The Journal of Immunology

BRIEF REPORT | AUGUST 15 2001

Cutting Edge: Bacterial Flagellin Activates Basolaterally Expressed TLR5 to Induce Epithelial Proinflammatory Gene Expression[1]

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J Immunol (2001) 167 (4): 1882–1885.
https://doi.org/10.4049/jimmunol.167.4.1882

Cutting Edge: Bacterial Flagellin Activates Basolaterally Expressed TLR5 to Induce Epithelial Proinflammatory Gene Expression[1]

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Flagellin, the structural component of bacterial flagella, is secreted by pathogenic and commensal bacteria. Flagellin activates proinflammatory gene expression in intestinal epithelia. However, only flagellin that contacts basolateral epithelial surfaces is proinflammatory; apical flagellin has no effect. Pathogenic *Salmonella*, but not commensal *Escherichia coli*, translocate flagellin across epithelia, thus activating epithelial proinflammatory gene expression. Investigating how epithelia detect flagellin revealed that cell surface expression of Toll-like receptor 5 (TLR5) conferred NF-κB gene expression in response to flagellin. The response depended on both extracellular leucine-rich repeats and intracellular Toll/IL-1R homology region of TLR5 as well as the adaptor protein MyD88. Furthermore, immunolocalization and cell surface-selective biotinylation revealed that TLR5 is expressed exclusively on the basolateral surface of intestinal epithelia, thus providing a molecular basis for the polarity of this innate immune response. Thus, detection of flagellin by basolateral TLR5 mediates epithelial-driven inflammatory responses to *Salmonella*. *The Journal of Immunology*, 2001, 167: 1882–1885.

The surface of the human intestine is densely colonized by a variety of largely commensal microbial species (1). Many of these microbes release molecules such as LPS, which has the potential of inducing proinflammatory gene expression if internal access to the host is gained. However, the epithelial cells that line the gastrointestinal tract are relatively unresponsive to LPS (2–5), consistent with the fact that they are continually exposed to this bacterial product but are not in a constant state of inflammation. In contrast, the more recently recognized proinflammatory bacterial product (or pathogen-associated molecular pattern) flagellin is a potent activator of intestinal epithelial proinflammatory gene expression (3, 5, 6). Although flagellin is secreted by commensal and pathogenic bacteria, flagellin promotes inflammation only if it crosses intestinal epithelia and contacts their basolateral membranes (3), thus explaining why commensal microbes can secrete flagellin into the lumen yet not induce inflammation.

Toll-like receptors (TLR)[4] are an evolutionarily conserved family of receptors that function in innate immunity via recognition of conserved patterns in bacterial molecules (for review, see Ref. 7). Given that the ligands for the majority of cloned TLRs have yet to be identified, we explored the possibility that a TLR would be a good candidate to mediate inflammatory responses to flagellin. Indeed, Hayashi et al. (8) recently screened bacterial products for ability to activate TLR5 and found that flagellin could activate NF-κB-mediated gene expression in TLR5-transfected cells. We report here that this function is specific to TLR5 in that of all known TLRs (1–10), only TLR5 could activate proinflammatory gene expression in response to flagellin. Further, TLR5 is expressed on the basolateral, but not apical, surface of model epithelia, thus providing a mechanism by which microbes that invade or translocate flagellin, but not commensal bacteria, induce intestinal epithelia to orchestrate an inflammatory response.

Materials and Methods

Cells and reagents

Model human intestinal epithelia were prepared by culturing the colonic cell line T84 on collagen-coated permeable supports as previously described (9). Model epithelia were used 6–14 days after plating and after verification that they had achieved a transepithelial electrical resistance of at least 1000 $\Omega \cdot cm^2$, thus indicating a high degree of surface polarity. COS-7, 293, and HeLa cell lines were cultured in DMEM with 10% FBS, 2 μM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transient transfections were conducted using Superfect (Qiagen, Chatsworth, CA) for COS and 293 cells and Effectene (Qiagen) for HeLa cells, according to the manufacturer's instructions. MG-262 was obtained from Affiniti (Exeter, U.K.). Purified flagellin was prepared as previously described (3).

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Received for publication May 30, 2001; accepted for publication July 2, 2001.

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[1] This work was supported by National Institutes of Health Grants DK-2792 (to A.T.G.) and DK-4762 (to J.L.M.).

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[4] Abbreviations used in this paper: TLR, Toll-like receptor; gD, glycoprotein D; Δ, deletion of amino acids.

