Injection of Flagellin into the Host Cell Cytosol by *Salmonella enterica* Serotype Typhimurium*

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Yao-Hui Sun, Hortensia García Rolán, and Renée M. Tsolis†
From the Department of Medical Microbiology and Immunology, University of California at Davis, Davis, California, 95616

Bacterial flagellins are potent inducers of innate immunity. Three signaling pathways have been implicated in the sensing of flagellins; these involve toll-like receptor 5 (TLR5) and the cytosolic proteins Birc1e/Naip5 and Ipaf. Although the structural basis of TLR5-flagellin interaction is known, little is known about how flagellin enters the host cell cytosol to induce signaling via Birc1e/Naip5 and Ipaf. Here we demonstrate for the first time the translocation of bacterial flagellin into the cytosol of host macrophages by the vacuolar pathogen, *Salmonella enterica* serotype Typhimurium. Translocation of flagellin into the host cell cytosol was directly demonstrated using β-lactamase reporter constructs. Flagellin translocation required the *Salmonella* Pathogenicity Island 1 Type III secretion system (SPI-1 T3SS) but not the flagellar T3SS.

Bacterial flagella are filamentous appendages on the cell surface that mediate bacterial motility. The filament of the flagellum is composed of ~20,000 flagellin subunits (1). Flagellin monomers of many bacterial species function as pathogen-associated molecular patterns and can be detected by host cells using both surface-localized toll-like receptor 5 (TLR5) and cytosolic Nod-like receptors (2–6). The interaction of flagellin with TLR5 at the cell surface has been characterized in detail, identifying the structural basis for the TLR5-flagellin interaction (7, 8). TLR5 sensing has been shown to be important in Legionnaires disease, a respiratory tract infection caused by the bacterium *Legionella pneumophila*, since human TLR5 polymorphisms correlated with disease susceptibility (7, 8). In the intestine, TLR5 localizes to the basolateral surface of enterocytes and to CD11c⁺ lamina propria dendritic cells (9, 10). In these locations, TLR5 is thought to be involved in sensing a breach of the intestinal mucosa by enteroinvasive pathogens, triggering an inflammatory response that limits systemic spread of the bacteria (11).

In addition to sensing by TLRs, flagellin was recently shown to be sensed by two different Nod-like receptors, Ipaf and Birc1e (also known as Naip5) (2, 4–6, 12). Ipaf transmits a proinflammatory signal in response to flagellin by activating the inflammasome, resulting in caspase 1-dependent activation of IL-1β, which is accompanied by cell death (pyroptosis) (13–17). Birc1e/Naip5 signals in response to flagellin, restricting intracellular growth of *L. pneumophila* by antagonizing the ability of the bacterium to avoid fusion with lysosomes and form a replicative phagosome (18). Several lines of evidence suggest that pathogens residing in a membrane-bound compartment of the cell, such as *L. pneumophila* and *S. Typhimurium* (*Salmonella enterica* serotype Typhimurium), may release flagellins from their vacuole into the host cytosol, which triggers signaling via Ipaf and Birc1e. First, *S. Typhimurium* and *L. pneumophila* mutants lacking flagellin elicit reduced caspase activation, IL-1β secretion, and cytotoxicity in macrophages (2, 4–6, 19). Second, mutants of *S. Typhimurium* lacking the SPI-1 T3SS and *L. pneumophila* mutants lacking a functional Type IV secretion system (T4SS) fail to trigger Ipaf-dependent signaling (4, 5, 19). Third, introduction of purified flagellins into the cytosol of macrophages using pore-forming toxins or detergents or by heterologous expression in *Escherichia coli* expressing listeriolysin O triggers caspase-1 activation and IL-1β activation by an Ipaf-dependent mechanism (2, 4–6, 20).

