesion glycoprotein-α (LFA-1), and 16 cycles for GAPDH and was followed by a 7-min final extension at 72°C. GAPDH was used as an internal standard for quantification of the total RNA. In each case, the number of amplification cycles achieved exponential amplification, wherein the amount of product formation was proportional to the concentration of starting cDNA (data not shown). The PCR products were subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Visual analysis and image-analyzing software (Gel-Doc 2000 system; Bio-Rad, Hercules, CA) were used for comparisons of band intensities. The primer pairs were as follows: for SOCS-3, 5′-GGA GAC TCC TGA GTT AAC ACT GGG-3′ and 5′-GAC CAG TTC CAG GTA ATT GCA TGG-3′ (197-bp fragment); for IL-1Ra, 5′-GCA AGC CTT CAG AAT CTG GGA TAC-3′ and 5′-CTC AGA TCA GTG ATG TTA ACT TCC-3′ (238-bp fragment); for MCP-3, 5′-ATG AGG ATC TCT GCC ACG CTT CTG-3′ and 5′-TGT CTA AGT ATG CTA TAG CCT CCT-3′ (269-bp fragment); for CNIL, 5′-TGA TGA GCT GCG GAC TGA CTT CAA-3′ and 5′-TGA TAG AGA CCG CAT CAT ACA TGA-3′ (276-bp fragment); for CCP-1, 5′-ACA TGG CCT TAC TTT CGA TCA AG G-3′ and 5′-CTG GCT TCA CAT TGA CAT TGC GCC-3′ (315-bp fragment); for LFA-1, 5′-GCT TCT TGG CAC AAG CTG GAA GAC-3′ and 5′-CAT ACA TTC CTG ATA GGC GGG ACG-3′ (347-bp fragment); and for GAPDH, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (452-bp fragment).

Western blot analysis. Western blot analyses were performed essentially as described previously (40). An anti-SOCS-3 antibody was purchased from Immuno-Biological Laboratories (Gunma, Japan) and used at 3 μg/ml. Phosphospecific antibodies to STAT1 and STAT3 were purchased from New England Biolabs (Beverly, MA) and used according to the manufacturer's protocol. Bands were analyzed using a GS-800 calibrated densitometer (Bio-Rad).

Statistical analysis. Each experiment was performed at least three times. The results are expressed as means ± standard deviations (SD). The data were analyzed by analysis of variance with Dunnett's test. P values of <0.05 were considered statistically significant.

RESULTS

Gene expression analysis by Atlas cDNA array. Our previous studies showed that SPI-2 participates in the up-regulation of IL-10 and COX-2 expression by affecting the signal transduction pathways in *Salmonella*-infected macrophages (40, 41). For the present study, to identify additional genes that are up-regulated in a SPI-2-dependent manner, we carried out gene expression analysis in macrophages infected with wild-type, *spiC* mutant, or *purB* mutant *Salmonella*. For this experiment, we used an Atlas Mouse 1.2 array, which contains 1,176 known genes. The *spiC* mutant, a strain carrying a nonpolar mutation in the *spiC* gene carried within SPI-2, is reported to be defective for intramacrophage survival (39). The *purB* mutant, which is defective in purine metabolism, also survives poorly in macrophages, similar to the *spiC* mutant (40). Therefore, we used the *purB* mutant as a control to test whether *spiC* defects are due simply to a reduction in intramacrophage survival. J774 macrophages were infected with the wild type or with each *Salmonella* mutant. RNAs was isolated at 5 h postinfection, converted to labeled cDNAs, and hybridized to the Atlas membranes. A summary of the genes whose expression was increased over 1.5-fold in macrophages infected with wild-type *Salmonella* compared to those infected with the *spiC* mutant is presented in Table 1. Of these 20 genes, 6 (shown in bold) had a >1.5-fold increase in expression in both wild-type- and *purB* mutant-infected cells compared to cells infected with the *spiC* mutant, suggesting that the up-regulation of these six

TABLE 1. Analysis of mRNA expression using Atlas cDNA arrays[c]

