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TLR5-mediated activation of p38 MAPK regulates epithelial IL-8 expression via posttranscriptional mechanism

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Yu, Yimin, Hui Zeng, Sean Lyons, Adam Carlson, Didier Merlin, Andrew S. Neish, and Andrew T. Gewirtz. TLR5-mediated activation of p38 MAPK regulates epithelial IL-8 expression via posttranscriptional mechanism. *Am J Physiol Gastrointest Liver Physiol* 285: G282–G290, 2003. First published April 17, 2003; 10.1152/ajpgi.00503.2002.—Toll-like receptors (TLRs) activate antimicrobial gene expression in response to detection of specific bacterial products. Relatively little is known about TLR5, the only TLR thought to be preferentially expressed by epithelial cells, beyond that it confers activation of the transcription factor NF-κB in a MyD-88 dependent manner in response to flagellin. Because TLRs, in general, are also thought to signal through members of the MAPK family, we examined flagellin-induced MAPK activation (via examining its phosphorylation status) and its subsequent role in expression of the chemokine IL-8 in polarized intestinal epithelia. Flagellin, like other proinflammatory stimuli (TNF-α, *Salmonella typhimurium*), activated p38 MAPK in a TLR5-dependent manner, whereas aflagellate bacteria or EGF did not activate this kinase. Although ERK1 and -2 were also observed to be activated in response to flagellin, their activation was not restricted to proinflammatory stimuli because they were also potently activated by aflagellate bacteria (*S. typhimurium* or *Escherichia coli*) and EGF (neither of which activate NF-κB in these cells). Pharmacological inhibition of p38 MAPK (by SB-203580) potently (IC_{50} = 10 nM) reduced expression of IL-8 protein (maximal inhibition, 75%) but had no effect on NF-κB activation, only slightly attenuated upregulation of IL-8 mRNA levels in response to flagellin, and did not effect IL-8 mRNA stability. Together, these results indicate that epithelial TLR5 mediates p38 activation and subsequently regulates flagellin-induced IL-8 expression independently of NF-κB, probably by influencing IL-8 mRNA translation.

flagellin; nuclear factor-κB; inflammation; toll-like receptor-5; mitogen-activated protein kinase; interleukin-8

EPITHELIAL CELLS THAT line the gastrointestinal tract are the front line of defense against the diverse population of commensal and potentially pathogenic microbes that thrive within the lumen of the human intestine. Although a major component of epithelial protection results from the physical exclusion of gut microbes, the defensive function of the intestinal epithelium is not limited to merely being a barrier. Rather, in response to pathogens, such as the gastroenteritis-causing pathogen *Salmonella typhimurium*, the intestinal epithelium activates the transcription of a panel of genes whose overall function appears to be the recruitment of immune inflammatory cells (13). Such a mucosal inflammatory response, which is the primary histopathologic characteristic of human *Salmonellosis* (26), generally results in clearance of the infection, thus avoiding the potential hazards of widespread bacterial dissemination in the host. The quintessential epithelial proinflammatory gene that drives mucosal inflammation is the chemokine IL-8, which serves to recruit neutrophils to the intestinal mucosa (15, 16).

We recently demonstrated (6, 9) that the primary means by which epithelial cells activate IL-8 expression in response to *S. typhimurium* is by the activation of epithelial toll-like receptor 5 (TLR5) via *S. typhimurium* flagellin monomers, which comprise flagella. Importantly, in T84 and Madin-Darby canine kidney model epithelia, expression of TLR5 is restricted to the basolateral membrane (6, 23), thus suggesting that TLR5-mediated proinflammatory gene expression may occur only on breach of the epithelium by bacteria or their products. TLR5 is one of 10 currently identified mammalian TLRs, which in general are thought to play a key role in innate immunity via the detection of conserved molecular patterns in microorganisms. In general, the 10 TLRs share much greater homology in their intracellular domains (toll-like IL-1 receptor domain) thought to be responsible for signaling than in their extracellular regions that are thought to function in ligand recognition (21). As such, there appears to be a considerable amount of conservation in the signal transduction pathways activated by different TLRs and, subsequently, in the changes in gene expression induced by the various members of this family. However, there are also some clear examples in differences in the signal transduction pathways used by different TLRs resulting in activation of distinct genes (27). Thus the signal transduction pathways need to be defined for each TLR and perhaps for different cell types.

Like other described TLRs, TLR5 utilizes the adaptor protein MyD88 and IL-1 receptor-associated kinase (IRAK) to activate a signal transduction cascade that

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results in the activation of the transcription factor NF-κB necessary for flagellin-induced effects on gene expression (6, 20). Although NF-κB family members play a diverse role in immune regulation and development and thus can be activated by various classes of stimuli in some systems (2), this transcription factor's central role in intestinal epithelia is known to be primarily in activation of proinflammatory gene expression (4). TLR2, -4, and -9 have also been demonstrated to activate members of the MAPK signaling cascade, particularly ERK1 and -2 and p38MAPK (21). In some experimental systems, these MAPK proteins have been shown to lead to activation of NF-κB, whereas in others, p38 MAPK has been shown to potentiate proinflammatory gene expression via posttranscriptional effects (14). The goal of this study was to examine the role of MAPK activation in flagellin-induced TLR5-mediated proinflammatory gene expression focusing primarily on the chemokine IL-8. We utilized both polarized model intestinal epithelia expressing endogenous TLR5 as well as MDCK and HeLa cells engineered to express TLR5, allowing both physiologic and mechanistic assessments of this signaling pathway. We observed that, whereas TLR5 activates both p38 and ERK1 and -2, p38 activation is more restricted to proinflammatory signaling. Furthermore, we observed that such p38 MAPK activation is not involved in TLR5-mediated NF-κB activation but rather regulates IL-8 expression by a posttranscriptional mechanism analogous to that described for p38 regulation of TNF-α expression (14).

