The Journal of Immunology

RESEARCH ARTICLE | MAY 15 2006

TLR5-Mediated Phosphoinositide 3-Kinase Activation Negatively Regulates Flagellin-Induced Proinflammatory Gene Expression[1] FREE

Yimin Yu; ... et. al

J Immunol (2006) 176 (10): 6194–6201.
https://doi.org/10.4049/jimmunol.176.10.6194

TLR5-Mediated Phosphoinositide 3-Kinase Activation Negatively Regulates Flagellin-Induced Proinflammatory Gene Expression[1]

Yimin Yu,* Shigenori Nagai,[†‡] Huixia Wu,* Andrew S. Neish,* Shigeo Koyasu,[†‡] and Andrew T. Gewirtz[2]*

Epithelial cells detect motile pathogens via TLR5 ligation of flagellin, resulting in rapid induction of antibacterial/proinflammatory gene expression. Although such flagellin-induced gene expression is quite transient, likely to avoid the negative consequences of inflammation, little is known regarding the molecular mechanisms that mediate its shutdown. We hypothesized that, analogous to the case for TLR4, phosphoinositide 3-kinase (PI3K) might negatively regulate TLR5 signaling. However, because PI3K is an essential positive mediator of some pathways of TLR-mediated gene expression, the opposite hypothesis was also considered. Herein, we observed that flagellin stimulation of epithelial cells indeed induced rapid (<30 min) PI3K activation, as evidenced by Akt phosphorylation, via a TLR5-mediated mechanism. Blockade of PI3K with wortmannin resulted in marked enhancement of flagellin-induced gene expression as assessed by measuring levels of inducible NO synthase, IL-6, and IL-8. Such enhancement of gene expression by PI3K inhibition correlated with prolonged activation of MAPK (p38 and ERK1/2) and was ablated under MAPK inhibition. Such effect of inhibiting PI3K with wortmannin was mimicked by the PI3K inhibitor LY294002, and, conversely, a constitutively active PI3K prevented p38 activation in response to flagellin. Last, to test the significance of these results in vivo, we measured flagellin-induced gene expression in PI3K knockout mice. PI3K-null mice displayed increased levels of flagellin-induced serum IL-6, KC (IL-8 homolog), and nitrite as compared with heterozygous littermates. Thus, TLR5's rapid activation of PI3K serves to limit MAPK signaling, thus limiting proinflammatory gene expression and reducing the potential negative consequences of proinflammatory gene expression. *The Journal of Immunology,* **2006, 176: 6194–6201.**

Toll like receptor-mediated gene expression plays a central role in host defense. For this reason, extensive effort has been placed on defining the molecular mechanisms by which TLRs signal. In large part, such efforts have focused in dissecting the general and specific signaling mechanisms by which each TLR activates a somewhat specific pattern of gene expression (1). We and others have contributed to such efforts by examining the signaling mechanisms by which TLR5, in response to its only known ligand flagellin, activates proinflammatory gene expression in intestinal epithelial cells, which under normal conditions have not been widely observed to exhibit proinflammatory gene expression in response to any TLR ligands other than flagellin (2). Such epithelial TLR5 signaling shares some of the generalities of TLR signaling defined in hemopoietic cells in that it relies upon MyD88-mediated activation of the NF-κB and MAPK signaling pathways (3–5). Like TLR4, but in contrast to TLR2, TLR5 also activates the JAK/STAT pathway by a protein synthesis-dependent pathway but, in doing so, uses different autocrine mediators than does TLR4 (6, 7). Like TLR7 and -9, but unlike TLR2 and -4, TLR5 signaling is independent of Toll-IL-1R domain-containing adapter protein (8). Thus, despite some shared mechanisms of TLR signaling, there are key differences in the specific mechanisms by which TLRs induce changes in gene expression.

In light of the potential danger posed by excessive proinflammatory signaling, there has recently been increased attention on defining mechanisms that turn off TLR signaling. TLR5 in particular would seem to have substantial potential to signal excessively in light of the large load of commensal flagellated bacteria in the gut and the expression of TLR5 on gut epithelial cells. Like proinflammatory gene expression induced by cytokines, there are "classic" methods of protein synthesis-dependent feedback inhibition in that some gene products of TLR signaling, namely IκBα and suppressor of cytokine signaling (SOCS),[3] attenuate transcription of proinflammatory gene expression via, respectively, the NF-κB and JAK/STAT pathways (9). Another mechanism of attenuating TLR-mediated proinflammatory gene expression uses IL-1R-associated kinase M, which is induced, activated, and negatively regulates TLR4 signaling in hemopoietic cells (10). An additional mechanism proposed to attenuate TLR4 signaling is via the activation of phosphoinositide 3-kinase (PI3K) in that genetic deletion of this lipid kinase, and in some studies pharmacologic blockade, results in enhanced responses to LPS (11, 12). However, PI3K has also been reported to play a critical role in activating cytokine expression mediated by TLR2 (13), TLR3 (14), and TLR9 (15). Thus, given PI3K's central, albeit varied and as yet unclear role in TLR

*Epithelial Pathobiology Division, Department of Pathology, Emory University School of Medicine, Atlanta, GA 30322; [†]Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan; and [‡]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi, Japan

Received for publication September 13, 2005. Accepted for publication February 21, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the National Institutes of Health via a center grant (R24 DK064399) and R-01 Grants DK061417 (to A.T.G.) and RO1AI51282 (to A.S.N.).

[2] Address correspondence and reprint requests to Andrew T. Gewirtz, Epithelial Pathobiology Division, Department of Pathology, Emory University School of Medicine, 105H WRB, 615 Michael Street, Atlanta, GA 30322. E-mail address: agewirt@emory.edu

[3] Abbreviations used in this paper: SOCS, suppressor of cytokine signaling; PI3K, phosphoinositide 3-kinase; CAp85, constitutive active p85 mutant; DN, dominant negative; iNOS, inducible NO synthase; MKK, MAPK kinase.

0022-1767/06/$02.00

signaling, the vital role played by epithelial TLR5 signaling in host defense, and the potential of TLR5 to drive mucosal inflammation in response to commensal microbes in inflammatory bowel disease, we sought to define the role of PI3K in regulating flagellin-induced gene expression in intestinal epithelial cells. We observed that activation of PI3K functions to limit TLR5-mediated proinflammatory gene expression via suppressing flagellin-induced MAPK activation.