Up-regulated gene product	GenBank accession no.	Protein category	WT/*spiC* ratio[a]	*purB*/*spiC* ratio[b]
Suppressor of cytokines signaling protein 3 (SOCS-3)	U88328	Modulators, effectors, and intracellular transducers	**6.7**	**5.5**
Interleukin-1 receptor antagonist (IL-1Ra)	M74294	Apoptosis-associated proteins	**5.5**	**4.1**
Macrophage mannose receptor	Z11974	Receptors (for ligands)	3.2	1.2
Monocyte chemoattractant protein 3 (MCP-3)	S71251	Extracellular cell signaling and communication	**3.2**	**2.1**
Cornichon-like protein (CNIL)	AB006191	Modulators, effectors, and intracellular transducers	**2.8**	**1.8**
Leukemia inhibitory factor (LIF)	X06381	Extracellular cell signaling and communication	2.5	0.8
TPA-induced sequence 11 (TIS-11)	M57422	Transcription factors and DNA-binding proteins	2.4	0.9
Hepatocyte growth factor (HGF)	X72307	Extracellular cell signaling and communication	2.3	1.0
5-Hydroxytryptamine receptor 1B receptor (5HT1B)	Z11597	Receptors (for ligands)	2.3	1.2
Lymphotoxin receptor (TNFR family)	U29173	Receptors (for ligands)	2.3	0.7
Heparin-binding EGF-like growth factor (HBEGF)	L07264	Extracellular cell signaling and communication	2.3	1.0
NADPH-cytochrome P450 reductase (CPR)	D17571	Apoptosis-associated proteins	2.1	1.2
T-cell death-associated protein (TDAG51)	U44088	Apoptosis-associated proteins	2.0	0.7
Hepatoma transmembrane kinase ligand	L38847	Extracellular cell signaling and communication	1.9	0.9
Inhibin alpha-subunit precursor (INHA)	X69618	Extracellular cell signaling and communication	1.8	0.9
Nerve growth factor beta precursor (NGFB)	K01759	Extracellular cell signaling and communication	1.8	1.0
Early growth response protein 1 (EGR-1)	M20157	Transcription factors and DNA-binding proteins	1.7	1.2
Cytotoxic cell protease 1 (CCP-1)	M12302	Apoptosis-associated proteins	**1.5**	**1.7**
Macrophage inflammatory protein 2 alpha (MIP2-α)	X53798	Extracellular cell signaling and communication	1.5	0.9
Leukocyte adhesion glycoprotein alpha (LFA-1)	M60778	Modulators, effectors, and intracellular transducers	**1.5**	**1.9**

[a] Ratio of relative mRNA expression levels in wild-type (WT)-infected and *spiC* mutant-infected macrophages.
[b] Ratio of relative mRNA expression levels in *purB* mutant-infected and *spiC* mutant-infected macrophages.
[c] Data shown in bold are for genes that were up-regulated >1.5-fold in wild-type-infected and *purB* mutant-infected macrophages compared to *spiC* mutant-infected macrophages.

genes is dependent on SpiC function and is not due to the intracellular proliferation of wild-type *Salmonella*.

SOCS-3, IL-1Ra, and MCP-3 are up-regulated in a SPI-2-dependent manner. To confirm the results of the cDNA array analysis, we used RT-PCR to examine the expression levels of the six SPI-2-dependent up-regulated genes, which encode SOCS-3, IL-1Ra, MCP-3, CNIL, CCP-1, and LFA-1. RNAs were extracted at 5 h postinfection. For the detection of these mRNAs, amplification was carried out for 22 cycles for SOCS-3, 25 cycles for IL-1Ra, 24 cycles for MCP-3, 35 cycles for CNIL and CCP-1, and 26 cycles for LFA-1, indicating that the expression level of SOCS-3 mRNA in *Salmonella*-infected macrophages is the highest among these genes. As shown in Fig. 1, the expression levels of the SOCS-3, IL-1Ra, and MCP-3 mRNAs were higher in macrophages infected with wild-type or *purB* mutant *Salmonella* than in those infected with the *spiC* mutant.

