Flagellin/TLR5 responses in epithelia reveal intertwined activation of inflammatory and apoptotic pathways

Hui Zeng,1,2* Huixia Wu,1,2* Valerie Sloane,1 Rheinallt Jones,1 Yimin Yu,1 Patricia Lin,2 Andrew T. Gewirtz,1 and Andrew S. Neish1

1Epithelial Pathobiology Unit, Department of Pathology and Laboratory Medicine, and 2Division of Neonatal-Perinatal Medicine, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia

Submitted 15 June 2005; accepted in final form 15 September 2005

Zeng, Hui, Huixia Wu, Valerie Sloane, Rheinallt Jones, Yimin Yu, Patricia Lin, Andrew T. Gewirtz, and Andrew S. Neish. Flagellin/TLR5 responses in epithelia reveal intertwined activation of inflammatory and apoptotic pathways. Am J Physiol Gastrointest Liver Physiol 290: G96–G108, 2006. First published September 22, 2005; doi:10.1152/ajpgi.00273.2005.—Flagellin, the primary structural component of bacterial flagella, is recognized by Toll-like receptor 5 (TLR5) present on the basolateral surface of intestinal epithelial cells. Utilizing biochemical assays of proinflammatory signaling pathways and mRNA expression profiling, we found that purified flagellin could recapitulate the human epithelial cell proinflammatory responses activated by flagellated pathogenic bacteria. Flagellin-induced proinflammatory activation showed similar kinetics and gene specificity as that induced by the classical endogenous proinflammatory cytokine TNF-α, although both responses were more rapid than that elicited by viable flagellated bacteria. Flagellin, like TNF-α, activated a number of antiapoptotic mediators, and pretreatment of epithelial cells with this bacterial protein could protect cells from subsequent bacterially mediated apoptotic challenge. However, when NF-κB-mediated or phosphatidylinositol 3-kinase/Akt proinflammatory signaling was blocked, flagellin could induce programmed cell death. Consistently, we demonstrate that flagellin and viable flagellate Salmonella induces both the extrinsic and intrinsic caspase activation pathways, with the extrinsic pathway (caspase 8) activated by purified flagellin in a TLR5-dependant fashion. We conclude that interaction of flagellin with epithelial cells induces caspase activation in parallel with proinflammatory responses. Such intertwining of proinflammatory and apoptotic signaling mediated by bacterial products suggests roles for host programmed cell death in the pathogenesis of enteric infections.

Salmonella; inflammation; Toll-like receptor; cDNA microarray

**Gram-negative bacteria** of the *Salmonella enteritidis* group are common human pathogens often isolated from cases of acute food-borne gastroenteritis in the United States and developing countries (49). *S. enteritidis* interaction with the intestinal epithelia provokes secretion of chemokines and subsequent luminal translocation of neutrophils (51). The result of this acute inflammatory response is the disruption of barrier integrity permitting transepithelial fluxes of ions and water as well as neutrophils. These events correlate clinically with an acute inflammatory diarrhea. Other enteric bacterial infections, such as those caused by *Shigella*, enteropathogenic *Escherichia coli*, and *Yersinia*, elicit similar responses (10).

Many of the epithelial responses to intestinal infections involve the transcriptional activation of inflammatory mediators such as chemokines, adhesion molecules, and antibacterial peptides. These gene products are controlled by the action of proinflammatory signaling cascades such as the NF-κB (Rel) and MAPK pathways that culminate in the activation of nuclear transcription factors and the initiation of de novo mRNA transcription. Indeed, work in our and other laboratories has identified NF-κB activation as a central event in the proinflammatory gene expression that mediates infectious enterocolitis (13, 16). Enterocytes (and most other cells) can perceive bacterial threats and activate the NF-κB pathway via a battery of transmembrane Toll-like receptors (TLRs) (35). These sentinel pattern recognition receptors are potently activated by pathogen-associated molecular patterns (PAMPs), complex molecules with a macromolecular structure limited to and characteristic of prokaryotic life, permitting stable and efficient recognition by eukaryotic TLRs (48).

Flagellin is a bacterial product that is generally considered a PAMP, with TLR5 as its physiological receptor in vertebrates (22). This pattern recognition receptor is present on the basolateral aspect of intestinal epithelial cells (15) and binds a 13-amino acid motif present in the flagellin protofilament but not accessible in polymerized flagella (46). In *S. typhimurium*, the flagellar protofilaments are 55-kDa monomers encoded by the two similar but not identical genes FliC and FliB (34). Approximately 20,000 subunits of flagellin assemble to form the extracellular filament structure that is necessary for bacterial motility. Flagellin is found on a wide variety of bacteria, and it is assumed that structural constraints necessary for motor function permit TLR5 recognition of flagellin encoded in a variety of organisms (47).