Materials and Methods

Cell culture

Human colon epithelial cell line T84 was cultured in DMEM/F12 medium supplemented with 5% FBS as previously described (16). Generation/maintenance of HeLa cells expressing wild-type or dominant mutant TLR5 was performed as described previously (5).

Reagents

Flagellin was purified from *Salmonella typhimurium* and purity verified as previously described (17, 18). Anti-phospho-p38, phospho-ERK, phospho-Akt, phospho-IκBα, phospho-MKK3/6 and anti-p38 Abs were purchased from Cell Signaling. Anti iNOS Ab was purchased from R&D Systems. Anti-IκBα Ab was purchased from Santa Cruz. Kinase inhibitors SB203580, PD98059, LY294002, and wortmannin were purchased from Calbiochem.

Plasmids and transfection

Constitutive active p85 mutant (CAp85)-expressing vector was generously provided by Dr. J. Cheng (Emory University, Atlanta, GA). HA-tagged p38 expression vector was kindly provided by Dr. X. Wang (Emory University, Atlanta, GA). Transient transfection was performed using Lipofectamine 2000 (Invitrogen). The ratio of HA-tagged p38 to CAp85 was 1:2 in co-transfection. PCDNA4.1 (Invitrogen) was used as empty vector (control). Transfection was performed according to the manufacturer's protocol. T84 cells were seeded 1 day before transfection at a density of 50% confluence. Flagellin stimulation was performed 24 h after transfection.

Western blotting

Cells were lysed in ice-cold lysis buffer (RIPA supplemented with protease inhibitor mixture (Roche)). Lysates were cleared by centrifugation and proteins were separated in SDS-PAGE and transferred to nitrocellulose membranes. After being blocked with PBS containing 0.1% Tween 20 and 5% milk, membranes were blotted to specific Abs. For repeated blotting, membranes were stripped in 0.2 M NaOH.

PI3K translocation (19)

Epithelial cells (15×10^6 cells per condition, pooling three wells of a six-well tissue culture plate) were treated with flagellin for 0–30 min, at which time cells were washed in PBS, scraped with a rubber policeman, and homogenized with a glass/Teflon homogenizer in ice-cold buffer containing 250 mM sucrose, 10 mM Tris (pH 7.5), 1 mM PMSF, 1 μg/ml leupeptin and pepstatin. The disrupted cell suspension was then centrifuged at $800 \times g$ for 10 min at 4°C. The supernatant was then subjected to centrifugation at $17{,}000 \times g$ for 45 min. Plasma membrane-containing pellets were resuspended in PBS, analyzed for protein concentration (using kit from Pierce), and subsequently diluted so that all samples had equal protein concentration. Samples were subsequently diluted 1/2 in SDS-PAGE loading buffer and subjected to immunoblot analysis using mAbs to the p85a subunit of PI3K (Santa Cruz) and Na^+/K^+ ATPase (Upstate Biotechnology) or anti-flagellin.

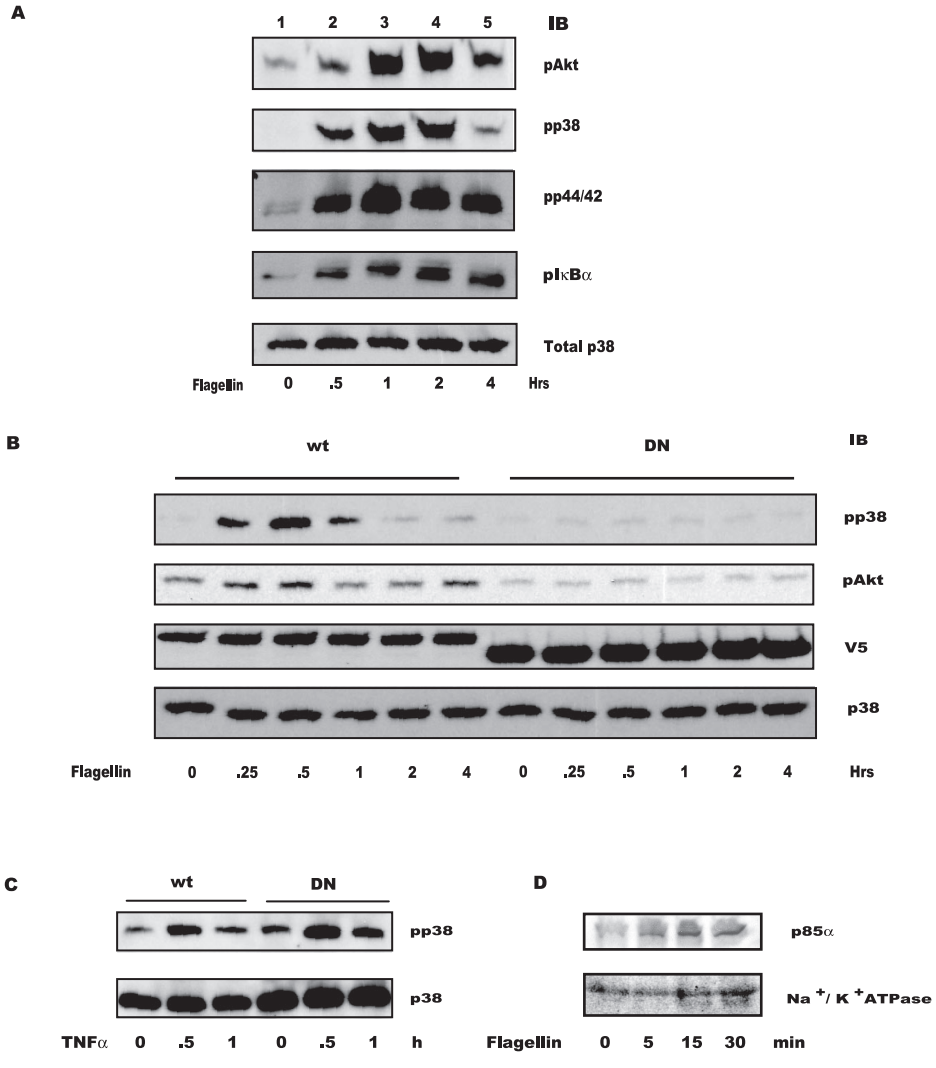

FIGURE 1. Flagellin activates PI3K via a TLR5-mediated mechanism. T84 cells (*A* and *D*) or HeLa cells stably transfected with V5-tagged wild-type or DN TLR5 (*B* and *C*) were treated with flagellin (100 ng/ml) for the indicated time, at which point cell lysates were generated and subjected to SDS-PAGE immunoblotting with indicated Abs. Unphosphorylated p38 Ab was used as loading control. *D*, Level of PI3K in plasma membrane fraction of cells disrupted at the indicated time following flagellin treatment.