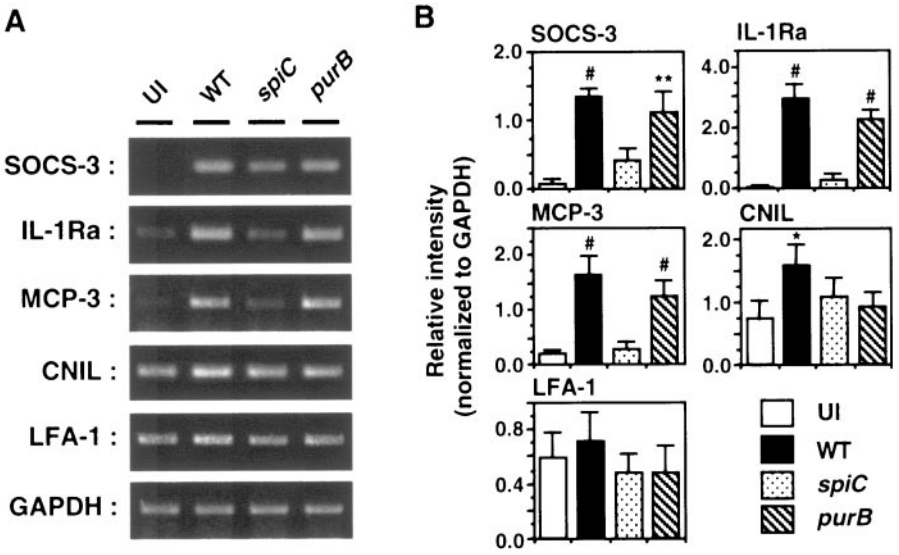

FIG. 1. Expression of SOCS-3, IL-1Ra, MCP-3, CNIL, and LFA-1 mRNAs in macrophages infected with *Salmonella*. (A) At 5 h postinfection with wild-type (WT), *spiC*, or *purB Salmonella*, total RNAs were extracted from the pooled macrophages, reverse transcribed, and amplified by PCR. The PCR products were separated in 1.5% agarose gels. (B) Expression of SOCS-3, IL-1Ra, MCP-3, CNIL, and LFA-1, normalized to GAPDH expression. Data represent the means ± SD of three independent experiments. The levels of SOCS-3, IL-1Ra, and MCP-3 mRNAs were significantly lower in cells infected with the *spiC* mutant than in cells infected with wild-type or *purB Salmonella*. *, $P < 0.05$; **, $P < 0.005$; #, $P < 0.001$ (significantly different from macrophages infected with *spiC Salmonella*). UI, uninfected.

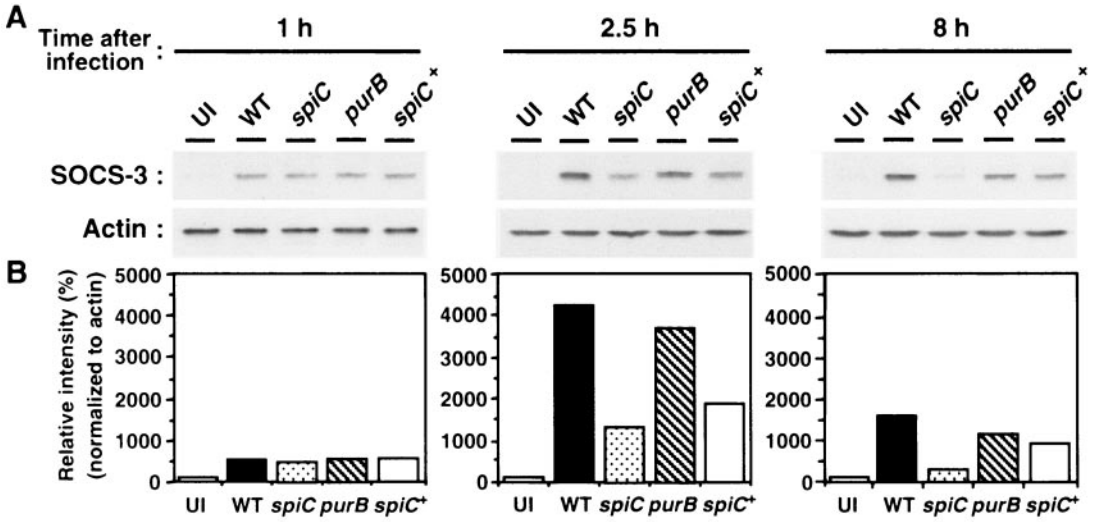

FIG. 2. Western blot analysis of SOCS-3 expression in macrophages infected with *Salmonella*. Cytosolic extracts from macrophages infected with wild-type (WT), *spiC*, or *purB Salmonella* or with *spiC Salmonella* carrying the *spiC* gene-containing plasmid pEG9127 (*spiC*$^+$) were prepared at the indicated times postinfection and were analyzed using an anti-SOCS-3 antibody. (A) Images of the original blots. (B) Levels of SOCS-3 normalized to actin levels. The graphs show percentages of the value for uninfected macrophages. UI, uninfected.

These results indicate that SpiC is involved in the specific up-regulation of the expression of these genes.

On the other hand, the expression patterns of CNIL and LFA-1 mRNAs by RT-PCR were different from the results of the cDNA array. The levels of CNIL mRNA induced by the *spiC* and *purB* mutants were lower than those induced by wild-type *Salmonella*, indicating that CNIL expression is dependent on the level of intracellular proliferation of *Salmonella*. The expression of LFA-1 mRNA was not different following infection with wild-type or *spiC* mutant *Salmonella*, and a signal for CCP-1 mRNA could not be detected even after 40 cycles (data not shown).