MATERIALS AND METHODS

Materials. Antibodies to p38, phospho-p38, phospho-ERK1, and -2, and phospho-c-Jun NH_2-terminal kinase (phospho-JNK) were purchased from Cell Signaling Technology (Beverly, MA). P38, and ERK1 and -2, inhibitors SB-203580 and U-0126, respectively, were from Biomol (Plymouth Meeting, PA). Positive controls for phospho-JNK antibodies were the cell lysates of stimulated astrocytes that Cell Signaling Technology sells for this purpose. JNK inhibitor I was purchased from Calbiochem. Anti-asialo GM1 (anti-ASGM1) was purchased from Wako Scientific (Osaka). Generation of aflagellate *S. typhimurium fliC/fljB* (also referred to as $fliC^{-}/flB^{-}$) from its wild-type parent SL3201 was previously described (25). Top10 *E. coli*, a common cloning strain derivative of *E. coli* Dh5α was purchased from Invitrogen (Carlsbad, CA). Flagellin was purified from *S. typhimurium*-conditioned media by anion/cation exchange chromatography and purity verified as previously described (9). Briefly, such flagellin does not activate any TLR other than TLR5 (6) and has <50 pg/ml LPS (18). Plasmid encoding V5-tagged TLR5 was a gift of Jongdae Lee (Scripps Institute, La Jolla, CA), and DN-TLR5 was made from this construct as previously described (6). All other reagents were purchased from Sigma (St. Louis, MO).

Model epithelia. Intestinal epithelial cell lines T84, HT-29cl19A, or CACO-2BBE were cultured on collagen-coated permeable supports as previously described (8). IL-8 secretion, activation of NF-κB-CAT reporter constructs, and IκB-α levels were measured via ELISA as previously described (5, 7).

MAPK assessments. Model epithelia were stimulated as described in figure legends, rinsed in cold HBSS, lysed (in PBS with 1% Triton X-100, 1 mM EDTA, 1 mM $NaVO_4$, 1 mM NaF) cleared by centrifugation (10 min at 5,000 *g* at 4°C), and assayed for phospho-p38, phospho-ERK, or total P38 by SDS-PAGE immunoblotting. Where indicated, densitometry was performed via scanning with Scion Image densitometry software (Scion, Frederick, MD).

IRAK. IRAK activity was assayed via in vitro kinase activity on the basis of the protocol by Croston et al. (3). Briefly, stimulated or control 5 cm^2 model epithelia were lysed in 250 μl 1% Triton-X-100, plus (in mM) 25 Tris·HCl, 150 NaCl, 1 DTT, 1 EDTA, 1 $NaVO_4$, 1 NaF, 10 $Na_4P_2O_7$, and complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Lysates were cleared via centrifugation (5,000 *g*; 10 min) and subsequently via overnight incubation with 100 μl protein A-Sepharose beads (Pierce Endogen, Rockford, IL). IRAK-1 was then immunoprecipitated via overnight incubation with 10 μg anti-IRAK Ab (Santa-Cruz Bioscience, Santa Cruz, CA) that had been preloaded onto 100 μl protein A-Sepharose beads followed by four washes in the above-described lysis buffer followed by a wash in (in mM) 50 HEPES, 10 NaCl, 1 DTT, 6 $MgCl^2$, and 1 $MnCl^2$. Immunoprecipitates were then incubated with 2 μg of myelin basic protein and 0.5 μl $ATP\gamma_{32}$ (New England Nuclear, Boston, MA). Phosphorylated major basic protein was then visualized/quantitated via SDS-PAGE autoradiography and Bio-Rad Gel Imaging System (Bio-Rad Laboratories, Hercules, CA).

Exogenous expression of TLR5. MDCK cell and HeLa cells were cultured in DMEM, supplemented with 10% FBS. For transient transfection, cells were seeded in 6-well plates a day before transfection at 30% confluency. Superfectin (Qiagen, Valencia, CA) was used to form DNA complexes, following manufacturer's instructions. For each well, 0.5 μg of wild-type plasmid combined with either 2 μg empty vector or 2 μg dominant-negative (DN)-TLR5 plasmid was mixed with Superfectin reagent. Empty plasmid was used as control. After 3 h incubation, normal medium was added for further incubation overnight. Transfected cells were incubated overnight with and experiments then performed within 18–24 h. For stable transfection, pEF6/V5-TLR5 was linearized by ScaI. Linearized plasmid DNA (5 μg) was added to MDCK cells in 100-mm culture dishes that were ~30% confluency with superfectin as transfection reagent. At 24 h after transfection, transfected cells were split at low density and cultured in DMEM supplemented with 5–10 mg/ml blasticidin (Invitrogen) until the appearance of foci. Foci were transferred to new a culture container and propagated. Expression of TLR5 was confirmed by Western blotting with V5 antibody (Invitrogen).

Real-time PCR. Total RNA was isolated from treated cells (TRIzol; Invitrogen) and then reverse transcribed (1.0 μg) from random hexamer primers by using Multiscribe Reverse Transcriptase (Applied Biosystems; Foster City, CA). Real-time quantitative PCR analysis was performed in triplicate on 1 μl of this reverse transcribed cDNA by using the SYBRgreen Real-Time PCR assay (Applied Biosystems, Foster City, CA). The PCR reactions and SYBRgreen detections were carried out in an iCycler iQ Real-Time Detection System (Bio-Rad) whose cycling conditions included preliminary incubations for 2 min at 50°C and for 10 min at 95°C, followed by 40 cycles of a 15-s denaturation at 95°C and a 1-min annealing/extension at 60°C. The 18S ribosomal RNA gene was used as an endogenous control allowing for cDNA amounts to be normalized. The primers for the IL-8 gene (forward: 5′-AAACCACCGGAAGGAACCAT-3′, reverse: 5′-GCCAGCTTGGAAGTCATGT-3′) were designed from the

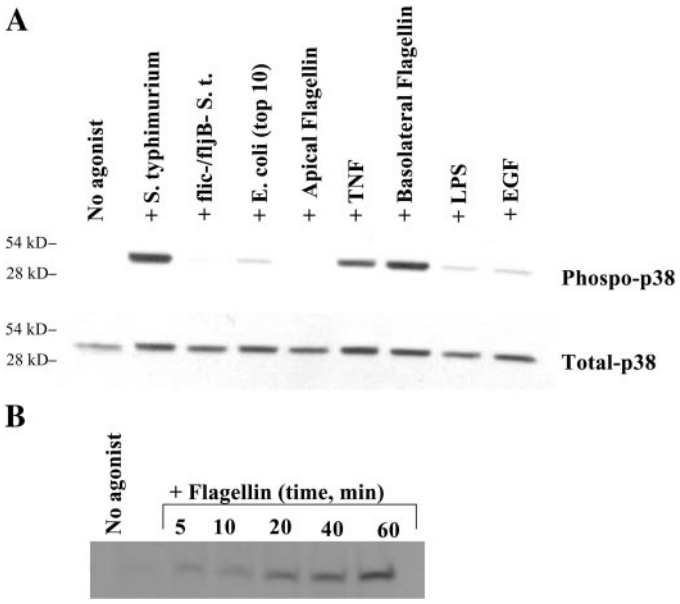


Fig. 1. Flagellin activates P38 MAPK in model intestinal epithelia. *A*: model epithelia were placed in HBSS and colonized apically with indicated bacteria or stimulated with flagellin (50 ng/ml), TNF (10 ng/ml), EGF (100 ng/ml), or LPS (10 μg/ml). Flagellin was applied to the indicated reservoir, whereas other agents were applied to both the apical and basolateral compartment. At 60 min later, p38 levels were assayed via immunoblotting. *B*: model epithelia were treated with 50 ng/ml flagellin for the indicated time. After stimulation, cells were lysed and immunoblotted for phospho-p38. Data are from a single experiment and representative of 5 experiments that showed an identical pattern of results.