Flagellin stimulation in vivo

Mice lacking p85α regulatory subunit of class IA PI3K (p85$\alpha^{-/-}$ mice) (19) were backcrossed to BALB/c background for 12 generations before generating homozygous mice. Heterozygous females and homozygous males were mated to generate p85$\alpha^{-/-}$ mice and p85$\alpha^{+/-}$ littermate controls. Groups ($n = 6$) of such PI3K-deficient and their littermate controls (20) were i.p. injected with 10 μg/mouse of purified flagellin. Blood was collected at 1.5, 3, and 6 h after flagellin stimulation (and 2 days before as baseline control, to avoid the blood loss that would be caused by taking four bleeds in a short time span). Serum IL-6 levels were determined by ELISA.

Cytokine ELISAs

IL-6 (mouse and human), IL-8, and KC, were measured with Duoset Ab pairs purchased from R&D Systems following their suggested protocol.

Nitrite measurement

Nitrite level was quantified in the supernatants using the Griess Reagent System (sensitivity, 100–1.56 μM) (Promega) according to the manufacturer's instructions.

Results

Flagellin activates PI3K/Akt signaling in intestinal epithelial cells via a TLR5-dependent mechanism

In light of the emerging role of PI3K in TLR signaling, we assayed this kinase's activation in response to the TLR5 ligand flagellin in the intestinal epithelial cell line T84 using measurement of levels of phospho-Akt as a well-defined indicator of PI3K activation. Significant levels of phosphorylation of Akt were observed within 30 min, peaked between 1 and 2 h, and remained elevated above baseline levels for at least 4 h (Fig. 1*A*). Such induction of Akt phosphorylation was completely blocked by the well-characterized PI3K inhibitor wortmannin (shown below), confirming it was indeed reflecting PI3K activity. The time course of such PI3K/Akt activation appeared similar to that of other kinases known to mediate induction of flagellin-induced gene expression consistent with the possibility that PI3K might positively or negatively influence these events. To investigate whether flagellin activates PI3K signaling via TLR5, we engineered HeLa cells to stably express either wild-type TLR5 or mutant TLR5 that had its intracellular domain deleted, making it unable to signal and furthermore function as a dominant negative (DN), thus blocking activation of the small amount of endogenous wild-type TLR5 expressed by these cells (4, 5). HeLa cells transfected with wild type, but not DN-TLR5, exhibited flagellin-induced activation of a control signal (p38) and PI3K/Akt signal in response to flagellin (Fig. 1*B*). To verify that DN-TLR5 had not simply nonspecifically shut down signaling per se as has been observed for some DN-TLRs, we verified that expression of DN-TLR5 did not affect p38 activation in response to the cytokine TNF-α (Fig. 1*C*). Last, we measured whether, analogous to other receptor-mediated activators of PI3K, flagellin might induce PI3K to translocate to the plasma membrane (where TLR5 is thought to be located). As shown in Fig. 1*D*, flagellin induced rapid (<5 min) translocation of PI3K to the cell membrane. Thus, flagellin induces PI3K signaling in intestinal epithelial cells via a TLR5-dependent mechanism.

PI3K activity attenuates flagellin-induced gene expression by limiting MAPK activation

To investigate the role of flagellin-induced PI3K activation in regulating flagellin-induced gene expression, we examined the effect of the PI3K kinase inhibitor wortmannin on the expression of inducible NO synthase (iNOS), IL-6, and IL-8 in response to flagellin. We observed that pretreatment of T84 cells with wortmannin resulted in markedly enhanced expression of iNOS, IL-6, and IL-8 in response to flagellin (Fig. 2). Such "superinduction" of iNOS expression was evident at the level of iNOS mRNA and protein and was observed with wortmannin concentrations was as low as 2 nM with maximal superinduction achieved at 10 nM. These results suggest that PI3K may negatively regulate flagellin-induced proinflammatory gene expression in intestinal epithelial cells.

