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Flagellin Is the Major Proinflammatory Determinant of Enteropathogenic *Salmonella*[1]

Hui Zeng,* Adam Q. Carlson,* Yanwen Guo,* Yimin Yu,* Lauren S. Collier-Hyams,* James L. Madara,† Andrew T. Gewirtz,* and Andrew S. Neish[2]*

The gastroenteritis-causing pathogen *Salmonella typhimurium* induces profound transcriptional changes in intestinal epithelia resulting in the recruitment of neutrophils whose presence is the histopathologic hallmark of salmonellosis. Here we used cDNA microarray expression profiling to define the molecular determinants that mediate such changes in model intestinal epithelia. Enteropathogenic *Salmonella* induced a classical proinflammatory gene expression program similar to that activated by the canonical proinflammatory agonist TNF-α. Nonproinflammatory bacteria, both commensals (*Escherichia coli*) and systemic pathogens (*S. typhi*), did not activate this expression profile. While *S. typhimurium* strains lacking the SPI-1-encoded type III system were fully proinflammatory, strains lacking the genes for the flagellar structural component flagellin were nearly devoid of proinflammatory signaling. Lastly, the epithelial proinflammatory response could be largely recapitulated by basolateral addition of purified flagellin. Thus, *S. typhimurium* flagellin is the major molecular trigger by which this pathogen activates gut epithelial proinflammatory gene expression. ***The Journal of Immunology*, 2003, 171: 3668–3674.**

Members of the genus *Salmonella* are an antigenically diverse group (>2000 serovariants) of Gram-negative facultative intracellular pathogens responsible for a wide spectrum of enteric and systemic disease in humans and other vertebrates. *Salmonella enteritidis* serovar typhimurium (*S. typhimurium*) is a common human pathogen isolated in many cases of acute food-borne gastroenteritis in the U.S. and developing countries (1).

The initial step in the pathogenesis of salmonellosis involves contact between the bacterium and the apical surface of the host epithelial cell. This interaction induces the host epithelium to orchestrate a classical acute inflammatory reaction (2). In the intestine, acute inflammation is manifested by the luminal translocation of neutrophils migrating along gradients of chemotactic chemokines secreted by the epithelium and by consequent disruption of epithelial barrier integrity that results in transepithelial fluxes of ions and water. These events correlate clinically with acute inflammatory diarrhea. Unpleasant as this response may be, it is necessary to clear the infection, otherwise the local infection may become systemic (3). Many of the epithelial proinflammatory responses to *Salmonella* infection involve the transcriptional activation of inflammatory mediators. Typically, most mediators are controlled by NF-κB and mitogen-activated protein kinase (MAPK)[3] signal transduction pathways. Indeed, work in our and other laboratories has identified NF-κB activation as a central event in the pathogenesis of infectious enterocolitis (4, 5).

Studies of the molecular interactions between *S. typhimurium* and human enterocytes often use cell culture models, since appropriate pathologic specimens are rarely obtained from patients early in the course of infection. The T84 cell line is a particularly well-suited in vitro model because it forms polarized monolayers when grown on apical supports, mimicking the physiological epithelial barrier (6). Furthermore, when exposed to pathogenic *Salmonellae*, these model epithelia recapitulate many in vivo responses, including altered gene expression (7–9).

Mechanistic investigation of the pathogenic determinants required to induce changes in epithelial gene expression have generally focused on several, presumably representative, proinflammatory effector genes. These studies have reported roles for *S. typhimurium* type III secretion system and for genes encoding flagella or regulating synthesis of flagella (10, 11). However, recent technical advances have permitted the analysis of eukaryotic gene expression in parallel and on a large scale. Using cDNA microarray analysis, the relative expression levels of several thousand genes can be compared in test and control cells simultaneously, thus affording a much broader look at the overall importance of specific determinants. In this report we used such gene expression profiling from model epithelia colonized with pathogens (intestinal or systemic), specific mutants, and commensal microbes to characterize the bacterial determinants that mediate *Salmonella* enterocolitis.

Materials and Methods

Bacterial culture and flagellin purification

S. typhimurium wild types, mutant strains, *Salmonella typhi*, and *Escherichia coli* 4 and 7 (normal gut strains; Table I) were prepared overnight under nonagitated microaerophilic conditions as previously described (7). *fliD*$^-$ (and parent) was a gift from Dr. R. Macnab (Yale University). Flagellin was purified from *S. typhimurium* wild type (SL3201) through sequential cation and anion exchange chromatography, and purity was verified as previously described (11, 12).