Purified flagellin can activate transcription and secretion of the proinflammatory chemokine IL-8 in vitro cell culture systems (45). Flagellin is also a potent activator of systemic inflammation in murine models (12), and, in humans, serum levels of the protein correlate with clinical severity of bacteremic shock syndromes (31). Interestingly, studies of circulating antibodies in the serum of human Crohn’s disease patients and in murine colitis models identified flagellin as a dominant antigen, suggesting a role for this bacterial protein in the immunopathogenesis of inflammatory bowel disease (32).

In our laboratory, we have shown that infection of model epithelia with live *S. typhimurium* elicited a classic proinflammatory transcriptional response involving NF-κB (16). Strik-
ingly, mutation of both *Salmonella* genes encoding flagellin (FliC and FljB) could totally abolish this response (17, 53), indicating that this PAMP is necessary for the proinflammatory responses to this pathogen. Because of the vital role flagellin plays in *Salmonella* pathogenesis (and probably other bacterial infections) as well as the ability of this protein to activate known proinflammatory genes, we sought to characterize the signaling pathways and transcriptional responses elicited by this exogenous prokaryotic protein. We report herein that flagellin is sufficient to recapitulate epithelial proinflammatory responses to *Salmonella* typhimurium. Furthermore, we demonstrate that flagellin also activates apoptotic signaling pathways. We also observe transcriptional upregulation of antiapoptotic factors that serve to arrest caspase activation in a NF-κB-dependant manner; however, when the NF-κB pathway is blocked, caspase activation proceeds to cell death. We speculate that this parallel activation may be a general feature of activators of innate immunity and that flagellin may play a previously under appreciated role in host monitoring of, and response to, microbes.

**MATERIALS AND METHODS**

**Reagents and constructs.** Flagellin (FliC) from wild-type *S. typhimurium* (SL3201) was purified through sequential cation and anion-exchange chromatography and used at the concentrations indicated in the figures (16). Purity was verified as previously described (15). Cycloheximide and wortmannin were obtained from Calbiochem (La Jolla, CA); MG-262 was from BioMol (Plymouth Meeting, PA), staurosporine was from Sigma (St. Louis, MO); 4′,6-diamidino-2-phenylindole dihydrochloride was from Molecular Probes (Eugene, OR), and TNF-α was from R&D Systems (Minneapolis, MN). Antibodies used included caspase 3, cleaved caspase 3, caspase 8, caspase 9, phospho-p38, phospho-Akt, phospho-IκBα, phospho-JNK, phospho-ERK, p38 (all from Cell Signaling; Beverly, MA), poly(ADP-ribose) polymerase (PARP; BD Pharmingen; San Diego, CA), IkBα (Santa Cruz Biotechnology; Santa Cruz, CA), cIAP-2 (R&D Systems), and β-actin (Sigma). Epitope-tagged antibodies included T7 (EMD Biosciences; Madison, WI) and V5 (Invitrogen; Carlsbad, CA). The pDsRed2 construct was obtained from BD Biosciences. The mutant IκBα pCMV4 T7-IκB-α S32/36A construct, containing Ser→Ala replacements at Ser32 and Ser36, was kindly provided by Dr. Dean W. Ballard (Vanderbilt University School of Medicine). The cellular inhibitor of apoptosis-2 (cIAP-2) expression vector pcDNA4.1-cIAP2 was constructed by cloning RT-PCR products into the pcDNA4.1A vector (Invitrogen).

**Cell culture and bacterial infection.** Model human intestinal epithelial cells (T84) were prepared on glass coverslips to 95% confluence were stimulated with purified flagellin. After experimental incubation, caspase 8 and caspase 9 activation was measured using APO-LOGIX (BD Biosciences; Piscataway, NJ), respectively, hybridized to microarrays, washed, and scanned. Three repeats for each experimental condition were performed. All experiments were compared with the same reference to allow the relative expression level of each gene to be compared across all experiments. After normalization, genes with intensity over four times of background mean were selected. To minimize variability, the mean of three independent experiments for each gene was calculated and used for final data clustering. Only genes that showed significant change (over 2-fold difference) were selected for further characterization. Cluster/Tree view (Michael Eisen, Stanford University; Stanford, CA) analytic software packages were used for hierarchical clustering.