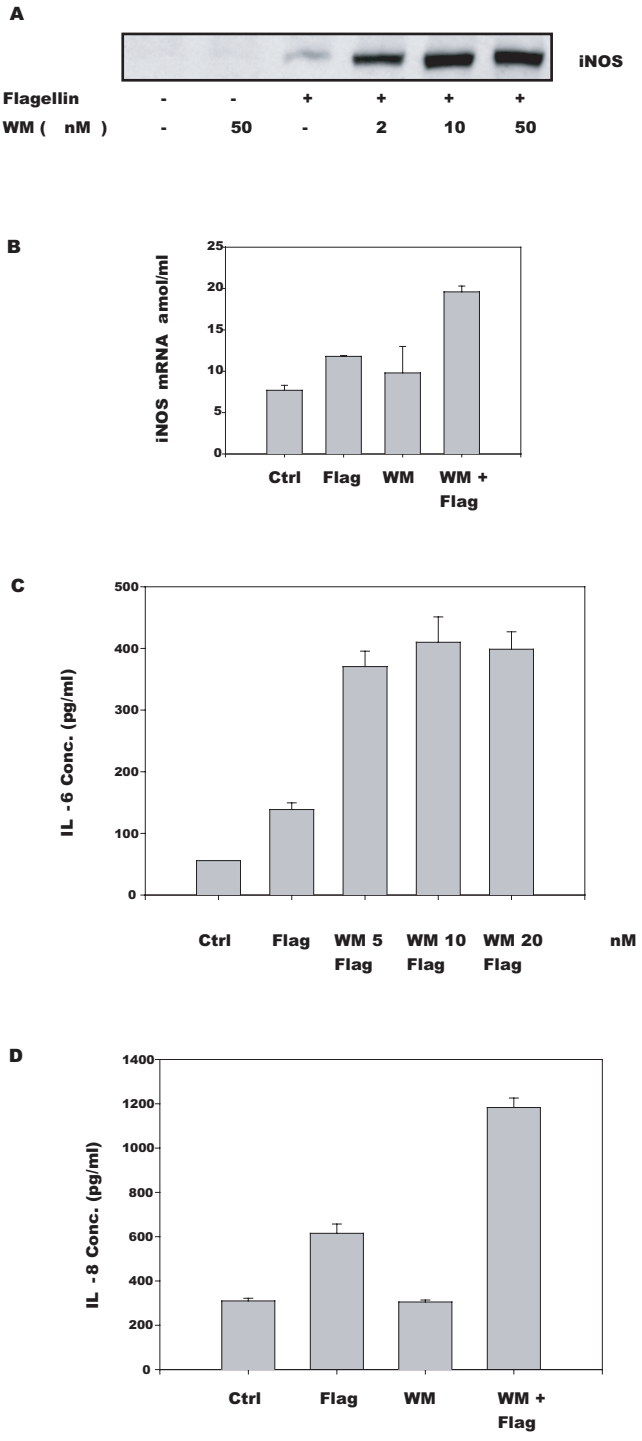

FIGURE 2. PI3K inhibitor potentiates flagellin-induced proinflammatory gene expression in intestinal epithelial cells. T84 cells were treated with 100 ng/ml flagellin in the presence of wortmannin (10 nM or indicated concentration) or vehicle (0.1% DMSO) for 6 h. *A*, iNOS protein was measured in cell lysates by SDS-PAGE immunoblotting. *B*, iNOS mRNA was measured via a commercially available hybridization-based quantitative assay. Supernatants were assayed for IL-6 (*C*) and IL-8 (*D*) by ELISA. Wortmannin was applied as a 30-min pretreatment. β-Actin was used as loading control.

To investigate the mechanism by which blockade of PI3K upregulated flagellin-induced iNOS expression, we further investigated the effects of wortmannin on signaling pathways that are activated by flagellin. Specifically, we measured the phosphorylation status of p38, p42/44, IκBα, and STAT (1 and 3) at various times following flagellin treatment in the presence or absence of wortmannin. Wortmannin, when applied by itself to epithelial cells, induced only a very slight activation of MAPK over a 4-h time course (data not shown). However, when administered with flagellin, wortmannin suppressed the attenuation of MAPK phosphorylation at later time points, resulting in markedly elevated levels of p38 and ERK1/2 activity 4 h after flagellin treatment. In contrast to its effect on MAPK augmentation, wortmannin treatment did not affect phosphorylation or degradation of IκBα indicating that PI3K blockade did not affect the NF-κB pathway within this time frame. Consistent with its enhancement of flagellin-induced proinflammatory gene expression in general and IL-6 in particular, wortmannin enhanced the delayed STAT activation, which is known to be mediated, in part, by this cytokine. To verify that these effects of wortmannin owed to PI3K blockade rather than a nonspecific effect of this compound, we next used an additional inhibitor of this kinase, LY294002. LY294002 pretreatment of intestinal epithelial cells (10 μM) also resulted in prolonged phosphorylation of flagellin-induced MAPK phosphorylation (Fig. 3*B*), further indicating that blockade of PI3K results in prolonged MAPK activation.

As a converse approach to blocking PI3K activity, we next examined the effects of augmented PI3K activity, which we achieved by introducing a constitutive active mutant of p85 (CAp85), the regulatory subunit of PI3K. T84 cells were cotransfected with CAp85-expressing vector or empty vector, along with the vector expressing epitope (HA)-tagged p38. Although transfection efficiency in these cells is low (~20%), use of the HA-tagged p38 allowed us to focus on phosphorylation of the exogenous p38, which was only present in cells that were transfected. In cells transfected with empty vector, phosphorylation of HA-tagged p38 was activated by flagellin. In the cells transfected with CAp85-expressing vector, phosphorylation of HA-tagged p38 was dramatically reduced (Fig. 4). That pharmacologic

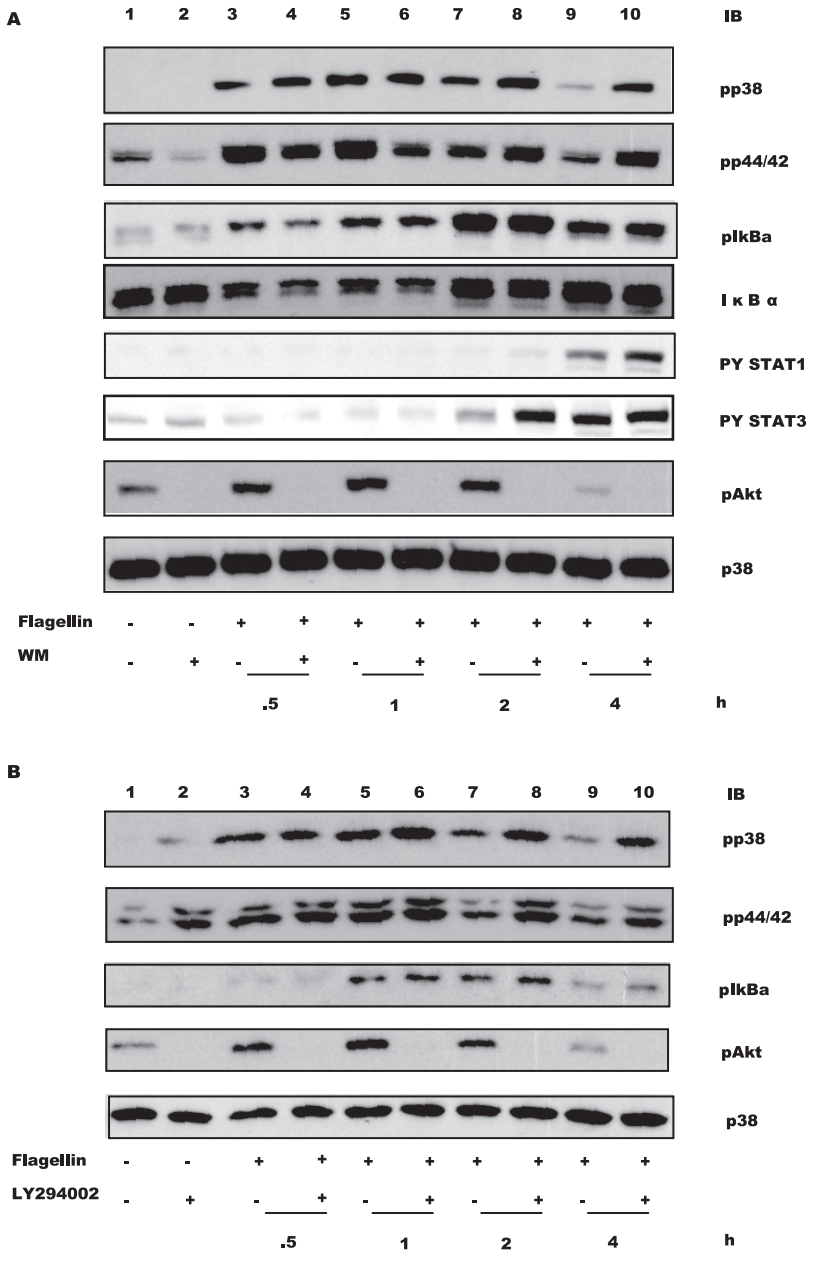

FIGURE 3. Multiple PI3K inhibitors result in potentiated MAP activation in response to flagellin. T84 cells were pretreated with 20 nM wortmannin (*A*) or LY294002 (*B*) for 30 min, followed by stimulation with flagellin (100 ng/ml) for the indicated period. Cell lysates were subjected to SDS-PAGE immunoblotting with indicated phosphospecific Abs. Blot with total p38 Ab was used as a control for equal loading.

