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Cutting Edge: FimH Adhesin of Type 1 Fimbriae Is a Novel TLR4 Ligand

Karen L. Mossman,* M. Firoz Mian,* Nicole M. Lauzon,* Carlton L. Gyles,† Brian Lichty,* Randy Mackenzie,* Navkiran Gill,* and Ali A. Ashkar2*  

Several TLR ligands of bacterial origin induce innate immune responses. Although FimH, the adhesin portion of type 1 fimbria, plays an important role in the pathogenicity of some Gram-negative bacteria, its ability to stimulate the innate immune system via TLR signaling remains unclear. In this study we report that FimH induces potent innate responses in a MyD88-dependent fashion. The FimH-induced innate activity was restricted to cells expressing TLR4. In addition, FimH was able to bind directly to TLR4. More importantly, cells unresponsive to LPS were responsive to FimH and the presence or absence of MD-2 and CD14 had no effect on FimH activity. Our data suggest that TLR4 is a functional receptor for the adhesin portion of bacterial type 1 fimbria. The Journal of Immunology, 2008, 181: 6702–6706.

Bacteria encode numerous pathogen-associated molecular patterns (PAMPs), including LPS, flagellin, peptidoglycan and bacterial DNA, that can activate the innate immune system via TLRs (1, 2). Upon ligand binding, TLRs initiate signaling via interactions between their cytoplasmic Toll/IL-1R (TIR) domain and cellular adaptor molecules. These pathways can be divided into common (MyD88-dependent) and specific (MyD88-independent) categories. TLR2, 5, 7–9, and 11 signaling is MyD88-dependent, while TLR3 and 4 signaling is MyD88-independent category. TLR2, 5, and CD14 had no effect on FimH activity. Furthermore, that type 1 fimbriae play an important role in pathogenesis. Type 1 fimbriae have long been implicated in bacterial urinary tract infections in humans (3) and have been the focus of many attempts to generate a vaccine against pathogenic Gram-negative bacteria (4, 5). In vivo studies indicate that type 1 fimbriae play an important role in pathogenesis. Furthermore, E. coli expressing both P and type 1 fimbriae were found to activate TLR4 in mucosal tissues (6). FimH, the adhesin portion of type 1 fimbriae, is involved in bacterial attachment to epithelial cells through interactions with mannose (5, 7). Although the role of FimH in pathogenicity is well appreciated, there is no report of FimH inducing innate host responses. In this study, we provide evidence that purified FimH is a protein ligand for TLR4 and induces potent proinflammatory cytokine production through MyD88. Furthermore, we show that FimH activity is independent of LPS signaling and can occur in cells unresponsive to this ubiquitous bacterial PAMP.

Materials and Methods

Cells, viruses, and reagents

RAW264.7, human embryonic lung (HEL) fibroblasts, and BJ fibroblasts were purchased from InVivoGen and maintained in DMEM with 10% FBS and 10 μg/ml blasticidin (293-hTLR4) or 10 μg/ml blasticidin and 50 μg/ml HygroGold (293-hTLR4-CD14/MD2). Vesicular stomatitis virus (VSV)-GFP expresses GFP from the viral promoter. LPS (L26–54) and polyinosinic-polycytidylic acid (polycylic acid) were purchased from Sigma-Aldrich.

Purification of FimH

The fimH gene from E. coli strain EC99 (7) was cloned into pQE-30 and expressed in BL-21-competent E. coli. FimH expression and purification were performed as previously described (8). LPS levels in FimH preparations were determined using a Limulus amebocyte lysate LPS detection kit as directed.

ELISA

TNF-α and IL-8 ELISAs were conducted using Quantikine murine kits from R&D Systems according to the manufacturer’s instructions. An unpaired t test was used to determine significant differences in cytokine production.

Preparation of peritoneal macrophages

Peritoneal macrophages were prepared from B6, MyD88−/−, and TLR4−/− mice by injecting 6 ml of warm RPMI 1640 into the peritoneal cavity and collecting the fluid after 10 min. Peritoneal macrophages were seeded at 1 × 105 cells/well in a 96-well plate, incubated for 1 h, and then washed to remove non-adherent cells.

RT-PCR

RNA was harvested using TRIZol (Invitrogen) as directed. RT-PCR was performed as previously described (9) using the following primers: TLR4 forward
MyD88 dependent.

