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RESEARCH ARTICLE
Host-Microbe Biology

Rapid Bladder Interleukin-10 Synthesis in Response to Uropathogenic *Escherichia coli* Is Part of a Defense Strategy Triggered by the Major Bacterial Flagellar Filament FliC and Contingent on TLR5

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ABSTRACT Urinary tract infection (UTI) caused by uropathogenic *Escherichia coli* (UPEC) engages interleukin-10 (IL-10) as an early innate immune response to regulate inflammation and promote the control of bladder infection. However, the mechanism of engagement of innate immunity by UPEC that leads to elicitation of IL-10 in the bladder is unknown. Here, we identify the major UPEC flagellar filament, FliC, as a key bacterial component sensed by the bladder innate immune system responsible for the induction of IL-10 synthesis. IL-10 responses of human as well as mouse bladder epithelial cell-monocyte cocultures were triggered by flagella of three major UPEC representative strains, CFT073, UTI89, and EC958. FliC purified to homogeneity induced IL-10 *in vitro* and *in vivo* as well as other functionally related cytokines, including IL-6. The genome-wide innate immunological context of FliC-induced IL-10 in the bladder was defined using RNA sequencing that revealed a network of transcriptional and antibacterial defenses comprising 1,400 genes that were induced by FliC. Of the FliC-responsive bladder transcriptome, altered expression of *il10* and 808 additional genes were dependent on Toll-like receptor 5 (TLR5), according to analysis of TLR5-deficient mice. Examination of the potential of FliC and associated innate immune signature in the bladder to boost host defense, based on prophylactic or therapeutic administration to mice, revealed significant benefits for the control of UPEC. We conclude that detection of FliC through TLR5 triggers rapid IL-10 synthesis in the bladder, and FliC represents a potential immune modulator that might offer benefit for the treatment or prevention of UPEC UTI.

IMPORTANCE Interleukin-10 is part of the immune response to urinary tract infection (UTI) due to *E. coli*, and it is important in the early control of infection in the bladder. Defining the mechanism of engagement of the immune system by the bacteria that enables the protective IL-10 response is critical to exploring how we might exploit this mechanism for new infection control strategies. In this study, we reveal part of the bacterial flagellar apparatus (FliC) is an important component that is sensed by and responsible for induction of IL-10 in the response to UPEC. We show this response occurs in a TLR5-dependent manner. Using infection prevention and control trials in mice infected with *E. coli*, this study also provides evidence that purified FliC might be of value in novel approaches for the treatment of UTI or in preventing infection by exploiting the FliC-triggered bladder transcriptome.

KEYWORDS flagella, urinary tract infection, uropathogenic *Escherichia coli*

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Urinary tract infections (UTI) are common illnesses, predominantly affecting women and causing more than ten million ambulatory visits per year in the United States alone (1). Expenditures aimed at the management of UTI account for approximately $3.5 billion in medical costs annually (2). Up to 80% of acute UTI cases are caused by uropathogenic *Escherichia coli* (UPEC) (3). Studies have shown key roles for virulence factors of UPEC, such as flagella, autotransporters, capsule, fimbriae, toxins, lipopolysaccharide (LPS), and siderophores, in UTI disease pathogenesis (4–7).

The innate immune signature of acute UPEC UTI is reviewed elsewhere (7); it encompasses various cytokines, including interleukin-10 (IL-10), that is upregulated in the bladder within a few hours of experimental infection in mice (8). IL-10 is secreted in urine of adults who exhibit symptomatic UTI (8, 9) and is induced in several *in vitro* models of UTI, including in monocytes and mast cells (10, 11) and bladder epithelial cell-monocyte cocultures (10), which are used to model host-pathogen interactions (12). IL-10 plays pleiotropic roles in defense against infection depending on the illness and the causal pathogen. Frequently, IL-10 facilitates immune suppression to moderate inflammatory mechanisms that can damage the host (13–16). The contribution of IL-10 to resolution of infection reflects its tightly controlled expression, which can be a key factor in determining disease outcome (17–19). Functionally, an absence of IL-10 in mice exacerbates the host's ability to control bacterial colonization during the innate phase of infection in the bladder (8). Reflecting its central regulatory role in many diseases and its ability to reduce tissue damage and protect tissue integrity, IL-10 is the subject of clinical trials for inflammatory diseases; however, its manipulation for benefit in a therapeutic setting remains experimental (20).

One facet in understanding the role of IL-10 in infectious disease is elucidation of microbial products that elicit production of this key regulator of innate immune responses. Bacterial virulence factors shown to induce the production of IL-10 in experimental disease models include M protein of *Streptococcus* (21), peptidoglycan-embedded lipopeptides and cell wall glycopolymers of *Staphylococcus* (22), and flagella of *Salmonella* (23) and *Yersinia* (24). For some other pathogens that trigger the production of IL-10, including *Helicobacter* and *Chlamydia*, links between virulence factors and IL-10 elicitation remain elusive. The nature of the host response encompassing IL-10 can also depend on the genus or species of origin from which the pathogen virulence factor is derived (25–32).