Although injection of effector proteins into host cells by the *S. Typhimurium* SPI-1 T3SS has been visualized microscopically (21, 22), it has not yet been demonstrated that in infected cells, bacteria are able to translocate flagellin from a membrane-bound compartment into the cytosol. In this report, we show that *S. Typhimurium* translocates flagellin into the cytosol of infected cells by a process that depends on the SPI-1 T3SS but is independent of the flagellar T3SS.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The *S. Typhimurium* strains used are derivatives of IR715, a spontaneous nalidixic acid-resistant mutant of ATCC14028 (23). An *invA* mutant (AJB75) was obtained by transduction of the *invA::TpophA* mutation from *S. Typhimurium* χ3642 using bacteriophage KB1int (24, 25). A *flgB* mutant was obtained by transduction of *flgB6148::MudJ* into IR715 from TH8460 (obtained from K. Hughes) using the bacteriophage P22HTint. A *flc::Tn10 fljB::MudJ* mutant (EHW26) was obtained from the Bäumler laboratory (26). For macrophage infection, *S. Typhimurium* was grown in Luria-Bertani (LB) broth to early logarithmic phase to maximize expression of the *Salmonella* pathogenicity island 1-encoded Type III secretion system (T3SS-1), as described previously (21).

**Plasmid Construction**—Plasmids are derivatives of the broad host range plasmid pBBR1MCS (27). Plasmids pFlagTEM-1, pStpA/FT, and pGST/FT (where FT indicates FlagTEM-1 fusions) have been described previously (21). In plasmid
pFlagTEM-1, the N-terminal signal sequence directing β-lactamase to the periplasm has been signal and replaced with a 3×FLAG epitope. To construct fusions of *S. Typhimurium* Phase I flagellin (FliC) to TEM-1, the *fliC* gene or portions containing the N-terminal 35, the N-terminal 48, or the C-terminal 225 amino acids were amplified from *S. Typhimurium*. These fragments were cloned into pFlagTEM-1 to generate constructs encoding FliC₁₋₄₉₅::FT, FliC₁₋₃₅::FT, FliC₁₋₄₈::FT, or FT::FliC₂₇₀₋₄₉₅. To express untagged FliC protein under control of the Trc promoter, fliC was amplified with NdeI/SalI extensions and cloned into pFlagTEM-1 digested with NdeI and XhoI. All constructs were confirmed by DNA sequencing.

**Isolation of Bone Marrow-derived Macrophages**—Bone marrow-derived macrophages (BMDMs) were isolated from C57BL/6 following standard protocols. Briefly, after aseptically obtaining femurs from the mice, the bone marrow cells were flushed out with 6 ml of cold RPMI 1690 medium (RPMI, Invitrogen). Cells were pelleted at 1,000 rpm for 10 min at 4 °C. The cells were resuspended in RPMI supplemented with 20% heat-inactivated fetal bovine serum, 1-ml conditioned medium, 1 mM glutamine, 1% nonessential amino acids, and 1% antibiotic-antimycotic (Invitrogen) and placed in Petri dishes at room temperature. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were collected from each well in 1 ml of PBS with a cell scraper. Data were acquired using an LSRII (Becton Dickinson), using 407 nm as excitation wavelength and bandpass filters 450/50 for detection of Cascade Blue and 545/60 for detection of AmCyan. Data were analyzed using FlowJo software (Tree Star, Inc).

**Western Blot**—After growing bacteria to mid-log phase, 1 mM IPTG was added for an additional 2 h to induce fliC expression. Bacterial supernatants were precipitated with 10% (w/v) trichloroacetic acid and resuspended in 1× Laemmli sample buffer. Bacteria were pelleted and resuspended in 1× Laemmli sample buffer and heated at 100 °C for 5 min. The total protein equivalent to 1 × 10⁷ colony-forming units or 1 ml of culture supernatant was loaded onto an SDS-PAGE gel and transferred to polyvinylidene difluoride membrane by electroblotting. FliC was detected using *Salmonella* Hi antiserum and goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP). FliC::FLAG-TEM-1 fusions were detected using anti-FLAG monoclonal antibody (1:5000, Sigma). Primary antibody binding was detected using a goat anti-mouse IgG antibody conjugated to HRP. HRP activity was detected with a chemiluminescent substrate (PerkinElmer Life Sciences).

**Macrophage Cytotoxicity**—The percentage of BMDMs death was determined by measuring lactate dehydrogenase (LDH) release at 1 and 4 h after infection using the CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI) following instructions provided by the manufacturer.