In further studies, we focused on SOCS-3 because the expression level of SOCS-3 mRNA in *Salmonella*-infected macrophages was the highest among those of the six up-regulated genes. In contrast, expression of other members of the SOCS family was not observed in response to *Salmonella* infection (Table 1 and data not shown). The expression of SOCS-3 protein in *Salmonella*-infected macrophages was quantified by Western blot analysis. As shown in Fig. 2, a *Salmonella*-induced increase in SOCS-3 expression was detectable at 1 h postinfection, but there was no significant difference between the wild-type and mutant forms of *Salmonella*. At 2.5 h postinfection, however, the level of SOCS-3 in wild-type *Salmonella*-infected macrophages was 3.1-fold higher than that in the *spiC* mutant-infected macrophages. The level of SOCS-3 protein peaked at 5 h postinfection (data not shown). Although the SOCS-3 level was decreased at 8 h postinfection, the difference in expression between the wild type and the *spiC* mutant was 5.7-fold. In addition to wild-type *Salmonella* infection, SOCS-3 expression in *purB* mutant-infected macrophages was higher than that in *spiC* mutant-infected cells.

We previously showed that the survival defect of the *spiC* mutant within macrophages at 18 h postinfection can be reversed by a single-copy plasmid harboring the *spiC* gene (pEG9127) (39). In this study, although complementation of the *spiC* mutant with pEG9127 did not completely restore the level of SOCS-3 expression to that found in wild-type *Salmonella*-infected macrophages, the level of SOCS-3 induced by the *spiC* mutant carrying pEG9127 was increased 1.4- and 3.4-fold compared to that induced by the *spiC* mutant at 2.5 and 8 h postinfection, respectively (Fig. 2). Taken together, these results indicate that the reduction of SOCS-3 expression by the *spiC* mutant is due to a defect in SpiC function.

Up-regulation of SOCS-3 expression due to SPI-2-dependent ERK1/2 activation. Subsequent studies focused on the signal transduction pathways that govern the *Salmonella*-induced expression of SOCS-3. Several observations have shown that the mitogen-activated protein kinase (MAPK) signal transduction pathways have a significant role in the induction of SOCS-3 expression (4, 10, 11). Therefore, we examined the effects of MAPK inhibitors on the expression of SOCS-3. Figure 3 shows that SOCS-3 expression in wild-type *Salmonella*-infected macrophages was blocked by PD98052, which inhibits MAP/ERK kinase, as well as by the p38 MAPK inhibitor SB203580, in a concentration-dependent manner. In contrast, SP600125, an inhibitor of c-Jun amino-terminal kinase, did not have an inhibitory effect. The results indicate that both the ERK1/2 and p38 MAPK signaling pathways may participate in the *Salmonella*-induced expression of SOCS-3.

We previously measured the levels of p38 and ERK1/2 phosphorylation in *Salmonella*-infected macrophages (40, 41). Wild-type *Salmonella* induced a higher level of p38 phosphorylation in macrophages than did the *spiC* mutant, but there was no significant difference between the *spiC* mutant and the *purB* mutant, which, like the *spiC* mutant, is defective for intramacrophage survival. Although the results with the p38 MAPK inhibitor suggest that the p38 MAPK pathway is involved in the *Salmonella*-induced expression of SOCS-3, our previous results imply that activation of the p38 MAPK pathway is not responsible for the SPI-2-dependent induction of SOCS-3 expression. On the other hand, the level of ERK1/2 phosphorylation in