Flagella of UPEC contribute to the pathogenesis of UTI in several ways, including through motility that is associated with bacterial ascension from the bladder to the kidneys, leading to the development of pyelonephritis (33, 34). Expression of flagella by UPEC has also been associated with enhanced urinary tract colonization, invasion of host cells (35, 36), survival inside macrophages (37), and biofilm formation (38, 39). The flagellar filament is synthesized as a polymerized product of >20,000 protein monomers, termed flagellin or FliC (usually encoded by *fliC*), as reviewed elsewhere (40). In mammals, flagella are characteristically sensed through Toll-like receptor 5 (TLR5), which recognizes FliC monomers but not flagellar filaments (41–45). FliC can also be detected by NLR family apoptosis inhibitory protein 5 (NAIP5) and Ipaf within the intracellular environment (46, 47). Initial observations suggested that TLR11 senses flagellin (48–51); however, it has since been established that binding of flagellin to TLR11 does not occur, and the responses of wild-type and TLR11-deficient mice to flagellin are similar (52). A detailed understanding of how FliC from UPEC engages innate immunity in the bladder during UTI is lacking (34, 53–55), and the potential contribution of FliC to rapid IL-10 induction in the bladder during UTI is unknown. In this study, we examined the role of FliC in the bladder innate immune response to UPEC, with a focus on early IL-10 induction and the role of TLR5 in the FliC-driven bladder defense response.

RESULTS

Effect of flagellar expression on UPEC-induced IL-10 in uroepithelial cell monocyte cocultures. In initial experiments testing the effect of differential UPEC flagellar

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
E. coli strains		
DH5α	Cloning strain; d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1*	Bethesda Research Laboratories
MC4100	*E. coli* K-12 strain, OR:H48	111
MC4100/p*flhDC*	MC4100 containing p*flhDC*; Kn^r	112
CFT073	Reference UPEC strain, O6:K2:H1 (ATCC 700928)	101
UTI89	Reference UPEC strain, O18:K1:H7	102
EC958	Reference ST131 UPEC strain, O25b:K100:H4	103
GU2139	CFT073/p*flhDC*; Kn^r	57
GU2639	CFT073Δ*fliC*; Kn^r	57
GU2671	UTI89Δ*fliC*; Kn^r	This study
EC958Δ*fliC*	EC958Δ*fliC*; Cm^r	68
CFT073Δ*4*	CFT073 with combined deletions Δ*fim*, Δ*foc*, Δ*pap1*, and Δ*pap2*	113
GU2647	CFT073Δ*4*/p*flhDC* (p*flhDC*); Kn^r	57
GU2642	CFT073Δ*4* Δ*fliC*; *fliC*$^-$ derivative of CFT073Δ*4*	57
GU2648	GU2642/p*flhDC*; Kn^r (for carrier control)	57
Plasmids		
p*flhDC*	*flhDC* operon from *Serratia* in pVLT33; Cm^r	112
pKD4	Template plasmid for *kan* gene amplification	104
pKD46	λ-Red recombinase expression plasmid	104
pCP20	FLP synthesis under thermal control	104

expression on IL-10 induction, we used liquid-grown wild-type (WT) and *fliC*-deficient CFT073 and *E. coli* MC4100 (deficient for flagella due to a frameshift mutation in the *flhD* master regulator [56]) and MC4100/p*flhDC* (p*flhDC* was used to confer a hyperflagellated state) (Table 1). Uroepithelial cell-monocyte cocultures exhibited a 7-fold increase in IL-10 at 5 h after infection with MC4100/p*flhDC* and a 4-fold increase for other infections (on average) versus noninfected controls (Fig. 1A). The level of IL-10 induced by MC4100/p*flhDC* compared to that of MC4100 WT was statistically significant ($P = 0.02$); there was no difference between CFT073 WT and CFT073Δ*fliC* strains under these conditions. We next tested bacteria grown on soft agar, which induces swarming associated with increased flagellin expression. In these assays, CFT073 WT induced significantly more IL-10 than the CFT073Δ*fliC* strain but less IL-10 than CFT073/p*flhDC* (Fig. 1B). Similar IL-10 responses occurred in cultures exposed to MC4100 WT and MC4100/p*flhDC*. Experiments comparing the responses of human cell cocultures to UPEC UTI89 and EC958 and their respective *fliC*-deficient derivatives showed equivalent trends in which higher levels of IL-10 were induced by UPEC expressing flagella than *fliC*-deficient mutants (Fig. 1C).

Measurement of IL-10 induction in response to UPEC CFT073 and derivatives was then undertaken using a multiplex assay to explore functionally opposed (e.g., IL-12p70, IL-2, and tumor necrosis factor alpha [TNF-α]) and related cytokines (e.g., IL-4 and IL-6) and thereby gain a broader picture of the immunological context of IL-10 induction (14). In contrast to the induction of IL-10 observed in the response to hyperflagellated UPEC CFT073/p*flhDC* compared to WT (and significantly lower levels than the CFT073Δ*fliC* strain), there were no changes in levels of IL-12p70 (Fig. 1D); however, statistically significant changes in several other cytokines, including IL-1α, -1β, -2, -4, -6, and TNF-α, and multiple chemokines (e.g., granulocyte colony-stimulating factor [G-CSF]) were detected (see Fig. S1 in the supplemental material). Hyperflagellation in *flhDC*-complemented UPEC strains and an absence of flagellar expression in *fliC*-deficient strains was confirmed using immunoblots for FliC (Fig. S2A), as previously described (57); in addition, motility assays showed phenotypes consistent with hyperflagellation in *flhDC*-complemented UPEC strains (Fig. S2B). Taken together, these data show that flagellar expression in CFT073 and other UPEC strains, including UTI89 and EC958, induces the production of IL-10 in uroepithelial cell monocyte cocultures as well as significant induction of several other functionally opposed and related cytokines.