0022-1767/01/$02.00

Construction of TLR expression plasmids

Full length human TLR cDNAs (TLRs 1–10) were cloned from a human fetal lung library into the pRKN. N-terminal epitope tag versions (gD.TLR) linking TLRs to the first 53 aa of HSV-1 glycoprotein D (gD) were constructed as previously described (10). The predicted amino acid sequence for TLR5 matched that previously published (11) except for the following substitutions: V233L, C352L and L616F. TLR5 deletion plasmids were constructed as follows: gD.TLR5 in pRKN were restricted with *Xho*I (deletion of aa (Δ) 1–191), *Eco*RV and *Mfe*I (Δ408–591), and *Bsr*GI (Δ695–858). The resulting cDNAs were gel purified and religated. Protein expression was confirmed by immunoprecipitation and immunoblotting using gD mAb (Genentech, South San Francisco, CA).

Luciferase reporter and other proinflammatory assays

293, COS, or HeLa cells were plated (in 12-well plates; 1×10^5 cells/well) and transfected 18 h later with expression plasmids and 0.5 μg luciferase reporter plasmid pGL3-ELAM.tk and 0.1 μg *Renilla* luciferase reporter vector as an internal control. After 24 h, cells were treated with flagellin (100 ng/ml) or TNF-α (60 ng/ml) for 6 h, and luciferase was assayed via the Dual-Luciferase system (Promega, Madison, WI). Data are expressed as relative luciferase activity representing the mean ± SE of duplicate experiments and were obtained by calculating the ratio of firefly luciferase activity and *Renilla* luciferase activity. IL-8 secretion, IκBα degradation, and IκBα phosphorylation were measured as previously described (12).

Fluorescence microscopy and FACS analysis

Confocal microscopy was performed on paraformaldehyde model epithelia as previously described (13). FACS analysis was performed on confluent T84 cells, disassociated with EGTA as previously described (13) using a goat polyclonal Ab to the N-terminal region of TLR5 and competing immunogen peptide from Santa Cruz Biotechnology (Santa Cruz, CA). Isolation of apically or basolaterally biotinylated proteins was performed as previously described (14). Western blotting of such lysates was performed with a 1/800 dilution of TLR5 rabbit polyclonal Ab directed at aa 154–280 purchased from Santa Cruz Biotechnology. Abs to E-cadherin and IFN-γ-inducible protein 82 were a gift from C. Parkos (Emory University, Atlanta, GA).

Results

Exposing the basolateral, but not apical, surface of model intestinal epithelia to purified flagellin potently elicits epithelial secretion of the neutrophil chemoattractant IL-8 (Fig. 1 and Ref. 3). Preventing NF-κB activation with the proteasome inhibitor MG-262 (15) blocked this IL-8 secretion by ~80% (data not shown). Further, basolateral flagellin induced NF-κB nuclear translocation (Fig. 1*B*) as well as rapid phosphorylation (Fig. 1*C*) and subsequent degradation and resynthesis of the inhibitor IκBα (Fig. 1*D*) in a manner similar to TNF-α, confirming that flagellin induces epithelial chemokine expression via the activation of NF-κB.

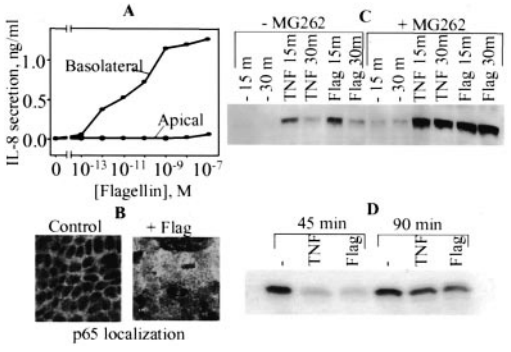

FIGURE 1. Basolateral flagellin activates epithelial chemokine secretion via an NF-κB-mediated mechanism. Model epithelia were stimulated with flagellin (Flag; 10^{-9} M or indicated concentration) or 20 ng/ml TNF-α. TNF-α and flagellin were applied basolaterally, except where indicated otherwise in *A. A,* IL-8 secretion was measured by ELISA 5 h after addition of flagellin to indicated surface. *B,* Model epithelia were fixed and immunostained for NF-κB (p65) 1 h after agonist addition. *C* and *D,* Model epithelia were lysed at the indicated time after agonist addition and analyzed via SDS-PAGE immunoblotting (C-anti-phospho-IκBα, D-anti-IκBα)