primer design software PrimerExpress, whereas the primers for the 18S-rRNA gene were obtained from the TaqMan Ribosomal RNA Control Reagents Kit (Applied Biosystems, Foster City, CA). Real-time PCR analysis was performed on both treated and untreated cells to establish relative levels of mRNA expression. The level of IL-8 expression in individually treated and untreated samples was first normalized by subtracting the mean value of the cycle threshold (C_t) for the 18S-rRNA gene from that of the IL-8 gene (ΔC_t). Relative levels of IL-8 expression were then determined by *1*) subtracting the individual ΔC_t values for untreated control samples from those of the treated cells ($\Delta\Delta C_t$) and *2*) expressing the final quantitation value as $2^{-\Delta\Delta Ct}$. Standard deviation of this relative mRNA level for a single treatment: $2^{(\Delta\Delta C \pm \{SD[\Delta Ctreated]2 + SD[\Delta Cuntreated]2\})}$. As variance of replicate analysis of each sample was far less than the variance of values from separate experiments, data are displayed as the means ± SE of three separate experiments.

Il-8 mRNA stability. Model epithelia were treated as indicated and subsequently treated with actinomycin D (5 μg/ml) or vehicle (0.1% DMSO) to stop further transcription. At various times afterward, IL-8 mRNA was quantitated via hybridization-based assay purchased from R&D Systems (Minneapolis, MN) following the manufacturer's instructions.

RESULTS

Activation of p38 MAPK in response to flagellin. Whereas it is well established that the transcription factor NF-κB is a major regulator of proinflammatory gene expression induced by flagellin and many other proinflammatory agonists, it has become apparent that MAPKs can also play an important role in regulating proinflammatory gene expression. To begin to examine the role of MAPKs in flagellin-induced proinflammatory gene expression, we first examined which of the well-defined MAPK members were activated by exposure to purified flagellin in polarized model intestinal epithelia. Activation levels of p38 MAPK, ERK1 and -2 and JNK were assessed via measuring their phosphorylation states via immunoblotting because this is a well-defined indicator of their level of enzymatic activity. We observed that either apical colonization by live *S. typhimurium*, which expressed flagellin, or the basolateral addition of purified flagellin both potently activated p38 MAPK, whereas colonization by a mutant strain that lacked genes for flagellin (*fliC/fljB*) did not (Fig. 1). The level of p38 activation induced by purified flagellin appeared similar to that induced by the potent proinflammatory cytokine TNF-α, whereas, in contrast, neither the growth factor EGF nor *Salmonella* LPS, which like aflagellate *S. typhimurium* does not induce IL-8 secretion (7, 9), did not activate this kinase. Analogous to what we have previously demonstrated for IL-8 secretion (6), flagellin added basolaterally but not apically, activated this kinase. The time course of flagellin-induced p38 activation was consistently biphasic with slight, but reproducibly detectable, activation at 5 min and a much more pronounced activation by 45 min and persisting for at least 2 h. In contrast, although the proinflammatory stimuli *S. ty-*

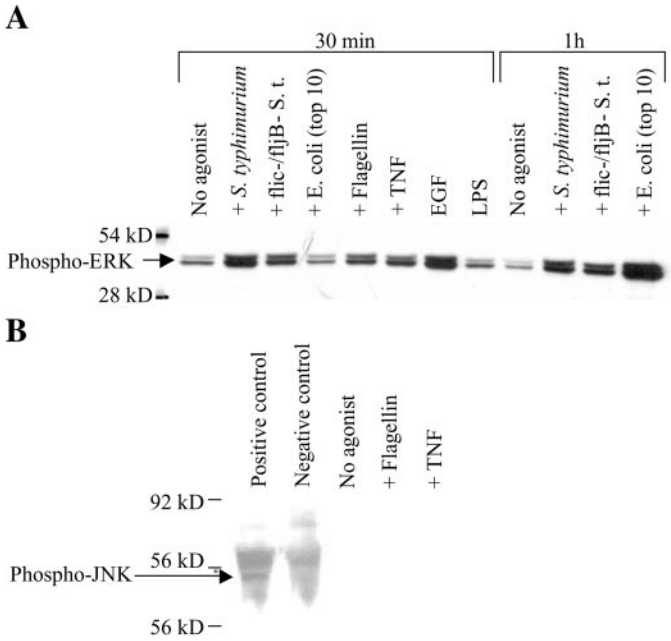


Fig. 2. Flagellin and nonproinflammatory stimuli activate ERK1 and -2. Model epithelia were treated as described in Fig. 1 and immunoblotted for phospho-ERK (*top*) or phospho-c-Jun NH_2-terminal kinase (JNK) (bottom). Positive and negative controls for JNK antibody were purchased from the manufacturer of the antibody. Data are from a single experiment and representative of 5 experiments that showed an identical pattern of results.

phimurium, purified flagellin, and TNF-α all induced activation of ERK1 and -2 (Fig. 2), activation of this kinase was not restricted to proinflammatory stimuli, because ERK1 and -2 were activated at least as well by EGF, aflagellate *S. typhimurium*, or *E. coli* although the aflagellate bacteria appeared to act somewhat slower. Neither flagellin nor TNF-α induced detectable activation of JNK consistent with our observation detailed below that JNK inhibition does not block IL-8 production, thus suggesting this kinase is not involved in mediating responses to such proinflammatory agonists in these cells. Lastly, we observed that the same pattern of MAPK activation was observed in multiple intestinal epithelial cell lines (HT29 and CaCo-2), indicating this pattern is a property of these stimuli's actions on intestinal epithelial cells in general as opposed to one cell line in particular (data not shown). Thus, whereas both ERK and p38 are activated in epithelia by flagellin, p38 activation was more closely associated with the proinflammatory responses induced by flagellin.