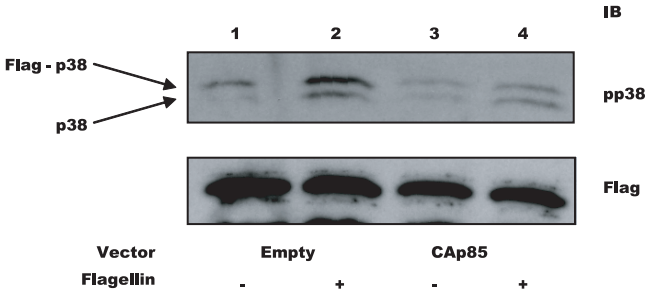

FIGURE 4. Constitutive active PI3K suppresses flagellin-induced p38 MAPK activation. T84 cells were transiently transfected with Flag-tagged p38 expressing vector with either pcDNA (Invitrogen; as empty control) or vector expressing CAp85. Transfected cells were stimulated with or without flagellin for 30 min. Cells were lysed, and SDS-PAGE immunoblotting was performed with the indicated Abs. Anti-Flag immunoblot was used as control for transfection and loading.

blockade of PI3K potentiates MAPK activation and constitutively active PI3K ablates p38 activation supports the notion that PI3K is a negative regulator in flagellin-induced MAPK activation.

We next sought to ascertain whether PI3K regulation of MAPK underlies the superinduction of flagellin-induced IL-6 and IL-8 expression observed under conditions of PI3K blockade. To address this question, we used pharmacologic inhibitors of MAPK (p38 inhibitor SB203580 and ERK1/2 PD90085) under conditions in which these inhibitors do not abolish flagellin-induced gene expression. Specifically, while treatment of epithelial cells with MAPK inhibitors concurrent with flagellin treatment blocks flagellin-induced gene expression, adding the p38 inhibitor 2 h following flagellin treatment (at which time MAPK activation begins to wane) did not effect flagellin-induced secretion of IL-6 (Fig. 5*A*). However, such delayed treatment with MAPK inhibitors substantially blocked the potentiation of IL-6 expression by wortmannin. Similarly, this p38 inhibitor also prevented wortmannin's enhancement of expression of iNOS (Fig. 5*B*). Thus, the negative regulation of flagellin-induced gene expression by PI3K is likely underlain by its negative regulation of MAPK activity.

MAPKs are activated by their upstream kinases, the MAPK kinase (MKK), with MKK3/6 being thought to play a role in activation of p38 (21). To investigate whether PI3K might act on p38 directly or the cascade that activates this kinase, we examined the effects of PI3K blockade, via wortmannin, on the phosphorylation of MKK3/6. Intestinal epithelial cells were treated with 10 nM wortmannin (or vehicle-DMSO) followed by flagellin stimulation. At various times following such treatment, phosphorylation of MKK3/6 was determined by SDS-PAGE immunoblotting via a phosphospecific Ab. As shown in Fig. 6, phosphorylation of MKK3/6 was indeed up-regulated in response to flagellin and while such up-regulation peaked at 1 h and waned thereafter, in the presence of wortmannin, maximal induction persisted for at least 4 h. Thus, PI3K likely negatively regulates an early event in the p38 MAPK pathway rather than directly regulates p38.

Absence of PI3K leads to enhanced/prolonged flagellin-induced gene expression in vivo

As a final approach to confirm the negative regulatory role of PI3K in flagellin-induced inflammatory responses and to assess its significance in vivo, we compared serum levels IL-6, KC (murine