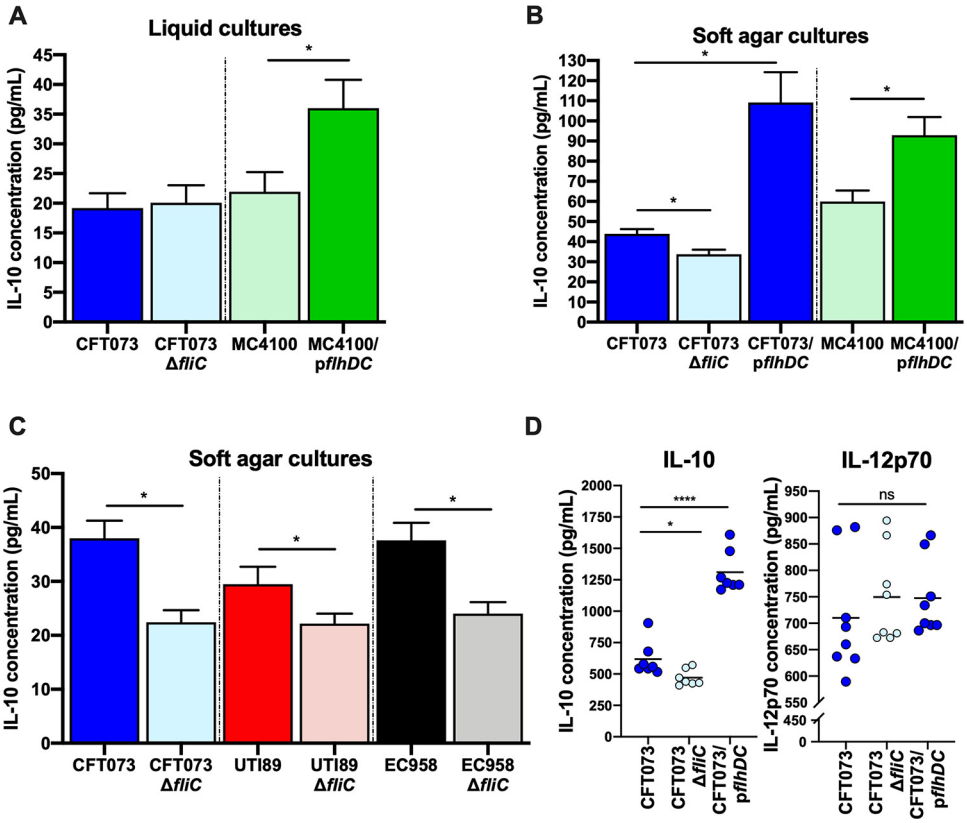


FIG 1 IL-10 production in uroepithelial cell monocyte cocultures challenged with UPEC CFT073 and other *E. coli* strains with altered flagellar expression. (A) Human 5637-U937 cocultures exposed to liquid-grown CFT073 and *fliC*-deficient CFT073 or MC4100 with or without p*flhDC* for hyperflagellation. Significance was determined by *t* test for MC4100 versus MC4100/p*flhDC* (*, $P = 0.02$). (B) Human cell cocultures exposed to soft-agar-grown CFT073 or *fliC*-deficient and p*flhDC* derivatives and MC4100 strains. Significance was determined by ANOVA for CFT073 strains and *t* test for MC4100 strains (*, $P = 0.02$). (C) Responses of human 5637-U937 cocultures to CFT073, UTI89, and EC958 (soft agar grown) and their *fliC*-deficient mutants. (D) Responses of IL-10 and the functionally opposed cytokine IL-12p70 in cocultures exposed to CFT073, CFT073Δ*fliC*, and CFT073/p*flhDC* strains according to multiplex analysis. Significance was determined by ANOVA with Tukey's *post hoc* analysis (*, $P < 0.05$). Additional responses of other cytokines and chemokines are illustrated in Fig. S1.

IL-10 responses of cell cultures to enriched flagella and purified FliC. Experiments examining the responses of uroepithelial cell monocyte cocultures to flagellum-enriched protein from CFT073 (isolated by shearing and ultracentrifugation) showed significant IL-10 responses to flagella from CFT073 WT (versus the CFT073Δ*fliC* mutant) and MC4100/p*flhDC* (versus MC4100 WT) (Fig. 2A). Subsequently, we measured IL-10 levels in response to FliC purified to homogeneity from CFT073Δ*4*/p*flhDC* using fast protein liquid chromatography (FPLC), because flagellum-enriched preparations contain trace amounts of other outer membrane proteins that could contribute to IL-10 induction (57). Pure FliC triggered significantly more IL-10 than the carrier control that was generated from the CFT073Δ*4* Δ*fliC* strain (Fig. 2B). Similar responses were observed for mouse macrophages but did not reach statistical significance due to higher basal levels of IL-10 detected in these experiments (data not shown). Taken together, these findings show that pure FliC stimulates significant IL-10 synthesis in human uroepithelial cell monocyte cocultures.

