Flagellin Suppresses Epithelial Apoptosis and Limits Disease during Enteric Infection

Matam Vijay-Kumar,* Huixia Wu,*
Rheinallt Jones,* George Grant,[†] Brian Babbin,*
Timothy P. King,[†] Denise Kelly,[†]
Andrew T. Gewirtz,* and Andrew S. Neish*

From the Department of Pathology and Laboratory Medicine,*
Epithelial Pathobiology Unit, Emory University School of
Medicine, Atlanta, Georgia; and the Gut Immunology Group,[†]
Rowett Research Institute, Bucksburn, Aberdeen, United Kingdom

Flagellin, the primary component of bacterial flagella, is a potent activator of toll-like receptor 5 (TLR5) signaling and is a major proinflammatory determinant of enteropathogenic *Salmonella*. In accordance with this, we report here that aflagellate *Salmonella* mutants are impaired in their ability to up-regulate proinflammatory and anti-apoptotic effector molecules in murine models of salmonellosis and that these mutants elicit markedly reduced early mucosal inflammation relative to their isogenic parent strains. Conversely, aflagellate bacteria were more potent activators of epithelial caspases and subsequent apoptosis. These phenomena correlated with a delayed but markedly exacerbated mucosal inflammation at the later stages of infection as well as elevated extra-intestinal and systemic bacterial load, culminating in a more severe clinical outcome. Systemic administration of exogenous flagellin primarily reversed the deleterious effects of *in vivo* *Salmonella* infection. These observations indicate that in *Salmonella* infection, flagellin plays a dominant role in activation of not only innate immunity but also anti-apoptotic processes in epithelial cells. These latter TLR-mediated responses that delay epithelial apoptosis may be as critical to mucosal defense as the classic acute inflammatory response. This notion is consistent with the emerging paradigm that specific TLR ligands may have a fundamental cytoprotective effect during inflammatory stress. (Am J Pathol 2006, 169:1686–1700; DOI: 10.2353/ajpath.2006.060345)

Intestinal epithelial cells are entrusted with a unique physiological role: although necessarily permeable to nu-

trients and fluids, the epithelial monolayer must partition a diverse commensal enteric normal flora as well as defend against overt enteric pathogens. The epithelium has well-studied physical and chemical barrier functions to manage this task.^{1,2} In addition, epithelial cells respond to and manage bacterial threats (or maintain stable commensal relationships) by using both transmembrane and intracytoplasmic pattern recognition receptors, such as Toll-like receptors (TLRs) and related Nod proteins. These sentinel receptors recognize and bind to MAMPs (microbial associated molecular patterns), which are conserved macromolecular motifs present in, and characteristic of, a wide range of microbes.³ MAMPs include complex carbohydrate components of prokaryotic cell walls (lipopolysaccharide and peptidoglycans), nucleic acids (double-stranded RNA and CpG-rich DNA), and proteins (flagellin).⁴

Ligand binding of all TLRs leads to initiation of several cytoplasmic signal transduction cascades that culminate with the appearance of activated transcription factors in the nucleus.⁵ These pathways include the Rel/nuclear factor (NF)- κ B, the MAP kinase, and the IRF pathways, all of which exploit rapid, transient posttranslational modifications of protein intermediates to transmit signals to the nucleus. These alarm pathways stimulate the transcriptional up-regulation of a battery of proinflammatory effector proteins including chemokines, cytokines, and adhesion molecules. For example, previous expression

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M.V.K. and H.W. share primary authorship and A.T.G. and A.S.N. share senior authorship.

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Address reprint requests to Andrew S. Neish, M.D., Department of Pathology, Emory University School of Medicine, Room 105F, Whitehead Bldg., 615 Michael St., Atlanta, GA 30322. E-mail: aneish@emory.edu.

profiling experiments from our and other laboratories demonstrated that purified flagellin interacting with its physiological receptor TLR5 was capable of initiating a proinflammatory transcriptional response almost identical to that induced by the canonical endogenous proinflammatory cytokine tumor necrosis factor- α .^{6–8} Regardless of the specific inciting ligand, up-regulation of proinflammatory effectors orchestrates circulating leukocyte recruitment and results in increased numbers of leukocytes entering affected tissues. It is generally accepted that whereas proinflammatory gene expression and the resulting acute inflammatory responses are necessary for the control/destruction of bacterial pathogens, this stereotypical tissue reaction is also causal of the clinical manifestations of enteric infection.⁹ Thus, MAMPs have been generally conceptualized as bacterial virulence factors—prokaryotic products that contribute to pathogenicity.

The *Salmonellae* are a group of gram-negative, facultative intracellular enteric pathogens causal of a spectrum of diseases in susceptible higher vertebrates.¹⁰ In human nontyphoidal *Salmonella* infection, the pathogen characteristically induces a classic acute proinflammatory response in infected intestinal mucosa.^{11,12} A combination of MAMPs and virulence factors translocated by type III secretion systems are known to contribute to the proinflammatory properties of this bacterium.^{13,14} We previously reported that flagellin is a key MAMP in epithelial responses to *Salmonella* infection *in vitro*. Mutation of the flagellin-encoding genes (*FlhC* and *FlhB*) in enteropathogenic *S. typhimurium* (SL3201) resulted in a viable and invasive microbe that was nearly devoid of proinflammatory activity at both the signaling and effector level.¹⁵ Importantly, we also showed that a mutant that secreted flagellin but was unable to assemble intact flagella (*FlhD*) was fully proinflammatory, indicating that loss of motility did not account for the inability to stimulate inflammation. Thus, flagellin is necessary to induce inflammatory signaling and effector responses, the cardinal features of human disease, in model *Salmonella* infection of cultured epithelia.

To address the physiological role of flagellin-mediated proinflammatory signaling during infection *in vivo*, we have now used two distinct sets of isogenically matched wild-type (WT) and flagellin-deficient (aflagellate) *Salmonella* strains in two distinct murine models of enteric salmonellosis. These isogenic strains presented the possibility of evaluating bacteria with and without a key MAMP, thus permitting loss-of-function analysis, an approach that is impossible with other MAMPs such as lipopolysaccharide and bacterial DNA that are required for viability. Unexpectedly, we observed that the aflagellate *Salmonella* mutants markedly increased disease severity. This aggravated clinical course corresponded to increased bacterial penetration from epithelia into systemic tissues. Importantly, enhanced bacterial translocation could be correlated with increased enterocyte apoptosis. These results expose an important cytoprotective component of TLR-mediated signaling induced during a bacterial-epithelial encounter and highlight its functional importance in the containment and control of gut-borne infections.