**Real-time quantitative PCR.** Total RNA (1.0 μg) from sample and reference T84 cells were reverse transcribed with a Taqman RT kit (Applied Biosystems; Foster City, CA). One microliter of the product was subjected to SYBR green Real-Time PCR assay (Applied Biosystems). Reactions were performed in triplicate and normalized to 18s rRNA. The level of expression for a given gene was first normalized by subtracting the mean value of the cycle threshold (Ct) with that of 18s rRNA (ΔCt). Relative levels of gene expression were then determined by subtracting the individual ΔCt values of samples with those of reference (ΔΔCt) and expressing the final quantification value as $2^{-ΔΔCt}$. Primers for the genes of interest were designed with PrimerExpress (Applied Biosystems), and their sequences are available upon request.

**Caspase staining and quantification.** T84 cells on permeable supports or IEC-6 cells grown on glass coverslips were stimulated with purified flagellin. After experimental incubation, caspase 8 and caspase 9 activation was detected using APO LOGIX carboxyfluorescein Caspase Detection Kits (Cell Technology; Minneapolis, MN) through irreversible binding of substrate to enzymatically active caspases in living cells. Cells were visualized by confocal microscopy (Zeiss LSM 510) at 505 nm. The numbers of caspase positive cells from 5 representative viewing fields including at least 500 cells were counted. Data are presented as one of three representative experiments. Caspase 8 and caspase 9 activities were also determined with luminescent substrates, using Caspase-Glo 8 and 9 Assays (Promega; Madison, WI) according to the manufacturer’s protocol, and quantified by a TD-20/20 luminometer (Turner Designs; Sunnyvale, CA).

**Programmed cell death assay.** Two different assays were applied to IEC-6 cells (both floating and attached cells) to detect apoptotic cells after treatment at various times. In the propidium iodide staining assay, cells were fixed in 70% ethanol and analyzed for DNA content by flow cytometry. With this method, the percentage of apoptotic cells was determined by quantification of the sub-G1 fraction of cells. For TdT-mediated DUTP nick-end labeling (TUNEL) staining, the In Situ Cell Death Detection Kit (Roche) was used according to the manufacturer’s instructions. Briefly, IEC-6 cells were harvested, fixed in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100–0.1% SDS-PAGE immunoblot analysis. Differently treated cells, as described in the figures, were collected and lysed in ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Sigma). Protein concentration was determined with the Bradford reagent (Bio-Rad; Hercules, CA). Equal amounts of proteins were loaded on SDS-PAGE and blotted with different antibodies as indicated, following standard protocols.

**Microarray analysis.** Details of cDNA microarray fabrication, hybridization, scanning, and labeling of RNA samples are described in Ref. 53. Briefly, total RNA from treated T84 cells (sample) and untreated T84 cells (reference) was prepared using TRIzol reagent (Invitrogen). The integrity of RNA transcripts was verified by gel electrophoresis. With the use of an Oligo-dT primer and Superscript II Reverse Transcriptase (Invitrogen), labeled cDNA was synthesized from 40 μg of total RNA. Reference and experimental RNA samples were labeled with Cy3- and Cy5-coupled dCTP (Amersham Biosciences; Piscataway, NJ), respectively, hybridized to microarrays, washed, and scanned. Three repeats for each experimental condition were performed. All experiments were compared with the same reference to allow the relative expression level of each gene to be compared across all experiments. After normalization, genes with intensity over four times of background mean were selected. To minimize variability, the mean of three independent experiments for each gene was calculated and used for final data clustering. Only genes that showed significant change (over 2-fold difference) were selected for further characterization. Cluster/Tree view (Michael Eisen, Stanford University; Stanford, CA) analytic software packages were used for hierarchical clustering.
sodium citrate, and labeled with TUNEL reaction mixture. In both assays, 10,000 fluorescent events were measured by a FACS flow cytometer (Becton Dickinson Immunocytometry Systems; San Jose, CA) for each sample. Flow cytometric data were analyzed using FlowJo software.

RESULTS

Flagellin elicits proinflammatory signaling in epithelial cells. We have previously utilized genetic “loss of function” experiments using Salmonella flagellin mutants to show that this protein was necessary for proinflammatory cellular responses (53). In a complementary “gain of function” approach, we sought to determine whether purified flagellin was sufficient to mediate the proinflammatory effects of the viable pathogen. We evaluated kinetics of established proinflammatory signal response pathways in T84 model epithelia activated by flagellin (applied basolaterally) and directly compared responses with those elicited by the canonical endogenous proinflammatory agonist TNF-α (also applied basolaterally). When fully polarized T84 model epithelia were stimulated with purified flagellin (100 ng/ml, a concentration comparable with that seen during coculture with live S. typhimurium), we observed rapid (within 5 min) activation of the NF-κB pathway, as measured by the appearance of phosphorylated IκBα and subsequent degradation of the unmodified form (Fig. 1). We also noted robust activation of p38 and JNK kinases of the MAPK signaling group as well as ERK and Akt kinase activation within 5 min of treatment with both flagellin and TNF-α. Notably, flagellin elicited a more prolonged phosphorylation of these effectors relative to TNF-α, particularly JNK and p38, but overall cellular responses to these proinflammatory agonists appeared highly similar. These results demonstrated the ability of purified flagellin, a prokaryotic protein, to activate cellular proinflammatory response pathways highly similar to those activated by an endogenous proinflammatory agonist.