IL-10 and related responses of the mouse bladder to FliC. We next analyzed the bladder response in mice that received either 30 μg of pure FliC from CFT073Δ*4*/p*flhDC* or the equivalent volume of carrier control generated from the CFT073Δ*4* Δ*fliC*/p*flhDC* strain. Transurethral delivery of FliC triggered significant production of IL-10 in the bladders of mice at 2 h postinoculation compared to that of control mice according to multiplex assay (Fig. 3A). Levels of IL-6, often associated with IL-10-regulated responses,

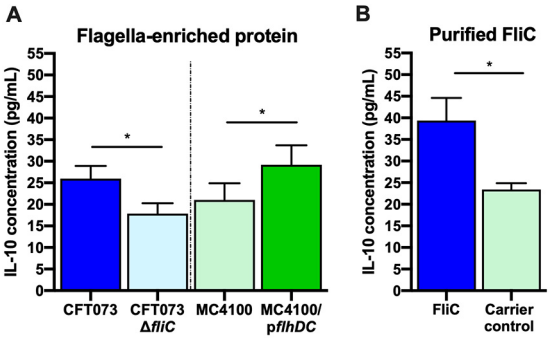


FIG 2 IL-10 production in human cells *in vitro* after stimulation with flagella and purified FliC from UPEC CFT073. (A) Human uroepithelial cell monocyte cocultures stimulated (5 h) with flagellum-enriched protein (1 μg) from CFT073 and CFT073Δ*fliC* strains or MC4100 with or without p*flhDC*. Significance was determined by *t* test for CFT073 strains (*, $P < 0.05$). (B) Monocytes stimulated (5 h) with purified FliC (1 μg) from CFT073Δ*4* strain or carrier control (generated from CFT073Δ*4*Δ*fliC*). Significance was determined by *t* tests (*, $P < 0.05$).

were also elevated (Fig. 3B), as were levels of IL-1α, IL-1β, and several chemokines, including monocyte chemoattractant protein 1 (MCP-1/CCL2), macrophage inflammatory protein 1α (MIP-1α/CCL3) and -β (MIP-1β/CCL4), and RANTES (CCL5) (Fig. 3). However, there were no significant changes in levels of IL-12p40, IL-12p70, TNF-α, IL-2, IL-4, IL-5, or IL-13 (Fig. S3). Data generated using enzyme-linked immunosorbent assay (ELISA) for IL-10 were consistent with elevated levels of IL-10, as detected by multiplex assay (Fig. S4). Thus, UPEC FliC causes rapid induction of IL-10 in the bladder with concurrent early responses for IL-6, IL-1, and multiple chemokines.

The FliC-responsive bladder transcriptome and dependency on TLR5. We next defined a more complete picture of the innate immune response of mouse bladder to FliC using RNA sequencing to comprehensively map the transcriptional responses that initiated with early IL-10 induction. Bladders of WT mice exposed to FliC or carrier control exhibited distinct global transcriptional signatures (Fig. 4A) that encompassed 1,400 significant gene responses, represented by 831 upregulated and 569 downregulated genes (Fig. 4B); significance criteria included a fold change of $\geq \pm 2.0$ and *q* value of <0.05, as described in Materials and Methods. Upregulated genes of particular interest in the context of IL-10 and innate immune activation included *il10* (3.3-fold), *il6* (3.2-fold), *il1a* (5.1-fold), *il1b* (10.9-fold), *ccl2* (14.0-fold), *ccl3* (11.5-fold), *ccl4* (9.6-fold), *ccl5* (2.6-fold), and *tnf* (15.5-fold); the responses of these genes are illustrated as absolute transcript abundance for control and FliC groups in Fig. 4C. The complete list of significant gene responses is listed in Data Set S1. The *tlr5* gene was significantly downregulated (2.3-fold). Notably, many transcriptional responses detected by RNA sequencing exhibited consistency with parallel translational activities detected in the bladder, including those for IL-10, -6, and -1 and chemokines, according to the multiplex protein assays (Fig. 3 and Fig. S4).

The top five canonical pathways (generated by Reactome analysis within innateDB [58] and ranked according to significance from overrepresentation analysis [ORA]) are summarized in Fig. 4D. These data highlight the extensive activation of networks related to cytokine signaling in the innate immune system and TLR cascades activated as a result of FliC treatment (complete list is in Data Set S1). Integrating Network Objects with Hierarchies (INOH) analysis identified similar strongly activated biological processes in FliC-treated WT mice, including TLR signaling, JAK STAT pathway activity, and GPCR signaling (Fig. 4D and Data Set S1). Taken together, these data illustrate an overall FliC-responsive bladder transcriptome that is characterized by extensive cytokine and TLR signaling and innate immune regulatory processes that are collectively engaged with early *il10* induction.