Materials and Methods

Materials and Reagents

Flagellin (FlhC) from WT *S. typhimurium* (SL3201, *flhB*[−]) was purified through sequential cation and anion-exchange chromatography.¹⁶ Purity was verified as previously described.¹⁷ Antibodies used include cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), RelA (Santa Cruz Biotechnology, Santa Cruz, CA), cIAP2, and anti-mouse lipocalin-2/NGAL (R&D Systems, Inc., Minneapolis, MN). Anti- β -actin, human leukocyte myeloperoxidase (MPO), streptomycin, and hexa-decyltrimethylammonium bromide were from Sigma (St. Louis, MO). All other reagents were from Sigma unless otherwise stated.

Bacterial Strains Used

WT *Salmonella enterica* serovars *typhimurium* (SL3201) phase variable and its isogenic mutant (aflagellate, phenotype:nonmotile:genotype: *flhC*[−]/*flhB*[−]) were grown under microaerophilic conditions as previously described.¹⁵ Hereafter, these strains are referred to as WT-1 and aflagellate-1. FlhD is a mutant in the flagellar hook protein and was a gift of Dr. R. Macnab (Yale University, New Haven, CT). WT *Salmonella enterica* serovars *enteritidis* (S1400/94) was originally isolated from a poultry infection and its isogenic mutant strain (aflagellate, phenotype:nonmotile:genotype: *FlhC::cam*^R) was constructed by insertional mutagenesis.¹⁸ Hereafter these strains are referred to as WT-2 and aflagellate-2. Bacterial stocks were inoculated into 10 ml of Luria-Bertani broth and incubated with agitation at 37°C overnight to give $\sim 10^9$ CFU ml^{−1}.

Murine Infections

Experimental Murine Enteritis

Pathogen-free female BALB/cj mice (6 to 8 weeks) were procured from Jackson Laboratories (Bar Harbor, ME). Animals were pretreated with streptomycin and then infected with *Salmonella* to induce colitis as described in Barthel and colleagues,¹⁹ with few modifications. On the day of the experiment, water and food were withdrawn 4 hours before treatment with 7.5 mg of streptomycin in 75 μ l of sterile water by oral gavage. Afterward, animals were supplied with water and food *ad libitum*. At 20 hours after streptomycin treatment, water and food were withdrawn again for 4 hours before the mice were infected with 10^8 CFU of *Salmonella typhimurium* by gavage (WT-1, aflagellate-1, or FlhD) and supplied with food and water. In some experiments, mice were pretreated with flagellin (20 μ g/mouse i.p.) 2 hours before challenge with *Salmonella*. At the indicated times after infection, urine and blood were collected, and ceca were removed and fixed immediately in 10% buffered neutral formalin for histopathological analysis or thoroughly washed and snap-

frozen for RNA isolation or homogenized for microbiological analysis by serial dilution on McConkey's agar plates. Assessment of acute histological injury was based on the semiquantitative scheme used by Barthel and colleagues.¹⁹ For each experiment, five individual mice were evaluated for a given condition, scores totaled, and SE calculated by two independent blinded observers. Animal experiments were approved by the Emory University and Rowett Research Institute institutional ethical committee and performed according to the legal requirements.

Experimental Murine Natural Infection

Specific pathogen-free C3H/HeN female mice were used (5 to 6 weeks) (Harlan, Blackthorn, UK). Mice were given a single dose of LB broth (0.1 ml) containing 10^8 CFU *S. enteritidis* (WT-2 or aflagellate-2) by gavage. Food intake, body weights, and appearance/condition were monitored twice daily and scored according to the schedule of Shu and colleagues.²⁰ Tissues were flushed to remove contents and nonadherent bacteria, followed by weighing and homogenization. Viable bacteria were enumerated by serial dilution on XLD agar plates.

Ileal Loop

Green fluorescent protein (GFP)-labeled aflagellate-2 contained a GFP plasmid construct with an ampicillin resistance gene insert. The bacterial stock was maintained frozen and grown under the same conditions as the unlabeled strain, except that all media contained ampicillin (50 μ g/ml). At 8 days after infection with unlabeled WT-2 or aflagellate-2, C3H/HeN mice were anesthetized (isoflurane), a ligated intestinal loop (2 to 8 cm from the ileocecal junction) prepared *in situ*, and GFP-aflagellate-2 (10^9 CFU) introduced. The mice were maintained under anesthesia at 35 to 37°C for up to 4 hours and then euthanized, and tissues removed for analysis.

Tissue Myeloperoxidase Activity

Neutrophil infiltration into tissue was quantified by measuring MPO enzyme activity (a marker for neutrophils). Briefly, after thorough washings to remove the contents, tissue (50 mg/ml) was homogenized in 0.5% hexadecyltrimethyl ammonium bromide (in 50 mmol/L phosphate buffer, pH 6.0), freeze-thawed three times, sonicated, and centrifuged. MPO was assayed in the clear supernatant (14 μ l) by adding 1 mg/ml diaminodiphenylmethane dihydrochloride and $5 \times 10^{-4}\%$ of H_2O_2 and the change in optical density measured at 450 nm. One U of MPO activity was defined as the amount that degraded 1.0 μ mol of peroxide/minute at 25°C.²¹

Serum Cytokine Assays

Mice were bled at 6, 12, 24, and 48 hours after infection through the retro-orbital plexus, and serum was isolated

by centrifugation. Serum KC and IL-6 assays were performed using mouse DuoSet enzyme-linked immunosorbent assay kits (R&D Systems) according to the manufacturer's instructions. Serum amyloid A was measured by enzyme-linked immunosorbent assay kit (sensitivity 10 ng/ml) procured from Bender MedSystems (Burlingame, CA) and Biosource (Camarillo, CA).