Flagellin elicits a proinflammatory transcriptional response in epithelial cells. To study the transcriptional program elicited by bacterial flagellin, we utilized the same epithelial model stimulated with proinflammatory agonists over 1–6 h and measured mRNA abundance by cDNA microarray. This time frame was chosen to evaluate primary, largely transcriptional changes in epithelial gene expression. The microarray system and data management methodology have been reported previously (53). Model epithelia were treated with 1) purified flagellin, applied basolaterally; 2) TNF-α, also applied basolaterally; and 3) the wild-type pathogen S. typhimurium (strain SL3201, from which we purified flagellin), cocultured apically with the monolayer.

Model epithelia were stimulated for 1, 2, 3, 4, 5, and 6 h. After experimental manipulation, RNA was prepared and subjected to microarray analysis. From the collected data, 263 genes reproducibly exhibited a greater than doubling differen-
tial expression; for display purposes, 103 genes showing significantly greater than twofold change relative to control are shown. The genes comprising the data set are represented in matrix form with the expression patterns of individual genes organized by hierarchical clustering (Fig. 2), and also listed as supplementary Table 1. This clustering method calculates the mathematical degree of relatedness between the expression patterns of each gene in an iterative fashion and arranges similar expression patterns adjacent to each other along the y-axis. A striking aspect of this series of experiments was the degree to which cellular responses to S. typhimurium could be recapitulated by endogenous (TNF-α) or exogenous (flagellin) proinflammatory protein agonists. Most of these genes have recognized roles in innate immune and inflammatory reactions, and many of them are regulated by the NF-κB transcription factor pathway (37). The characteristic differences between early induced genes [often acute inflammatory mediators such as IL-8 and chemokine, CXC motif, ligand 3 (CXCL3)] and later induced genes [typically chronic mediators such as interferon regulatory factor 1 (IRF-1) and IFN-α receptors 1 and 2] were clearly apparent. Overall, TNF-α- and flagellin-mediated upregulation was more rapid than induction elicited by S. typhimurium, consistent with the kinetics of bacteria-mediated activation of NF-κB reported earlier (16).

Flagellin mediates antiapoptotic effects in epithelial cells. In addition to transcriptional activation of genes with well-known roles in cellular inflammation, flagellin also activated a panel of antiapoptotic mediator genes including cIAP-1, cIAP-2, and A20 (indicated in red in Fig. 2). These genes are rapidly induced in a NF-κB-dependent manner (7) and can inhibit caspase activation and subsequent proapoptotic signaling (9, 27). For example, the IAPs are ubiquitin ligases that can bind to and induce degradation of activated caspases (9), whereas A20 is a zinc finger protein that disrupts assembly of proapoptotic adaptor molecules to the TNF receptor (23). The unexpected appearance of antiapoptotic mediator genes induced by an exogenous stimulus led us to confirm this regulation by real-time PCR, cIAP-1, cIAP-2, and A20 antiapoptotic proteins are well known TNF-α-responsive genes; as shown in Fig. 3A, flagellin is also a potent inducer. Given the very high levels of cIAP-2 transcript induced by flagellin, we also verified expression of this antiapoptotic protein by Western blot analysis (Fig. 3B). Purified flagellin (FliC) induced this antiapoptotic protein with kinetics consistent with the microarray analysis.