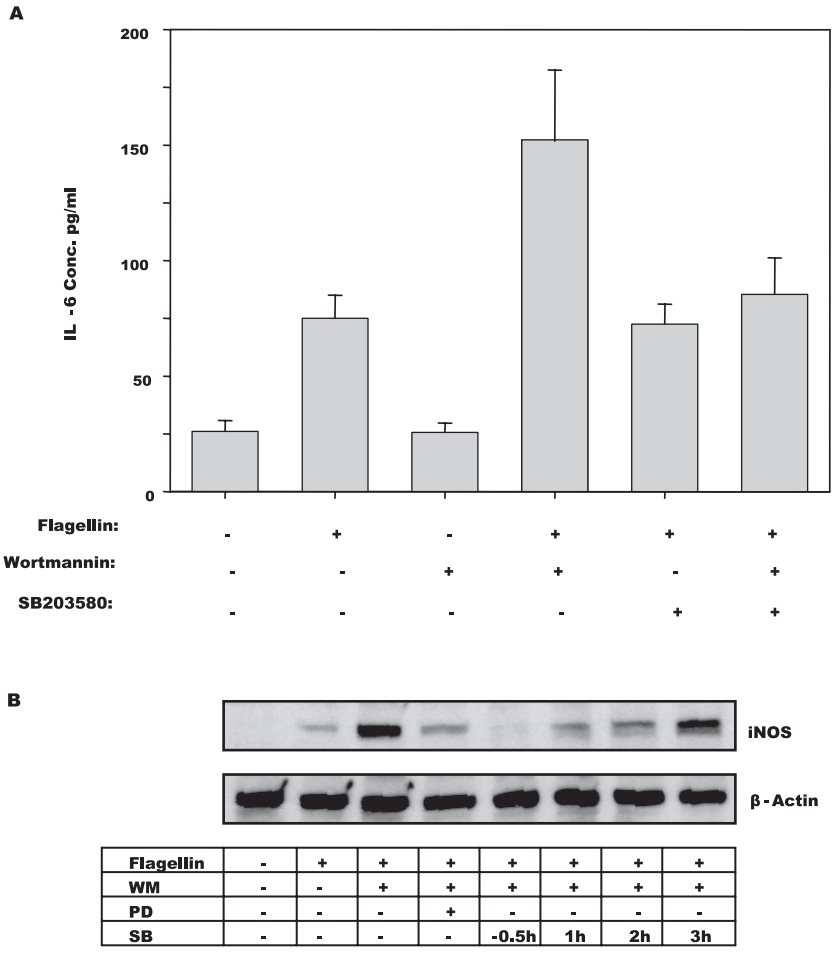

FIGURE 5. Potentiation of flagellin-induced proinflammatory gene expression by wortmannin is suppressed by blocking p38 and ERK1/2 signaling. T84 cells were stimulated with 100 ng/ml flagellin in the presence or absence of PD90085 or SB203580. *A*, SB 203580 or vehicle (DMSO) was added 2 h following flagellin treatment. Conditioned medium was collected in three parallel experiments at 6-h and [IL-6] was determined via ELISA. *B*, Cells were pretreated (0.5 h before stimulation) with PD90085 or treated with SB203580 at the indicated time before or following stimulation with flagellin. Cell lysates were analyzed for expression of iNOS by SDS-PAGE immunoblotting.

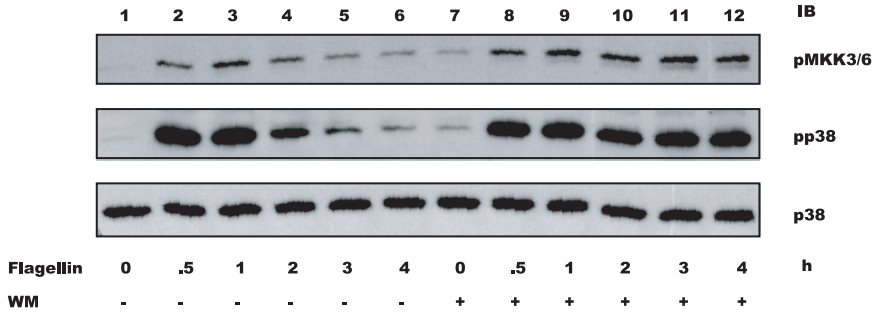

FIGURE 6. Wortmannin enhancement of p38 activation correlates with enhancement of MKK3/6. T84 cells were pretreated with wortmannin (10 nM) or vehicle (DMSO) for 30 min followed by treatment with flagellin for the indicated time. Cell lysates were subjected SDS-PAGE immunoblotting using indicated phosphospecific Abs. Levels of total p38 served as a loading control.

equivalent of IL-8), and nitrite (as a surrogate indicator of iNOS), which are known to be elevated in response to flagellin (3), in PI3K-deficient mice (due to engineered deletion of p85α) or heterozygous littermate controls (we also measured levels of IL-10 but did not observe significant induction in either mouse strain). Control and PI3K-deficient mice were systemically treated with flagellin via i.p. injection. Although such responses to flagellin do not necessarily reflect responses of only epithelial cells, we have observed that, at least for IL-6, they are largely the result of cytokines produced by nonhemopoietic cells (22). Mice were administered 10 μg of flagellin i.p., and blood samples were collected at 1.5, 3, and 6 h. IL-6 levels were measured by ELISA. PI3K-deficient mice displayed higher levels of serum IL-6 and KC particularly at 3 and 6 h, thus mimicking the in vitro results seen with pharmacologic PI3K inhibition (Fig. 7). PI3K-deficient mice displayed higher levels of nitrite at 6 h. Thus, both in vitro and in vivo, PI3K activation plays an important negative regulatory role in limiting flagellin-induced proinflammatory gene expression.

Discussion

TLRs are the primary means by which multicellular organisms can rapidly detect the presence of microbes and, subsequently, rapidly activate the expression of a panel of genes that protect the host from the immediate danger and prepare it to better handle similar challenges in the future (i.e., promote adaptive immunity). Many of the genes induced upon TLR ligation have their expression go from virtually undetected to highly expressed to undetectable again within a period of hours. Such rapid induction of these genes is mediated by distinct parallel signaling pathways, particularly NF-κB, MAPK, and STAT, which via distinct mechanisms regulate the transcription, mRNA stability, and translation of these "proinflammatory" genes. The use of multiple mechanisms to rapidly activate expression of these genes underscores their importance to host defense and persons carrying defects in the TLRs or signaling molecules that mediate their activation exhibit increased susceptibility to a variety of pathogens. An apparent reason for the need to have such multifaceted means of up-regulating of proinflammatory gene expression is that such gene expression is normally kept quite low as it seems to pose substantial danger to the host in that an increasingly growing list of disease states are being associated with and/or characterized by inflammation (23).

A consequence of having multiple pathways of regulating proinflammatory gene expression is the need to be able to rapidly down-regulate each pathway, thus minimizing potential negative consequences of inflammation. In this regard, multiple gene products of TLR activation such as SOCS and IκBα, whose expression accompanies that of proinflammatory genes, function in shutting down TLR-mediated gene expression. Another mechanism that has been proposed to be "built in" to TLR signaling cascades to down-regulate TLR activation is activation of PI3K, although studies of PI3K role in signal transduction by TLR2, -3, -4, and -9 have observed a variety of pro- and anti-inflammatory actions mediated by this lipid kinase (for review, see Ref. 11). Herein, we observed that, using both pharmacologic and genetic manipulation, TLR5-mediated PI3K plays a role in negatively regulating proinflammatory gene expression in intestinal epithelial cells.

PI3K's negative regulation of TLR5-mediated gene expression was mediated primarily via its effects on the MAPK pathway in that PI3K blockade prolonged MAPK activation but did not appear to significantly affect NF-κB activation in response to flagellin. However, it should be noted that NF-κB activation is indeed necessary for flagellin-induced gene expression as proteosomal blockade of NF-κB activation ablates induction of all of the genes assayed in our studies (4) (data not shown). Because PI3K-mediated negative regulation of p38 correlated with levels of activation of MKK3/6, a known upstream activator of p38, it seems likely that PI3K acts on one or more members of the MKK family, which is a place in the TLR signaling cascade in which the MAPK signaling cascade has bifurcated from the NF-κB signaling pathway. Although PI3K blockade also led to enhanced STAT activation, this was likely a consequence of enhanced production of proteins that mediate such STAT activation rather than acting on the STAT signaling pathway per se. Why one might expect such mediators might also promote NF-κB activation, such effects may be counteracted by increased induction of negative regulators of the NF-κB pathway. In contrast to SOCS, IL-1R-associated kinase M, and IκBα, PI3K may negatively regulate proinflammatory signaling cascades via a pathway that does not require new protein synthesis. As one of the major targets of PI3K negative regulation, p38 MAPK, is thought to primarily effect flagellin-induced proinflammatory gene expression primarily via effects on mRNA translation (24), it seems reasonable to speculate that TLR5-mediated PI3K activation functions as a means to rapidly shut off translation of genes that contain 5′ AU-rich untranslated elements. Such ability to rapidly shut down translation would seem to allow for substantially quicker turning off of gene expression than simply shutting down transcription.