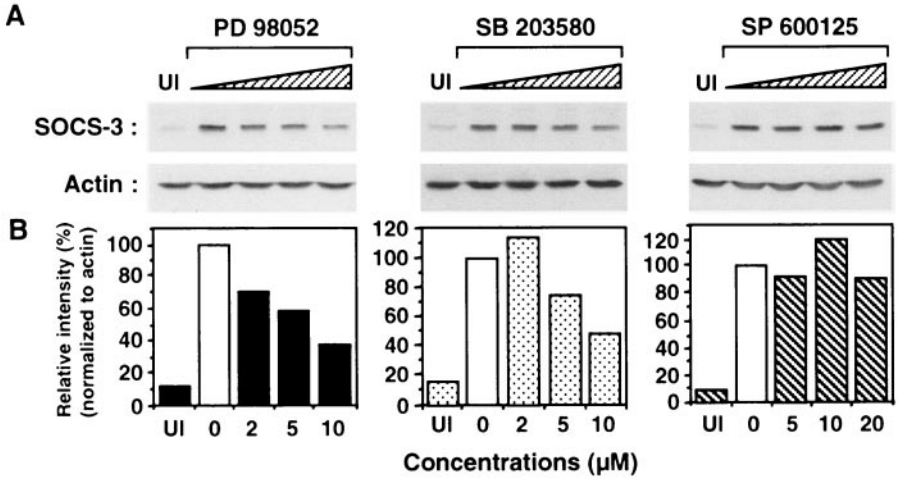

FIG. 3. Effects of inhibitors of ERK1/2 (PD98052), p38 MAPK (SB203580), and Jun N-terminal kinase (SP600125) on *Salmonella*-induced expression of SOCS-3. Macrophages were infected with wild-type *Salmonella* in the presence of the indicated concentrations of inhibitors or 0.1% DMSO (solvent control). At 5 h postinfection, cytosolic extracts were prepared and analyzed using an anti-SOCS-3 antibody. (A) Images of the original blots. (B) Levels of SOCS-3 normalized to actin levels. The graphs show percentages of the value for untreated macrophages. Both PD98052 and SB203580 dose-dependently reduced the expression of SOCS-3. UI, uninfected.

wild-type and *purB* mutant *Salmonella*-infected macrophages was higher than that in *spiC* mutant-infected macrophages. Taken together, these results demonstrate the involvement of SPI-2 in the activation of ERK1/2 but not p38 MAPK. This activation of ERK1/2 leads to the increase in SOCS-3 expression induced by wild-type or *purB* mutant *Salmonella*.

Involvement of PGE_2 in SPI-2-dependent expression of SOCS-3. It has been reported that PGE_2, a product of the COX pathway, is involved in the induction of SOCS-3 expression in peripheral blood mononuclear cells (15). Our previous study showed that *Salmonella* causes a SPI-2-dependent activation of ERK1/2 that leads to COX-2 expression, resulting in an increase in PGE_2 production in macrophages. Therefore, the influence of COX-2 in SPI-2-dependent SOCS-3 expression was examined using NS-398, a selective COX-2 inhibitor. As shown in Fig. 4A, treatment with NS-398 (10 μM) at a dose that completely blocks PGE_2 production (data not shown) decreased SOCS-3 expression in wild-type *Salmonella*-infected macrophages, but the level did not drop to that seen for PD96052-treated macrophages. This indicates that COX-2 is partially involved in SPI-2-dependent SOCS-3 expression through ERK1/2 activation.

To further clarify the involvement of PGE_2 in SOCS-3 expression, *Salmonella*-infected macrophages were treated with