Peritoneal macrophages from B6 and FimH or LPS to another murine membrane protein, IL-15R-gated goat anti-human IgG Ab (Bethyl Laboratories). Binding of the immobilized with PBS and Tween 20, the captured TLR4-Fc was detected using a HRP-conju-

and reverse, 5′-CTGGACACCTCTCAGTGTCC-3′ and 5′-GCCAGAAGCCTGAAATGGAGG-3′; MD-2 forward and reverse, 5′-GAGCTCAGAAGCACTTATGTTGGTC-3′ and 5′-GGTGTGTTAGTGGACAAACTCC-3′; GAPDH forward and reverse, 5′-CGAGTCAACGGATTTGGTCGTA-3′ and 5′-AGCCTTCTCCATGGTGTTAGG-3′.

Luciferase assays

293, 293-hTLR4, and 293-hTLR4-CD14-md2 cells seeded in 24-well dishes were cotransfected with pCMV-βgal (50 ng; Clontech) and pNFκB-luc (500 ng; Clontech). Sixteen hour later, cells were left untreated (control) or treated with FimH (2 μg/ml) or LPS (10 ng/ml). Six hours later, cells lysates were harvested and luciferase assays were performed as directed (BD Biosciences). Data were analyzed using GraphPad.

**FimH-TLR4 interaction/binding assay**

High-binding ELISA plates (Costar) were coated with increasing concentrations of FimH or LPS overnight at 4°C, and then blocked with 2% BSA in PBS for 1 h before incubation with 2 μg/ml human TLR4-Fc fusion protein. After three washes with PBS and Tween 20, the captured TLR4-Fc was detected using a HRP-conju-

To determine whether FimH alone can activate macrophages, we purified recombinant FimH (Fig. 1A) and tested the protein on RAW264.7 murine macrophages, which express all TLRs except TLR5. We measured production of TNF-α (Fig. 1B) and NO (Fig. 1C) in response to FimH and poly(I:C) treatment. FimH induced both TNF-α and NO. Because MyD88 null mice fail to clear P and type 1 fimbriated E. coli infection, we examined the role of MyD88 in FimH-mediated TNF-α and NO production.

Peritoneal macrophages from MyD88+/− mice were treated with FimH and the levels of TNF-α and NO were measured. MyD88−/− peritoneal macrophages were unresponsive to FimH, whereas B6 peritoneal macrophages induced significant amounts of TNF-α and NO (Fig. 1, D and E).

**FimH elicits an antiviral response in cells expressing TLR4**

Because both P and type 1 fimbriae signal through TLR4 (6), we examined whether FimH alone possesses this activity. We used either cells from TLR4−/− mice or two nonimmortalized, nontransformed human fibroblast lines that either express TLR4 (BJ foreskin fibroblast) or do not (HEL lung fibroblast) (9). The rationale for using a human cell type devoid of TLR4 was that human cells do not express TLR11, a murine-specific TLR that recognizes PAMPs from FimH+ UPEC (11). In addition to cytokine secretion (Fig. 2A), an antiviral state assay using VSV-GFP was used (Fig. 2B) where an antiviral state capable of blocking VSV-GFP replication is indicated by the absence of GFP fluorescence. All cells tested responded to poly(I:C) (Fig. 2). However, peritoneal macrophages from TLR4−/− mice were unresponsive to both LPS and FimH (Fig. 2A). At concentrations of LPS ≥10 ng/ml, both BJ fibroblasts and B6 MEFs responded to LPS, confirming the presence of TLR4. In contrast, HEL fibroblasts failed to respond to LPS, even at 1000-fold higher concentrations (10). Similarly, whereas no antiviral activity was observed in HEL fibroblasts treated with FimH, FimH induced a complete antiviral response in both BJ fibroblasts and B6 MEFs (Fig. 2B). Complete abrogation of responsiveness to FimH was observed at 30 ng/ml and 10 ng/ml in B6 MEFs and BJ fibroblasts, respectively (data not shown).

**FimH-induced innate immunity is not due to LPS or other bacterial contaminants**

We next tested the levels of LPS in our column-purified recombinant FimH preparations using a standard Limulus amoebocyte lysate assay. The samples had very low LPS contamination, with 4–9 pg of LPS per μg of FimH protein. This level of LPS contamination would account for a maximum of 1.7 pg of LPS per 250 ng of...
FimH. Two hundred fifty ng/ml FimH demonstrates complete antiviral activity whereas 1 ng/ml LPS does not. To verify that contamination with LPS or other bacterial components is not responsible for the FimH activity, we produced preparations from bacteria that contained either the FimH-expressing plasmid or a control plasmid. Although samples with or without FimH were prepared in exactly the same manner and had similar levels of LPS, only FimH\(^+\)/H11001 preparations provided antiviral protection in BJ fibroblasts (Fig. 3A) or B6 MEFs (data not shown) and induced TNF-\(\alpha\)/H9251 in peritoneal macrophages from B6 mice, but not TLR4\(^{-/-}\)/H11002 mice (Fig. 3B) or human PBMCs (data not shown). Moreover, enzymatic digestion or heat inactivation of FimH significantly abrogated its activity (Fig. 3C).