Comparative analysis of TLR5-deficient mice enabled delineation of the bladder responses of WT mice that are contingent on TLR5; this identified a total of 809 genes

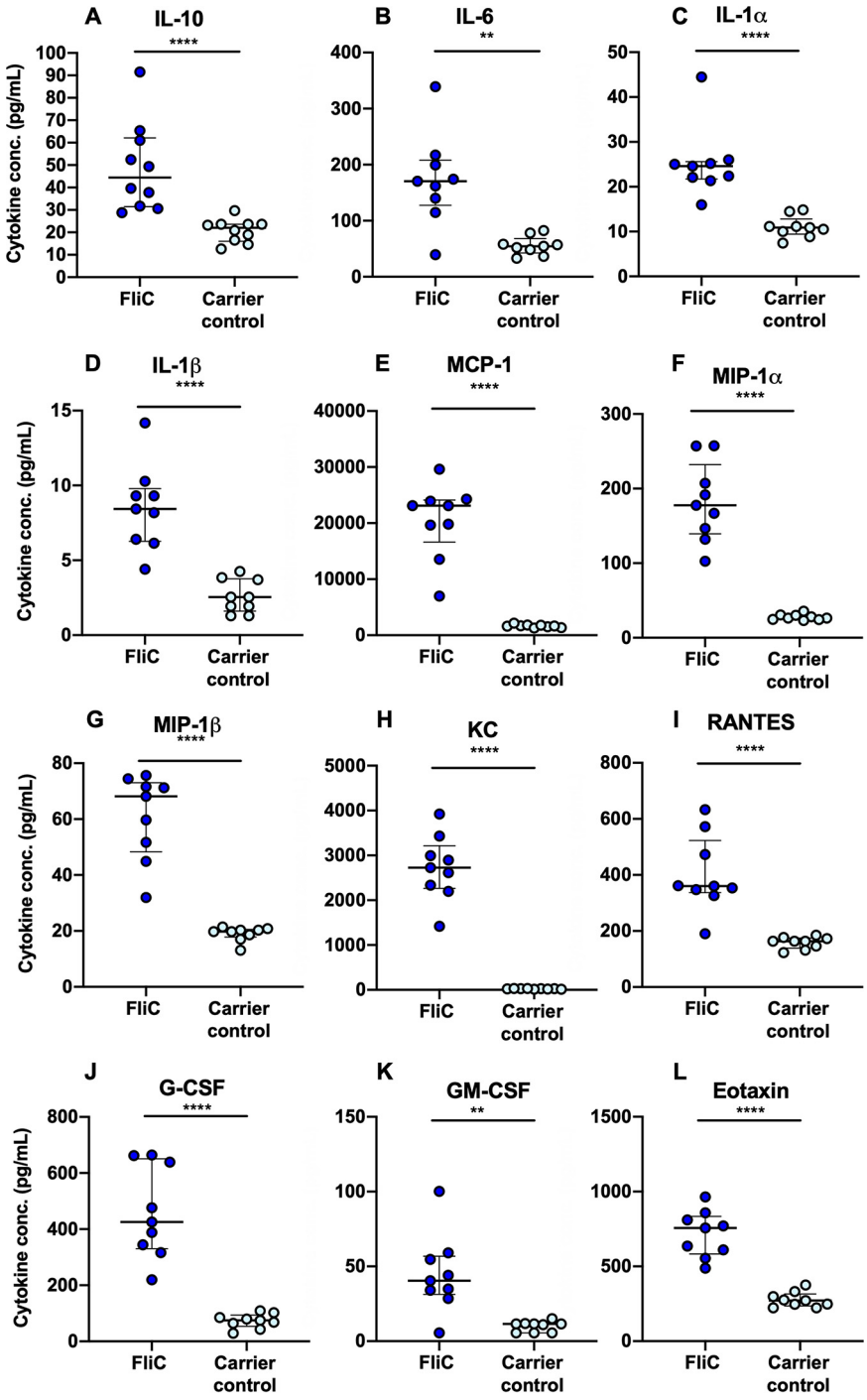
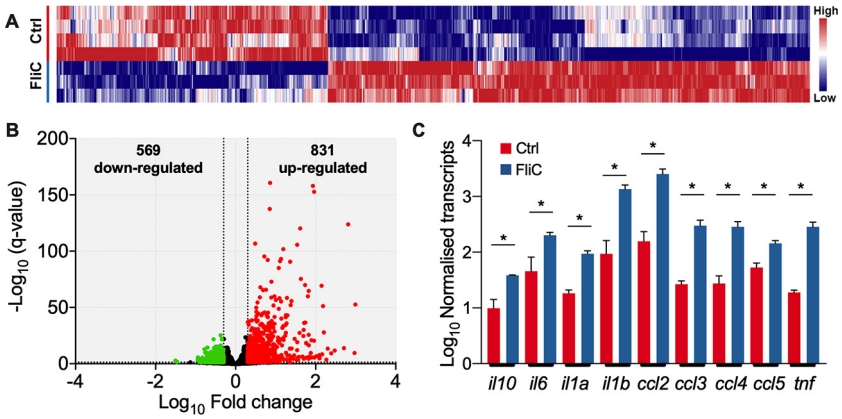


FIG 3 Bladder IL-10 and other cytokine responses in mice treated with purified FliC from UPEC CFT073Δ*4* strain. Multiplex analysis of IL-10 and other cytokines in bladder homogenates at 2 h following transurethral delivery of 30 μg FliC or carrier control. Significance was determined by *t* test for FliC versus the control (*, $P < 0.05$; ****, $P < 0.0001$). All cytokines that exhibited significantly altered expression are shown, with additional multiplex data (for nonsignificant factors) provided in Fig. S3. Data shown represent at least 2 independent experiments with separate groups of mice ($n = 9$ [at least] per group).