*Epithelial Pathobiology Unit, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322; and †Pritzker School of Medicine, University of Chicago, Chicago, IL 60637

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[2] Address correspondence and reprint requests to Dr. Andrew S. Neish, Emory University School of Medicine, 105-F Whitehead Building, 615 Michael Street, Atlanta GA 30322. E-mail address: aneish@emory.edu

[3] Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; TTSA, type III secretion apparatus.

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Table I. *Bacterial strains used in this study*

Strains	Characteristics	Source/Ref. No.
S. typhimurium		
SL3201	Wild-type; phase variable (*fljB/fliC*)	11
SL3201 *fliC*$^-$	*fliC*::Tn10	11
SL3201 *fljB*$^-$	*fljB*::MudJ	11
SL3201 *fliC*$^-$/*fljB*$^-$	*fliC*::Tn10 *fljB*::MudJ	11
flhD$^-$	*flhD*::Tn10 from KK2040	11
KK1004	Wild-type of *fliD*$^-$	13
fliD$^-$	*fliD*::*kan*	13
SR11	Wild-type of *invA*$^-$	14
invA$^-$	*invA*::kan	14
TML-83	Wild-type of *invG*$^-$	15
invG$^-$	*invG*::kan	15
SL1344	Wild-type of Δ*spi-1*, mouse-virulent strain	10
Δ*spi-1*	Δ*spi-1*::kan	10
E. coli		
E. coli 7	Aflagellate *E. coli* commensal	11
E. coli 4	Flagellated *E. coli* commensal	11
S. typhi		
S. typhi	Wild type	Clinical isolate

Cell culture

Model intestinal cells (T84) were prepared on 5-cm^2 permeable filters as described previously (6) and were used 9–14 days after plating and achieving a stable transepithelial resistance of >1000 Ω·cm^2. Monolayers were washed and equilibrated with HBSS at 37°C for 15 min. For bacterial treatments, bacterial cultures were washed, concentrated, and applied to the apical aspects of cells at a multiplicity of infection of 30 organisms/cell (4). After 1 h, monolayers were washed to remove nonadherent bacteria and replaced with HBSS for the duration of the experiment. For other treatments, 5 ng/ml flagellin and 3 ng/ml TNF-α were applied to the basolateral aspect of cells (11).

Preparation of custom arrays: inflammatory chip

A set of 650 individual bacterial clones carrying target genes of interest was picked from the Sequence Validated Human cDNA Library (ResGen, Carlsbad, CA). These clones together with several human housekeeping genes and yeast genes (Incyte, Palo Alto, CA) were reinoculated into a new set of seven 96-well plates containing Luria-Bertoni medium with ampicillin. Target cDNA inserts (0.5–2 kb) were amplified directly from bacterial culture by PCR using universal primers. The resulting PCR products were purified and checked for size and yield by agarose gel electrophoresis. Three copies of five *Arabidopsis thaliana* cDNAs (Stratagene, La Jolla, CA) were distributed throughout printing plates for normalization. PCR products were suspended in 50% DMSO for printing.

Glass slides were coated with poly-L-lysine following the protocol reported previously (P. Brown's laboratory, http://cmgm.stanford.edu/pbrown/protocols/index.html) and were used as solid substrates for microarray printing. Arrays were printed under temperature and humidity control with an Affymetrix 417 robotic arrayer (Affymetrix, Santa Clara, CA).

RNA isolation, labeling, and hybridization

Total RNA from samples (treated T84 coculture) and references (untreated T84) was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Labeled cDNA was synthesized from 40 μg of total RNA and 0.125 ng of mRNA from each *A. thaliana* spike. Sample and reference RNA samples were labeled with Cy5- and Cy3-coupled dCTP (Amersham Pharmacia Biotech, Piscataway, NJ), respectively. After labeling, the probes were purified, concentrated, and combined with competitors in hybridization buffer (5× SSC, 0.2% SDS, and 25% formamide) and then pipetted onto a prehybridized chip. The array was covered with a 22 × 22-mm coverslip and incubated overnight at 42°C. Following hybridization the array was washed for 4 min with each of the following: 2× SSC with 0.1% SDS at 42°C, 1× SSC with 0.1% SDS, and 0.1× SSC at room temperature. Arrays were dried by centrifugation and immediately scanned with an Affymetrix 418 scanner.