Cell Culture

Primary rat intestinal epithelial cells (IEC-6) and CaCo2 cells were maintained as described.⁶ Monolayers were washed twice with Hank's balanced salt solution at 37°C and equilibrated for 15 minutes before treatment. For bacterial treatments, bacterial cultures were washed, concentrated, and applied to the apical aspects of cells at an multiplicity of infection of 30 as described.¹⁵

Immunoblotting

For lipocalin-2 immunoblot assessment, serum and urine samples were diluted 1:10 and 1:2, respectively, in loading buffer, subjected to 4 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting, and probed with biotinylated anti-mouse-lipocalin-2/NGAL monoclonal antibody (0.2 μ g/ml). For immunoblot of cell lysates, cells were lysed in ice-cold buffer containing 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN). Immunoblot was performed as described.⁶

Electrophoretic Mobility Shift Assay of NF- κ B

Nuclear extracts from untreated and treated cells were incubated with double-stranded ³²P-labeled oligonucleotide probes containing consensus-binding sequences for NF- κ B (5'-AGTTGAGGGGACTTTC-CAGGC-3') separated by electrophoresis and visualized by autoradiography.

Real-Time Quantitative Polymerase Chain Reaction (PCR)

Total RNA from mouse cecum were prepared using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcriptase (RT)-PCR was performed as described.⁶ Primers for the genes of interest were designed with Primer-Express (Applied Biosystems, Foster City, CA), and their sequences are available on request.

Fluorogenic Caspase Staining

IEC-6 cells were grown on glass coverslips as described earlier. Bacterial suspensions were distributed as 400- μ l beads over a piece of parafilm, with the coverslip applied in an inverted manner. This experimental design was to eliminate confounding results potentially caused by set-

ting and accumulation of immotile mutants. Caspase-8, -9, and -3 activation was detected using the APO LOGIX carboxyfluorescein caspase detection kit (Cell Technology, Mountain View, CA). Positive cells were visualized by confocal microscopy (Zeiss LSM 510) at 505 nm.

Cell Death Assay

IEC-6 cells after treatment (floating and adherent), were collected at various times for terminal dUTP nick-end labeling (TUNEL) staining; the *In Situ* Cell Death Detection Kit (Roche Diagnostics Corp.) was used according to the manufacturer's instructions. In brief, IEC-6 cells were harvested and fixed in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate, and labeled with TUNEL reaction mixture. For morphological evaluation of TUNEL positivity, cells were co-cultured with bacteria in an inverted manner as described above and visualized by confocal microscopy (Zeiss LSM 510) at 505 nm. For analysis of bacteria-induced apoptosis by flow cytometry, 10,000 fluorescent events were measured for each sample by a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) using the Cell Quest software. Data were analyzed using FlowJo software.

Immunofluorescence Analysis

After experimental treatments CaCo2 cells grown on 35-mm culture dishes were fixed in 4% paraformaldehyde or methanol and permeabilized in 0.2% Triton X-100/phosphate-buffered saline. Cells were stained as previously described.¹⁷

Statistical Analysis

The statistical method was Student's *t*-test with either one-tailed or two-tailed distribution as appropriate.

Results

Aflagellate Salmonellae Initially Elicit Reduced Inflammation in an Acute Model of Enteric Salmonellosis but Ultimately Cause Enhanced Disease Severity

To better understand the role of flagellin in *Salmonella* infections *in vivo*, WT *Salmonella* strains were compared with isogenic aflagellate mutants in an acute model of *Salmonella* enteritis. Recently, Hardt and colleagues^{19,22–24} showed that clinical isolates of *S. typhimurium*, which normally cause little intestinal inflammation in mice, will induce a robust neutrophilic colitis highly reminiscent of human Salmonellosis when mice are pretreated with streptomycin. We in-

fecting BALB/c mice in this manner using a WT *S. typhimurium* clinical isolate (SL3201—designated WT-1) and an isogenic mutant lacking both flagellin genes (SL3201—*fljC*[−]/*fljB*[−] designated aflagellate-1). We observed that mice infected with WT-1 developed a watery diarrhea within 12 hours after colonization. The incidence of diarrhea was significantly less in response to infection with an isogenic aflagellate mutant (incidence of diarrhea was 60 versus 20% in response to WT-1 and aflagellate-1 mutant, respectively; *P* < 0.05). Moreover, histopathological analysis showed that mice infected with WT-1, but not the aflagellate-1, exhibited neutrophil infiltration accompanied by mild submucosal edema as early as 6 to 12 hours after infection (Figure 1A). These histological changes closely correlated with tissue MPO (Figure 1B) and tissue injury score (Figure 1C), which increased within this time frame only in response to the WT-1 strain. The elicitation of a strong rapid inflammatory response by the WT-1, but not aflagellate-1, strain was not only evident by examining intestinal parameters but was also reflected systemically with elevated serum keratinocyte-derived chemokine (KC—murine homolog of IL-8) (Figure 1D), serum levels of lipocalin-2/NGAL (Figure 1F), and serum amyloid A [known to represent a general marker of acute inflammation (Supplementary Figure 1A at <http://ajp.amjpathol.org>)].^{25,26} Similarly, urinary excretion of lipocalin was significantly higher in mice challenged with WT-1 (Supplementary Figure 1B at <http://ajp.amjpathol.org>). Thus, consistent with *in vitro* results that indicate flagellin is a dominant proinflammatory determinant of enteropathogenic *Salmonella*, aflagellate *S. typhimurium* induced markedly less acute inflammation in a murine model of Salmonellosis.