Flagellin-induced p38 MAPK activation mediated by TLR5/IRAK. Two different molecules have been reported to function as receptors for flagellin, namely TLR5 and the ganglioside ASGM1 (6, 10, 17). Thus we then sought to determine what aspect of flagellin-induced p38 activation could be accounted for by flagellin activation of TLR5. We generated MDCK cells that stably express TLR5 and compared their p38 activation to control MDCK transformed with antibiotic resistance gene only (Fig. 3*A*). MDCK express some endogenous TLR5 and thus, as expected, exhibited detectable p38 activation in response to flagellin. However, the magnitude of the response was much greater in MDCK expressing TLR5 indicating flagellin-induced p38 MAPK activation was, at least in part, directly attributable to TLR5. We then sought to use our previously described DN-TLR5 construct to investigate whether functional TLR5 signaling is necessary for flagellin-induced p38 MAPK activation. First, we attempted to generate MDCK cells that stably expressed DN-TLR5. However, cells that survived initial drug selection (indicating they expressed DN-TLR5) consistently died a couple of days later suggesting that DN-TLR5 may interfere with a necessary growth/survival signaling pathway in these cells. Thus we utilized transient transfection to verify the role of TLR5 in mediating flagellin-induced p38 activation. Specifically, we utilized HeLa cells that allow high transfection rates and do not seem to have a functional endogenous TLR5 signaling pathway. HeLa cells were transfected with either control vector [red fluorescence protein (RFP) to monitor transfection efficiency], TLR5 alone, or TLR5 and DN-TLR5. Counting of fluorescent cells indicated a transfection efficiency of 55–60%. RFP transfectants exhibited enhanced p38 phosphorylation in response to TNF but not flagellin, whereas either agonist activated p38 in cells expressing TLR5 (Fig. 3*B*). Such TLR5 conferment of p38 activation in response to flagellin was blocked by DN-TLR5, whereas TNF-induced p38 activation (negative control) was unaffected, confirming the role of TLR5 in flagellin-induced MAPK activation.

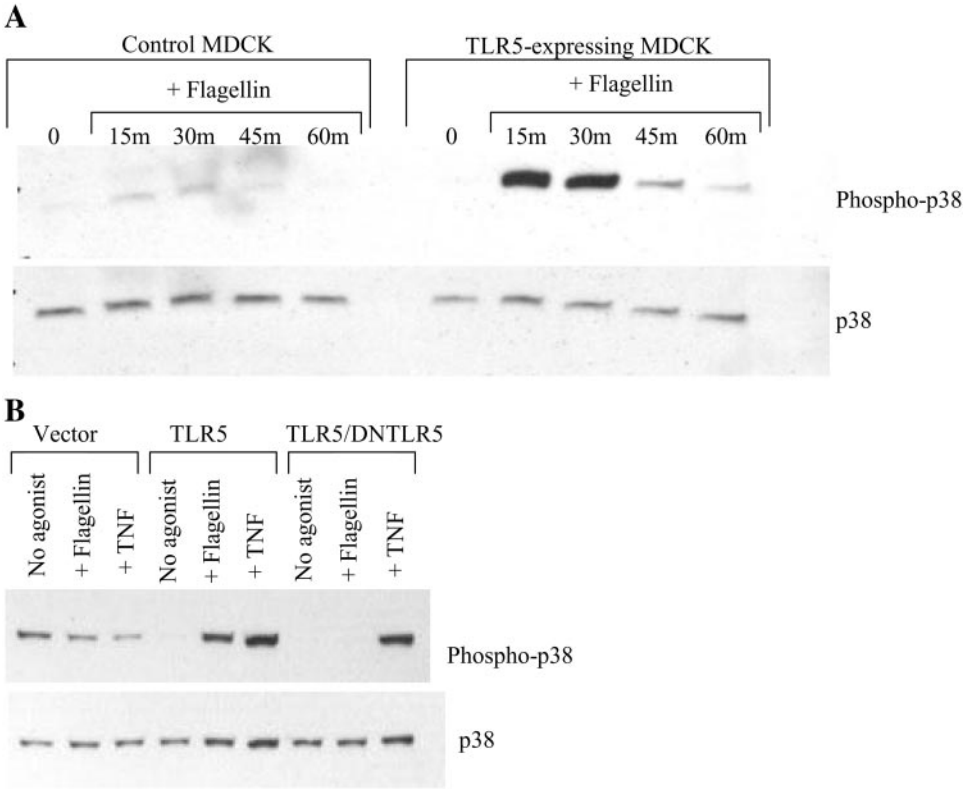


Fig. 3. Expression of TLR5 confers flagellin-induced MAPK activation. *A*: control Madin-Darby canine kidney (MDCK) cells or MDCK cells stably transfected with toll-like receptor-5 (TLR5) were stimulated with flagellin (50 ng/ml) for the indicated time and lysed, and p38 MAPK activation was measured via immunoblotting. *B*: HeLa cells were transiently transfected with vector-only TLR5 or TLR5/dominant-negative (DN)-TLR5. At 18 h later, cells were stimulated with flagellin (1,000 ng/ml) or TNF-α (100 ng/ml), and p38 activation was assayed 30 min later. Data are from single experiments and representative of 3 experiments that showed an identical pattern of results.

Fig. 4. T84 model epithelia do not exhibit p38 activation in response to asialo GM1 (ASGM1) ligation. T84 model epithelia were treated apically or basolaterally with flagellin (1 μg/ml) or rabbit anti-ASGM (10 μg/ml) for 1 h. Cells were washed thoroughly with HBSS and then lysed, and p38 activation was assayed via immunoblotting. Immunoblotting with a murine antibody detects only phospho-p38, whereas immunoblotting with a polyclonal antibody detects both phospho-p38 and the ASGM1 antibody that remained bound to the cells. Data are from a single experiment and representative of many experiments that showed an identical pattern of results.

Flagellin activation of ASGM1 can be simulated by ligation of ASGM1 by use of a commercially available anti-ASGM1 antibody (22). Because ASGM1 can be on either the apical or basolateral membrane, we investigated whether we might ligate this molecule and activate p38 with either flagellin or anti-ASGM1. Although very high concentrations of apical, or of course basolateral, activated p38 (albeit modest activation for apical flagellin), neither apical nor basolateral anti-ASGM1 (even when used at high concentrations) activated p38 (Fig. 4) although immunoblotting with the rabbit antibody, and subsequent detection by an anti-rabbit secondary antibody, revealed that such anti-ASGM1 had bound both the apical or basolateral surface with sufficient avidity to resist extensive washing. These results indicate that flagellin's activation of p38 MAPK in such model intestinal epithelia is indeed mediated by activation TLR5 rather than ASGM1.