Although our studies relating to TLR5 signaling have primarily focused on genes that promote immune cell recruitment, we have also recently observed that TLR5 activation also activates both pro- and antiapoptotic signaling pathways in intestinal epithelial cells (25). Although NF-κB activation was observed to underlie much of the antiapoptotic signaling, the proximal signaling events by which TLR5 activates the caspases that promote apoptosis remain largely unknown. That inhibition of PI3K promotes flagellin-induced caspase 3 activation without an apparent effect on NF-κB activation (which in these cells prevents caspase activation/apoptosis) suggests that the prolonged p38 activation we observe in response to PI3K inhibition might also play a role in mediating the increased apoptosis associated with PI3K blockade. Indeed, recent work by Kim et al. (26) demonstrates a role for p38 in activating caspase 3 in response to a physiologic inducer of apoptosis,

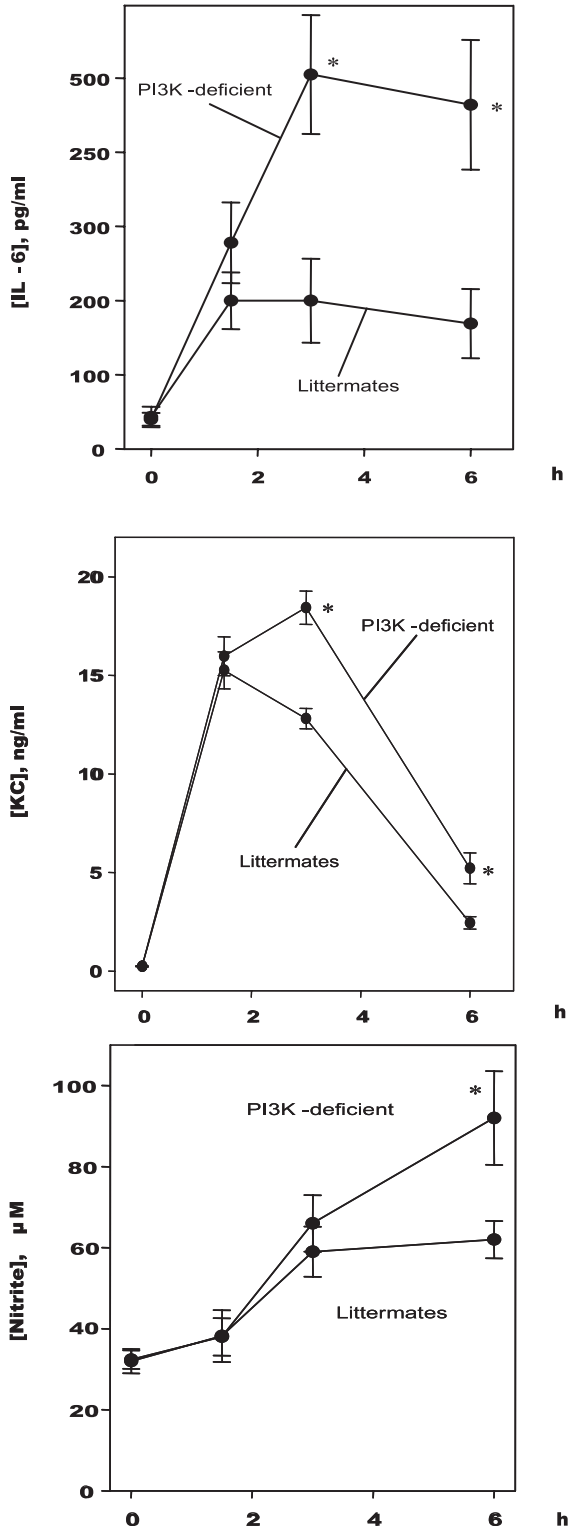

FIGURE 7. Genetic loss of PI3K results in increased flagellin-induced proinflammatory gene expression in vivo. PI3K-deficient mice and heterozygous littermate controls (described in *Materials and Methods*) were i.p. injected with 10 μg of flagellin per mouse ($n = 6$). Serum was isolated at the indicated time point and assayed for IL-6 and KC via ELISA. Nitrite was assayed by use of the Griess reagent (*, $p < 0.05$, PI3K-deficient vs littermate controls).

namely *Clostridium difficile* toxin A. Given that growth and spread of many intracellular pathogens requires that the infected host cells be viable, we speculate that having p38 both promote apoptosis and increase expression of proinflammatory cytokines may be a signaling mechanism to allow infected cells to simultaneously reduce microbe dissemination and increase immune cell recruitment, whereas, in contrast, increasing NF-κB activation would promote both proinflammatory gene expression and cell survival. We would presume a variety of factors would regulate which type of response might be warranted in response to a particular pathogen.

Upon considering the ubiquitous nature of microbes and their products, it is not difficult to imagine a variety of scenarios in which there might be activation of TLR-mediated gene expression that could be potentially dangerous to the host. TLR5 seems particularly at risk for being activated excessively due to its expression on epithelial cells in general and in particular intestinal epithelial cells, which normally live among a large population of commensal bacteria, which include flagellated bacteria that have the potential to activate TLR5 (4). In support of such a potentially detrimental role for TLR5, a DN TLR5 polymorphism has recently been shown to be negatively associated with Crohn's disease, suggesting that TLR5-mediated gene expression can promote development of Crohn's disease (27). This finding underscores the importance of understanding mechanisms that normally serve to limit TLR signaling. Although such mechanisms will likely prove to be quite complex, as herein, TLR5-mediated activation of PI3K is one key element of this process.