In contrast to responses seen at early times (24 hours or less), by 48 hours after administration of *S. typhimurium*, the flagellin-deficient mutant appeared substantially more virulent. Specifically, approximately half of the mice infected with the aflagellate mutant displayed overt peritonitis and intussusception of bowel segments (incidence of peritonitis 0 and 60% and intussusception 0 and 40%, respectively, for mice infected with WT-1 or aflagellate-1; *P* < 0.05). Furthermore, gross cecal contraction, reduced weight, and pallor, the previously observed macroscopic hallmarks of this Salmonellosis model,¹⁹ were far more apparent in mice receiving the aflagellate-1 as opposed to the WT-1 (Figure 2A, top; and Supplementary Figure 1C at <http://ajp.amjpathol.org>). This gross phenotype was observed in 13 and 87%, respectively, of mice infected with WT-1 or aflagellate-1, *P* < 0.05. In addition, cecal histopathology induced by aflagellate bacteria was more severe with increased numbers of crypt abscesses as well as massive edema (Figure 2A, bottom), again highly correlated with increased mucosal MPO (Figure 1B) and tissue response (Figure 1C). At these time points, aflagellate-1 infection also stimulated greater systemic markers of inflammation: serum KC, IL-6, and lipocalin-2/NGAL (Figure 1, D–F). Furthermore, at 48 hours, mice challenged with aflagellate-1 exhibited significantly higher numbers of bacteria adherent to or within the cecal mucosa, relative to mice infected with WT-1 (Figure 2B), confirming the infectivity

Figure 1. Aflagellate *S. typhimurium* exhibits enhanced but delayed inflammation in a murine model of enteric Salmonellosis. BALB/cj mice were pretreated with streptomycin and infected as indicated with *Salmonella* (WT-1, aflagellate-1, and *FliD*) and sacrificed at 6, 12, 24, and 48 hours after infection as described in Materials and Methods. **A:** H&E-stained cecal mucosal images (12 hours after infection) of control (streptomycin alone), WT-1, and aflagellate-1. **Arrow** denotes neutrophil influx. **B:** Cecal MPO activity at different time points. **C:** Quantification of intestinal inflammation. Sections were graded on a semiquantitative scale as described in Materials and Methods. Data are aggregate of five mice for each time point. **D** and **E:** Enzyme-linked immunosorbent assay for serum KC (**D**) and IL-6 (**E**). **F:** Representative immunoblot for serum lipocalin-2/NGAL from three mice in each group. * $P < 0.05$; ** $P < 0.01$. Original magnifications, $\times 40$.

of aflagellate-1 and suggesting reduced bacterial clearance. Thus, by 48 hours after infection, as compared with WT-1, aflagellate-1 resulted in a more severe disease process.

Importantly, the enhanced clinical effects of aflagellate bacteria were not seen with immotile bacteria. We infected mice with the *FliD* mutant, which ablates the hook protein of the flagellar apparatus and prevents assembly of

Figure 2. Cecal pathology of *Salmonella* infection after streptomycin pretreated. **A:** Top: gross cecal appearance; bottom: H&E-stained cecal images (48 hours after infection) with indicated strain. **Inset:** Crypt abscess in aflagellate-1-infected mice cecum. **B:** Adherent and invaded cecal bacterial load (*Salmonella*) at 48 hours after infection. * $P < 0.05$. Original magnifications, $\times 10$; $\times 40$ (inset).

a functional flagellum. The mutant is immotile but expresses abundant levels of flagellin protein. In cell culture assays, this mutant was shown to be almost as proinflammatory as WT *Salmonella*.¹⁵ In the mouse model, *FliD* infection resulted in less clinical response than WT bacteria, eliciting significantly reduced cecal MPO and only baseline levels of systemic KC (Figure 1, B and D, and Figure 2A), indicating that the enhanced clinical effects of the aflagellate *Salmonella* were not the result of immotility of the bacteria.

Aflagellate *Salmonella* Are Hypervirulent in Vivo in C3H/HeN Mice

We next studied a model of enteric *Salmonellosis* based on oral infection in C3H/HeN mice, which, unlike many inbred mouse strains, have isoforms of the gene *Slc11a1*⁺ that are associated with relatively high natural resistance to *Salmonella*. To address the role of flagellin in this form of enteric *Salmonellosis*, we again compared the outcome of infection with a WT *Salmonella enterica serovar enteritidis* isolate (designated WT-2) or its isogenic aflagellate mutant *fliC:cam^R* derivative (designated aflagellate-2). C3H/HeN mice were infected with a single dose of 10^8 organisms and monitored for up to 12 days after infection. Bacterial colonization was prevalent in ileal, cecal, and colonic tissues (Table 1) and was accompanied by crypt hyperplasia, edema of submucosa, and PMN infiltration into the lamina propria and crypts (Figure 3A, i–iv). No differences in intestinal colonization were seen with the WT-2 or aflagellate-2 *Salmonella* at 6 and 12 days. Systemic infection, quantified by the numbers of bacteria recovered from spleen and liver, was at a low level at 6 days after infection (Table 1). However, by 12 days after infection the numbers of bacteria found in peripheral organs, including extra-reticuloendothelial tissues such as kidney, was significantly greater in aflagellate-2-infected mice (Table 1) and correlated with a more severe disease manifested by greater weight loss (data not shown) and increased ileal MPO levels (Figure 3B). In aflagellate-2-infected mice the liver and spleen weights were significantly

Table 1. Tissue-Associated *Salmonella* (Log₁₀CFU/g) and Proportion of Samples Positive for *Salmonella* in C3H/HeN Mice 12 Days after Infection with 10^8 CFU of WT-2 or Aflagellate-2

	WT-2		Aflagellate-2	
	6 Days	12 Days	6 Days	12 Days
Ileum	5.10 ± 0.37 (100)	3.56 ± 2.16 (53)	3.37 ± 1.03 (40)	4.03 ± 2.69 (65)
Cecum	8.57 ± 1.63 (100)	8.03 ± 2.10 (100)	6.38 ± 0.66 (100)	8.94 ± 2.31 (100)
Colon	8.33 ± 1.74 (100)	8.77 ± 2.24 (89)	6.91 ± 1.12 (100)	8.32 ± 2.19 (100)
Mesenteric lymph nodes	5.50 ± 0.45 ^a (100)	3.57 ± 0.89 ^b (89)	4.67 ± 0.20 ^a (100)	4.58 ± 0.84 ^a (100)
Liver	3.12 ± 1.74 ^a (73)	3.21 ± 1.27 ^a (76)	3.89 ± 0.73 ^a (100)	4.60 ± 1.37 ^b (94)
Spleen	4.98 ± 0.31 ^a (82)	3.70 ± 1.28 ^b (87)	4.40 ± 0.58 ^{ab} (100)	4.75 ± 0.83 ^a (100)
Kidneys	1.41 ± 0.34 ^a (10)	1.47 ± 0.39 ^a (18)	1.64 ± 0.64 ^a (30)	2.66 ± 1.09 ^b (88)

Values in a row with distinct superscripts differ significantly ($P \leq 0.05$). Values are mean $\pm$ SE; numbers in parentheses correspond to percent positives.

higher than in WT-2-infected mice, indicative of greater bacterial infiltration of these reticuloendothelial tissues (Supplementary Figure 2, A and B, at <http://ajp.amjpathol.org>).

