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Salmonella Secreted Factor L Deubiquitinase of Salmonella typhimurium Inhibits NF-κB, Suppresses IκBα Ubiquitination and Modulates Innate Immune Responses

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Salmonella enterica translocates virulent factors into host cells using type III secretion systems to promote host colonization, intracellular bacterial replication and survival, and disease pathogenesis. Among many effectors, the type III secretion system encoded in Salmonella pathogenicity island 2 translocates a Salmonella-specific protein, designated Salmonella secreted factor L (SseL), a putative virulence factor possessing deubiquitinase activity. In this study, we attempt to elucidate the mechanism and the function of SseL, in vitro, in primary host macrophages and in vivo in infected mice. Expression of SseL in mammalian cells suppresses NF-κB activation downstream of IκBα kinases and impairs IκBα ubiquitination and degradation, but not IκBα phosphorylation. Disruption of the gene encoding SseL in S. enterica serovar typhimurium increases IκBα degradation and ubiquitination, as well as NF-κB activation in infected macrophages, compared with wild-type bacteria. Mice infected with SseL-deficient bacteria mount stronger inflammatory responses, associated with increased production of NF-κB-dependent cytokines. Thus, SseL represents one of the first bacterial deubiquitinases demonstrated to modulate the host inflammatory response in vivo. The Journal of Immunology, 2008, 180: 5045–5056.

S. typhimurium infections rank among the most common bacterial diseases, ranging from self-limiting gastroenteritis (food poisoning) to severe systemic infection (typhoid fever) (1–3). Studies using mice in which specific cytokine genes have been ablated indicate that innate immune responses represent the first line of defense against S. typhimurium (4–7). Both innate and adaptive immune response are regulated by the NF-κB/REL family of transcription factors. In unstimulated cells, NF-κB-family transcription factors exist as homo- or heterodimers bound to IκB-family proteins, which typically sequester NF-κB-family transcription factors in the cytosol, preventing their access to the nucleus and thereby maintaining NF-κB in an inactive state (8). Degradation or proteolytic processing of IκB-family proteins represents the pivotal event that triggers NF-κB activation. In the canonical and the noncanonical NF-κB-signaling pathway, phosphorylation and activation of IκB kinases (IKK)β and α, respectively, trigger the recruitment of the multiprotein ubiquitin ligase stem cell factor complex (SCFβ-TrCP). Activation of IKKβ leads to phosphorylation of IκBα, which is then ubiquitinated by SCFβ-TrCP and degraded by the 26S proteasome, allowing NF-κB to enter the nucleus. In the nonclassical pathway, phosphorylated IKKα recruits the SCFβ-TrCP to polyclubiquitinate the NF-κB precursor p100, which is then processed into the mature subunit p52-NF-κB (9, 10).

Ubiquitin-dependent destruction of IκB-family proteins is opposed by deubiquitinating (DUB) enzymes. CYLD and A20 are two of the best-studied DUB enzymes in mammals, which negatively regulate NF-κB upstream of IKK (11, 12). Some types of pathogenic bacteria encode virulence factors that interfere with NF-κB activation and ubiquitination, thus blunting signaling events involved in host defense. For example, AvrA, the bacterial effector from Salmonella, deubiquinates IκBα inhibiting NF-κB activation (13). The OspG protein kinase of Shigella flexneri has also been shown to bind various ubiquitin-conjugating enzymes belonging to the SCFβ-TrCP complex, and prevent phospho-IκBα degradation (14). Another example of a bacterial virulence factor that interferes with NF-κB activation is the YopJ protein expressed in Yersinia species. YopJ is homologous to Cysteine protease and was reported to hydrolyze ubiquitin and SUMO (a ubiquitin-like protein) (15–17). YopJ was also shown to act as an acetyltransferase that blocks phosphorylation sites on MAPK6 and IKKβ kinases implicated in NF-κB activation (18, 19). The physiological relevance of YopJ in the pathogenic mechanism of Yersinia has been demonstrated in experiments comparing the lethality of wild-type (WT) and YopJ-deficient bacteria (20). Chlamydia

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Abbreviations used in this paper: IKK, IκB kinase; SCF, stem cell factor; DUB, deubiquitinating enzyme; WT, wild type; SseL, Salmonella secreted factor L; LB, Luria-Bertani; Kan, kanamycin; HA, hemagglutinin; PARP, poly(ADP-ribose) polymerase; IP, immunoprecipitation; p.i., postinfection; AMC, 7-amino-4-methylcoumarin; PMN, polymorphonuclear neutrophil; m.o.i., multiplicity of infection; hsp, heat shock protein; SUMO, small ubiquitin-related modifier; NEDD8, neural precursor cell-expressed developmentally down-regulated 8.
trachomatis encodes two DUBs, which have been shown in vitro to hydrolyze thioester bonds that attach ubiquitin and NEDD8 (a ubiquitin-like protein) to target proteins (21). However, the role of these DUBs in the pathobiology of Chlamydia has not been established. Indeed, the cellular function of bacterial DUB remains to be elucidated.

Recently, an effector protein of Salmonella, SseL, was suggested to contribute to systemic virulence during murine typhoid-like diseases (22), possibly by promoting delayed macrophage cytotoxicity (23). Additionally, SseL was recently demonstrated to have DUB activity (23) in vitro and in cells (23), but the physiological relevance of this enzymatic function in Salmonella pathogenesis was not previously elucidated. In the present study, we functionally characterized the mechanism of SseL in cultured host cells and in infected mice. Altogether, our data indicate that the DUB activity of SseL suppresses Ixβ ubiquitination and degradation, preventing subsequent NF-κB activation, and that SseL modulates host inflammatory responses in vivo during infection.

Materials and Methods

Bacteria strains and cell culture

Bacteria strains include WT Salmonella enterica serovar typhimurium LT2 and 14028s strains (provided by Dr. D. G. Guiney), and the sseL:Kan mutant lacking the STM2287 gene. Nonagitated microaerophilic bacterial cultures were grown overnight in Luria-Bertani (LB) medium (modified with 300 mM NaCl), with or without kanamycin (Kan; 25 μg/ml), and/or ampicillin (50 μg/ml). Typically, bacteria were subcultured (1:20 to 1:100 v/v) for 2–3 h in fresh medium, then washed twice in sterile PBS before being diluted (0.22 μM) LB medium for in vivo and in vitro infection experiments.