Flagellin-induced NF-κB activation is not specific to intestinal epithelial cells. Rather, NF-κB-mediated responses also occur in monocytic cells (16) as well as in such well-characterized cell lines as COS cells and 293 cells (Fig. 2*A*). We used the latter cell types to investigate whether TLR mediates NF-κB activation in response to flagellin. We observed that transfection of 293 cells with a dominant-negative TLR effector protein MyD88 (Δ152–296) that blocks most TLR responses (17, 18) also prevented NF-κB activation in response to flagellin, but not TNF-α (negative control), suggesting that a TLR was mediating NF-κB activation in response to flagellin (Fig. 2*B*). Thus, flagellin was likely activating an endogenous TLR(s) in 293 cells.

To investigate which TLR(s) transduce proinflammatory signals in response to flagellin, we tested each known TLR for ability to confer responsiveness to flagellin. First, we sought a cell type that did not activate NF-κB in response to flagellin and found HeLa cells fit this criteria (Fig. 2*A*). Next, we transfected HeLa cells with cDNA for TLRs 1–10 and verified, via flow cytometry, that each TLR was expressed on the cell surface (data not shown). Subsequently, the ability of each transfectant to induce an NF-κB reporter gene in response to flagellin was measured. HeLa cells transfected with TLR5 exhibited ~10-fold activation of NF-κB-mediated gene expression, whereas no activation was observed for HeLa transfected with any of the other TLRs (Fig. 3*A*). Like the responses mediated by endogenous TLRs, flagellin-induced NF-κB activation in TLR5-expressing HeLa was prevented by co-expressing dominant-negative MyD88 (Fig. 3*B*). Mutant TLR5 that lacked either certain regions of the extracellular LRR or the intracellular Toll/IL-1R homology region of TLR5 were unable to induce this response, indicating that both of these domains of TLR5 are required to signal in response to flagellin (Fig. 3*C*). TLRs lacking their intracellular domains act as dominant-negative effectors of wild-type TLRs (presumably via soaking up ligand or preventing dimerization of wild-type proteins). We observed such dominant negative activity from TLR5 mutants that lacked intracellular domains (Δ695–858), but not from analogous mutants of TLR4 (Fig. 3*D*). Together, these results indicate that activation of proinflammatory gene expression in response to flagellin is specifically mediated by TLR5 in agreement with the report of Hayashi et al. (8).

Having determined that TLR5 can activate proinflammatory gene expression, we next examined whether this receptor was expressed on the surface of the intestinal epithelial cells that we used to originally identify the proinflammatory activity of flagellin. Using flow cytometry, we observed a 10-fold shift in the fluorescence

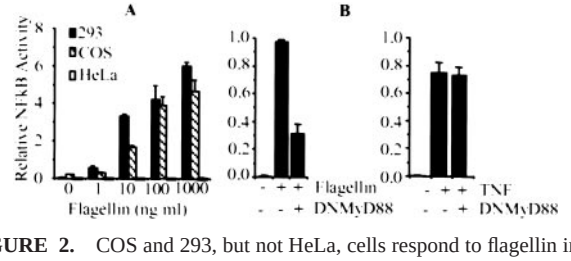

FIGURE 2. COS and 293, but not HeLa, cells respond to flagellin in an MyD88-dependent manner. Cells were treated as indicated and NF-κB activation measured via a reporter gene assay as described in *Materials and Methods*. *A,* Indicated cell type and flagellin concentration were used. *B,* 293 cells were transfected with 0.25 μg dominant-negative MyD88 (+) or empty vector (−) and treated with 100 ng/ml flagellin or with 60 ng/ml TNF-α.