**Measurement of IL-1β Secretion**—IL-1β was measured in culture supernatants of infected BMDMs using an enzyme-linked immunosorbent assay kit (e-Bioscience, San Diego, CA) following the manufacturer’s instructions. Macrophages were primed with lipopolysaccharide as described above, and supernatants were collected 24 h after infection.

## RESULTS

Although the inability of *Salmonella* flagellin mutants to activate the inflammasome provides indirect evidence for translocation of flagellin into the host cell cytosol during infection, flagellin has not yet been detected directly in the cytosol of infected cells. To detect this translocation event occurring during infection, we employed a β-lactamase reporter system utilized previously by our group to visualize translocation of the *Salmonella* Typhi Type III secretion effector SipA (26). For this purpose, we expressed *S. Typhimurium* FliC from a derivative of the broad host range plasmid pBRR1MCS, pFlagTEM-1, in...
two flagellins, elicited neither IL-1β release nor cell death, consistent with earlier reports that flagellin expression is required for these cellular responses to Salmonella infection (2, 4).

We next expressed S. Typhimurium flagellin from the same promoter, with TEM-1 β-lactamase fused to its C-terminus. By loading S. Typhimurium-infected macrophages with the cell-permeable β-lactamase substrate CCF2/AM (28), cytosolic FliC::TEM-1 fusion protein can be detected. Cleavage of CCF2 by the β-lactamase reporter results in a shift in the fluorescence emission maximum from 520 (green) to 447 nm (blue) so that cells in which cytosolic translocation of FliC has occurred appear blue by fluorescence microscopy. At 4 h after infection of macrophages, a fusion of the full-length FliC protein to TEM-1 (FliC1–495::FT) could be detected in the cytosol of 77.5% of infected macrophages (Fig. 2A). This translocation was dependent on the SPI-1 T3SS as an invA mutant defective in the T3SS did not translocate the FliC1–495::FT fusion. Translocation was reduced but not abolished in a flgB mutant defective in the flagellar basal body, showing that the flagellar T3SS is not required for translocation of flagellin into the cytosol of infected cells. The number of cells containing translocated flagellin was similar to the number found for the SPI-1 T3SS effector SipA (Fig. 2A).

It has been shown that the N terminus of FliC is required for its secretion and assembly into the flagellar structure (29). The first 48 amino acids are sufficient for secretion of FliC into Salmonella culture supernatants (30). We therefore tested whether the FliC secretion domain is sufficient for its translocation into infected host cells. Fusions of the first 35 (FliC1–35::FT) or 48 (FliC1–48::FT) N-terminal amino acids of FliC to TEM-1 were sufficient for secretion into the supernatant of S. Typhimurium cultures (Fig. 2B). Inactivation of the SPI-1 T3SS by an invA mutation did not abolish secretion either of the full-length FliC fused to TEM-1 or of the N-terminal fusions of FliC to TEM-1, although the amount of protein in the supernatant was reduced (Fig. 2B). Inactivation of the flagellar T3SS by mutation of flgB, encoding a component of the basal body rod structure, also reduced, but did not abrogate, secretion of the N-terminal fusions of FliC to TEM-1. The reduction in FliC::FT translocation observed cells infected with the flgB mutant may be due to overexpression of chromosomally encoded flagellin in the flgB mutant (data not shown), which competes with the FliC::FT fusion protein for secretion (Fig. 2A). A fusion of the C-terminal 225 amino acids of FliC to the C terminus of TEM-1 (FliC270–495::FT) was expressed in S. Typhimurium but secreted in small amounts by the invA mutant. As expected, secretion of the SPI-1 T3SS effector SipA was eliminated by the invA mutation but not by the flgB mutation. These results show that either a functional SPI-1 T3SS or a functional flagellar T3SS is sufficient for secretion of N-terminal fusions of FliC in liquid cultures.

Although FliC1–35::FT and FliC1–48::FT were secreted to the culture supernatant, these domains did not mediate wild type levels of flagellin translocation into host cells (Fig. 2C). FliC1–48 mediated a low level of translocation of the TEM-1 reporter that was 3% of the level observed with full-length flagellin. FliC270–495, containing the binding site for the flagellar chap-
erone FliS (31), did not mediate translocation. These results suggest that domains of FliC in addition to the N-terminal secretion signal are required for translocation into the host cell.