Fig. 2. Transcriptional response of polarized model epithelia to proinflammatory stimuli. Cells were treated with TNF-α (3 ng/ml, applied basolaterally), wild-type (WT) Salmonella typhimurium (SL3201, applied apically), and purified flagellin (5 ng/ml, applied basolaterally) for 1, 2, 3, 4, 5, and 6 h. Flagellin (FliC) was purified from the SL3201 strain. Data are a time course of expression profiles of 103 genes differentially regulated by proinflammatory stimuli with ≥2-fold induction. The expression patterns of individual genes were organized by uncentered correlation complete linkage hierarchical clustering. Data are presented as a matrix; each row represents an individual array element (gene), and each column represents an experimental condition. Red designates relative upregulation, whereas green designates downregulation, in a semicontinuous fashion (see scale, bottom left). The intensity of the color corresponds to the mean ratio of transcript abundance of the experimental condition relative to the transcript abundance of the untreated cell line (T84). The green vertical bar delineates the “bacterially enhanced” group of genes, whereas the red bar denotes “flagellin-enhanced” genes. Antiapoptotic effector genes are shown in red.
Fig. 3. Flagellin mediates antiapoptotic effects in epithelial cells. A: real-time PCR quantification of 3 selected antiapoptotic effector genes: cellular inhibitor of apoptosis (cIAP)1, cIAP2, and A20. Cells were stimulated with flagellin as described in Fig. 2 for the indicated time points. Data are shown as fold upregulation relative to basal (unstimulated) levels. B: immunoblot for cIAP-2. T84 cells were stimulated with flagellin for the times indicated. Actin is included as a loading control. C: IEC-6 cells were stimulated with flagellin (100 ng/ml) for 4 h before challenge by the addition of viable Escherichia coli for an additional 6 h at a multiplicity of infection (MOI) of 30. Caspase activation was detected and quantitated with a luminescent caspase 8 substrate. Data are from a single experiment and representative of 3 experiments that showed an identical pattern of results. D: IEC-6 cells were treated as in C. Caspase 8 activation in viable cells was detected with an APO LOGIX carboxyfluorescein (FAM) caspase detection kit, and nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Data are from a single experiment and representative of 3 experiments that showed an identical pattern of results. E: IEC-6 cells were treated with flagellin as in C and then stimulated with 1 μM staurosporine (STS) for 4 h. Caspase activation was detected and quantitated with a luminescent caspase 9 substrate. Data are from a single experiment and representative of 3 experiments that showed an identical pattern of results.
To test whether flagellin-induced gene expression could protect cells from subsequent apoptotic stimuli, we used a nontransformed epithelial model, rat IEC-6 cells. These cells are commonly used in studies of epithelial cell apoptosis (6). IEC-6 epithelial cells were pretreated for 4 h with flagellin (this duration elicited the maximum induction of antiapoptotic genes; Fig. 2) and subsequently challenged them by incubation with the intestinal commensal bacteria *E. coli* for 6 h. Non-pathogenic *E. coli* was used to stimulate multiple TLR/PAMP receptor ligand pairs simultaneously without the presence of type III secretion system (TTSS) effectors that might be present in pathogens. Under conditions where cells were not treated with flagellin, this coculture with *E. coli* induced potent caspase 8 activation within 6 h, as measured quantitatively with luminescent substrates and morphologically with fluorescent caspases substrates (Fig. 3, C and D). However, cells pretreated with flagellin before identical *E. coli* coinoculation showed a marked reduction in caspase-positive cells in both assays. Flagellin treatment alone for a total of 10 h showed no activation. To determine flagellin-mediated cytoprotective ef-
ffects against agents that stimulate the intrinsic, mitochondrial pathway, we performed similar experiments utilizing the apoptotic activator staurosporine (11). Pretreatment of IEC-6 cells with flagellin reduced caspase 9 stimulated by staurosporine (as expected, staurosporine had no effect on caspase 8 activity; data not shown), although the effects were not statistically significant (Fig. 3E). Collectively, these data indicate that the flagellin-induced epithelial transcriptional program shown in Fig. 2 involves an antiapoptotic component.

Flagellin can induce caspase activation and apoptosis in cells under blockade of NF-κB. Given the similarity of proinflammatory signaling between TNF and flagellin, we attempted to determine whether flagellin could also positively influence apoptotic processes in the manner of the TNF receptor. TNF-α signaling has a dualistic nature: although it is potently proinflammatory and strongly induces antiapoptotic genes, TNF-α is also an activator of the extrinsic pathway of apoptotic activation through caspase 8 activation (50). Usually, the concurrent activation of proinflammatory pathways, especially NF-κB, results in upregulation of antiapoptotic effector genes, which aborts caspase action and resultant cell death (27). Activation of apoptotic pathways by ligands/TLRs is poorly defined, although many studies have evaluated the TNF/TNF receptor (death receptor) interaction (50).

To investigate a potential role of flagellin in proapoptotic signaling, we examined T84 cells stimulated with basolateral flagellin over a time course for evidence of caspase activation. We utilized specific antibodies to evaluate activation of initiator caspases of the extrinsic pathway (caspase 8) and intrinsic pathway (caspase 9) as well as executioner caspases (caspase 3) and substrates (PARP) in Western blots. These antibodies react with the processed forms of the specific caspases, a proteolytic event necessary for activation of enzymatic function, whereas PARP cleavage is another marker of the execu-
tion phase of apoptosis. With treatment with flagellin alone, trace appearances of the cleaved/activated forms of caspases were seen over 4–8 h (Fig. 4A). In cells under blockade of the NF-κB pathway with proteasomal inhibitors (MG-262), activation of caspases (caspase 8, caspase 9, and caspase 3) was markedly more intense than that with flagellin or MG-262 alone, most noticeable with caspase 8. In cells treated with wortmannin (an inhibitor of P13K/Akt kinase) flagellin potentially induced caspase-3 cleavage, suggesting a role for this pathway in flagellin-mediated caspase activation (Fig. 4B).