We then sought to investigate the relationship between p38 MAPK activation and other signals generated by interaction of TLR5 and flagellin. First, we examined the relationship between P38 MAPK and IRAK-1, a kinase associated with TLR signaling in general (11) and that has been shown to be activated by TLR5 in monocytic cells (20). We observed that, indeed, IRAK-1 is also activated in response to flagellin in polarized epithelial cells as measured by an in vitro kinase assay (Fig. 5). IRAK-1 activation is generally thought to be a more proximal signal than MAPK activation. Consistent with this notion, the time course of IRAK activation appeared fairly similar to that of p38 activation with modest activation after 5 min and more robust activation by 1 h. Furthermore, activation of IRAK-1 was not affected by the p38 inhibitor SB-203580 (average inhibition was <5% in 2 experiments) suggesting that activation of IRAK-1 is not dependent on p38 MAPK activation.

p38 regulates IL-8 expression via a posttranscriptional mechanism. We then considered the consequences of MAPK activation on the epithelial expression of the proinflammatory chemokine IL-8. We observed that the well-characterized p38 inhibitor SB-203580 exhibited a concentration-dependent inhibition of flagellin-induced IL-8 secretion (Fig. 6). A similar level of inhibition was observed for IL-8 secretion induced by TNF-α, which, like flagellin, induces activation of p38 MAPK.

ERK1 and -2 inhibitor U-0126 also blocked flagellin-induced IL-8 secretion suggesting that, although not specific to proinflammatory stimuli, the ERK signaling pathway is nonetheless required for such induced proinflammatory gene expression. Consistent with the

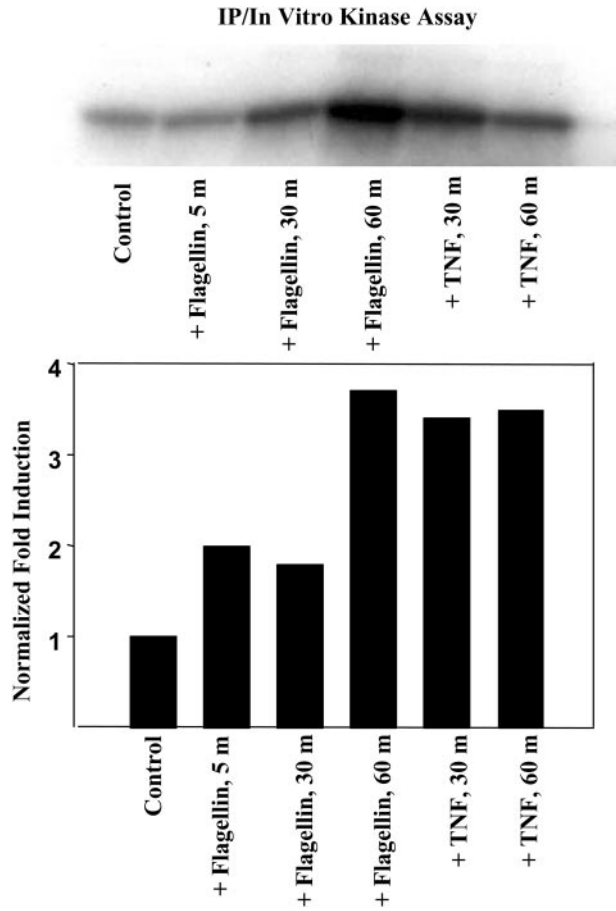


Fig. 5. Flagellin-induces IL-1 receptor-associated kinase (IRAK) activation in model epthelia. Model epithelia were stimulated basolaterally with 50 ng/ml flagellin. At the indicated time point, cells were permeabilized and IRAK activation was measured by immunoprecipitation (IP)/in vitro kinase assay as described in MATERIALS AND METHODS. Bar graph is the fold activation of kinase activity normalized to the level of IRAK in the immunoprecipitates as assessed by immunoblot/densitometry. Data are from a single experiment and representative of 3 experiments that showed similar results.

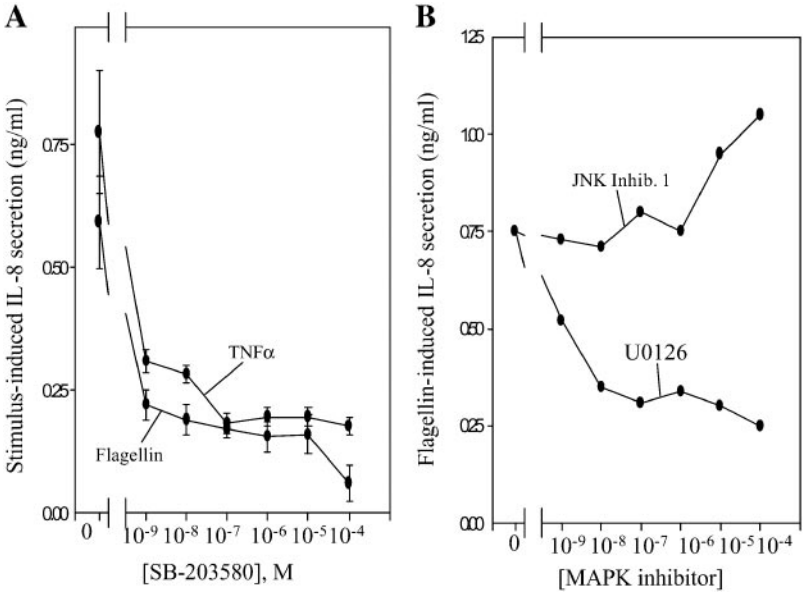
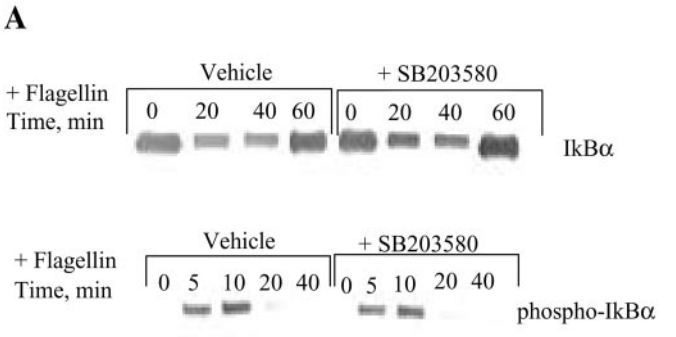