HEK293N, HEK293T, HeLa, and RAW264.7 cells were cultured in DMEM, while THP-1 cells were maintained in RPMI 1640 medium, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Mammalian cells were cultured at 37°C in 5% CO₂/air.

Murine macrophage cultures

Bone marrow cells were harvested from leg bones of sacrificed 8-wk-old inbred pathogen-free female BALB/c mice (Harlan Breeders). Bone marrow cells were cultured at 37°C in RPMI 1640 medium with 10% FBS. CSF-1 (10 ng/ml; Sigma-Aldrich) was added to medium for 1 wk to promote differentiation. Adherent macrophages in 100-cm² plates were used for bacterial infection and replication assays.

Plasmids

The complete open reading frame of sseL DNA was PCR-amplified from S. typhimurium LT2 genomic DNA (American Type Culture Collection), using the primers SseL:forward (5′-GGAAATCATGAAATATGTGTAATTACATTTACAAGGACAAATAGTGGTGACGTGGAGCTGTCAC-3′) and SseL:reverse-Myc (5′-CTCTTACTACGACCTGGGACATGCACGT-3′). After enzymatic digestions, the resulting DNA fragment and pCMV-β-gal vector were digested with EcoRI and XhoI. After DNA purification, the amplified PCR fragment was ligated into pWSK29 to give pWSK-sseL.

Generation of Salmonella SseL mutants

S. enterica serovar typhimurium strain 14028s was used for mutant generation using the one-step gene inactivation method of Datsenko and Wanner (24). Briefly, STM2287 was replaced by a Kan-resistance gene (Kan*) by generating PCR primers SseLp1 (5′-CGGTTACATATGACGATTTCTTCTTCACTACGACCTGGGACATGCACGT-3′), SseLp2 (5′-GATACAAGGCTTAACTGGGATACGTTCTGTATGTC-3′), SseLp3 (5′-GTTCTCTCACTTATTACATGCAGTGGTGGGACATGCACGT-3′), and SseLp4 (5′-GTTCTCTCACTTATTACATGCAGTGGTGGGACATGCACGT-3′), containing p1 and p2 FLP recognition target sites flanking the Kan* gene of the template plasmid pFDK, and the 50-nt extensions homologous to the region flanking sseL. (underlined). sseL::Kan mutants were also used for p22 phage transductions to independently generate additional Salmonella sseL::Kan mutant strains, which were selected on Kan LB plates. All experiments were confirmed with at least two independently derived sseL:Kan mutants.

To restore sseL, the sseL::Kan mutant bacteria were electroporated with pWSK-sseL plasmid and cells were selected on Kan and ampicillin LB plates. The presence of sseL was verified by PCR using sseL internal primers (CD-SseL:forward, and SseL:reverse-Myc).

Luciferase gene reporter assays

HEK293T cells were seeded into 96-well plates and transfected the next day by using Superfect transfection reagent (Qiagen), holding total DNA content constant, using pcDNA3Myc vector, as described in Ref. 25. At 72 h posttransfection, cells were left untreated or were treated with TNF-α (10 ng/ml) or IL-1β (20 ng/ml) for 6 h, and transcriptional activity was assessed 3 days after transfection. Data are expressed as fold induction relative to basal activity (mean ± SD; n = 3).

Immunoblotting, cellular fractionation, and immunoprecipitation

For immunoblotting, control and transfected HEK293N cells or RAW264.7 cells were lysed in radioimmunoprecipitation assay buffer containing 20 μg/ml Na3VO4, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 5 mM NaF, 1 mM PMSF, 1 mM DTT, and 1× protease inhibitor mix (Roche). Clarified lysates, normalized for protein content (50 μg), were separated by SDS-PAGE (8% gels), and transferred to nitrocellulose membranes. Blots were analyzed using anti-FLAG (1:1000; Santa Cruz Biotechnology) and anti-c-Myc (1:1000; Santa Cruz Biotechnology), anti-heat shock protein 60 (hs60; Santa Cruz Biotechnology), anti-FLAG, and anti-b-actin (Sigma-Aldrich), anti-hemaggglutinin (HA), and anti-Myc (Roche) Abs. Detection was accomplished using ECL (Amersham Biosciences). Polyclonal antiserum for SseL (BR40) was generated in New Zealand rabbits using recombinant protein immunogens.

Caspase activity and cell death assays

For immunoprecipitations, transfected HEK293T or RAW264.7 cells were lysed in immunoprecipitation (IP) buffer (50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1% Triton X-100, 1 mM DTT, 10% glycerol, 1× protease inhibitor mix (Roche), 20 μg/ml Na3VO4, 20 μg/ml leupeptin, 1 mM PMSF, and 20 mM N-ethylmaleimide. Cell lysates (0.5 ml) were incubated with 2 μg of anti-FLAG or anti-IκBα Abs prelinked to 25 μg of protein G and A Sepharose (25 μl each) for 4 h or overnight at 4°C. Samples were then washed four times, boiled in Laemmli buffer, and analyzed by SDS-PAGE/immunoblotting. Alternatively, lysates were directly analyzed by immunoblotting after normalization for total protein content. Abs used for Ag detection included anti-IκBα (Santa Cruz Biotechnology), anti-mono- and polyubiquitin (clone FK2; Biomol), anti-HA, and anti-c-Myc (Roche), anti-FLAG, and anti-b-actin Abs (Sigma-Aldrich).

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Cell death of control and infected RAW264.7 cells was detected by staining with trypan blue (0.1% (w/v) in DMEM medium; Sigma-Aldrich). Cells were counted using a hemocytometer to determine the ratio of dead to live cells ($n/10^3$ 100 cells per determination).

Measurement of p65 nuclear translocation and p65 DNA-binding activity

Nuclear p65 translocation was monitored in HEK293N cells grown in 100-cm$^2$ plates, transfected with 10$\mu$g of DNA, then cultured with or without TNF-$\alpha$ for various times. Relative amounts of p65 DNA-binding activity were also measured from RAW264.7 cells or primary macrophages before or at various times after bacterial infection. Nuclear extracts were prepared using a kit (ActiveMotive), and relative amounts of p65 protein in nuclear extracts were quantified by ELISA, according to the manufacturer’s instructions (p65 ActivELISA; Imgenex). Data were normalized for cell number and assays performed in triplicate. The specificity of the assay was verified by adding excess DNA containing the p65-binding site as a competitor for NF-$\kappa$B (data not shown), subtracting noncompetitive binding from values.