To further study caspase activation, carboxyfluorescein (FAM) caspase substrates were employed. Polarized T84 cells were stimulated with flagellin and/or MG-262 as above, treated with the fluorescent substrates, and evaluated by fluorescence microscopy. Occasional fluorescent cells were seen within 6 h of flagellin treatment alone with substrates specific for both caspase 8 and caspase 9 (Fig. 4, C and D). Similar activation was observed in cells treated with MG-262. However, the combination of flagellin stimulation and NF-κB blockade revealed a marked quantitative upregulation of caspase 8 and caspase 9 activation, a pattern highly consistent to the data obtained with anti-active caspase antibodies.

To demonstrate that viable Salmonella could activate apoptotic signaling in cultured model epithelia, we cocultured live wild-type Salmonella with IEC-6 cells and evaluated caspase 8 and caspase 9 activation with fluorescent substrates (Fig. 4E). High levels of caspase 8 activation was seen within 12 h postinoculation with the wild-type strain, whereas activation of caspase 9 was less significant. These data are consistent with prior evaluation of Salmonella-mediated apoptotic activation (28) and suggests that Salmonella-mediated apoptotic stimulation is predominately via the extrinsic pathway.

Collectively, these data indicate that flagellin and flagellated bacteria can initiate caspase activation, a process that is markedly enhanced when proinflammatory (survival) pathways are blocked. Further experiments were undertaken to determine whether this caspase activation proceeded to cell death. We observed that at 6 h of flagellin stimulation, no cell death occurred in IEC-6 epithelial cells, as measured by an increase in the sub-G1 DNA fraction (Fig. 5A), as would be expected with the simultaneous and unimpeded activation of the NF-κB pathway and secondary arrest of activated caspses. However, during flagellin stimulation under conditions of NF-κB blockade (with the proteasome inhibitor MG-262), IEC-6 cells showed evidence of programmed cell death. The sub-G1 fraction was comparable with that induced by staurosporine. Proteasome inhibitors are commonly used to augment apoptosis during flagellin stimulation under conditions of NF-κB blockade. This mutant cannot be degraded by proinflammatory stimuli and also acts as a dominant negative substrate for inhibitor of κB, kinase (IKK), effectively and selectively inhibiting the NF-κB pathway. IEC-6 cells were transfected (with expression confirmed by Western blot) and assayed for apoptosis by TUNEL staining or immunoblot for activated caspase 3 with and without stimulation of the cells with flagellin (Fig. 5, B and C). Whereas the mutant IkBα construct alone had relatively little affect on TUNEL positively, transfected cells exposed to flagellin for 12–24 h showed a more than doubling of the numbers of apoptotic cells.

To support the notion that specific inhibition of the NF-κB pathway would allow flagellin-induced caspase activation to proceed to irreversible cell death, we transfected cells with an IkBα mutant bearing Ser to Ala transitions (S32/36A) on the phosphorylation motif. This mutant cannot be degraded by proinflammatory stimuli and also acts as a dominant negative substrate for inhibitor of κB, kinase (IKK), effectively and selectively inhibiting the NF-κB pathway. IEC-6 cells were transfected (with expression confirmed by Western blot) and assayed for apoptosis by TUNEL staining or immunoblot for activated caspase 3 with and without stimulation of the cells with flagellin (Fig. 5, B and C). Whereas the mutant IkBα construct alone had relatively little affect on TUNEL positively, transfected cells exposed to flagellin for 12–24 h showed a more than doubling of the numbers of apoptotic cells.
Flagellin activates the extrinsic pathway in a TLR5-dependent fashion. Proinflammatory signaling elicited by flagellin requires TLR5. To investigate whether flagellin-elicited apoptotic responses are also TLR5 dependent, we utilized a cell line stably transfected with TLR5 that can confer the ability to secrete IL-8 in response to flagellin (the parent cell line does not react to flagellin) (52). These cells were stimulated with flagellin with and without cycloheximide (a protein synthesis inhibitor) for 4–6 h and evaluated with immunoblots for activated caspases. As expected, strong caspase 3 and caspase 8 activation was only seen in the presence of both flagellin and cycloheximide, at 4–6 h (Fig. 6, A–C). Caspase 9 activation was not consistently observed with these reagents. The control HeLa cell line, without the transfected TLR5, did not show any caspase activation under identical conditions. Taken together, these results indicate that