Image processing and data analysis

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. For the analysis of scanned microarray images, ImaGene (Biodiscovery, Marina Del Rey, CA) was used to compute Cy3 and Cy5 fluorescence intensities and identify technically inadequate spots. Normalization, Cy5/Cy3 fluorescence ratio calculation, and log transformation of the raw data were conducted with GeneSight (Biodiscovery). Normalization was based on dividing all values by the median signal intensity of the population of *A. thaliana* control elements. Hybridization signals with fluorescence intensities in either channel <4 times local background were excluded from further analysis. To minimize variability, the mean of three independent experiments for each gene was calculated and used for final data analysis. Only genes that showed significant change (>2-fold difference) were selected for further hierarchical clustering with the Cluster/TreeView analytic package (Eisen, Stanford University, Stanford, CA)

Real-time quantitative PCR

Total RNA (1.0 μg) from sample and reference T84 cells was reverse transcribed with the TaqMan RT kit (PE Applied Biosystems, Foster City, CA). One microliter of the product was subjected to SYBR Green real-time PCR assay (PE Applied Biosystems). Reactions were performed in triplicate and normalized to 18S ribosomal RNA. The level of expression for a given gene was first normalized by subtracting the mean value of the cycle threshold (C_t) with that of the 18S rRNA (ΔC_t). Then relative levels of gene expression were determined by subtracting the individual ΔC_t values of samples with those of reference ($\Delta\Delta C_t$) and expressing the final quantitation value as $2^{-\Delta\Delta Ct}$. Primers for the genes of interest were designed with PrimerExpress (PE Applied Biosystems); their sequences are available upon request.

Western blots

To evaluate IκB-α phosphorylation, T84 cells were colonized by bacteria in the presence of a proteasome inhibitor (MG262, 250 nM). Cell lysates were prepared as previously described (4) and immunoblotted with phospho-IκB Ab (Cell Signaling Technology, Beverly, MA). To detect IκB-α degradation, T84 cells were pretreated with cyclohexamide (10 ng/ml) for 30 min before colonization. Since IκB-α turnover is rapid, this protein synthesis inhibitor (cyclohexamide) was added to block new IκB synthesis (4, 5). These lysates were probed with Ab to IκB-α (Santa Cruz Biotechnology, Santa Cruz, CA). T84 cell lysates were also prepared and immunoblotted with Abs to phospho-p38 MAPK, phospho-stress-activated protein kinase/c-Jun NH_2-terminal (JNK), and p38 MAPK (Cell Signaling Technology).

Results

To study the initial events in the *Salmonella* mucosal infectious process, we used an in vitro epithelial model cocultured with bacteria over 1–6 h. This time frame was chosen to evaluate primary transcriptional changes in epithelial gene expression. We established a database of genes induced or suppressed in T84 model epithelia when cocultured with live *Salmonella* over 1, 2, 3, 4, 5,

and 6 h by use of a commercial high density (>8000 genes) cDNA microarray platform (Incyte, Palo Alto, CA). This resulted in a reasonably comprehensive survey of eukaryotic genes differentially regulated by direct interaction with this pathogen. From this database and published data, we fabricated a custom array of >600 potentially informative genes. Our custom array permitted multiple repeats of the same experimental condition, increasing the validity of the results and facilitating the analysis of multiple experimental conditions.

We sought to compare epithelial expression profiles elicited by a panel of viable pathogenic *Salmonella*, nonpathogenic bacteria, and an endogenous proinflammatory stimulus. In previous experiments almost all genes that were differentially regulated by infection in the first 6 h were apparent at the 4 h point. Thus, this duration of model infection was used in subsequent analyses. We generated expression profiles from a series of experiments with five isolates of pathogenic *S. typhimurium*, two commensal *E. coli* isolates, and *S. typhi*, all infected on the physiological apical surface of the model epithelia. To compare the expression profiles generated from multiple experiments we used a hierarchical clustering algorithm. Expression profiles from experimental conditions (i.e., different bacterial treatments) were clustered, and the calculated relationships were displayed with a dendrogram (Fig. 1*a*). Not unexpectedly, well-defined proinflammatory pathogens (wild-type *S. typhimurium* strains) and the nonpathogens (commensal *E. coli* isolates) segregated into two very distinct clusters (designated with red and blue branches, respectively). Note that the TNF-elicited expression profile is present within the proinflammatory experimental cluster group. The expression matrix reveals a subset of induced genes that discriminates between the proinflammatory and nonproinflammatory groups. This set of genes (29 genes) is operationally designated the proinflammatory gene cluster and is indicated by a red bar, with the expanded matrix and gene names shown. Notably, *S. typhi*, the causal agent of typhoid fever, clustered among the nonproinflammatory group, although a modest up-regulation of inflammatory mediators was observed. This is consistent with the in vivo behavior of this organism that is able to penetrate the mucosal physical and immune barriers to gain systemic access without causing significant intestinal inflammation (9, 16).