Expression and purification of SseL catalytic domain

SseL (179–341) and the corresponding SseL (C285A) mutant cloned into pET21b were expressed in $\textit{Escherichia coli}$ (BL21 (DE3) RP), and purified by liquid chromatography using Ni-NTA agarose (Qiagen) followed by mono-Q, then concentrated with buffer exchange on an Amicon Ultra filtration apparatus (molecular mass of 10,000 Da). The proteins were exchanged into 50 mM Tris (pH 8.0), 1 mM DTT. Both proteins were obtained with 90–95% of purity, as determined by SDS-PAGE gel stained with Gelcode Blue (Pierce). Gel filtration using Superdex 200 suggests that SseL (211–341) has a monomeric structure.

Ubiquitin protease assays

Purified SseL WT or C285A catalytic domains, or human rSENP8 were incubated with ubiquitin-7-amino-4-methylcoumarin (ubiquitin-AMC), small ubiquitin-related modifier (SUMO)-AMC, or neural precursor cell-expressed developmentally down-regulated 8 (NEDD8)-AMC with or without addition of 8 $\mu$M ubiquitin-aldehyde (Boston Biochem) in 20 mM HEPES-KOH (pH 7.5) and 1 mM DTT. The resulting liberated fluorogenic AMC was measured continuously at 23°C for 1 h using an Analyst HT fluorometer. Relative fluorescence units were converted to picomoles of AMC using a standard curve of relative fluorescence units vs AMC concentration. We assume that 100% of purified SseL catalytic domain was active. $K_m$ and $V_{max}$ were determined using a nonlinear regression method to fit the Michaelis-Menten equation: $V = (V_{max} - V_0)(S)/(K_m + (S)) + V_0$, where $V$ = initial catalytic rate, in nanomoles of AMC per minute; (S) = concentration of the substrate in nanomoles or micromoles; $V_0$ = limiting value of $V$ without (S);

![Figure 1](https://www.jimmunol.org/)

**FIGURE 1.** SseL inhibits NF-$\kappa$B activation downstream of the IKK complex and upstream of p65 and p52 NF-$\kappa$B. A, HEK293T cells were transfected with empty vector (200 ng), or plasmids encoding SseL or SseL C285A mutant (SseL C/A). NF-$\kappa$B activity induced by TNF-$\alpha$ was monitored by gene reporter assays (mean ± SD; $n = 3$). B, NF-$\kappa$B activity was also induced by transfecting HEK293N cells with expression plasmids (200–300 ng) encoding CD4/TLR4 or MyD88, in combination with empty vector (200 ng) or plasmids encoding SseL or YopJ. Cells transfected with IL-1R1-encoding plasmid or empty vector were cultured with IL-1$\beta$. B–F, To map the site of inhibition within the NF-$\kappa$B pathway, similar transfection experiments were conducted using expression plasmids encoding Bcl-10 (B), TRAF6, Cardiak, TRAF2 (C), CD40 (D), IKK$\beta$, IKKa (E), or p65 and p52 (F). G, β-Catenin transcriptional activity was induced by cotransferring a β-catenin-luc reporter gene plasmid (100 ng) and β-catenin-encoding plasmid (500 ng) with plasmids encoding either SseL or YopJ or pcDNA3Myc, holding total transfected DNA at 1 $\mu$g. H, Expression of transfected TRAF2, TRAF6, Cardiak, IKK$\beta$, IKKa, and SseL-Myc was verified after cotransferection with SseL plasmid or pcDNA3Myc by immunoblotting (C–, untransfected cells). Arrowheads and asterisks denote specific and nonspecific bands, respectively.

**Measurement of p65 nuclear translocation and p65 DNA-binding activity**

Nuclear p65 translocation was monitored in HEK293N cells grown in 100-cm$^2$ plates, transfected with 10 $\mu$g of DNA, then cultured with or without TNF-$\alpha$ for various times. Relative amounts of p65 DNA-binding activity were also measured from RAW264.7 cells or primary macrophages before or at various times after bacterial infection. Nuclear extracts were prepared using a kit (ActiveMotive), and relative amounts of p65 protein in nuclear extracts were quantified by ELISA, according to the manufacturer’s instructions (p65 ActivELISA; Imgenex). Data were normalized for cell number and assays performed in triplicate. The specificity of the assay was verified by adding excess DNA containing the p65-binding site as a competitor for NF-$\kappa$B (data not shown), subtracting noncompetitive binding from values.

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$V_{\text{max}}$ = a limiting value of $V$ at sufficient high or saturating ($S$). The fitting procedure was performed using PRISM software.

**Bacterial infection of RAW 264.7 and bone marrow murine macrophages**

RAW 264.7 cells were seeded at $3.5 \times 10^6$ cells, and murine macrophages at $8 \times 10^6$ cells in 100-mm dishes, then infected 24 h or 7 days later, respectively. Bacterial invasion and intracellular replication assays were performed as previously described (25).

**Bacterial infection of mice**

A streptomycin pretreatment model was used using inbred pathogen-free female BALB/c mice (Harlan Breeders) at 8 –10 wk age as described in Ref. 26. Animals were euthanized by i.p. injection of 1 ml of avertin (BD Biosciences) at various times postinfection (p.i.). Organs were harvested aseptically for histological examination and colony counts. All animal experiments were conducted according to local animal care advisory committee guidelines.

**Determination of TNF-$\alpha$, IL-12p70, and IL-1$\beta$ levels in serum and supernatant**

Serum was collected from mice 4 and 9 days postinfection. Supernatants of infected RAW264.7 cells infected with various *Salmonella* strains were collected at various times p.i. Cytokines levels were measured by ELISA kits from eBioscience, according to the manufacturer’s protocol. Three mice were used per condition, and each sample was measured in duplicate expressing results as means $\pm$ SE.

**FIGURE 2.** SseL inhibits RelA nuclear translocation upon TNF-$\alpha$ stimulation. A, HEK293N cells were cotransfected with empty vector (−) or SseL plasmid (+). At 48 h posttransfection, cells were treated with TNF-$\alpha$ (10 ng/ml) for 6 h. Nuclear and cytosolic extracts were subjected to immunoblot analysis with Abs specific for RelA, caspase-3, and PARP (as markers of cytosolic and nucleus extracts, respectively). B, Alternatively, nuclear extracts were analyzed by ELISA for RelA protein (mean $\pm$ SD; $n = 3$) (data are representative of several experiments; $n \geq 3$).