Acknowledgments

We thank Matam Vijay-Kumar for helpful discussions and Sean Lyons and Danile Moore for technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat. Rev. Immunol.* 4: 499–511.
2. Gewirtz, A. T. 2003. Intestinal epithelial Toll-like receptors: to protect. And serve? *Curr. Pharm. Des.* 9: 1–5.
3. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410: 1099–1103.
4. Gewirtz, A. T., T. A. Navas, S. Lyons, P. J. Godowski, and J. L. Madara. 2001. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J. Immunol.* 167: 1882–1885.
5. Yu, Y., H. Zeng, S. Lyons, A. Carlson, D. Merlin, A. S. Neish, and A. T. Gewirtz. 2003. TLR5-mediated activation of p38 MAPK regulates epithelial IL-8 expression via posttranscriptional mechanism. *Am. J. Physiol.* 285: G282–G290.
6. Toshchakov, V., B. W. Jones, P. Y. Perera, K. Thomas, M. J. Cody, S. Zhang, B. R. Williams, J. Major, T. A. Hamilton, M. J. Fenton, and S. N. Vogel. 2002. TLR4, but not TLR2, mediates IFN-β-induced STAT1α/β-dependent gene expression in macrophages. *Nat. Immunol.* 3: 392–398.
7. Yu, Y., H. Zeng, M. Vijay-Kumar, A. S. Neish, D. Merlin, S. V. Sitaraman, and A. T. Gewirtz. 2004. STAT signaling underlies difference between flagellin-induced and tumor necrosis factor-α-induced epithelial gene expression. *J. Biol. Chem.* 279: 35210–35218.
8. Horng, T., G. M. Barton, R. A. Flavell, and R. Medzhitov. 2002. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* 420: 329–333.
9. Elliott, J., and J. A. Johnston. 2004. SOCS: role in inflammation, allergy and homeostasis. *Trends Immunol.* 25: 434–440.
10. Kobayashi, K., L. D. Hernandez, J. E. Galan, C. A. Janeway, Jr., R. Medzhitov, and R. A. Flavell. 2002. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* 110: 191–202.
11. Fukao, T., and S. Koyasu. 2003. PI3K and negative regulation of TLR signaling. *Trends Immunol.* 24: 358–363.
12. Fukao, T., M. Tanabe, Y. Terauchi, T. Ota, S. Matsuda, T. Asano, T. Kadowaki, T. Takeuchi, and S. Koyasu. 2002. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat. Immunol.* 3: 875–881.
13. Arbibe, L., J. P. Mira, N. Teusch, L. Kline, M. Guha, N. Mackman, P. J. Godowski, R. J. Ulevitch, and U. G. Knaus. 2000. Toll-like receptor 2-mediated NF-κB activation requires a Rac1-dependent pathway. *Nat. Immunol.* 1: 533–540.
14. Sarkar, S. N., K. L. Peters, C. P. Elco, S. Sakamoto, S. Pal, and G. C. Sen. 2004. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. *Nat. Struct. Mol. Biol.* 11: 1060–1067.

15. Park, Y., S. W. Lee, and Y. C. Sung. 2002. Cutting edge: CpG DNA inhibits dendritic cell apoptosis by up-regulating cellular inhibitor of apoptosis proteins through the phosphatidylinositide-3′-OH kinase pathway. *J. Immunol.* 168: 5–8.
16. Gewirtz, A. T., K. A. Reed, D. Merlin, A. S. Neish, and J. L. Madara. 2002. Modeling microbial-epithelial interactions in the intestine. *Methods Microbiol.* 31: 377–396.
17. Gewirtz, A. T., P. O. Simon, Jr., C. K. Schmitt, L. J. Taylor, C. H. Hagedorn, A. D. O'Brien, A. S. Neish, and J. L. Madara. 2001. *Salmonella typhimurium* translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J. Clin. Invest.* 107: 99–109.
18. McSorley, S. J., B. D. Ehst, Y. Yu, and A. T. Gewirtz. 2002. Bacterial flagellin is an effective adjuvant for $CD4^+$ T cells in vivo. *J. Immunol.* 169: 3914–3919.
19. Gouraud, S., A. Laera, G. Calamita, M. Carmosino, G. Procino, O. Rossetto, R. Mannucci, W. Rosenthal, M. Svelto, and G. Valenti. 2002. Functional involvement of VAMP/synaptobrevin-2 in cAMP-stimulated aquaporin 2 translocation in renal collecting duct cells. *J. Cell Sci.* 115: 3667–3674.
20. Suzuki, H., Y. Terauchi, M. Fujiwara, S. Aizawa, Y. Yazaki, T. Kadowaki, and S. Koyasu. 1999. Xid-like immunodeficiency in mice with disruption of the p85α subunit of phosphoinositide 3-kinase. *Science* 283: 390–392.
21. Tibbles, L. A., Y. L. Ing, F. Kiefer, J. Chan, N. Iscove, J. R. Woodgett, and N. J. Lassam. 1996. MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. *EMBO J.* 15: 7026–7035.
22. Sanders, C. J., D. A. Moore, I. R. Williams, and A. T. Gewirtz. 2005. Nonhematopoietic cells link innate and adaptive immune responses to bacterial flagellin via early cytokine production. *FASEB J.* 19: A939.
23. Abreu, M. T., and M. Arditi. 2004. Innate immunity and Toll-like receptors: clinical implications of basic science research. *J. Pediatr.* 144: 421–429.
24. Kontoyiannis, D., M. Pasparakis, T. T. Pizarro, F. Cominelli, and G. Kollias. 1999. 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.
25. Zeng, H., H. Wu, V. Sloane, R. Jones, Y. Yu, P. Lin, A. T. Gewirtz, and A. S. Neish. 2005. Flagellin/TLR5 responses in epithelia reveal intertwined activation of inflammatory and apoptotic pathways. *Am. J. Physiol.* 290: G96–G108.
26. Kim, H., E. Kokkotou, X. Na, S. H. Rhee, M. P. Moyer, C. Pothoulakis, and J. T. Lamont. 2005. *Clostridium difficile* toxin A-induced colonocyte apoptosis involves p53-dependent $p21^{WAF1/CIP1}$ induction via p38 mitogen-activated protein kinase. *Gastroenterology* 129: 1875–1888.
27. Gewirtz, A. T., M. Vijay-Kumar, S. R. Brant, R. H. Duerr, D. L. Nicolae, and J. H. Cho. 2006. Dominant-negative TLR5 polymorphism reduces adaptive immune response to flagellin and negatively associates with Crohn's disease. *Am. J. Physiol.* In press.