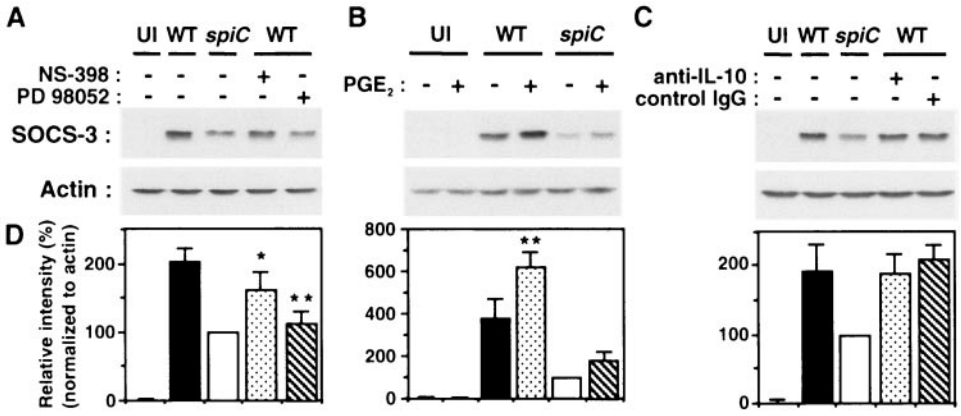

FIG. 4. Involvement of PGE_2 in *Salmonella*-induced expression of SOCS-3. (A) Effect of the COX-2 inhibitor NS-398 on *Salmonella*-induced expression of SOCS-3. Macrophages were infected with wild-type *Salmonella* in the presence of NS-398 (10 μM), the ERK1/2 inhibitor PD98052 (10 μM), or 0.1% DMSO (solvent control). At 5 h postinfection, cytosolic extracts were prepared and analyzed using an anti-SOCS-3 antibody. (B) Effect of PGE_2 on *Salmonella*-induced expression of SOCS-3. Macrophages were infected with wild-type *Salmonella* in the presence of PGE_2 (10 μM). At 5 h postinfection, cytosolic extracts were prepared and analyzed using an anti-SOCS-3 antibody. (C) Effect of a neutralizing anti-IL-10 antibody on *Salmonella*-induced expression of SOCS-3. Macrophages were infected with wild-type *Salmonella* in the presence of an anti-IL-10 antibody (4 μg/ml) or an irrelevant immunoglobulin G (IgG; 4 μg/ml). At 5 h postinfection, cytosolic extracts were prepared and analyzed using an anti-SOCS-3 antibody. (D) Levels of SOCS-3 normalized to actin levels. The graphs show percentages of the value for *spiC* mutant-infected macrophages. Data represent the means ± SD of three independent experiments. The results show that PGE_2, but not IL-10, is involved in SOCS-3 expression. *, $P < 0.05$; **, $P < 0.001$ (significantly different from untreated macrophages infected with the wild-type strain). UI, uninfected.

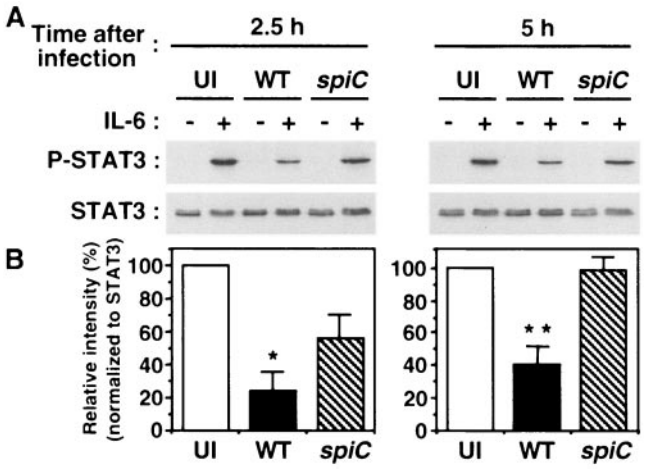

FIG. 5. Effect of *Salmonella* infection on IL-6-stimulated STAT-3 phosphorylation in macrophages. After infection with wild-type (WT) or *spiC Salmonella*, macrophages were treated for 20 min with 40 ng/ml IL-6. Thereafter, cytosolic extracts were prepared and analyzed using anti-phospho-STAT-3 and anti-STAT-3 antibodies. (A) Images of the original blots. After analysis using an anti-phospho-STAT-3 antibody (top), the membranes were stripped and reprobed with an antibody to STAT-3 (bottom). (B) Densitometric analysis of the amounts of phospho-STAT-3 normalized to the amounts of STAT-3 in the same samples. The graphs show percentages of the value for uninfected macrophages. Data represent the means ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.001$ (significantly different from macrophages infected with *spiC Salmonella*). UI, uninfected.

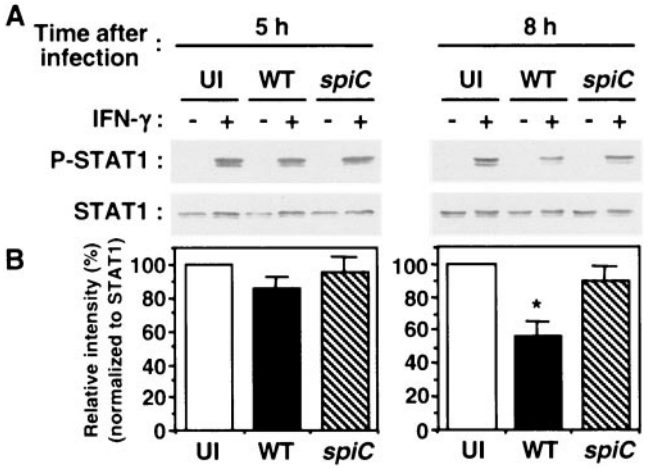

FIG. 6. Effect of *Salmonella* infection on IFN-γ-stimulated STAT-1 phosphorylation in macrophages. After infection with wild-type (WT) or *spiC Salmonella*, macrophages were treated for 20 min with 100 U/ml IFN-γ. Thereafter, cytosolic extracts were prepared and analyzed using anti-phospho-STAT-1 and anti-STAT-1 antibodies. (A) Images of the original blots. After analysis using an anti-phospho-STAT-1 antibody (top), the membranes were stripped and reprobed with an antibody to STAT-1 (bottom). (B) Densitometric analysis of the amounts of phospho-STAT-1 normalized to the amounts of STAT-1 in the same samples. The graphs show percentages of the value for uninfected macrophages. Data represent the means ± SD of three independent experiments. *, $P < 0.005$ (significantly different from macrophages infected with *spiC Salmonella*). UI, uninfected.