**FIGURE 3.** SseL catalytic domain cleaves ubiquitin-AMC in vitro. A, Michaelis-Menten analysis of SseL catalytic domain (CD-SseL) activity was performed by incubating CD-SseL or CD-SseL C285A (CD-SseL C/A) (3.6 $\mu$M) with increasing amounts of ubiquitin-AMC for 1 h, and measuring hydrolysis of ubiquitin-AMC continuously to determine the values for $k_{\text{cat}}$, $K_m$, and $V_{\text{max}}$, as indicated (a representative experiment is shown; $n \geq 3$). B, Various concentrations of isopeptidase T were incubated with ubiquitin-AMC (20 $\mu$M) (Ub-AMC) as positive control for deubiquitinating activity, or with ubiquitin-AMC and ubiquitin-aldehyde (8 $\mu$M) (Ub-Ald) or with SUMO-1-AMC (20 $\mu$M) to verify the specific deubiquitin activity of isopeptidase T. C, Various concentrations of CD-SseL were incubated with ubiquitin-AMC (20 $\mu$M) or SUMO-AMC (20 $\mu$M) as described above to verify the specificity of the enzymatic activity of CD-SseL. D, Logarithmic dilutions of purified CD-SseL and SENP8 (used as positive control for specific deNEDDylation activity) were incubated with NEDD8-AMC (3 $\mu$M) as substrate and deNEDDylation activity was measured by the release of fluorescent AMC (mean $\pm$ SEM; $n = 3$). Specific de-NEDDylation activity was verified by adding NEDD8-aldehyde (NEDD8-Ald) (8 $\mu$M) (data are representative of several experiments; $n \geq 3$).
NF-κB downstream of the IKK complex by impairing IκBα degradation and ubiquitination. A, IκBα degradation is impaired by SseL expression. HEK293T cells were cotransfected with a plasmid encoding IκBα in combination with SseL or SseL C285A mutant-encoding (SseL C/A) plasmid or with pcDNA3Myc empty vector for 24 h before TNF-α stimulation (30 ng/ml). Cell lysates were subjected to immunoblot analysis with Abs recognizing IκBα (top) or phospho-IκBα (middle). Membranes were reprobed using Abs to hsp60 for loading control (bottom). B, SseL expression inhibits IκBα ubiquitination. HEK293T cells were cotransfected as described above, and treated with TNF-α (30 ng/ml) (left). Cell lysates normalized for protein content were IP using anti-FLAG mAbs. Immunoprecipitates or cell lysates were analyzed by immunoblotting (IB) using Abs to FLAG epitope (top), ubiquitin (second and fourth panels), and IκBα (third panel). As specificity controls for the experiment, HEK293T cells were transfected with plasmids encoding FLAG-tagged IκBα (lanes 1, 2) or untagged IκBα (lane 3). Immunoprecipitates were performed using anti-FLAG (lanes 1, 3) or a control IgG Ab (lane 2). IPs and lysates were analyzed by immunoblotting. Arrowheads and asterisks denote specific and nonspecific bands, respectively.

Results

SseL prevents NF-κB activation downstream of IKK complex

The bacterial virulence-associated effector proteins YopJ and AvrA inhibit the innate immune response by suppressing NF-κB signaling pathways upstream and downstream of the IKK complex, respectively (15, 27). By analogy to these bacterial proteins, we hypothesized that SseL may also inhibit NF-κB activity, through its DUB activity. To explore this possibility, we first performed NF-κB reporter gene assays using HEK293T cells transfected with plasmids encoding SseL or SseL C285A (SseL C/A), in which the predicted active site cysteine was mutated to alanine. Cells were cotransfected, with a luciferase reporter gene plasmid containing NF-κB-binding sites within its promoter. SseL significantly inhibited NF-κB activity stimulated by treatment of cells with TNF-α and IL-1β cytokines, while SseL C285A did not (Fig. 1, A and B, and data not shown). NF-κB activity was also inhibited by SseL when induced by transfection of a constitutively active LPS receptor TLR4 (CD4:TLR4 fusion protein) (Fig. 1B). As expected, YopJ suppressed TLR4-mediated NF-κB activity (Fig. 1B). Expression of SseL-Myc and equivalent protein loading were verified by immunoblotting using anti-Myc and anti-FLAG mAbs, respectively (data not shown). Representative experiments are shown; n ≥ 3.

Histopathology

At various days p.i., spleens, livers, and intestine from infected and control animals were fixed in zinc-buffered formalin (Z-fix; Anatech), and embedded in paraffin. Organs were then sectioned at 0.5 mm, stained with H&E, and analyzed blindly. The pathological scores were quantified by an established method (26), averaging six fields per sample, based on variables of polymorphonuclear neutrophil (PMN) infiltration into various locations, including gut lumen (scant, 2; moderate, 3; dense, 4), intestinal surface epithelium (yes, 1; no, 0), submucosa (no extravascular PMN = 0; single extravascular PMN = 1; PMN aggregates = 2). Statistical analysis was performed by comparing total pathological scores using Mann-Whitney U and Kruskall-Wallis nonparametric tests. Values of p < 0.05 were considered as statistically significant.
SseL appears to operate at a downstream point of convergence of diverse NF-κB activation pathways. Because essentially all NF-κB activation pathways converge on IKK, we tested the ability of SseL to inhibit NF-κB activity induced by overexpression of the kinase components of this complex, IKKα and IKKβ, using the same NF-κB reporter gene approach. SseL significantly inhibited NF-κB activity induced by either IKKα or IKKβ (Fig. 1E), suggesting it operates at the level of or downstream of kinases. In contrast, YopJ did not inhibit NF-κB activity induced by IKKβ, consistent with previous reports showing that YopJ blocks NF-κB activation upstream of the IKK complex (30). Thus, SseL targets a more distal point in NF-κB activation pathways than YopJ. Although SseL suppressed NF-κB activity induced by IKKα and IKKβ, it did not suppress NF-κB reporter gene activity induced by overexpression of p65-RelA or p52-NF-κB2 (Fig. 1F), indicating that SseL functions upstream of these transcription factors that comprise subunits of NF-κB heterodimer complexes that are competent to bind DNA (31). The effects of SseL on NF-κB transcriptional activity, however, were selective, in as much as SseL did not suppress the activity of other transcription factors measured in similar reporter gene assays, such as β-catenin/T cell factor or p53 (Fig. 1G, and data not shown). Furthermore, in experiments where SseL was cotransfected with plasmids encoding TRAF2, TRAF6, Cardiak, IKKβ, or IKKα, we confirmed that the effect on NF-κB activation was not due to suppression of protein expression (Fig. 1H).