(652 upregulated, 157 downregulated), including *il10*, that depend on TLR5 for their activation or repression in response to FliC; these are illustrated according to topology analysis of key nodes in Fig. 5 (59). Heat maps and a volcano plot representing the FliC-responsive bladder transcriptome of WT and TLR5-deficient mice are shown in Fig. S5. The complete list of bladder transcriptional responses and biological pathways



D

Reactome	ORA (P-adj)	Gene-products
Cytokine Signaling in Immune system	36/194 (<0.005)	Csf2; Fcgr1; Gbp2; H2-Q7; Icam1; Ifit1; Il1a; Il1b; Il1r2; Il1rn; Il2ra; Il6; Irak3; Irf1; Irf7; Irf9; Isg15; Lyn; Map2k6; Map3k8; Mt2; Myd88; Nfkb1; Nfkb2; Nod2; Oas1a; Oas2; Oas3; Oasl1; Ptpn2; Ripk2; Socs1; Sp100; Stat5a; Ube2l6; Usp18;
Innate Immune System	61/450 (<0.005)	Adcy1; Adcy7; Adcy8; Adcy9; Birc2; Birc3; Cd14; Cd180; Clec5a; Ddx58; Defb1; Fcgr1; Fcgr2b; Fgf23; Fgr; Hc; Hck; Ifi205; Ifih1; Ikbke; Il6; Irak3; Irf1; Irf7; Kit; Lbp; Lcp2; Ly96; Lyn; Malt1; Map2k6; Mapk11; Mapk13; Mef2c; Mefv; Mnda; Mndal; Myd88; Nfkb1; Nfkb2; Nfkbia; Nfkbib; Nlrp3; Nod2; Phlpp1; Rasgrp1; Ripk2; Rps6ka1; Saa1; Saa2; Saa3; Socs1; Tlr12; Tmem173; Tnfaip3; Traf2; Trem1; Treml4; Trim25; Vav2; Zbp1;
Toll-Like Receptors Cascades	26/127 (<0.005)	Birc2; Birc3; Cd14; Cd180; Ikbke; Il6; Irak3; Irf7; Lbp; Ly96; Map2k6; Mapk11; Mef2c; Myd88; Nfkb1; Nfkb2; Nfkbia; Nfkbib; Nod2; Ripk2; Rps6ka1; Saa1; Saa2; Saa3; Socs1; Tlr12;
INOH	**ORA (P-adj)**	**Gene-products**
Toll-like receptor signaling pathway	11/40 (<0.005)	Cd14; Lbp; Ly96; Map2k6; Mapk11; Mapk13; Myd88; Nfkb1; Nfkbia; Nfkbib; Nfkbie;
JAK STAT pathway and regulation	27/204 (<0.005)	Csf2; Csf3; Csf3r; Cxcr2; Ikbke; Il10; Il13ra1; Il1a; Il1b; Il1r2; Il2ra; Il4ra; Il6; Kit; Lif; Map2k6; Map3k8; Mapk11; Mapk13; Pak3; Prkag3; Ripk2; Rps6ka1; Socs1; Stat5a; Tnf; Tnfsf10;
GPCR signaling	25/243 (<0.05)	Adcy7; Adcy8; Adcy9; Bmp10; Csf2; Csf3; Cxcr2; Dll1; Fgf23; Fzd2; Gng2; Il10; Il1a; Il1b; Il6; Inhba; Lif; Ntf3; Rgs16; Rgs18; Rgs9; Tnf; Tnfsf10; Wnt5a; Wnt9a;

FIG 4 Bladder transcriptome in WT mice in response to pure FliC from UPEC CFT073Δ*4*. (A) Heat map of transcriptional changes in mouse bladder in response to 30 μg pure FliC (in 50 μl carrier) or equivalent volume of carrier control (Ctrl) (2-h exposure). (B) Volcano plot of the total number and the breadth of fold change of transcriptional response of genes exhibiting significantly altered expression (fold change of $\geq\pm2.0$, *q* value of <0.05) in the bladder response to pure FliC. (C) Normalized transcript abundances for *il10* and several other genes encoding cytokines in the FliC-treated and carrier control groups (bars represent the means ± SEM; an asterisk denotes a fold change of $\geq\pm2.0$ and *q* value of <0.05. (D) Top canonical biological pathways, according to Reactome (upper) and Integrating Network Objects with Hierarchies (INOH) analysis (lower).