Fig. 6. Inhibition of p38 MAPK blocks IL-8 secretion. *A*: model epithelia were stimulated with 50 ng/ml flagellin or TNF-α in the presence of indicated concentration of p38 inhibitor SB-203580, whereas maintaining constant vehicle concentrations (0.01% DMSO). *B*: model epithelia were stimulated with 50 ng/ml flagellin in the presence of indicated concentration of ERK inhibitor (U-0126) or JNK inhibitor (JNK Inhibitor 1) while maintaining constant vehicle concentrations (0.01% DMSO). At 5 h after stimulation, basolateral media were assayed for IL-8 via ELISA. Data in *A* are means ± SE of 3 parallel experiments, whereas those in *B* are the means of a representative experiment performed in duplicate.

failure of flagellin to induce JNK phosphorylation, JNK inhibition did not reduce flagellin-induced IL-8 expression indicating this kinase's activity is not crucial in this signaling pathway. We then considered possible mechanisms whereby blockade of MAPK might so attenuate IL-8 expression focusing on p38 MAPK, the MAPK most specific to proinflammatory stimuli. In light of a number of reports that MAPKs regulate the NF-κB pathway and the major role of this transcription factor in regulating IL-8 transcription, we measured whether p38 MAPK inhibition affected activation of the NF-κB pathway in model epithelia. As such, epithelia are very difficult to transfect, the activation of the NF-κB pathway is best assessed in such epithelia by examining the signaling events that immediately precede and regulate the activation of this proinflammatory transcription factor, namely the phosphorylation and degradation of IκB-α. We observed that the concentration of SB-203580 (10 μM) that maximally inhibited IL-8 secretion had no effect on these signaling events that mediate NF-κB activation (Fig. 7). Furthermore, by using HeLa cells transiently transfected with TLR5 and a synthetic NF-κB reporter gene construct whose activity is regulated solely by this transcription factor, we observed that neither the NF-κB activation induced by TNF-α nor by flagellin in TLR5 transfected cells was significantly inhibited by SB-203580. These results indicate that inhibition of p38 MAPK blocks IL-8 expression independent of effects on NF-κB activation.

Activation of p38 MAPK has been shown to increase both the translation rate and mRNA stability of genes such as TNF-α, which contain a 5′ AU-rich element (ARE) (1, 12, 14). Because the IL-8 gene also contains a 5′ARE, we investigated whether p38 inhibition suppressed IL-8 expression via affecting levels of IL-8 mRNA level via quantitative real-time PCR. IL-8 mRNA levels are very low in unstimulated epithelia but increased ~150-fold 90 min after the addition of purified basolateral flagellin or TNF-α. By 3 h after such stimulation, IL-8 mRNA levels had moderately declined in the flagellin-treated cells but remained fully elevated in the TNF-α-treated cells. For neither stimulus at neither time point did we observe a decline in IL-8 mRNA levels significant enough to explain the reduction in IL-8 secretion in epithelia under p38 inhibition (Fig. 8). Because p38 inhibition had only a modest effect on the induction of IL-8mRNA, we then



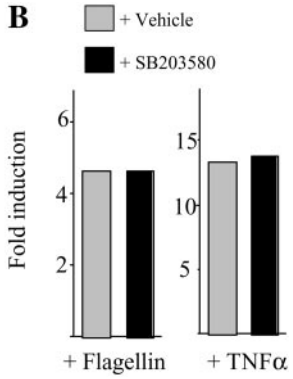


Fig. 7. Inhibition of p38 MAPK does not affect NF-κB activation. *A*: model epithelia were stimulated with 50 ng/ml flagellin in 10 μM SB-203580 or vehicle (0.01% DMSO) for indicated times at which whole cell lysates were obtained and analyzed for levels of IκB-α and phospho-IκB-α via immunobotting. *B*: HeLa cells were transiently transfected with TLR5 and NF-κB-CAT, stimulated with 50 ng/ml flagellin or TNF-α, and CAT activity was measured as described in MATERIALS AND METHODS. Data are from a single experiment and representative of 3 experiments that showed an identical pattern of results.

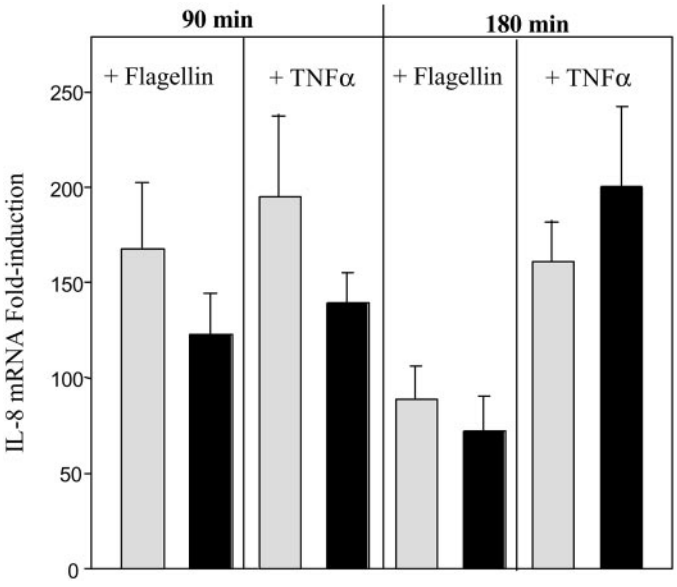


Fig. 8. Inhibition of p38 MAPK has only a modest affect on induction of IL-8 mRNA. Model epithelia were stimulated with either flagellin 50 ng/ml or TNF-α (10 ng/ml) for the indicated time in the presence of 10 μM SB-203580 or vehicle (0.01% DMSO). RNA was isolated and relative levels of IL-8 mRNA were quantitated as described in MATERIALS AND METHODS. Grey bars, treatment by vehicle; black bars, treatment by the p38 inhibitor. Data are means ± SE of 3 separate experiments assayed in triplicate.

examined the effect of p38 inhibition on Il-8 mRNA stability. Model epithelia were treated with flagellin in the presence or absence of SB-203580 for 90 min, treated with actinomycin D to shut off further transcription and IL-8 mRNA quantitated by a well-characterized commercial hybridization-based quantitative assay. We did not observe a difference in the relative decay rates of IL-8 transcript in such cells (Fig. 9) indicating that p38 inhibition was not significantly affecting IL-8 mRNA half-life. Lastly, we utilized trypan blue exclusion to assess whether p38 inhibition was simply toxic to these cells. SB-203580 did not have a substantial effect on trypan blue exclusion by T84 cells (cells showing positive trypan blue staining was 6 vs. 8% for control cells vs. those treated with 10 mM SB-203580 for 5 h, respectively). Together, these results indicate p38 inhibition likely suppresses TLR5-mediated IL-8 expression via reducing IL-8 translation analogous to observations made about genes such as TNF that also have a 5′ARE (14).