Fig. 6. Flagellin activates caspase 8 (extrinsic pathway) in a Toll-like receptor (TLR)5-dependent manner. A: HeLa cells with and without a stably transfected TLR5 expression plasmid were stimulated with flagellin (100 ng/ml), cycloheximide (CHX; 5 μg/ml), or a combination for the indicated durations. Whole cell lysates were subjected to immunoblots with the indicated activated caspase antibodies. V5 is an epitope tag of the transfected TLR5. B and C: densitometric analysis of caspase 8 (B) and caspase 3 (C) immunoreactivity. Cleaved caspase 8 and caspase 3 bands were scanned with Bio-Rad Imaging Densitometer GS-700 and quantitated with Bio-Rad Quantity One acquisition software.
flagellin can activate the extrinsic pathway of caspase processing through its physiological TLR5 receptor.

We have shown that flagellin induces antiapoptotic proteins as part of its transcriptional program, with cIAP-2 being the most highly induced example. We have also shown that when survival pathways are inhibited, TLR5-mediated signaling can activate caspases. We hypothesize that in most flagellin-TLR5 interactions, these antiapoptotic proteins inhibit caspase activation and permit inflammation. To obtain mechanistic data, we overexpressed cIAP-2 under conditions where flagellin induces apoptotic activation, by blockade of the NF-κB pathway with proteasome inhibitors, as demonstrated in Fig. 5C. As shown in Fig. 7, cIAP-2 reduced executioner caspase 3 cleavage by ~50% under these conditions.

DISCUSSION

Flagellin is increasingly recognized as a bacterial inducer of innate and adaptive immunity in mammals. It also serves to stimulate cellular defensive responses in plants [acting through a specific pattern recognition receptors (FLS2)] (18, 19, 55) and invertebrates (43), suggesting a wide range of eukaryotic life has evolved mechanisms to perceive this protein and react to it as a threat. In our experiments, flagellin largely recapitulated the transcriptional response induced by viable S. typhimurium. The responses are also highly similar to those responses induced by TNF-α, a classical endogenous promoter of innate immunity/inflammation. The differences that were observed, such as the preferential activation of inducible nitric oxide synthase and more prolonged MAPK (both JNK and p38) phosphorylation by flagellin, presumably stems from different receptor utilization (TNF receptor vs. TLR5) and distinct receptor specific secondary signaling intermediates (i.e., MyD88) that ultimately control downstream transcriptional responses (35). Our current data showing the essentially similar transcriptional responses of flagellin and TNF-α underscore the importance of acute neutrophilic inflammation in control of infection by flagellated organisms.

Although signaling cascades such as NF-κB are generally understood as components of the proinflammatory response mediating upregulation of cytokines, chemokines, adhesion molecules, etc., it is becoming increasingly apparent that integral components of this response are protein modifiers of...
TNF-H9251, can initiate apoptotic signaling via the extrinsic pathway (7, 27). Certain inducers of inflammation, such as TNF-α, can initiate apoptotic signaling via the extrinsic (caspase 8) pathway (2). The TNF receptor proteins employ differential utilization of adaptor proteins to control the bifurcation of proapoptotic and proinflammatory signaling. Receptor-bound TNF receptor-associated death domain protein (TRADD) may interact with TNF receptor-associated factor (TRA2F) to activate downstream IKK and NF-κB, or it may bind the death effector domain-containing adaptor FAS-associated death domain protein (FADD) to initiate caspase 8 processing (50). Evidence is beginning to emerge that indicates that the TLRs can also mediate both proinflammatory and proapoptotic signaling in a similar fashion. TLR4/LPS interactions have been demonstrated to induce apoptotic pathways in macrophages under conditions of proteasomal blockade, and TLR4-deficient macrophages were shown to be resistant to Yersinia-induced apoptosis (21). Aliprantis et al. (3) reported that TLR2/bacterial lipoprotein interactions activated the extrinsic pathway (caspase 8) via signaling involving MyD88 and subsequent recruitment of FADD, indicating that TLRs can act as “death receptors.” Proapoptotic signaling via TLR4 has been described in macrophages (25, 41), involving the TLR interacting adaptor protein inducing IFN-β (TRIF). TRIF-null macrophages show reduced TLR4-dependent apoptosis, and over-expressed TRIF itself is proapoptotic (42). Our data indicate that flagellin-induced signaling can also mediate activation of the proapoptotic cascades. The potential roles of MyD88, FADD, and TRIF in transducing caspase activation via TLR5 are under investigation.