activated in response to FliC in a TLR5-dependent manner (i.e., WT versus TLR5-deficient mice) is provided in Data Set S2 (also provides a complete list of TLR5-independent responses). Unexpectedly, this analysis also revealed 591 genes that exhibited significantly altered expression in response to FliC independent of TLR5; this comprised 591 genes (179 upregulated and 412 downregulated genes; i.e., responses exclusive to FliC-treated WT versus carrier-treated WT mice and absent from a comparison of FliC-treated WT versus FliC-treated TLR5-deficient mice). A summary of the top 30 gene responses triggered by FliC via TLR5-dependent and -independent mechanisms is provided in Table 2. A visual summary of the responses in the form of a Venn diagram is provided in Fig. S6. The cellular context of TLR5-dependent and -independent responses identified genes with altered expression in the significantly activated TLR signal transduction pathway, as defined by innateDB and KEGG (Fig. 6). Taken together, these data establish that *il10* transcriptional activation is part of a rapid bladder defense strategy in response to FliC, which occurs in a TLR5-dependent manner; additionally, *il10* is part of a broader response that is initiated concurrently with an assembly of other TLR5-dependent, as well as TLR5-independent, transcriptional responses.

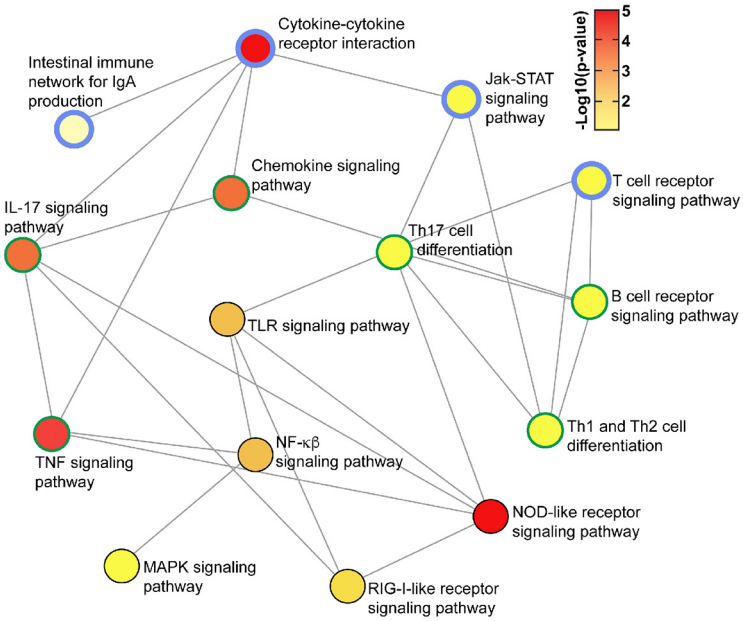


FIG 5 Topology network of interactive elements of the TLR5-dependent, FliC-responsive bladder transcriptome. The network highlights key nodes that include *il10* (blue edge) at the top of the network and the nodes that are directly (green edge) and indirectly (black edge) associated with *il10*-containing nodes. The network incorporates significant elements of cytokine-cytokine receptor interactions, IL-17 and chemokine signaling, lymphocyte signaling and differentiation, and underlying signaling pathways, such as those for NF-$\kappa\beta$ and MAPK. Images were derived using Network Analyst (59) and based on KEGG ontologies, with colors related to the significance of pathway activation.

Controlling UPEC UTI through FliC-mediated innate immunity. After establishing a comprehensive transcriptional picture of the bladder innate immune signature generated in response to FliC, we next examined whether this signature could be exploited for infection control. For this, mice were administered 30 μg FliC into the bladder at 2 h prior to, or 24 h after, infectious challenge with UPEC, and bacterial loads were subsequently determined (24 h later). Mice that received prophylactic FliC had 80% fewer UPEC in the bladder than control mice that received carrier alone ($P = 0.028$) (Fig. 7A). Similarly, mice that received FliC therapeutically exhibited 90% fewer UPEC in the bladder than control mice ($P = 0.039$) (Fig. 7B). There were no significant differences in the numbers of UPEC in urine or kidneys of mice between the FliC treatment groups and carrier control groups (Fig. S7). Of note, mice treated prophylactically with the carrier control exhibited significantly more UPEC in urine and kidneys than mice treated therapeutically with carrier control (similar trends were noted for mice treated with FliC) (Fig. S7). Taken together, these data provide experimental evidence that the immune regulatory activity induced by FliC in the bladder can be harnessed to enhance the ability of the host to control UPEC locally in the context of both pre- and postexposure to FliC.