DISCUSSION

TLRs play a central role in innate immunity. Specifically, their detection of bacterial products and subsequent activation of antibacterial signaling pathways helps permit eukaryotic survival among the enormous prokaryotic biomass that surround them. Additionally, TLRs may also play a role in a number of chronic inflammatory diseases (24). Thus understanding the molecular mechanisms by which various TLRs signal is germane to understanding human health. This study investigated the mechanism by which TLR5 signals in intestinal epithelial cells in response to its only known ligand, flagellin. Specifically, we investigated the role of MAPKs in flagellin-induced proinflammatory gene expression focusing on the neutrophil chemoattractant IL-8. We measured activation of these kinases in physiologically relevant polarized model epithelia and observed robust activation of p38 MAPK in response to flagellin. Furthermore, we observed that this activation was directly attributable to flagellin's interaction with TLR5. Activation of p38 appeared to follow activation of IRAK consistent with the recent report that TLR5 physically associates with IRAK, suggesting its activation is likely a proximal event in the TLR5 signaling pathway (19). In support of a central role for IRAK in TLR5-mediated signaling, we also observed that DN-IRAK blocked activation of NF-κB (DN-IRAK blocked flagellin-induced NF-κB activation in TLR5-transfected HeLa cells by ~80%).

The ability of flagellin and various other agonists to activate p38 MAPK correlated with their ability to induce expression of IL-8. In contrast, we observed that these proinflammatory agonists as well as a cytokine agonist and aflagellate bacteria, which do not induce IL-8, all activated ERK. This observation is consistent with the notion that p38 MAPK is a "stress-induced kinase" (one if its previously accepted names), whereas ERK activation is a more general signal that may function in both growth factor and stress-induced signaling. By such a paradigm, ligation of basolateral intestinal epithelial TLR5 would indeed seem an appropriate stress signal, because it would indicate that the gut epithelium has been breached by enteric microbes. However, a growth factor-type signal might be an appropriate "all is well" signal in response to apical colonization by commensal microbes, thus providing a

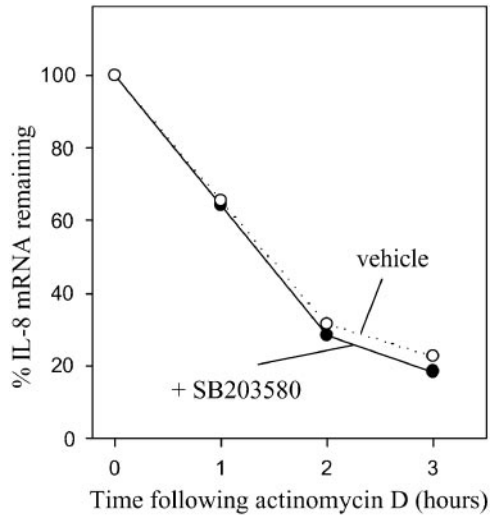


Fig. 9. Inhibition of p38 MAPK does not alter IL-8 mRNA half-life. Model epithelia were treated with 10 μM SB-203580 or vehicle (0.01% DMSO) and stimulated with flagellin for 90 min at which time a new transcription was stopped via the addition of actinomycin D. IL-8 mRNA levels were subsequently measured over the indicated time course as described in MATERIALS AND METHODS. Data are the means of a single experiment and representative of 3 experiments that showed an identical pattern of results.

rationale for why aflagellate and commensal bacteria might elicit only ERK activation. However, whereas ERK signaling is used broadly by many signaling pathways, it is likely still necessary for IL-8 expression because ERK inhibitors potently blocked IL-8 expression induced by flagellin or TNF.

Transcription factor NF-κB is known to play a major role in regulating the expression of many proinflammatory genes in general and IL-8 expression in model intestinal epithelia in particular. Whereas some studies have reported a role of MAPKs in activating NF-κB, particularly studies that overexpress these kinases, our results do not indicate such a role for the MAPK activity induced by flagellin or TNF-α. Specifically, we observed that inhibition of p38 activity suppresses production of IL-8 but yet did not affect NF-κB activation nor the signaling events immediately proximal to its activation. Furthermore, p38 inhibition appeared to have only modest effects on IL-8 mRNA levels. The modest effect of p38 inhibition of IL-8 mRNA induction could result through an effect of p38 on IL-8 mRNA stability as has been reported for TNF-α (1) and another AU-rich element containing transcripts or could result from effects of the p38 pathway on elements in the IL-8 promoter other than those responsive to NF-κB. In support of the latter possibility, reporter assays done with the native IL-8 promoter (instead of synthetic NF-κB construct) were moderately suppressed by p38 inhibition (by ~50%). In contrast to this modest suppression of IL-8 mRNA induction by p38 inhibition, we and others (7) have previously demonstrated that blockade of NF-κB activation concomitantly inhibits both IL-8 secretion and IL-8 mRNA accumulation. Thus our results indicate that, whereas NF-κB regulates IL-8 transcription, the p38 MAPK pathway acts downstream, and perhaps parallel, of this process. These observations are consistent with the recent report that activation of p38 is able to increase the efficiency of translation of genes that contain 3′ untranslated AU-rich elements (14).

Whereas we and others (5, 7) have previously observed a very strong correlation between levels of IL-8 secretion and IL-8 mRNA, it seems expedient for cells, particularly intestinal epithelial cells to exert an additional level of regulation over such a gene. While IL-8 plays an important role in recruiting neutrophils that clear mucosal infections, these neutrophils are also largely responsible for inducing the clinical manifestations of self-limiting, locally contained, infections such as human *Salmonellosis*. Thus intestinal epithelial cells would seek to recruit polymorphonuclear neutrophils as soon as possible once a pathogen is detected but also shut down continued neutrophil recruitment once the perturbing agonist is cleared. Having p38 MAPK regulate translation would seem to provide a means to boost translation rates and, conversely, shut off production more quickly than would be possible for a gene whose expression was controlled solely at the transcriptional level. Such rapid control would seem to prevent systemic infection and the uncontrolled inflammation that characterizes some chronic diseases.