Ultimately, greater mortality was observed with aflagellate-2 infection, which was particularly evident in younger/smaller mice (Figure 3C). Thus, in two independent murine models of enteric Salmonellosis, aflagellate bacteria caused a more severe clinical response.

WT-2 and aflagellate-2 may differentially affect the ability of the epithelia to exclude bacteria. To evaluate this idea, ligated ileal loops were constructed in mice infected with each strain at 8 days after infection, and GFP-tagged *Salmonella* was instilled for an additional 4 hours to track epithelial invasion/translocation. Fixed ileal tissue sections were stained with both intestinal membrane and nuclear probes, and the numbers of GFP-expressing bacteria within the epithelium and lamina propria were enumerated. We observed significantly higher numbers of GFP *Salmonella* invading gut tissues via villus and crypt epithelia and entering the lamina propria [Figure 4, A and B (i–iii)] of mice infected with aflagellate-2. This enhanced transepithelial translocation correlated directly with the greater systemic bacterial load observed in aflagellate-2-infected mice. Furthermore, as both the number of GFP bacteria in the gut lumen and/or those closely adhered to the gut tissues were not significantly different between WT-2 and aflagellate-2-infected mice (data not shown), it is likely that enhanced uptake and/or break-out from the gut tissues into submucosal/systemic tissues explains the elevated systemic infection in aflagellate-infected mice.

Exogenous Flagellin Reduces the Tissue Reaction Induced by *S. typhimurium*

Our data show that the absence of flagellin on a viable pathogen resulted in a more severe clinical phenotype. We next sought to determine whether the tissue effects could be reversed or attenuated by administration of purified flagellin monomers, the physiological ligand that activates TLR5. We observed that purified flagellin given systemically (20 $\mu\text{g}/\text{mouse}$ i.p.) 2 hours before oral inoculation of the aflagellate-1 could attenuate host tissue response, as assessed by gross and histopathological parameters, to levels associated with infection with WT-1 bacteria (Figure 5A, i and ii; Supplementary Figure 1C at <http://ajp.amjpathol.org>). Consistently, cecal MPO and serum KC levels at 48 hours after infection were significantly reduced after exogenous flagellin pretreatment (Figure 5B, i and ii). Furthermore, exogenous flagellin was able to substantially reduce the re-

Figure 3. Aflagellate *S. enteritidis* causes a hypervirulent phenotype in model systemic murine infection. **A:** Inflammatory responses in C3H/HeN mice after exposure to *S. enteritidis* (WT-2 or aflagellate-2). H&E-stained frozen sections. **i:** Ileum, 6 days after infection showing normal crypt histology. **ii–iv:** Ileum, 12 days after infection showing edema (arrows) (ii) and cellular infiltrate between crypts (iii) and (iv) jejunum, 12 days after infection showing the presence of neutrophils (arrows) in the lamina propria (LP) and transepithelial migration of PMNs into crypt abscesses. **B:** Ileal MPO activity. Aflagellate-2-infected versus control, or WT-2, * $P < 0.05$. **C:** Survival (percent) of C3H/HeN mice after oral infection with 10^8 CFU with WT-2 or aflagellate-2 as indicated. Scale bars = 10 μm (i, ii); 10 μm (iii); 25 μm (iv).

Figure 4. Aflagellate *Salmonella* invades more efficiently than WT. **A:** Counts of GFP-labeled invaded bacteria. **B:** Localization of GFP-labeled *Salmonella enteritidis* (aflagellate-2) in mouse ileum 4 hours after inoculation of an isolated ligated loop with 10^9 bacteria. Ligated loops were performed 8 days after infection with WT-2 or aflagellate-2. Representative confocal images of resin-embedded tissue sections showing the presence of bacteria within epithelial cells and LP of upper villus (**i**), mid villus (**ii**), and crypt (**iii**) regions. Intestinal membranes labeled with *Datura stromonium lectin* (DSL) and nuclei labeled with DAPI. * $P < 0.05$. Scale bars = 10 μ m.

covered bacterial load (Figure 5B, iii). In addition, we found that flagellin pretreatment could reduce the level of systemic KC and intestinal MPO induced by WT-1 flagellated *Salmonella* (Figure 5B, i and ii). These data indicate that the proinflammatory responses to flagellin are not maximal during the infectious process and can be further attenuated with additional flagellin. Given the widely observed ability of flagellin-induced gene remodeling in intestinal epithelial cells,^{27,28} this result suggests that the reversion of histological injury by flagellin is the consequence of TLR5-mediated transcriptional responses.

Aflagellate Salmonella Induces Increased Apoptosis within the Epithelial Mucosa

In the C3H/HeN model with aflagellate *S. enteritidis*, evaluation of intestinal epithelia throughout the course of the infection did not reveal clear-cut morphological differences in cellular inflammation between the WT-2-infected mice (although MPO levels were significantly higher in aflagellate-2-infected mice at 12 days after infection, implying a greater neutrophilic infiltrate; Figure 3B). However, strikingly increased numbers of apoptotic bodies were observed within ileal epithelia infected with the aflagellate-2 (Figure 6A, i and ii). Next, we sought to determine whether increased epithelial apoptosis was occurring within the time frame of the acute enteritis model using BALB/cj mice and *S. typhimurium*. Immunohistochemical stain for activated caspase-3 showed a markedly greater amount of immunoreactivity in the cecal tissues of mice infected with aflagellate-1 bacteria relative to infection with WT-1 at 12 hours after infection, the time point before increased inflammation was observed (Figure 6B, i–iii). Denudation and/or epithelial erosions were not observed. Apoptosis is well known as an intrinsic process by which individual cells can be eliminated while sparing overall tissue integrity,²⁹ and caspase activation is established as an inhibitor of inflammatory pathways.^{30,31}

Past analyses demonstrated anti-apoptotic genes, including cIAP1 and cIAP2, were potentially induced by

flagellin and could suppress apoptosis *in vitro*.⁶ We used qRT-PCR analysis of infected mucosa to study transcriptional responses during early infection (6 to 12 hours). Consistently, we found that aflagellate-1 had a reduced ability to induce transcript levels of these proteins (Figure 6C), suggesting that diminished proinflammatory signaling resulted in a proapoptotic state *in vivo*.