DISCUSSION

Activation of caspase-1 and pyroptotic cell death in S. Typhimurium-infected macrophages has been shown to depend on Ipaf and ASC (32). Recent studies have shown that S. Typhimurium mutants lacking flagellin fail to activate this pathway and do not cause cell death (2, 4). In addition to flagellin, activation of the inflammasome also required a functional SPI-1 T3SS, suggesting that flagellin is secreted into the cytosol by this system (4). However, it has not been demonstrated that flagellin is actually translocated from the vacuole containing Salmonella into the host cell cytosol. Therefore, the translocation of flagellin into the cytosol of Salmonella-infected cells shown here provides an important link between the data obtained with bacterial flagellin mutants and results obtained with macrophages from Ipaf<sup>−/−</sup> and ASC<sup>−/−</sup> mutant mice that show a reduced responsiveness to flagellin delivered to the cytosol via transfection reagents (2, 4). Thus, this proinflammatory mechanism may have important consequences for in vivo infection, for example, by preventing systemic spread of Salmonella from the intestine. Caspase-1<sup>−/−</sup> mice exhibited reduced survival after oral infection with S. Typhimurium, and ASC<sup>−/−</sup> and Ipaf<sup>−/−</sup> mice had slightly higher systemic spread to the lymph nodes from the intestine (33), suggesting that activation of the inflammasome by cytosolic flagellin may have important implications for responses to invasive bacteria. In mice, S. Typhimurium was found in CD11c<sup>+</sup> dendritic cells in the lamina propria (10). Since S. Typhimurium induces caspase-1-dependent apoptosis of dendritic cells via the SPI-1 T3SS, these cells may sense cytosolic flagellin translocation by Salmonella and trigger proinflammatory responses (34). The Peyer’s patches of the ileum and the lamina propria would appear to be the most likely anatomical site for sensing of cytosolic flagellin since S. Typhimurium represses flagellin expression at systemic sites such as the mesenteric lymph nodes and spleen (35, 36).

The results of our study reveal mechanistic details of FliC egress into the cytosol of infected cells. The SPI-1 T3SS was absolutely required for localization of flagellin to the cytosol, whereas no strict requirement of the flagellar T3SS was observed (Fig. 2). These findings are consistent with those of Miao et al. (4), who tested a large number of mutants with specific defects in the flagellar apparatus for their cytotoxicity in macrophages. These investigators found that mutants lacking a functional flagellar apparatus were able to trigger cytotoxicity and IL-1β secretion when flagellin was expressed ectopically (4). Further, the inability of secretion-competent fusions of flagellin to TEM-1 β-lactamase to be translocated into infected cells (Fig. 2) argues against the idea that flagellin may be secreted into the vacuole containing Salmonella and then leak nonspecifically through holes in the vacuole created by the SPI-1 T3SS. Rather, the requirement for additional sequences beyond the secretion signal for cytosolic translocation is similar to what has been observed for some T3SS effectors that are injected into the host cell. For the Salmonella T3SS effector SspH2, translocation into host cells required the N-terminal 143 amino acids, which includes two domains conserved among a group of translocated effectors (37). Similarly, for the Yersinia T3SS effectors YopE and YopH, the N-terminal domain required for translocation (50 or 71 amino acids) was longer than that required for secretion into culture supernatants (15 or 17 amino acids) (38).

A previous report has described the release of flagellin into cells by a different mechanism. Gewirtz and colleagues (39) showed that S. Typhimurium colonizing the apical side of epi-
thelial monolayers is able to mediate flagellin transcytosis to the basolateral aspect. The translocation of flagellin into the macrophage cytosol observed by these investigators is mechanistically distinct from the cytotoxic translocation of flagellin reported here since in the previous study, flagellin was localized to membrane-bound vacuoles rather than being free in the cytosol. Further, flagellin transcytosis was dependent on a third T3SS, encoded on Salmonella pathogenicity island 2 (SPI-2 T3SS), and was independent of the SPI-1 T3SS (39). Taken together, these results show that flagellin interacts with the innate immune system at multiple levels to induce proinflammatory responses during Salmonella infection.

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