PGE_2. The level of SOCS-3 expression in wild-type- or *spiC* mutant-infected macrophages was increased 1.7-fold by treatment with PGE_2, whereas the treatment of uninfected macrophages with PGE_2 had no effect on SOCS-3 expression (Fig. 4B). Together with the results in Fig. 4A, this shows that endogenous PGE_2 is partially involved in *Salmonella*-induced SOCS-3 expression.

Macrophages infected with *Salmonella* have been reported to release IL-10 in a SPI-2-dependent manner (40). Because IL-10, like PGE_2, is capable of stimulating SOCS-3 expression (8), it was of interest to study whether the SPI-2-dependent expression of IL-10 is involved in the up-regulation of SOCS-3 expression in *Salmonella*-infected macrophages. Neutralization of endogenous IL-10 with an antibody showed that it was not responsible for SOCS-3 expression (Fig. 4C). This indicates that SPI-2-dependent IL-10 production does not affect *Salmonella*-induced SOCS-3 expression.

Effects of SOCS-3 on cytokine signaling. The activation of cytokine receptors by ligand binding activates JAKs, which then phosphorylate STAT proteins. SOCS-3 is reported to inhibit cytokine signaling by blocking the phosphorylation of STAT-1 (35) and STAT-3 (4). Therefore, to examine the effect of SPI-2-dependent SOCS-3 expression on cytokine signaling, we assessed the induction of STAT-1 and STAT-3 tyrosine phosphorylation by IFN-γ and IL-6, respectively. Western blotting using phospho-specific antibodies (Fig. 5 and 6) showed that the phosphorylation of both STATs was readily detected in uninfected macrophages upon stimulation. The level of IL-6-induced STAT-3 phosphorylation at 2.5 h postinfection in wild-type *Salmonella*-infected macrophages was approximately twofold lower than that in macrophages infected with the *spiC* mutant (Fig. 5). Also, the degree of reduction was almost the same at 8 h postinfection. The level of IFN-γ-induced STAT-1 phosphorylation at 8 h postinfection in wild-type *Salmonella*-infected macrophages was decreased up to 60% compared to that in macrophages infected with the *spiC* mutant, but in contrast to the case for STAT-3, there was no significant difference in the phosphorylation of STAT-1 between wild-type- and *spiC* mutant-infected cells at 5 h postinfection (Fig. 6). Thus, the inhibition of IL-6 and IFN-γ signaling via the JAK/STAT signal pathway correlated with SPI-2-dependent SOCS-3 expression. These results suggest that SOCS-3 plays a significant role in the intracellular growth of *Salmonella* by inhibiting the cytokine activation of macrophages.

DISCUSSION

Our previous studies have shown that SPI-2 modulates IL-10 and COX-2 expression in macrophages by affecting signal transduction pathways. Our present studies using cDNA array analysis further show the involvement of SPI-2 in the *Salmonella*-induced expression of SOCS-3 in macrophages. Specifically, *Salmonella* causes SPI-2-dependent activation of the ERK1/2 signaling pathway, leading to the up-regulation of SOCS-3 expression. In addition, we found that the SPI-2-dependent expression of SOCS-3 inhibits cytokine signaling by blocking the phosphorylation of STAT proteins.

The cDNA array analysis showed that the expression of SOCS-3, IL-1Ra, and MCP-3 mRNAs in *Salmonella*-infected macrophages is up-regulated in a SPI-2-dependent manner.

IL-1Ra and MCP-3 are expressed and secreted by various types of cells, including monocytes and macrophages (12, 42). IL-1Ra release is believed to attenuate the effects of IL-1, a potent proinflammatory cytokine, through binding to the IL-1 receptor. Since IL-1α expression is reported to increase in macrophages in response to *Salmonella* infection (40), it is thought that IL-1Ra production affects the function of macrophages by inhibiting the actions of IL-1α. MCP-3 belongs to the C-C chemokines, which are cytokines involved in cell recruitment during inflammation and carcinogenesis. Recent studies have also shown that MCP-3 may act as a regulator of cellular differentiation (1, 7). We are now examining the roles of IL-1Ra and MCP-3 in SPI-2 function. In the present study, because the expression level of SOCS-3 mRNA was the highest among these three genes, we focused on SOCS-3 and examined the regulation of its expression and its role in *Salmonella*-infected macrophages.