Aflagellate Salmonella Induces Increased Apoptosis in Cultured Epithelial Cells

We next sought to compare apoptotic activation elicited by flagellate WT-1 and isogenic aflagellate-1 in the context of model infection in cultured primary epithelial cells. Epithelial monolayers were infected with bacteria at a multiplicity of infection of 30, a ratio that extensive past work has shown to mediate maximal signaling and transcriptional activation.^{16,17,32} Caspase activation of the extrinsic pathway (caspase-8) occurred within hours in cells infected with WT-1; however, the aflagellate-1 revealed a markedly increased degree and number of positive cells, with faster kinetics. Likewise, activation of caspase-9 (intrinsic pathway) and caspase-3 (executioner pathway) were slightly delayed but showed an identical acceleration and intensification when infected with aflagellate-1 (Figure 7A).

Caspase activation is reversible; providing survival pathways are intact, transcriptional up-regulation of anti-apoptotic genes can arrest caspases at several control points, preventing the cell from proceeding to cell death. For example, purified flagellin can activate caspases, but this activation is transient, and no programmed cell death is observed.⁶ To test whether the caspase activation induced in experimental infection was transient or proceeded to apoptosis, we performed similar co-culture experiments and assayed the cells for apoptosis by TUNEL stain. This method detects apoptosis-specific DNA strand breaks and is thus a marker of irreversible cell dismantling. It has been previously reported that WT enteropathogenic *Salmonella typhimurium* can induce apo-

Figure 5. Exogenously administered flagellin can reverse tissue injury caused by aflagellate *Salmonella*. BALB/cj mice infected as described in Materials and Methods. **A:** Gross (i) and histological (ii) details (H&E stained) of the ceca at 48 hours after infection either with WT-1 or aflagellate-1 alone or with flagellin pretreatment. Gross cecal pathology was not different in WT-1 infection with or without flagellin pretreatment. **B:** Cecal MPO (i), serum KC (ii), and cecal bacteria (iii) counted on selective McConekey agar plates (tetracycline, 13 μ g/ml), 48 hours after infection without or with flagellin (20 μ g/mouse, i.p.) treatment 2 hours before infection with aflagellate-1. ** $P < 0.01$.

ptosis in cultured epithelia at 12 to 18 hours of co-culture.³³ We were able to replicate this finding; moreover, the aflagellate-1 mutants exhibited marked acceleration and intensity of TUNEL positivity (Figure 7B).

Aflagellate *Salmonella* Fails to Induce Survival Pathways and Effector Genes in Cultured Epithelial Cells

We have demonstrated that purified flagellin can activate a NF- κ B-responsive reporter gene and that block-

ade of NF- κ B, either pharmacologically with proteasome inhibitors or with a dominant-negative I κ B construct, potentiates epithelial cell apoptosis, suggesting that flagellin-induced NF- κ B can function as an important anti-apoptotic survival pathway.⁶ To determine whether aflagellate bacteria were proapoptotic because of the failure to induce the NF- κ B pathway, we infected cells and assayed for NF- κ B activation. As expected, and consistent with previously published data, WT bacteria induced both NF- κ B nuclear translocation and DNA binding during a 4-hour infection (Figure 7, C and D). In contrast, the aflagellate mutants showed a greatly reduced induction of this signaling pathway in both assays.

WT *Salmonella* infection is a potent transcriptional inducer of well-known anti-apoptotic factors.⁶ We tested if aflagellate mutants fail to up-regulate these cytoprotective genes in model epithelium, as suggested by *in vivo* data (Figure 6C). In immunoblots for the highly expressed anti-apoptotic effector cIAP-2, we demonstrated that aflagellate-1 is a weaker inducer of this protein, and this decreased expression corresponds with enhanced activation of executioner caspase-3 (Figure 7E). These observations are consistent with the inability of these bacteria to activate the NF- κ B and MAPK pathways and, thus, the anti-apoptotic proteins that normally serve to inactivate caspases.¹⁵

Flagellin-induced survival gene expression may protect cells from concurrent and/or subsequent proapoptotic signaling. We show that *in vivo* administration of flagellin attenuates clinical and histopathological effects of infection with aflagellate *Salmonella* (Figure 5). To determine whether this treatment could attenuate apoptotic signaling *in vitro*, IEC-6 cells were pretreated with purified flagellin before a proinflammatory stimulus, ie, aflagellate-1 mutant. Previous work has shown that maximal induction of anti-apoptotic gene expression occurs within 4 hours of flagellin stimulation.⁶ Cells were then challenged with aflagellate-1 for 6 hours, and caspase activation was quantified with luminescent substrates for executioner caspase-3. As shown in Figure 7F, flagellin pretreatment reduced epithelial apoptosis elicited by aflagellate-1, relative to caspase induced in untreated cells.

Collectively, from this series of *in vivo* and *in vitro* experiments, we conclude that in loss-of-function experiments, aflagellate bacteria are able to elicit increased caspase activation and apoptosis, and in gain-of-function experiments, this caspase activation can be inhibited by exogenous flagellin. Aflagellate bacteria do not activate the NF- κ B pathway and only weakly induce survival genes; hence, concurrent apoptotic stimuli are able to proceed unimpeded. Perception of flagellin present on invading flagellated *Salmonella* by the host epithelial cells, and consequent inhibition of epithelial cell apoptosis may be an important mechanism limiting both the translocation of invading bacteria from epithelial tissues to the lamina propria and systemic tissues as well as the tissue pathology associated with infection-driven inflammation.