Reporter gene assays of NF-κB activity were then confirmed using other methods. Upon degradation of IkB-family proteins, NF-κB enters the nucleus. We therefore anticipated that expression of SseL in cells would inhibit nuclear entry of NF-κB. Accordingly, relative levels of nuclear NF-κB (p65 Rel-A subunit) were assessed before and after TNF-α stimulation in control or SseL-expressing cells by immunoblot analysis of nuclear extracts. In control-transfected cells, TNF-α induced a striking increase in the relative amount of nuclear p65-RelA compared with unstimulated cells (Fig. 2A). In contrast, levels of nuclear p65-RelA were unchanged in SseL-expressing cells after TNF-α stimulation. Probing the blots with Abs to nuclear (PARP) and cytosolic (caspase-3) marker proteins confirmed successful cell fractionation. Analysis of the cytosolic fraction showed similar levels of RelA in all samples, implying that most of the total cellular pool of RelA remained cytosolic under the conditions of these TNF-α stimulation experiments. The ability of SseL to suppress nuclear import of RelA was also examined by an ELISA method using nuclear fractions from SseL- and control-transfected cells, before vs after TNF-α stimulation (Fig. 2B), thereby independently confirming the results obtained by immunoblotting.

SseL is a deubiquitinating enzyme

The profile-profile algorithm FFAS03 matched the C-terminal ≈ 200 aa of SseL to a family of Ulp1 proteases and to other proteins from the sentrin-specific protease family with high confidence (Z score of 14.0, corresponding to a false-positive rate of <1%) (23). In addition, SseL was recently reported to be a cysteine protease, which hydrolyzes synthetic substrate ubiquitin-AMC, and branched ubiquitin polymers (23). Because the active site was predicted by three-dimensional modeling to include cysteine 285, we expressed in bacteria and purified the predicted catalytic domain of SseL (279–341) (CD-SseL), and C285A mutant (CD-SseL C/A), then tested the activity of these proteins using in

**FIGURE 5.** SseL does not deubiquitinate TRAF2 or TRAF6. A, SseL-Myc, ubiquitin-FLAG and TRAF2-HA or empty vectors were cotransfected into HEK293T cells after 24 h, cells were treated with TNF-α (30 ng/ml) for 30 min and cell lysates were normalized for protein content before performing immunoprecipitations using anti-HA mAbs (IP-HA). Immunoprecipitates (IPs) were then subjected to immunoblots (IB) using anti-HA and anti-FLAG specific Abs to assess the expression and the ubiquitination of TRAF2, respectively. Expression of ubiquitin-FLAG and SseL-Myc was verified by immunoblotting of total lysates (Lysates) using anti-FLAG and anti-Myc mAbs, respectively. B, At 24 h posttransfection with the indicated plasmids or empty vectors, cells were treated with MG132 (40 μM) for 1 h. Cell lysates were immunoprecipitated using anti-HA mAbs (IP-HA). Immunoprecipitates (IP-HA) were immunoblotted with anti-HA- and anti-FLAG-specific Abs to verify the expression and the ubiquitination of TRAF6, respectively. Transfected ubiquitin-FLAG and SseL-Myc levels were verified by immunoblots with anti-FLAG and anti-Myc Abs, respectively. Equivalent protein loading was verified by immunoblotting using anti-β-actin mAbs (data not shown). Data are representative of several experiments (n ≥ 3). Asterisks denote nonspecific bands. Arrow indicates HA-tagged TRAFs.
TNF-α-induced degradation and ubiquitination. In control cells, TNF-α-stimulation led to IKK complex, we hypothesized that SseL opposes IkBα ubiquitination, thus inhibiting its proteasome-mediated degradation. To test this hypothesis, we first compared IkBα levels by immunoblot analysis in HEK293T cells cotransfected with plasmids encoding IkBα and either SseL or C285A (or empty control plasmid), then stimulated cells with TNF-α to induce IkBα degradation. In control cells, TNF-α induced a rapid decline inIkBα levels, decreasing to just barely detectable levels within 45 min. By comparison, in SseL-expressing cells, IkBα levels remained essentially unchanged throughout the time-course of TNF-α stimulation evaluated. Mutation of cysteine 285 abolished this effect (Fig. 4A). Expression of SseL did not affect phosphorylation of IkBα. In both control- and SseL-transfected cells, TNF-α-induced phosphorylation of IkBα with a peak of phosphorylation seen at 10–20 min following TNF-α stimulation (Fig. 4A). We conclude therefore that SseL impairs cytokine-induced IkBα degradation but not phosphorylation.

To assess the effect of SseL expression on IkBα ubiquitination, HEK293T cells were cotransfected as above and stimulated by TNF-α, then IkBα was recovered from cell lysates by immunoprecipitation and ubiquitin was detected by immunoblotting. Ubiquitinylated species of various molecular masses were detected in IkBα immune complexes from TNF-α-treated cells (Fig. 4B). In contrast, ubiquitinylation of IkBα was not detected in SseL-expressing cells. Analysis of IkBα expression levels from immunoprecipitates or total lysates verified that SseL expression impairs its TNF-α-induced degradation, as expected (Fig. 4B). Although reducing IkBα ubiquitinylation, a general defect in ubiquitinylation of cellular proteins was not observed in SseL-expressing cells, as determined by immunoblot analysis of cell lysates using anti-ubiquitin Abs (Fig. 4B, bottom panel). Altogether, these data indicate that SseL inhibits IkBα degradation and ubiquitination.

Activation of the canonical NF-κB pathway often requires Lys63 polyubiquitination of TRAF2 and TRAF6 to mediate their activation of kinases leading to IkBα degradation (32). To determine whether SseL expression also targets other ubiquitination events acting upstream of IkBα, we assessed the effect of SseL expression on the expression and ubiquitination of TRAF2 and TRAF6. Expression of SseL did not reduce expression or polyubiquitination of TRAF2 or TRAF6 (Fig. 5). These results therefore suggest a specific effect of SseL on IkBα ubiquitination.