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DISCLOSURES

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REFERENCES

1. **Brook M, Sully G, Clark AR, and Saklatvala J.** Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade. *FEBS Lett* 483: 57–61, 2000.
2. **Chen F, Castranova V, and Shi X.** New insights into the role of nuclear factor-kappaB in cell growth regulation. *Am J Pathol* 159: 387–397, 2001.
3. **Croston GE, Cao Z, and Goeddel DV.** NF-kappa B activation by interleukin-1 (IL-1) requires an IL-1 receptor-associated protein kinase activity. *J Biol Chem* 270: 16514–16517, 1995.
4. **Elewaut D, DiDonato JA, Kim JM, Truong F, Eckmann L, and Kagnoff MF.** NF-kappa B is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria. *J Immunol* 163: 1457–1466, 1999.
5. **Gewirtz AT, McCormick B, Neish AS, Petasis NA, Gronert K, Serhan CN, and Madara JL.** Pathogen-induced chemokine secretion from model intestinal epithelium is inhibited by lipoxin A4 analogs. *J Clin Invest* 101: 1860–1869, 1998.
6. **Gewirtz AT, Navas TA, Lyons S, Godowski PJ, and Madara JL.** Cutting edge: bacterial flagellin activates basolaterally expressed tlr5 to induce epithelial proinflammatory gene expression. *J Immunol* 167: 1882–1885, 2001.
7. **Gewirtz AT, Rao AS, Simon PO Jr, Merlin D, Carnes D, Madara JL, and Neish AS.** *Salmonella typhimurium* induces epithelial IL-8 expression via Ca^{2+}-mediated activation of the NF-kappaB pathway. *J Clin Invest* 105: 79–92, 2000.
8. **Gewirtz AT, Reed KA, Merlin D, Neish AS, and Madara JL.** Modeling microbial-epithelial interactions in the intestine. *Methods Microbiol* 31: 377–396, 2002.
9. **Gewirtz AT, Simon PO Jr, Schmitt CK, Taylor LJ, Hagedorn CH, O'Brien AD, Neish AS, and Madara JL.** *Salmonella typhimurium* translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J Clin Invest* 107: 99–109, 2001.
10. **Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, and Aderem A.** The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410: 1099–1103, 2001.
11. **Jacinto R, Hartung T, McCall C, and Li L.** Lipopolysaccharide- and lipoteichoic acid-induced tolerance and cross-tolerance: distinct alterations in IL-1 receptor-associated kinase. *J Immunol* 168: 6136–6141, 2002.
12. **Jijon HB, Panenka WJ, Madsen KL, and Parsons HG.** MAP kinases contribute to IL-8 secretion by intestinal epithelial cells via a posttranscriptional mechanism. *Am J Physiol Cell Physiol* 283: C31–C41, 2002.
13. **Jung HC, Eckmann L, Yang SK, Panja A, Fierer J, Morzycka-Wroblewska E, and Kagnoff MF.** A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J Clin Invest* 95: 55–65, 1995.
14. **Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, and Kollias G.** Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* 10: 387–398, 1999.
15. **McCormick B, Hofman P, Kim J, Carnes D, Miller S, and Madara J.** Surface attachment of *Salmonella typhimurium* to intestinal epithelia imprints the subepithelial matrix with gradients chemotactic for neutrophils. *J Cell Biol* 131: 1599–1608, 1995.

16. **McCormick BA, Colgan SP, Archer CD, Miller SI, and Madara JL.** Salmonella typhimurium attachment to human intestinal epithelial monolayers: transcellular signalling to subepithelial neutrophils. *J Cell Biol* 123: 895–907, 1993.
17. **McNamara N, Khong A, McKemy D, Caterina M, Boyer J, Julius D, and Basbaum C.** ATP transduces signals from ASGM1, a glycolipid that functions as a bacterial receptor. *Proc Natl Acad Sci USA* 98: 9086–9091, 2001.
18. **McSorley SJ, Ehst BD, Yu Y, and Gewirtz AT.** Bacterial flagellin is an effective adjuvant for $CD4^+$ T cells in vivo. *J Immunol* 169: 3914–3919, 2002.
19. **Mizel SB and Snipes JA.** Gram-negative flagellin-induced self-tolerance is associated with a block in interleukin-1 receptor-associated kinase release from toll-like receptor 5. *J Biol Chem* 277: 22414–22420, 2002.
20. **Moors MA, Li L, and Mizel SB.** Activation of interleukin-1 receptor-associated kinase by gram-negative flagellin. *Infect Immun* 69: 4424–4429, 2001.
21. **O'Neill LA.** The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci STKE* 2000: RE1, 2000.
22. **Ratner AJ, Bryan R, Weber A, Nguyen S, Barnes D, Pitt A, Gelber S, Cheung A, and Prince A.** Cystic fibrosis pathogens activate Ca^{2+}-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. *J Biol Chem* 276: 19267–19275, 2001.
23. **Reed KA, Hobert ME, Kolenda CE, Sands KA, Rathman M, O'Connor M, Lyons S, Gewirtz AT, Sansonetti PJ, and Madara JL.** The *Salmonella typhimurium* flagellar basal body protein FliE is required for flagellin production and to induce a proinflammatory response in epithelial cells. *J Biol Chem* 277: 13346–13353, 2002.
24. **Sabroe I, Parker LC, Wilson AG, Whyte MK, and Dower SK.** Toll-like receptors: their role in allergy and non-allergic inflammatory disease. *Clin Exp Allergy* 32: 984–989, 2002.
25. **Schmitt CK, Darnell SC, and O'Brien AD.** The attenuated phenotype of a *Salmonella typhimurium* flgM mutant is related to expression of FliC flagellin. *J Bacteriol* 178: 2911–2915, 1996.
26. **Takeuchi A.** Electron microscope studies of experimental *Salmonella* infection. *Am J Pathol* 50: 109–119, 1967.
27. **Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S, Williams BR, Major J, Hamilton TA, Fenton MJ, and Vogel SN.** TLR4, but not TLR2, mediates IFN-β-induced STAT1α/β-dependent gene expression in macrophages. *Nat Immun* 3: 392–398, 2002.