Figure 6. Aflagellate *Salmonella* elicit increased apoptosis in a murine model of infection. **A:** C3H/HeN-*S. enterica* system. **i:** Representative H&E images showing apoptotic bodies (arrows) in the lower villus/crypt regions of ileum in control C3H/HeN mice or animals infected with WT-2 or aflagellate-2, 12 days after infection. **ii:** Quantification of apoptosis 12 days after infection. **B:** BALB/c-*S. typhimurium* system. Caspase-3 staining in mouse ceca 12 hours after infection with WT-1 or aflagellate-1. Data are aggregate of five mice infected with the indicated strain. **i:** Control, **ii:** aflagellate-1. **iii:** Quantification of positive cells from (10 hpf) in BALB/cj mice cecum. **C:** qRT-PCR of cIAP-1 and cIAP-2 transcript in ceca infected with WT-1 or aflagellate-1. Data are aggregate of five mice infected with the indicated strain. qRT-PCR reactions were performed in triplicate for each sample and normalized to 18s ribosomal RNA. The relative levels of RNA were determined by comparing with the uninfected group * $P < 0.05$, ** $P < 0.01$. Scale bars = 10 µm.

Figure 7. Aflagellate *Salmonella* are more potent inducers of caspases and apoptosis *in vitro*. **A:** Fluorescent microscopy of IEC-6 cells showing active caspase -8, -9, and -3 staining, after infection with WT-1 or aflagellate-1 for 2, 4, and 8 hours. **B:** TUNEL staining of IEC-6 cells 4, 8, and 12 hours after infection with WT-1 or aflagellate-1. **C:** Immunofluorescence detection of RelA in Caco-2 cells exposed to either *S. enteritidis* WT-2 or aflagellate-2 for 2 hours. **D:** Electrophoretic mobility shift assay for NF-κB activation in Caco-2 cells exposed to indicated bacteria for 2 hours. **E:** T84 cells were treated basolaterally for 8 hours with flagellin (100 ng/ml) or apically with WT-1 or aflagellate-1 (multiplicity of infection 30). Cell lysates were subjected to immunoblot with indicated antibodies, and β-actin was used as a loading control. **F:** *In vitro* cytoprotective effect of flagellin. IEC-6 cells were pretreated with flagellin (10 ng/ml) for 4 hours and subsequently infected with aflagellate-1 for 6 hours. Caspase-3 activation was quantified in cell lysates using luminescent substrate Caspase-Glo 3/7 assay (Promega). zVAD-FMK and staurosporine were included as positive controls for blocking or activating apoptosis, respectively. Data are representative of five experimental repeats.

Discussion

In this study, we have used two distinct murine enteritis models and two separate serovars of pathogenic *Salmonella*, each bearing isogenic mutations that result in loss of surface flagellin. We show that in both cases, the aflagellate mutants elicited increased, albeit with delayed kinetics, local mucosal reaction/systemic infection. Given that the experimental definition of a virulence factor necessitates "that loss of the factor by the bacterium results in a decrease in its ability to cause disease,"³⁴ our data describe a rare case (in animal pathogens) of a bacterial component that when ablated, results in increased disease, thus fulfilling the genetic definition of an avirulence factor.^{35,36} Our study gives mechanistic insights into this phenomenon, which may be relevant to many forms of infectious disease. In both *in vivo* models and *in vitro* cell culture, we found that aflagellate *Salmonella* were expect-

edly defective in induction of proinflammatory signaling pathways and effector genes but elicited strikingly accelerated epithelial apoptosis. Consistently, we have shown that purified flagellin induces anti-apoptotic genes and can reduce caspase activity *in vitro*.⁶ We hypothesize that the accelerated apoptosis plays a role in the increased tissue reaction/systemic invasion caused by aflagellate *Salmonella* *in vivo* (diagramed in Figure 8). Plausibly, the failure of aflagellate *Salmonella* to stimulate early proinflammatory and/or antibacterial gene expression permits these bacteria to delay innate immune detection and establish a foothold within the gut mucosa, thereby promoting their ability to gain access to the lamina propria and resident and/or newly recruited immune cells therein. In parallel, the failure of epithelial cells to respond to aflagellate bacteria with anti-apoptotic and other aspects of cytoprotective signaling accelerates caspase activa-

Figure 8. Diagram, see Discussion.

tion/apoptosis that further compromises the anti-microbial response and potentially facilitates bacterial release to the subepithelial tissues. Consistent with this latter suggestion, recent data have shown that TLR/MyD88 signaling is an important pathway limiting bacterial translocation to the lamina propria and, hence, susceptibility to systemic Salmonellosis.²⁴ Thus, we conclude that flagellin-induced signaling plays a previously unappreciated role in infectious disease pathogenesis, orchestrating not only well-characterized recruitment of phagocytes but also cytoprotective effects that ultimately limits the apoptotic response of intestinal epithelial cells.

Aflagellate Bacteria in Model Infection

Differences in the pathogenesis of infection with WT and aflagellate *Salmonella* have been described previously. Stecher and colleagues³⁷ evaluated a nonflagellated *S. typhimurium* mutant (fliGHI) in the streptomycin pretreatment model system in C57BL6 mice, reporting that aflagellate organisms efficiently colonized the cecum by 10 hours, showed increased systemic dissemination, and, highly consistent with our results, showed that these mutants elicited markedly less cellular inflammation and histologically demonstrable tissue injury throughout the first 48 hours after infection. These experiments did not examine longer time points nor apoptosis.³⁷ Schmitt and colleagues³⁸ evaluated survival in mice (also highly susceptible C57BL6) infected with *S. typhimurium* bearing a FljB-null genotype, as well as the FljC/FljB mutants used in our study, finding a modest increase in mortality. In a chicken model, an aflagellate *S. typhimurium* mutant (FlIM) showed an enhanced ability to establish a systemic infection. Also consistent with our findings, this strain elicited reduced IL-1 β up-regulation and PMN infiltration into the gut in early stages of infection.³⁹ Interestingly, flagellin perception is important in containment of bacte-

rial dissemination in pathogen-plant interactions.⁴⁰ Li and colleagues⁴¹ reported that flagellated *Pseudomonas syringae* activated specific nonhost responses in *Arabidopsis*, whereas aflagellate mutants induced markedly weaker responses and, as a consequence, proliferated within the plant to a greater extent and ultimately caused a more severe disease.