It is evident that epithelial cells exposed to a microbial challenge can eventuate in cellular proinflammatory responses or in programmed cell death. Natural infection can vary in numbers of microbes, duration of interaction, associated organisms, concurrent state of innate or adaptive immune activation, and undoubtedly many other variables. It is probably accurate to say that proinflammatory and proapoptotic activation both occur rapidly and reliably during the initial interactions of bacterial organisms with a potential host. The cellular end point of these biochemical events depends whether proinflammatory or proapoptotic signaling “gains the upper hand.” We propose that epithelial cells, once they perceive bacterial flagellin in an inappropriate location (i.e., intracellular or basolateral) via the TLR5 receptor, initiate in parallel tandem pathways, both proinflammatory, conducted along sequential transfer of covalent modifications, and apoptotic, conducted along proteolytic cascades (Fig. 8). In most cases, proinflammatory upregulation of antiapoptotic effectors, such as the IAP molecules, serves to arrest apoptotic pathways before activation of executioner caspases and irreversible cell damage occurs. In the event of inhibition of proinflammatory signaling and consequent transcription, caspase activation could occur unimpeded and terminate in the dismantling of the cell. Such inhibition is now recognized to play an important role in the pathogenesis of certain bacterial infections. Yersinia possess a translocated effector protein, YopJ, with demonstrated effects against NF-κB and MAPK survival pathways, which allows them to induce macrophage apoptosis in a TLR4-dependent manner, efficiently eliminating an immunomodulatory cell (54). Salmonella are well known inducers of apoptosis in macrophages (28, 29) and can induce apoptosis in HT-29 epithelial cell cultures after at least 24 h of coculture with wild-type Salmonella (36). These bacteria also possess a YopJ homolog, AvrA, which has been demonstrated to inhibit NF-κB and promote apoptosis in epithelial cells (8). While it is possible that such bacterial effectors may be directly activating caspases, prokaryotic proteins and small molecules that specifically inhibit proinflammatory pathways would not be proapoptotic unless accompanied by a concurrent signal that set caspase processing in motion. Because flagellin and other PAMP induced signaling through the TLRs fulfill this requirement, it will be interesting to determine whether the presence of these molecules is required for bacteria-mediated apoptosis.

Flagellin is emerging as a key regulator of innate and adaptive immunity against flagellated pathogens (40). Why are cells wired to respond to flagellin (and probably most proinflammatory agonists) in part by activation of the apoptotic program? It has been suggested that apoptosis itself may represent the earliest form of “immunity,” whereas the responses we now consider “innate immunity (i.e., inflammation)” are a later evolutionary development (4, 26). Colonial, multicellular eukaryotes may have utilized a form of programmed cell death to eliminate infected cells without lethal effects to the whole organism. Indeed, such a strategy is common in plant immunity and in lower metazoans such as nematodes (1), and it is well known that PAMPs including flagellin, peptidoglycan (PGN), and LPS are potent inducers of apoptosis in these organisms (30, 44). Pattern recognition receptors may have evolved to altruistically eliminate a threatened cell via activation of apoptotic proteolytic cascades or, if
such drastic steps are unwarranted, induce a parallel biochemical signaling pathway the results in synthesis and release of soluble mediators including chemotactic chemokines and antimicrobial peptides.

How flagellin (an extracellular agonist) stimulates the intrinsic/mitochondrial pathway is not clear, although potential mechanisms can be envisioned. Bid is a proapoptotic member of the Bcl-2 family of apoptotic regulators (33). These proteins, defined by the “BH3” domain, can heterodimerize with each other; a relative excess of pro- over antiapoptotic members is thought to permit formation of a mitochondrial “pore,” allowing the escape of cytochrome c (20) and subsequent initiation of the intrinsic pathway. Bid is regulated by cleavage and activation by caspase 8, thus providing an amplifying link from extrinsic proapoptotic signals to intrinsic pathways.

Transient Bid cleavage was observed maximally in T84 cells at 3 h of flagellin treatment, but, again, the exposure of cells to flagellin under conditions where survival genes could not be upregulated (MG-262) resulted in a strong appearance of the cleaved, 15-kDa active form, tBid (data not shown). Further experiments to characterize this process are underway.

Our data demonstrating that flagellin pretreatment and cIAP-2 overexpression can reduce caspase activation (in normal cells with intact proinflammatory pathways) elicited by a separate potentially injurious stimulus are consistent with our current understanding of the role of antiapoptotic effectors (27). Recent work has also suggested that constitutive TLR overexpression can reduce caspase activation (in normal epithelial cells by a commensal strain. Escherichia coli-mediated killing. Proc Natl Acad Sci USA 98: 2735–2739, 2001.