SseL deficiency increases IkBα degradation and NF-κB activation in Salmonella-infected macrophages

To delineate the physiological relevance of SseL in Salmonella pathogenesis, we generated a nonpolar, in-frame deletion of sseL in S. enterica serotype typhimurium (∆sseL), in which the sseL gene was replaced with a Kan’m cassette using the “Red Swap” method (24). Loss of the allele and replacement by Kan’ gene were verified by PCR using gene-specific primers (Fig. 6A), and independent phage p22 transductants were selected for further study. Ablation of the gene encoding SseL did not alter the growth properties of S. typhimurium in either rich or nutrient-poor media (data not shown), indicating that SseL is not required for bacterial growth. Ablation of sseL did not impair the ability of S. typhimurium to invade and replicate in macrophages (Fig. 6B), despite the observation that SseL expression is induced upon exposure to macrophages (Fig. 6C) (22).

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**FIGURE 6.** Ablation of sseL does not impair survival and replication of Salmonella in macrophages. A. Generation of sseL mutant Salmonella strains. Salmonella ΔsseL mutant colonies were generated and analyzed for sseL disruption and for Kan’ expression by PCR; C−: negative control; C+: S. typhimurium WT genomic DNA. B. Expression of SseL in RAW264.7 cells infected with S. typhimurium but not ΔsseL mutant Salmonella was verified by immunoprecipitation followed by immunoblotting using polyclonal antiserum directed against SseL, after normalization of lysates for protein contents (a representative experiment is shown; n ≥ 3). C. S. typhimurium 14028 WT, and ΔsseL were used to infect RAW264.7 cells (m.o.i. = 10), and the numbers of intracellular CFU were assessed at various times postinfection (p.i.) (a representative experiment is shown; mean ± SEM; n ≥ 3).
To further explore whether SseL impairs degradation of endogenous IκBα in the context of actual infection, we assessed the endogenous level of IκBα expression in RAW264.7 cells infected with 14028s WT and ΔsseL Salmonella strains. Immunoblot analysis indicated that ablation of sseL decreased IκBα expression, as early as 4 h p.i. (Fig. 7A), and this effect was sustained over the time course of Salmonella infection examined. As determined by scanning densitometry, IκBα was 75% lower in cells infected with ΔsseL at 4 h and 40% lower at 8 h, compared with WT bacteria. Thus, ablation of sseL increases Salmonella-induced IκBα degradation in infected macrophages.

As an additional control for these experiments, we also generated transformants of the mutant ΔsseL Salmonella strain in which sseL was restored in trans by introducing a bacterial plasmid (ΔsseL plus pWSK-sseL) (Fig. 7B). Primary mouse bone marrow macrophages were infected with WT, ΔsseL, or ΔsseL plus pWSK-sseL Salmonella strains. At 8 h after invasion, infection with WT bacteria induced a ~4-fold increase in NF-κB DNA-binding activity, whereas a ~8-fold induction was detected in ΔsseL-infected cells, approximately double the amount seen in macrophages infected with WT (Fig. 7C). In contrast to ΔsseL strain, bacteria in which sseL was reconstituted by transformation with a pWSK-sseL-plasmid stimulated essentially the same levels of NF-κB activity as WT bacteria (Fig. 7C). These genetic complementation experiments therefore confirm a direct cause-and-effect role for sseL in controlling NF-κB activity in the context of infection of primary murine macrophages with S. typhimurium. They also argue against artifacts related to the Kan' insertional mutagenesis procedure.

In addition to NF-κB activity, we also observed a 2–fold increase in production of an NF-κB-dependent cytokine IL-1β in the supernatant of RAW264.7 cells infected with ΔsseL strain vs WT or ΔsseL plus pWSK-sseL Salmonella strains (Fig. 7D). These results thus provide additional confirmation of the inhibitory effect of SseL on NF-κB activity in the context of Salmonella infection.

In addition to measuring nuclear NF-κB DNA-binding activity and levels of NF-κB-dependent cytokine, immunoblot analysis of RelA in cytosolic vs nuclear fractions also showed a difference in expression levels.
between macrophages infected with WT vs ΔsseL bacteria, such that 63–67% less RelA remained in the cytosol and 21–32% more RelA accumulated in the nuclei of cells infected with ΔsseL bacteria (as determined by scanning densitometry, data not shown). Results were confirmed using several ΔsseL strains obtained by independent p22 phage transduction (data not shown).

To explore whether the effect of the ΔsseL mutant on IκBα expression and NF-κB activity might be secondary to cytotoxicity mediated by Salmonella infection, we measured cell death in RAW264.7 macrophages infected with WT, ΔsseL, or ΔsseL plus pWSK-ΔsseL Salmonella strains. At each time point measured p.i. (8, 12, and 24 h), SseL-expressing Salmonella strains induced only ~10% more cell death than ΔsseL bacteria (Fig. 7E), suggesting that a difference in cell viability cannot account for the effects on NF-κB and IκBα.

**SseL deficiency increases IκBα degradation and ubiquitination in Salmonella-infected macrophages.**

Next, the effects of WT and ΔsseL bacteria were compared with respect to IκBα ubiquitination in infected primary murine macrophages. Compared with WT Salmonella, ΔsseL bacteria infection led to increased IκBα degradation and ubiquitination in infected cells, as shown by IκBα protein expression (measured by immunoblotting) and ubiquitination (determined by immunoprecipitation of IκBα, followed by immunoblot analysis with anti-ubiquitin Ab). In contrast, introduction in trans of sseL in ΔsseL mutant bacteria (ΔsseL plus pWSK-ΔsseL) restored IκBα expression and ubiquitination to the same levels seen in WT-infected cells (Fig. 8). These genetic complementation experiments therefore also verify in infected primary macrophages that SseL is directly responsible for IκBα stabilization and deubiquitination in the context of Salmonella infection.

**Ablation of sseL gene increases the in vivo inflammatory response to Salmonella infection.**

We used a mouse model to investigate the role of SseL in enteric salmonellosis (33, 34). First, the lethal dose of *S. typhimurium* 14028s was determined in BALB/c mice by orally injecting serial dilutions of bacteria. Because inoculation of ≥10⁷ WT bacteria induced 100% death by 5 days, mice were orally infected with 2 × 10⁶ WT bacteria. At 4 and 9 days p.i., organs were harvested aseptically for colony counts, and organ lysates were diluted before plating on selective LB plates. Bars indicate geometric means (a representative experiment is shown; n ≥ 3). B, Mice were infected with WT or ΔsseL mutant bacteria and tissues scored for inflammation as described in Materials and Methods. Livers from WT-infected mice display fewer granulomas (a, arrows) than ΔsseL mutant (b). Spleens from ΔsseL-infected mice display numerous granulomatous lesions in the red pulp area (d, arrows), whereas they were not detected in the WT-infected mice (c). C, Average numbers of granulomas in liver per microscopy field and per group of mice (mean ± SD; n = 3). Original magnification is indicated (×200 or ×400).