To evaluate the effects of secreted bacterial products, toxins, metabolic wastes, and potential cell culture artifacts, we performed a series of experimental infections in which wild-type pathogenic *Salmonella* (strain SL3201) was physically separated from the apical epithelial surface by a 0.2-μm pore size filter. The inoculum size and duration of coculture were unchanged. Under these conditions intact viable bacteria were unable to physically contact the model epithelia, but small molecules could freely permeate the apical compartment. Separated pathogenic bacteria (designated in Fig. 1*a* as SL3201 Sep(aration)) were unable to elicit the typical proinflammatory response, as evidenced by clustering within the nonproinflammatory group. Taken together, this analysis of wild-type bacteria indicates that enteropathogenic *S. typhimurium* elicits

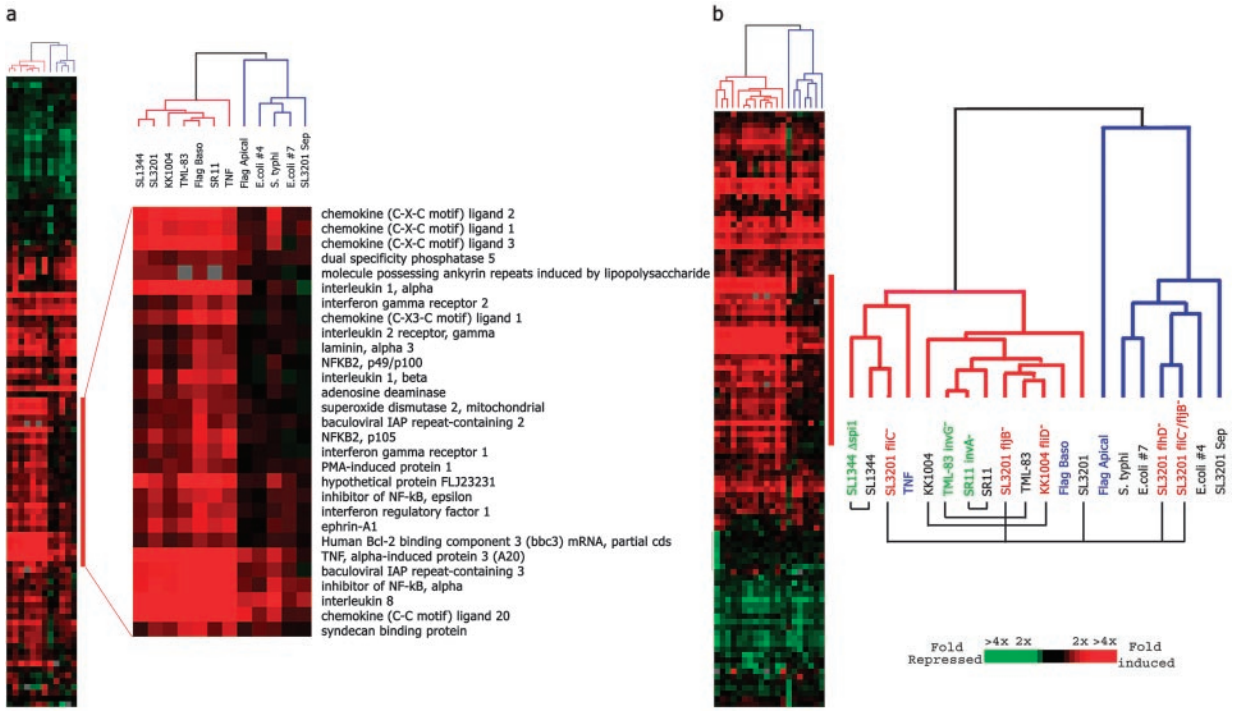

FIGURE 1. Hierarchical clustering of expression profiles of epithelial cells treated with bacteria and soluble agonists with informative genes (up or down 2-fold or greater). Data are presented as a matrix; each row represents an individual array element (gene), and each column represents an experimental condition. Gray indicates missing or technically inadequate data. Red designates relative up-regulation, while green designates down-regulation in a semicontinuous fashion (see scale, *bottom right*). The intensity of the color corresponds to the mean ratio of transcript abundance of the experimental condition relative to that of the untreated cell line (T84). Dendrograms indicate the degree of relationship among expression profiles elicited by bacterial strains/mutants and proinflammatory agonists. The relatedness of any two expression profiles is indicated by the length of the dendrogram branches; the shorter the branch, the more closely related the profiles are. All data points are at 4 h of stimulation, and branches of the proinflammatory cluster are designated with red; branches of the nonproinflammatory cluster are blue. *a*, Relationships of expression profiles elicited with wild type bacteria and soluble agonists. Genes of the proinflammatory cluster are designated with a red bar, and an enlarged matrix with gene names is shown. *b*, Relationships of expression profiles elicited by mutant bacteria. Wild-type bacteria are shown in black, flagellar mutants in red, type III mutants in green, and soluble proinflammatory agonists (TNF and flagellin) in blue. The enlarged dendrogram lists bacterial strains/agonists; black brackets beneath the bacterial names indicate isogenic relationships.

a largely, but not wholly, proinflammatory response that is contact dependant.

We next employed a loss of function approach to evaluate proinflammatory determinants. Expression profiles were generated from model epithelia infected with bacteria bearing mutations in candidate proinflammatory determinants along with their isogenic parent strains. These profiles were reclustered against the reference wild-type non- and proinflammatory bacterially elicited profiles established in Fig. 1*a*, and are displayed in Fig. 1*b*. We anticipated that this approach would assign mutants to a definitive category.