Apoptosis and Inflammation in Enteric Infection

Apoptosis, or programmed cell death, is a morphologically distinct, genetically defined intrinsic mechanism by which individual cells can eliminate themselves while primarily preserving the surrounding cells.^{29,42} Apoptosis is a common cellular response to bacterial infection, playing a key role in the pathogenesis of bacterial infections such as Yersinia plague and systemic anthrax, in which the target cell is the macrophage.⁴³ Epithelial apoptosis induced by bacteria has also been implicated in the pathogenesis of enteric infection.⁴⁴ Extensive mucosal apoptosis and epithelial damage has been described in experimental and clinical cases of *Shigella* infection.^{45,46} Intriguingly, *Shigellae* are aflagellate pathogens. Potentially, this aggravated clinical picture may be mediated by lack of ligands such as flagellin that play an important role in mediating cytoprotective responses. *Salmonella* are potent inducers of apoptosis in macrophages,⁴⁷ although the role of apoptosis in epithelial-*Salmonella* interactions has only been described *in vitro*. Previously, we have shown that a type III secreted effector of *Salmonella*, AvrA, could induce apoptosis when overexpressed in epithelial cells.⁴⁸ Kagnoff and colleagues^{33,49} have shown in *Salmonella* epithelial co-culture experiments that viable organisms induced apoptosis after a surprisingly prolonged interval of 12 to 18 hours and suggested that delayed epithelial apoptosis provides sufficient time for the epithelia to generate survival/defensive responses, as well as a safe interval for bacteria to up-regulate genes (ie, SPI-2) necessary for survival in the intracellular environment. The end result of this highly evolved equilibrium between host and pathogen is self-limited enteritis. Our data indicate that flagellin-mediated signaling accounts for this apoptotic delay via activation of survival factors. In the absence of flagellin, the usual equilibrium is disturbed, and suppression of apoptosis does not occur. Apoptotic activation is well known to repress proinflammatory signaling via caspase-mediated degradation of signaling intermediates such as IKK β and p65.^{30,31,50} Again, the failure to control intracellular caspase activity would be expected to paralyze the early proinflammatory response of the gut epithelia during the early stages of infection, thus allowing bacterial proliferation and unchecked bacterial invasion/dissemination to subepithelial and systemic tissues.

Studies of classic inflammatory responses clearly show that a subset of the acute inflammatory effectors includes anti-apoptotic survival proteins such as cIAP1, cIAP2, cFLIP, and A20.^{50–52} NF- κ B-dependent transcriptional up-regulation of these proteins arrests any concurrent caspase activation, allowing inflammation without

cell death. This interrelationship was demonstrated in the intestine by Karin and colleagues⁵³ using mice conditionally null in intestinal epithelial NF- κ B, which when challenged with a local hypoxic challenge, triggered massive epithelial apoptosis. In this case, the epithelium was constitutively unable to activate prosurvival pathways when confronted with an environmental stress (hypoxia). These events explain the apoptosis commonly recognized during pharmacological inhibition of NF- κ B or when this pathway is blocked by the action of specific bacterial effector proteins.⁵⁴ Aflagellate *Salmonella* (indeed any invasive *Salmonella* and many other pathogens) are able to initiate caspase activation in the epithelial barrier, likely because of type III effectors, secreted toxins, metabolic stress, and so forth, but the lack of a proinflammatory response, necessarily including survival gene up-regulation, permits progression to excessive local apoptosis. Our data suggest MAMP-TLR interactions may have an important role in activation of survival pathways *in vivo*, which results in attenuating virulence in certain infections.

Cytoprotective Effects of Exogenous Flagellin

Cytoprotection denotes a given agent or gene product that can reduce apoptosis or necrotic cell death (either in cell culture or *in vivo*) in response to a stressor. An emerging idea in intestinal biology is that TLR signaling can in fact be cytoprotective.^{27,55} Rakoff-Nahoum and colleagues⁵⁶ demonstrated that mice with intestines cleared of normal flora, and thus MAMPs, were markedly more sensitive to the chemical colitogen dextran sodium sulfate and that the mucosal injury mediated by this compound could be ameliorated by oral administration of MAMPs such as lipoteichoic acid and lipopolysaccharide. In addition, this study demonstrated that these protective effects were lost in TLR2- and TLR4-null mice, implicating TLR signaling as the protective mechanism. Several genes were identified as candidate protective genes, although known anti-apoptotic effectors were not described in this study. Consistently, we have demonstrated that administration of flagellin and poly(I:C) (a synthetic double-stranded RNA and ligand for TLR3) can also ameliorate dextran sodium sulfate colitis (M. Vijay-Kumar and A.T. Gewirtz, submitted).

Beneficial effects of probiotic bacteria can be recapitulated by isolated MAMPs. For example, unmethylated probiotic CpG DNA has been shown to ameliorate dextran sodium sulfate colitis. Furthermore, the observed protective effects were lost in TLR9-null mice, directly implicating TLR signaling in intestinal cytoprotection.^{57,58} TLR signaling from MAMPs has also been shown to directly inhibit apoptotic processes in neutrophils.⁵⁹ Other MAMP-inducible genes such as stromal growth factors and angiogenic factors have reparative functions, and may be protective in the sense of accelerating restitution. Interestingly, flagellin, but not other MAMPs, can induce cell cycle entry and proliferation in cultured fibroblasts.⁶⁰ In addition to directly activating anti-apoptotic gene expression, flagellin may also activate a variety of other

cytoprotective mechanisms (eg, heat-shock proteins, trefoil factors) whose actions might further, indirectly, reduce epithelial cell apoptosis. Taken together a picture is emerging that the mucosa—epithelial cells, immunocompetent resident cells in the lamina propria, or both—can perceive the abundant MAMPs in the intestinal lumen and induce a transcriptional response resulting in enhanced cyto-defenses. Potentially, luminal administration of TLR ligands such as flagellin may induce cytoprotective responses in this situation and act as a palliative.

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