**FIGURE 8.** SseL prevents IκBα degradation and ubiquitination in infected mouse primary macrophages. Murine primary macrophages were infected with WT, ΔsseL, or ΔsseL plus pWSK-ΔsseL bacteria (m.o.i. = 5), for 30 min. Cells remained untreated for negative control (Ctrl−) or were treated with TNF-α 30 min (30 ng/ml) for positive control (Ctrl+). Cell lysates were normalized for protein contents and subjected to IP using anti-IκBα mAbs. IκBα expression levels and ubiquitination status were analyzed from the immunoprecipitates and from the lysates by immunoblotting (IB) using anti-IκBα, anti-ubiquitin mAbs, respectively. Probing with anti-β-actin mAbs served as a loading control. Data are representative of several experiments (n ≥ 3).

**FIGURE 9.** Inactivation of sseL increases inflammatory response to *Salmonella* in mice. A, Bacterial enumeration in infected livers and spleens 4 and 9 days p.i. Streptomycin-treated mice were infected orally with 2 × 10⁷ WT or ΔsseL mutant bacteria. At 4 and 9 days p.i., organs were harvested aseptically for colony counts, and organ lysates were diluted before plating on selective LB plates. Bars indicate geometric means (a representative experiment is shown; n ≥ 3). B, Mice were infected with WT or ΔsseL mutant bacteria and tissues scored for inflammation as described in Materials and Methods. Livers from WT-infected mice display fewer granulomas (a, arrows) than ΔsseL mutant (b). Spleens from ΔsseL-infected mice display numerous granulomatous lesions in the red pulp area (d, arrows), whereas they were not detected in the WT-infected mice (c). C, Average numbers of granulomas in liver per microscopy field and per group of mice (mean ± SD; n = 3). Original magnification is indicated (×200 or ×400).
10^3 WT or ΔsseL S. {\textit{typhimurium}} 14028s WT bacteria, and animals were sacrificed from days 3 to 9 p.i. Bacterial migration into liver and spleen after oral administration was comparable for both groups of mice, similar to those previously reported (33). However, clear differences in the host reactions to WT vs ΔsseL bacteria were noted. At 4 days p.i., livers of mice infected with ΔsseL bacteria displayed 4-fold more granulomas than livers from mice exposed to WT bacteria (Fig. 9B, a and b, and C). Similarly, the spleens of mice infected with ΔsseL bacteria revealed the presence of granulomas, whereas these lesions were not detected at this stage of infection in the spleens of mice infected with WT bacteria (Fig. 9B, c and d).

In the intestine, common features of {\textit{Salmonella enterocolitis}} include intestinal infiltration of PMNs and mononuclear inflammatory cells, associated with epithelial mucosal and submucosal erosion, desquamation, and destruction (33). An established histological scoring method (26) was used to compare the degree of pathological changes in the intestines of mice infected with WT vs SseL-deficient S. {\textit{typhimurium}}. At day 4 p.i., PMN infiltration into the lumen and into the submucosa and surface epithelium of the intestine was more extensive in mice infected with SseL-deficient bacteria compared with WT bacteria (Fig. 10A). At days 4 and 9 p.i., we also observed higher levels of several NF-κB-inducible cytokines (TNF-α, IL-12, IL-1β) in the serum of mice infected with ΔsseL compared with WT-infected mice (Fig. 10B). These results thus demonstrate that inactivation of sseL enhances the in vivo inflammatory response to S. {\textit{typhimurium}}.

Interestingly, animals infected with ΔsseL bacteria died earlier than mice infected with WT 14028s bacteria. For example, at 7 days p.i., ~30% of mice infected with ΔsseL bacteria were dead, whereas none of the mice infected with WT bacterial had died by this time (Fig. 10C). Thus, ablation of the gene encoding SseL results in more rapid lethality under the experimental conditions of salmonellosis tested here, suggesting that excessive host inflammatory responses to SseL-defective bacteria may contribute to lethality.

**Discussion**

Because NF-κB pathways make key contributions to host defense mechanisms, the activation of NF-κB is tightly controlled by endogenous regulators acting at several sites. Ubiquitination regulates at least four steps in NF-κB pathways, including targeting IκBα for degradation, processing of NF-κB precursors to produce p50/p52 subunits of NF-κB, and activation of kinases such as receptor-interacting protein or the regulatory subunit of the IKK complex IKKγ (NEMO) and activation of TRAF-family adapter proteins (TRAF2, TRAF6) (9, 35, 36). Ubiquitin is a 76-aa protein that can be covalently attached to target proteins through an isopeptide bond, between the C terminus of ubiquitin and the ε-amino group of a lysine residue in the target proteins (9, 37). Ubiquitin contains seven lysine residues, but ubiquitin chains linked on Lys^48 and Lys^63 are the best characterized, to date. Whereas Lys^48-linked polyubiquitin chains represent a signal for proteosomal degradation of modified substrates such as IκBα, Lys^63-linked polyubiquitination acts as scaffolds to assemble signaling complexes and regulates protein localization, protein kinase activation, DNA repair, or transcription through proteosome-independent mechanisms (38, 39). However, both Lys^48 and Lys^63-mediated ubiquitination play roles in regulating NF-κB activity (10, 35, 38, 40).

Several examples have been elucidated whereby pathogens inhibit or limit the duration of NF-κB activation, thus blunting host responses. Many animal and plant pathogenic bacteria use type III and/or IV secretion systems to inject effector proteins into host cells, where they subvert host signaling cascades, such as ubiquitination and/or NF-κB activation (41, 42). For example, the \textit{Yersinia} type III effector protein YopJ blocks signaling of the MAPK kinase and NF-κB pathways by acetylating and/or deubiquitinating host MAPK kinases and NF-κB-signaling kinases (TRAF6),...
thereby inactivating innate immune pathways (16–19). The Salmonella AvrA protein and AopP, a recently identified secreted effector from Aeromonas salmonicida (spp. Salmonicidae), were also shown to inhibit activation of NF-κB downstream of IKK/β (27, 43). AvrA has recently been characterized as a DUB that removes ubiquitin from IκBα and β-catenin, thereby preventing their degradation and subsequent NF-κB activation (13).