Our data showed that a high level of SOCS-3 expression was induced in wild-type *Salmonella*-infected macrophages compared to that in macrophages infected with the *spiC* mutant. In contrast, the expression of other members of the SOCS family was not observed in response to *Salmonella* infection. These findings suggest that SpiC influences the signal transduction pathway involved in SOCS-3 expression. It has been reported that SOCS-3 induction occurs not only through cytokines but also through LPS and gram-positive bacteria (4, 35, 36) and that specific inhibitors of the MAPK signal transduction pathway can block the induction of SOCS-3 expression in macrophages by LPS, bacterial CpG-DNA, and tumor necrosis factor alpha (4, 10, 11). Our results showed that the inhibition of ERK1/2 or p38 MAPK blocked *Salmonella*-induced SOCS-3 expression, supporting the involvement of both the ERK1/2 and p38 MAPK signaling pathways in this process. We previously showed that wild-type *Salmonella* induces the phosphorylation of both ERK1/2 and p38 MAPK in macrophages, but we could only find evidence for SPI-2 involvement in ERK1/2 phosphorylation (40, 41), indicating that the up-regulation of SOCS-3 expression could occur through a SPI-2-dependent activation of ERK1/2. Thus, the signal transduction pathway that governs SOCS-3 expression was similar to that regulating COX-2 expression that we previously reported (41). However, more research is needed to clarify the mechanism by which SPI-2 affects the ERK1/2 signaling pathway.

IL-10 and PGE_2 are also known to induce SOCS-3 expression (8, 15), and PGE_2 promotes SOCS-3 expression by elevating intracellular cAMP levels (15). Macrophages infected with *Salmonella* have been reported to release IL-10 and PGE_2 in a SPI-2-dependent manner (40, 41). Therefore, we examined the involvement of these endogenous factors in SPI-2-dependent SOCS-3 expression, and we found that PGE_2 is partially involved in *Salmonella*-induced SOCS-3 expression but is unaffected by IL-10. This agrees with the finding that *Leishmania donovani*-induced expression of SOCS-3 is independent of IL-10 (2). It is thought that the involvement of PGE_2 in SOCS-3 expression in *Salmonella*-infected macrophages is mediated by a PGE_2-dependent increase in cAMP levels (41).

Two members of the SOCS family, SOCS-1 and SOCS-3, have been associated with an inhibition of the IFN-γ response (33, 35). Cytokines, especially IFN-γ, are essential contributors to macrophage activation, which promotes the effective killing of some intracellular pathogens (3, 17, 38). It has been reported that the intracellular pathogens *Listeria monocytogenes* (36) and *Leishmania donovani* (2) stimulate the induction of SOCS-3 in macrophages and that a decrease of IFN-γ-stimulated STAT-1 phosphorylation correlates with the induction of SOCS-3. Imai et al. (20) also showed that *Mycobacterium bovis* infection induces the production of SOCS-1 and SOCS-3 and inhibits IFN-γ-stimulated phosphorylation of STAT-1 in macrophages. This information suggests that SOCS-1 or SOCS-3 expression affects host defenses against intracellular pathogens by inhibiting IFN-γ signaling.

Because SOCS-3 has been reported to inhibit cytokine signaling by blocking the phosphorylation of STAT-1 (35) or STAT-3 (4), we examined whether *Salmonella*-induced SOCS-3 expression affects the induction of STAT-1 and STAT-3 phosphorylation by IFN-γ and IL-6, respectively. The levels of phosphorylation of both STATs in wild-type *Salmonella*-infected macrophages were significantly decreased compared with those in *spiC* mutant-infected macrophages, suggesting that SPI-2-dependent SOCS-3 expression may play a significant role in the intracellular growth of *Salmonella* by inhibiting macrophage activation in response to IFN-γ. In addition to the results of in vitro experiments with macrophages, we found that the expression of several proinflammatory cytokines, including IFN-γ and IL-6, and SOCS-3 is increased in the livers and spleens of mice infected with *Salmonella* (data not shown). Further studies should clarify the role of SOCS-3 in the establishment of a systemic infection by *Salmonella* in vivo.

In conclusion, a cDNA array analysis showed that SOCS-3 is up-regulated in a SPI-2-dependent manner in *Salmonella*-infected macrophages. The up-regulation of SOCS-3 expression was due to SPI-2-dependent activation of the ERK1/2 signaling pathway. The up-regulation of SOCS-3 expression also participated in the inhibition of STAT-1 and STAT-3 tyrosine phosphorylation in response to IFN-γ and IL-6, respectively. Thus, the induction of SOCS-3 and its inhibition of cytokine signaling may help explain the role of SPI-2 in *Salmonella* virulence.

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