Because of the potency of flagellin in activating proinflammatory signals, we first analyzed a series of *S. typhimurium* strains bearing mutations in structural and regulatory components of the flagellar apparatus. Flagellin protein can be encoded by either of two structural genes, *fliC* and *fljB* (17). We evaluated mutants bearing disruptions in 1) *fliC*, *fljB*, or both flagellin structural genes; 2) *flhD*, the flagellar operon master regulator that prevents both the basal body and the flagellar filament from being expressed; and 3) *fliD*, a filament capping protein that allows secretion of flagellin monomers, but does not assemble a functional flagellar filament, thereby rendering the bacterium immotile.

Strikingly, aflagellate *S. typhimurium* mutants (*fliC*$^-$/*fljB*− and *flhD*$^-$) were located within the nonproinflammatory cluster. Their profiles were very similar to each other and were more closely related to that of nonpathogens than to their isogenic parent *Salmonella* strain and TNF (Fig. 1*b*). These bacteria were almost completely unable to up-regulate the proinflammatory cluster of genes (red bar). Mutants in either flagellar gene (*fliC*$^-$ or *fljB*$^-$) induced fully proinflammatory responses, and their profiles were closely related to the expression profile elicited by their parent strains, indicating the interchangeability of these genes with respect to proinflammatory signaling. Importantly, the immotile *fliD*$^-$ mutant was also fully proinflammatory, consistent with the idea that the proinflammatory nature of flagellin is a property of the protein, rather than a functional consequence of bacterial motility. Of note, both *E. coli* isolate 4 and *S. typhi* are flagellated, but not proinflammatory, consistent with the hypothesis that apical application of this protein is not, by itself, proinflammatory. Flagellin purified from this strain of *E. coli* was previously shown by us to have potent proinflammatory potential when applied basolaterally (11). *S. typhi* flagellin has been shown to be potently proinflammatory to human cells as well (18). These flagellated strains may fail to translocate flagellin across the epithelia to the basolateral aspect where its receptor, Toll-like receptor 5 (TLR5), is located, and as a result, cannot elicit a proinflammatory response.

In light of this requirement of flagellin expression for *S. typhimurium* induction of proinflammatory gene expression, we next asked what portion of the overall change in gene expression that is induced by *S. typhimurium* could be recapitulated by purified flagellin. Purified *S. typhimurium* flagellin was added to the apical or basolateral side of polarized epithelia, and the expression profiles were compared (Fig. 1*a*). Flagellin applied basolaterally elicited an expression profile largely similar to that observed following direct colonization with pathogenic *Salmonella*, indicating that flagellin is not only necessary, but is also sufficient, for the inflammatory gene expression elicited by this enteric pathogen. In contrast, flagellin applied apically, which mimics a condition that presumably exists when commensal flagellated bacteria colonize the mucosal surface of the gut, was clearly nonproinflammatory, indicating that the ability to translocate flagellin across the epithelium is a major proinflammatory determinant.