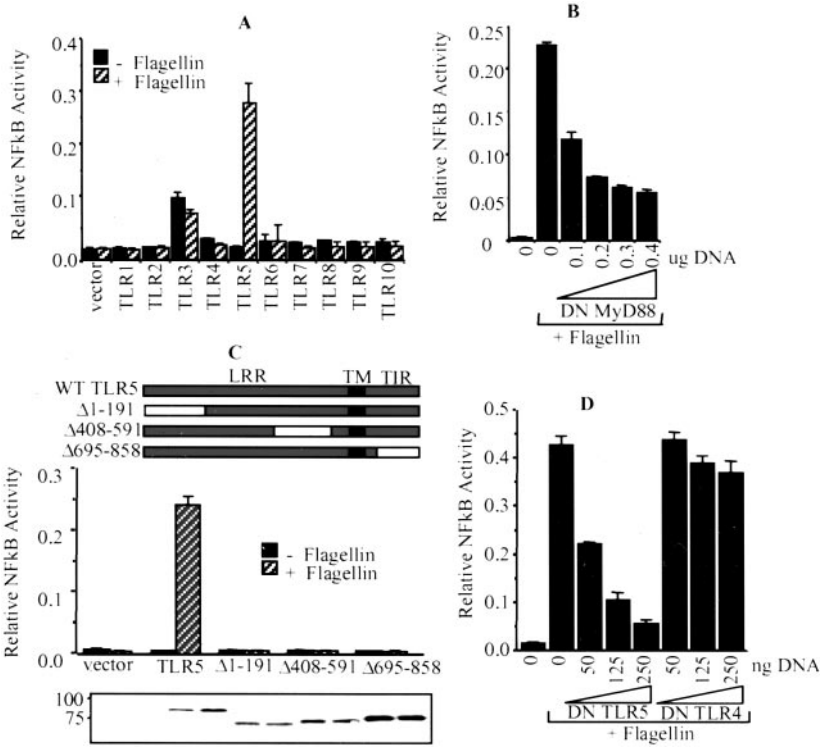

FIGURE 3. TLR5 mediates NF-κB activation in response to flagellin. NF-κB activation was measured in transfected HeLa cells in response to 100 ng/ml flagellin. *A*, Cells were transfected with 0.25 μg of the indicated TLR. *B*, Cells were transfected with 0.25 μg TLR5 and with increasing amounts of dominant-negative (DN) MyD88 as indicated. *C*, Cells were transfected with 0.25 μg wild-type (WT) or TLR5 deletion mutants (TM). *Top*, Schematic showing deletions. *Bottom*, Immunoblot showing mutant proteins were expressed. *D*, Cells were transfected with wild-type TLR5 (0.25 μg) as well as the indicated amount of dominant-negative TLR5 (Δ695–858) or dominant-negative TLR4. LRR, Leucine-rich repeat; TIR, Toll/IL-1R homology region of TLR5.

of intestinal epithelial cells labeled with TLR5 (Fig. 4*A*) compared with secondary Ab alone or with an isotype Ab to TLR1 (data not shown). The labeling was largely reduced by the immunogen TLR5 peptide, indicating that labeling was specific. Next, we examined the polarity of TLR5 expression on model epithelia by confocal microscopy. Model epithelia were double-labeled for actin and TLR5. Whereas apical sections (i.e., those at or above the perijunctional ring) showed staining only for actin, basolateral sections showed membrane staining for both actin and TLR5, indicating that surface expression of TLR5 is restricted to the basolateral membrane (Fig. 4*B*). Similarly, orthogonal (X-Z) image reconstruction showed that surface TLR5 was below the apical surface and appeared particularly prominent on the basal surface. Analogous to our FACS results, no staining was visible with control (secondary Ab only), and TLR5 staining was largely blocked by the competing peptide. Lastly, we examined the polarity of TLR5 expression on intestinal epithelia via cell surface-selective biotinylation. Epithelia were treated apically or basolaterally with cell-impermeant sulfobiotin, quenched, lysed, and immunoprecipitated with streptavidin; and immunoprecipitates were probed by SDS-PAGE/immunoblotting with anti-TLR5. TLR5 was expressed exclusively on the basolateral surface and migrated as a doublet of ~100 kDa (Fig. 5*C*) likely due to differential glycosylation. As controls (not shown), we verified that IFN-γ-inducible protein 82 and E-cadherin were preferentially expressed on the apical and basolateral surfaces, respectively, as previously described (19, 20). Thus, the polarity of TLR5 expression matches the polarity of the response to flagellin, further supporting the notion that flagellin activation of intestinal epithelia is mediated by TLR5.