Here, we provide evidence that SseL, a recently identified DUB produced by S. enterica serovar typhimurium (23), inhibits cellular NF-κB activity and impairs IκBα degradation and ubiquitination, thus suppressing multiple upstream signaling pathways that converge on IκBα to induce NF-κB activation. SseL is an Salmonella pathogenicity island 2 effector encoded by the regulatory system ssrB and translocated into host cells by the type III secretion system during infection (22). Additionally, the amino acid primary sequence of SseL shows similarities with known DUBs in yeast (Ulp1), Chlamydia trachomatis (ChlDAUB1), human adenovirus L3, and Homo sapiens (SENP1) (23).

Our enzymatic analysis demonstrated that the catalytic domain of purified SseL possesses intrinsic deubiquitinating activity, requiring Cys285, as previously shown but reported as Cys262 by Rytkonen et al. (23) due to a difference in assignment of the N terminus of the SseL protein deduced from genomic sequencing. We measured a K_m of ~10 μM for the ubiquitin substrate, using the purified catalytic domain of SseL, compared with ~1.5 μM reported by others for the full-length protein (23). This difference suggests the conserved ~140 aa N-terminal domain of SseL makes contributions to the catalytic efficiency of this bacterial protease. The specificity of SseL appears to be highly selective for ubiquitin, as other substrates containing ubiquitin-related molecules, NEDD8 and SUMO, were not cleaved. The previous report by Rytkonen et al. (23) characterized the DUB activity of the full-length SseL in vitro and in cells, finding higher activity against Lys63-linked ubiquitin chains compared with Lys48. In contrast, we observed inhibitory effects of SseL on ubiquitinylnation of IκBα (predominantly Lys48 ubiquitylation) but not TRAF2 or TRAF6 (predominantly Lys63). Thus, our findings favor a role for SseL in hydrolysis of Lys48-linked ubiquitin chains, but do not exclude a role also for Lys63-conjugated substrates.

Our results directly link the DUB activity of SseL to suppression of IκBα ubiquitination and degradation in epithelial cells and macrophages, showing that cysteine 285 is required for DUB activity and for the effects on NF-κB activity. The modulation of NF-κB activity by SseL was detected by expressing SseL in HEK293T cells by transfection, and also in RAW264.7 cells and primary cultured mouse macrophages by infection with WT and sseL-deficient Salmonella strains. These findings contrast with a previous report using I774 murine macrophage cells, which failed to detect effects of SseL deficiency on IκBα levels, and which found only a slight increase in production of NF-κB-inducible cytokine TNF-α in I774 cultures following cell infection with WT vs sseL-deficient strains (23). However, those studies did not directly measure NF-κB activity. Additionally, these differences could be due to the peculiarities of the transformed cell lines used and technical differences related to culture conditions, reagents, and the multiplicity of infection (m.o.i.) used. Unlike a prior report (23), we observed only a slight cytotoxic effect of SseL in infected RAW264.7 macrophages. This cytotoxic effect was evident at later stages of infection and did not account for the inhibition of IκBα degradation and NF-κB activity reported here.

When produced in HEK293T cells, SseL prevents phospho-IκBα degradation and ubiquitination triggered by TNF-α stimulation. Moreover, from comparisons of WT and ΔsseL Salmonella in macrophage infection experiments, we deduced that SseL stabilizes IκBα expression. Thus, based on the data presented here, and on the observation that SseL binds mono- and polyubiquitinated proteins (23), we propose that SseL may bind ubiquitinated IκBα after its phosphorylation, removing ubiquitin conjugates, and preventing IκBα recognition and degradation by the 26S proteasome, thereby limiting NF-κB activation.

A role for SseL in interfering with activation of NF-κB in vivo is also supported by the observation that the inflammatory response induced upon infection was more severe with the ΔsseL mutant than with the WT Salmonella strain. The elevated inflammatory response included PMN infiltration into intestinal tissues, granulomatous lesions in the liver, increased production of NF-κB-inducible cytokines in the serum, and more rapid death. These findings were confirmed using at least two independently derived ΔsseL mutant clones.

The increased rate of demise of mice infected with ΔsseL suggests that the inflammatory response to the pathogen contributes to lethality, particularly because little difference was observed in bacterial colonization of liver and spleen in vivo, and measurement of bacterial invasion and growth in macrophages in culture were also similar. In fact, using more sensitive competition experiments where knockout and WT bacteria are coinoculated into mice, it was recently suggested that ΔsseL bacteria have a slight competitive disadvantage with respect to early in vivo dissemination (22). Thus, the inability to inhibit NF-κB seems to have rendered SseL-deficient Salmonella more virulent, as defined by lethality in mice.

In both intestinal and systemic forms of Salmonella infection, there is a clear selective advantage for bacteria to maintain long-term viability of the host, increasing the transmissibility of the organism. The hallmark of Salmonella epidemiology in natural settings is the establishment of the carrier state to maintain the bacteria within the host population (44). Thus, by suppressing NF-κB, WT Salmonella reduces the host inflammatory response, which might promote more extensive tissue invasion or extend the long-term survival and replication of these pathogenic bacteria by maintaining a viable host for propagation. This “stealth” capability would also favor establishment of chronic carriers, providing a reservoir from which to seed new infections. Similar to SseL, the AvrA protein, also impairs NF-κB activation by impairing deubiquitination and subsequent degradation of IκBα (13), but studies of AvrA Salmonella in mice have not focused on NF-κB-driven or inflammatory events. OspG, a kinase produced by S. flexneri also prevents phospho-IκBα degradation and subsequent NF-κB activation. In accordance with the phenotype of mice infected with ΔsseL Salmonella, inactivation of OspG increases the host inflammatory response to S. flexneri infection (14).

It remains to be determined whether the ubiquitin hydrolase activity of SseL is relevant to cellular substrates beyond IκBα, which might dysregulate other components of the host immune response. The AvrA protease, for example, was reported to reduce ubiquitin-mediated degradation of both IκBα and β-catenin (13). Thus, while other substrates may exist, we propose that the suppression of NF-κB activation by SseL is at least one mechanism that endows pathogenic bacteria with virulence capabilities in specific contexts of host-pathogen interactions, the full consequences of which remain to be determined.

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