We next tested a series of mutants in the *Salmonella* SPI-1 type III secretion apparatus (TTSA), a multicomponent bacterial surface structure necessary for bacterial invasion in cultured epithelial cells and the histological and physiological manifestations of enteritis in animal models (19). We studied mutants (*invA*$^-$ and *invG*$^-$) that harbor disruptions in individual components of the TTSA. These mutants do not secrete effectors, but the TTSA is otherwise structurally intact. The third mutant, Δ*spi1*, is a deletion in the entire SPI-1 pathogenicity island, including TTSA structural components and all the genetically contiguous effector proteins. The structurally intact, but functionally inactivated, mutants elicited expression profiles that were still clearly proinflammatory and in both cases tightly clustered with profiles from their respective parent strains. The *spi-1* deletion mutant also clearly elicited a proinflammatory response from infected epithelial cells (Fig. 1*b*). Thus, mutations in the TTSA, while noticeably altering the expression profiles of genes elicited by apical infection of model epithelia, were clearly dispensable as proinflammatory determinants in this system.

Validation of reported data was accomplished by an independent method of mRNA measurement, quantitative real-time PCR. We selected four differentially expressed genes for confirmatory analysis: IL 8 (highly inducible), inhibitor of apoptosis protein repeat-containing 3 (modestly induced), anterior gradient protein 2 (down-regulated), and activating transcription factor 3 (specifically induced by bacteria). Ten samples of derived RNA from the same experimental preparation were subjected to side-by-side comparison by quantitative real-time PCR and array hybridization (Fig. 2). These experiments indicate a robust concordance of mRNA abundance across multiple experimental stimulations. Thus, independent characterization of mRNA levels by this sensitive technique confirmed the validity of our array system.

We next investigated whether the differences in the ability of commensal, pathogenic, and mutant bacteria to elicit changes in epithelial gene expression might be explained by their relative abilities to activate proinflammatory signaling events in model epithelia. Specifically, we measured the abilities of these bacterial strains to activate the NF-κB and MAPK signaling pathways. Activation of early steps in the NF-κB pathway was assessed by measuring the phosphorylation and degradation of IκB-α. We observed significant IκB phosphorylation in T84 cells incubated with proinflammatory strains (Fig. 3*a*). Furthermore, proinflammatory strains induced degradation of IκB, whereas nonproinflammatory strains did not (Fig. 3*b*).

Significant differences in the activation of proinflammatory MAPKs (p38 and JNK) were also observed between proinflammatory and nonproinflammatory bacteria. Specifically, phosphorylation of both p38 and JNK MAPKs were consistently observed in T84 cells apically colonized with proinflammatory bacterial strains, but not in those colonized with nonproinflammatory strains (Fig. 3*c*). Thus, the gross inability of aflagellate *S. typhimurium* strains to activate proinflammatory gene expression in model epithelia appears to result from the inability to activate well-defined signaling pathways, MAPK and NF-κB, both of which can be activated by ligation of TLR5 (20, 21).

Discussion

In this report we used expression profiling by cDNA microarray to dissect the proinflammatory determinants of enteropathogenic *S. typhimurium*. Extensive studies evaluating host responses to various pathogens have been performed with both cDNA and oligonucleotide array platforms (22–25). Generally, these studies have described a stereotypical core of induced genes, largely associated with innate immunity/inflammation, but have also characterized meaningful detectable differences in expression profiles elicited by distinct classes of organisms and pathogen-associated molecular