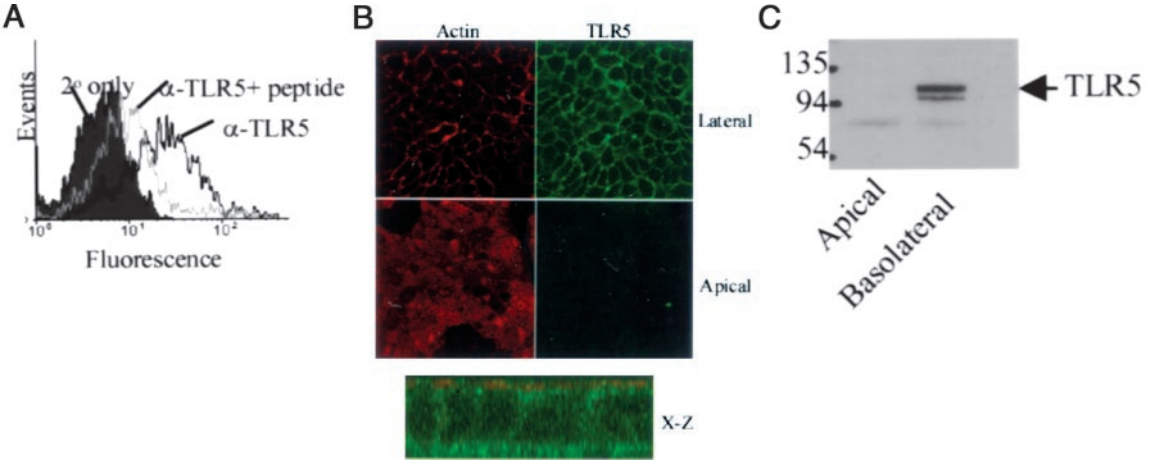

FIGURE 4. TLR5 is expressed on model epithelia and polarized to the basolateral surface. *A*, Surface expression of TLR5 on T84 cells was analyzed by FACS as described in *Materials and Methods*. *B*, Model epithelia were fixed, stained with rhodamine/phalloidin (to label actin) and an Ab to TLR5, and analyzed by confocal microscopy (Cactin = red; TLR5 = green). *C*, Model epithelia were exposed to biotin at the indicated surface and solubilized; biotinylated proteins were isolated by streptavidin beads and analyzed via Western blotting using an Ab to TLR5.

Discussion

Activation of proinflammatory signaling pathways in epithelial cells, especially the NF-κB pathway, is an essential component of innate immunity (21). TLRs play a key role in such innate immune pathways via recognition of conserved microbial molecular patterns, thus activating immune inflammatory responses. However, because components of both pathogenic and commensal bacteria can activate TLRs, host tissues that are normally densely colonized by commensal bacteria must be able to distinguish commensal from pathogenic organisms to avoid being in a constant state of inflammation. For example, bacterial flagellins that are secreted by both pathogenic and commensal bacteria have the potential to activate epithelial chemokine secretion but can only do so upon translocation from the luminal domain (where commensal strains are located) to the basolateral membrane domain (3). Such translocation of flagellin can be mediated by pathogens such as *Salmonella typhimurium*, but not by commensal *Escherichia coli* strains. This report identifies the molecular mechanism by which intestinal epithelia distinguish between basolateral and apical flagellin, namely, the basolateral expression of TLR5 that serves as the flagellin receptor. In addition to its expression on colonic epithelium, TLR5 is expressed in internal tissues including heart, brain, spleen, kidney, and testis, suggesting a wide role for TLR5 in host defense (11). Supporting this notion, mutations in TLR5 result in an increased susceptibility of mice to *Salmonella* infection (11).

TLR5 expressed on the basolateral surface of intestinal epithelia ought to be able to detect the invasion of a large variety of microbes and is positioned to do so at a very early step in the invasive process. Importantly, this innate immune mechanism could be activated not only by invasive pathogens but also by commensal organisms that had somehow, perhaps opportunistically, breached the epithelium. Recruitment of inflammatory cells in response to TLR5-mediated chemokine secretion could thus prevent systemic penetration of such locally invading microbes, hence preventing the dire consequences that occur when pathogenic or commensal bacteria achieve systemic infection in humans. Interestingly, *S. typhimurium* may translocate flagellin independent of bacterial invasion (3) and thus may use TLR5 as a mechanism to induce intestinal inflammation and the associated diarrhea that aids in their dissemination to new hosts. Additionally, ligation of basolateral TLR5 by flagellin secreted from commensal organisms may occur in states of epithelial barrier dysfunction such as inflammatory bowel disease and thus may play a role in inducing or exacerbating the inflammation that characterizes these disorders.

Acknowledgments

We thank Kol Zarember, Sanjeev Satyal, Walter Darbonne, and Melanie Mark for helpful discussions; Peter Schow for help in FACS analysis; and the Genentech Cloning and Sequencing Groups for excellent technical assistance.

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