FimH signals via TLR4 in the absence of Md-2 and CD14 and binds directly to TLR4

TLR4 and MD-2 are indispensable for LPS responses (12–15). To confirm that FimH signaling via TLR4 is independent of...
LPS signaling, we used the well established 293 cells stably transfected with either TLR4 alone (LPS unresponsive) or with TLR4 and MD-2/CD14 (LPS responsive). We first confirmed the expression of TLR4, Md-2, and CD14 (Fig. 4A). We then transiently transfected 293, 293/TLR-4, or 293/TLR-4/Md2/CD14 cells with a plasmid expressing luciferase from a TAT-like promoter fused to multiple copies of the NF-κB consensus sequence. FimH induced luciferase expression in both 293/TLR4 and 293/TLR4/Md2/CD14 cells, whereas LPS only induced luciferase in 293/TLR4/Md2/CD14 cells (Fig. 4C). It is well known that LPS signaling induces the production of IL-8. To confirm that FimH can induce the production of IL-8 in the absence of LPS signaling, we measured the levels of IL-8 production in 293/TLR4 cells. FimH was able to induce IL-8 production in the absence of Md-2 or CD14 (Fig. 4C). To determine whether FimH can directly bind TLR4, we performed an in vitro protein binding assay (Fig. 4D) using recombinant TLR4 fused to the Fc fragment of human IgG1 (TLR4-Fc; R&D Systems). A strong dose-dependent binding of immobilized FimH to TLR4 was observed. LPS, a known ligand for TLR4, was used as control. FimH, a defining feature of pathogenic bacteria. UPEC are able to express P, type 1, S, and F1C fimbriae. The type 1 fimbriae gene cluster contains 9 genes (FimA–FimI), of which four gene products, FimA, FimF, FimG, and FimH, form the fimbriae (16, 17). FimH is located at the tip and is involved in recognition by mannose. Recent crystal structure determination of FimH complexed with oligomannose-3 highlighted the feasibility of using natural and engineered mannose antagonists to block bacterial invasion and growth and biofilm formation (18). Previous studies using intact UPEC showed that both P and type 1 fimbriae signal through TLR4 to elicit chemokine production and neutrophil infiltration (6). In this study, we demonstrate that purified FimH protein is able to stimulate cytokine production through TLR4. These observations suggest that whereas UPEC use FimH to bind mannose on uroepithelial cell surfaces, the innate immune response is simultaneously recognizing FimH in a TLR4-dependent fashion. Absence of TLR4 on the host cells or FimH on the bacteria leads to significant reduction in the ability of UPEC to infect the urinary tract (data not shown).

The potent stimulatory activity of FimH is very unlikely to be due to contamination or synergy with LPS because of the following: 1) the amount of LPS required to induce an antiviral state in responsive cells was ~1000-fold higher than the levels of contaminating LPS in FimH preparations; 2) control preparations from bacteria not expressing FimH but processed identically to those expressing FimH had no stimulatory activity; 3) enzymatic digestion and heat inactivation of FimH significantly abrogated its activity; 4) the stimulatory activity of FimH

**Discussion**

Bacteria encode many diverse PAMPs that serve as potent stimulators of the innate immune system. Adhesive pili, or fimbriae,
was similar in LPS-responsive and -unresponsive cells, suggesting that FimH signals via TLR4 independently of MD-2; and 5) future purification of FimH by fast protein liquid chromatography, with no detectable LPS, had no effect on the potency of FimH activity (data not shown). Furthermore, whereas FimH has potent antiviral activity in an in vivo HSV-2 infection model, we detected no antiviral activity against high levels of LPS (data not shown).

The ability of the host to distinguish specific bacterial ligands, such as FimH, from common bacterial ligands, such as LPS, is critical to ensuring an effective host response to pathogenic organisms while maintaining tolerance to commensal organisms. Thus, it is not surprising that mucosal epithelial cells express TLR4 but not CD14, a coreceptor required for LPS recognition (19). As such, FimH serves as a potent stimulator of the innate immune response via interaction with TLR4 independently of LPS, allowing for recognition of pathogenic E. coli.

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Disclosures

A patent for the use of FimH as an innate microbicidal and antitumor agent has been filed.

References