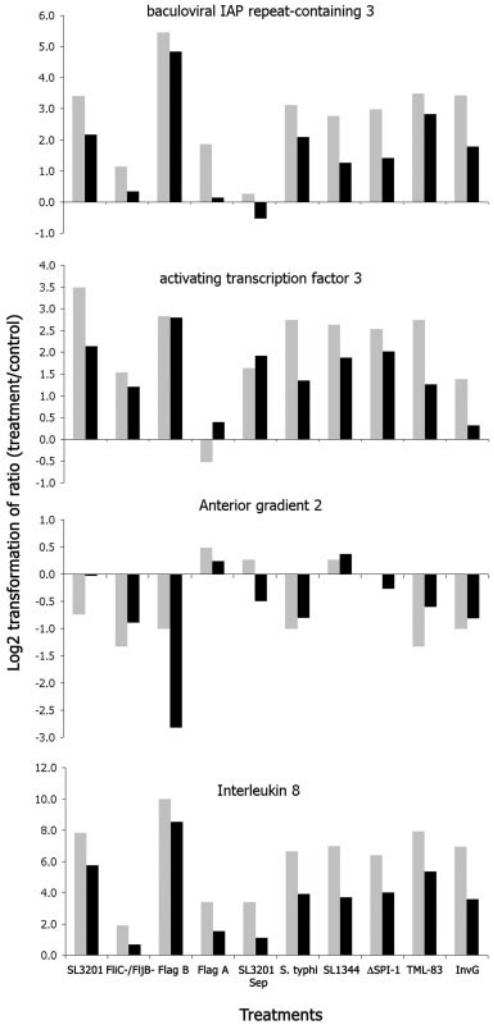

FIGURE 2. Verification of mRNA levels of four differentially expressed genes with real-time PCR. Log 2 transformations of the sample/reference ratio for the same set of samples from quantitative RT PCR assay (gray) and microarray experiments (black) were calculated and compared.

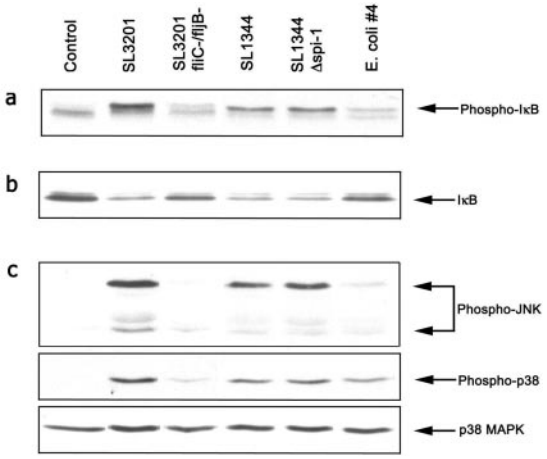

FIGURE 3. Activation of NF-κB and MAPK pathways with various proinflammatory and nonproinflammatory strains. Polarized T84 cells were apically colonized with the indicated organisms for 1 or 3 h. *a*, Immunoblot detecting IκB phosphorylation after 1-h colonization in the presence of a proteasome inhibitor (250 nM MG262). *b*, Immunoblot showing degradation of IκB-α in the presence of a protein synthesis inhibitor (10 ng/ml cycloheximide) for 3 h. *c*, Immunoblots showing phosphorylation of stress-activated protein kinase/JNK and p38 MAPK after 1 h of bacterial colonization. Total p38 MAPK served as an internal control for sample loading.

pattern (PAMPs). PAMPs are conserved microbial surface structures, such as LPS, that serve as activating ligands for eukaryotic pattern recognition receptors (TLRs) (26). However, specific proinflammatory determinants of pathogenic *Salmonella* in characteristically LPS-unresponsive polarized epithelia have not been evaluated with expression profiling.

To model enteropathogenesis, our experiments used viable organisms cocultured with model epithelia on the apical surface. Our major unexpected finding was the degree to which the epithelial proinflammatory responses to *S. typhimurium* could be ascribed to a single protein, flagellin. Purified *S. typhimurium* flagellin stimulated a proinflammatory program with striking overlap with the response elicited by viable pathogenic *S. typhimurium* and the canonical endogenous cytokine TNF-α. Furthermore, a strain of bacteria with mutations in both flagellin genes (*fljB*$^{-}$/*fliC*$^{-}$) was virtually devoid of proinflammatory signaling, as was a regulatory mutant that abolished the expression of the entire flagellar apparatus.

In *Salmonella*, flagellin is a 55-kDa monomeric protein encoded by *fliC* and *fljB* that assembles to form the filament structure of the flagellar apparatus necessary for bacterial motility. Flagellin is generally considered to be a PAMP. The protein has been shown to potently induce several proinflammatory effector genes in human epithelial cells (27, 28), and aflagellate *Salmonella* do not stimulate IL-8 secretion (11, 29, 30). The physiologic receptor for flagellin in vertebrates is TLR5 (31), which is present on the basolateral surface of intact epithelial cells and thus is sheltered from the flagellin-rich luminal environment (21). Flagellin is found on a wide variety of bacterial pathogens, and it is assumed that structural constraints necessary for the motor function permit TLR5 recognition of flagellin from a variety of organisms (26). Our observation that aflagellate bacteria fail to activate proinflammatory signaling when applied to the apical or basolateral aspect of model epithelia indicates that other remaining surface PAMPs, including LPS, were insufficient to activate these responses in polarized epithelial cells. Furthermore, we did not detect any proinflammatory response of epithelial cells (T84) to commercial LPS from *Salmonella* (data not shown). The LPS receptor, TLR4, has been reported to reside in an intracellular location in epithelial cells, perhaps as a monitor of invading or endocytosed organisms (32). The flagellin/TLR5 axis may act as a monitor of extracellular proinflammatory threats.

The widespread ability of eukaryotes to detect the presence of flagellin and interpret this as a danger or non-self signal is an emerging theme. Similar to the vertebrate gut, external plant cells are exposed to a microbe-rich environment, and flagellin is recognized by a specific receptor (FLS2) that elicits defensive responses (33, 34). Interestingly, plant symbionts, such as *Rhizobium* sp., have divergent flagellar sequences that are not recognized by the FLS receptor (35), suggesting the possibility of flagellin variants in vertebrate pathogens/commensals that are transparent to the innate immune system. Flagellin is also a potent activator of antimicrobial defensin expression in *Drosophila* (36) and will activate *Limulus* amebocytes (our unpublished observation). Consistent with our data, *Salmonella* strains with regulatory and structural mutations in the flagellar apparatus have a diminished ability to elicit acute inflammation in bovine models. Interestingly, these aflagellate mutants also show increased systemic virulence in the mouse model of typhoid fever, which may reflect the inability of systemic innate and adaptive immunity to detect and control the organism (37). Similarly, differential use of independent flagellar genes (phase

variation), such as *fliC* and *fljB*, in *Salmonella* may reflect a strategy to evade immune detection. In other bacteria, such as *Vibrio* and *Helicobacter* sp., flagellin is apparently hidden beneath the outer membrane (26).

Purified flagellin applied apically (luminally) does not activate proinflammatory pathways, presumably because the isolated protein cannot be translocated across the apical epithelial barrier to access the basolateral receptors. Flagellated nonpathogens, such as *E. coli*, do not elicit inflammatory responses, demonstrating, not surprisingly, that commensal *E. coli* lacks determinants that confer enteropathogenicity. However, all flagellin-expressing *Salmonella* tested, including an immotile mutant (*fliD*$^-$), were proinflammatory, indicating that enteropathogenic *Salmonella* possess additional virulence determinants that allow flagellin to access its receptor. Hypothetically, a specific interaction between enteropathogenic *Salmonella* and the apical epithelium induces receptor-mediated or fluid phase endocytosis and permits transcytosed flagellin access to the basolateral aspect of the cells, similar to the mechanisms by which apical Ags are internalized and trafficked to the basolateral class II HLA molecules (38, 39). Alternatively, bacterial interactions may influence tight junctions and allow paracellular leaking of soluble flagellin. Such processes may account for the delay in activation of many proinflammatory effectors we observed by live *Salmonella* relative to soluble agonists. Also, this difference in kinetics may be the result of active, but transient, repression of cellular responses by a distinct bacterial signal. Furthermore, nonproinflammatory bacteria are not necessarily nonpathogenic, as exemplified by *S. typhi*. This may be due to the absence or masking of proinflammatory determinants or by active repression of proinflammatory signaling. Taken together, flagellin is necessary, but not sufficient, as a proinflammatory determinant.

In contrast, the SPI-1 TTSA was apparently dispensable for proinflammatory signaling in our experimental system. We analyzed mutants with nonfunctional, but structurally intact, TTSAs (*invA*$^-$ and *invG*$^-$), and a mutant (Δ*spi1*) with a deletion of the entire SPI-1 pathogenicity island. Each of these mutants is defective in generally accepted assays of pathogenicity, including a virtually absent ability to invade cultured cells and induce histological acute inflammation and fluid secretion in bovine models of infection (19, 40). These mutants were only minimally reduced in proinflammatory potential, especially relative to the aflagellate strains. These type III mutants have been shown to be noninvasive, but still capable of translocation of flagellin across epithelial monolayer (data not shown). Thus, while the TTSA is necessary for enteropathogenesis of the intact organism, epithelial cells are clearly able to perceive pathogenic organisms and mount a response in the absence of this structure and its attendant functions.

In summary, we have identified flagellin as a major proinflammatory determinant of enteropathogenic *Salmonella*. As sensitivity to flagellin is an emerging theme in mucosal innate immunity and throughout metazoan life, it will be interesting to see whether future studies reveal a role for flagellin in other infectious and idiopathic inflammatory disorders of the intestinal tract.

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