DISCUSSION

This study was aimed at defining whether UPEC flagella, typically associated with motility and bacterial adherence, are sensed by the bladder innate immune system as part of a defense strategy utilizing IL-10 to control infection (8). The principle finding is that detection of the major flagellar filament of UPEC, FliC, within the bladder causes a very rapid local response, resulting in IL-10 synthesis. This study also shows that the bladder IL-10 response induced by FliC is contingent upon signaling through TLR5; this study does not show that IL-10 induction is the main effect of FliC but rather that high-resolution mapping of the FliC-responsive bladder transcriptome provides new, comprehensive details of how IL-10 is part of a broader bladder response to the major flagellar filament protein. Combined with the diversity of UPEC strains (of defined

TABLE 2 Top 30 genes in the bladder transcriptional response to FliC that are altered in expression via TLR5-dependent and -independent mechanisms

Gene	Fold change[a]	*P* value	Annotation
Upregulated TLR5-dependent response			
ccl20	980.3	4.94E−56	Chemokine (C-C motif) ligand 20
ngp	934.1	5.55E−12	Neutrophilic granule protein
slc6a14	647.2	5.13E−128	Solute carrier family 6, member 14
sprr2e	510	1.85E−16	Small proline-rich protein 2E
adgrf1	298	3.19E−14	Adhesion G protein-coupled receptor F1
saa1	245	1.33E−11	Serum amyloid A1
sprr2d	199.8	5.15E−16	Small proline-rich protein 2D
saa2	185.1	1.70E−05	Serum amyloid A1
ltf	159.9	7.92E−31	Lactotransferrin
abcc8	152	1.45E−54	ATP-binding cassette, subfamily C member 8
olfm4	145.6	1.06E−10	Olfactomedin 4
gm16685	140.2	6.06E−73	Predicted gene, 16685
gm5483	123.7	1.11E−07	Predicted gene, 5483
sprr2h	123.4	5.96E−10	Small proline-rich protein 2H
slc26a4	104.4	8.14E−09	Solute carrier family 26, member 4
Downregulated TLR5-dependent response			
fam131b	−4.7	2.84E−03	Family with sequence similarity 131, member B
oprk1	−4.8	3.69E−04	Opioid receptor, kappa 1
pnmal2	−4.9	1.38E−05	PNMA-like 2
bach2	−5.1	5.86E−17	BTB and CNC homology, basic leucine zipper transcription factor 2
gm4869	−5.3	1.05E−08	Predicted gene, 4869
slc16a14	−5.3	2.76E−12	Solute carrier family 16 (monocarboxylic acid transporters), member 14
gas1	−5.4	4.31E−05	Growth arrest specific 1
rbbp8nl	−5.4	2.87E−06	RBBP8 N-terminal like
oprd1	−6	1.89E−15	Opioid receptor, delta 1
alkal1	−6.1	1.88E−03	ALK and LTK ligand 1
evx2	−6.8	1.71E−07	Even-skipped homeobox 2
fam47e	−6.9	1.42E−03	Family with sequence similarity 47, member E
gm15513	−7.4	1.83E−03	Predicted gene, 15513
foxn1	−7.6	1.69E−05	Forkhead box N1
gm37711	−8.8	9.75E−08	Predicted gene, 37711
Upregulated TLR5-independent response			
mrgpra2b	124.57	1.02E−06	MAS-related GPR, member A2B
mir351	27.64	2.83E−06	MicroRNA 351
igkv12-89	22.77	6.51E−03	Immunoglobulin kappa chain variable 12-89
gbp6	12.18	2.48E−21	Guanylate binding protein 6
gm9378	10.82	8.14E−05	Predicted gene 9378
gm24245	9.86	3.22E−03	Predicted gene 24245
fam26f	9.05	5.11E−20	Family with sequence similarity 26, member F
gm43305	7.47	6.25E−03	Predicted gene 43305
c030013C21Rik	6.46	1.47E−07	RIKEN cDNA C030013C21 gene
slpi	6.12	2.06E−21	Secretory leukocyte peptidase inhibitor
clca3b	6.08	7.96E−03	Chloride channel accessory 3B
ang4	5.86	2.09E−03	Angiogenin, ribonuclease A family, member 4
rem2	5.51	3.73E−03	rad- and gem-related GTP binding protein 2
ldoc1	5.51	7.72E−05	Regulator of NFKB signaling
gm8818	4.66	9.33E−04	Predicted pseudogene 8818
Downregulated TLR5-independent response			
gm34583	−6.32	4.17E−05	Predicted gene 34583
pcdhb2	−6.41	1.96E−03	Protocadherin beta 2
5830418P13Rik	−6.45	1.90E−03	RIKEN cDNA 5830418P13 gene
slc6a11	−6.45	5.46E−03	Solute carrier family 6 (neurotransmitter transporter, GABA), member 11
gm43480	−6.49	2.26E−03	Predicted gene 43480
gsdmc	−6.55	1.64E−05	Gasdermin C
slitrk3	−7.03	6.16E−04	SLIT and NTRK-like family, member 3
ucp3	−7.24	2.00E−05	Uncoupling protein 3 (mitochondrial, proton carrier)
gbp2b	−7.57	1.13E−02	Guanylate binding protein 2b
lrtm1	−8.08	5.20E−03	Leucine-rich repeats and transmembrane domain 1
ascl1	−8.15	3.06E−04	Achaete-scute family bhlh transcription factor 1
htr4	−8.88	3.22E−05	5 Hydroxytryptamine (serotonin) receptor 4
gm35507	−29.13	3.14E−03	Predicted gene 35507
otop1	−29.48	7.62E−03	Otopetrin 1
mmd2	−31.89	2.08E−04	Monocyte to macrophage differentiation-associated 2

[a]Fold change refers to gene expression in the bladders of WT mice treated with FliC relative to carrier control.

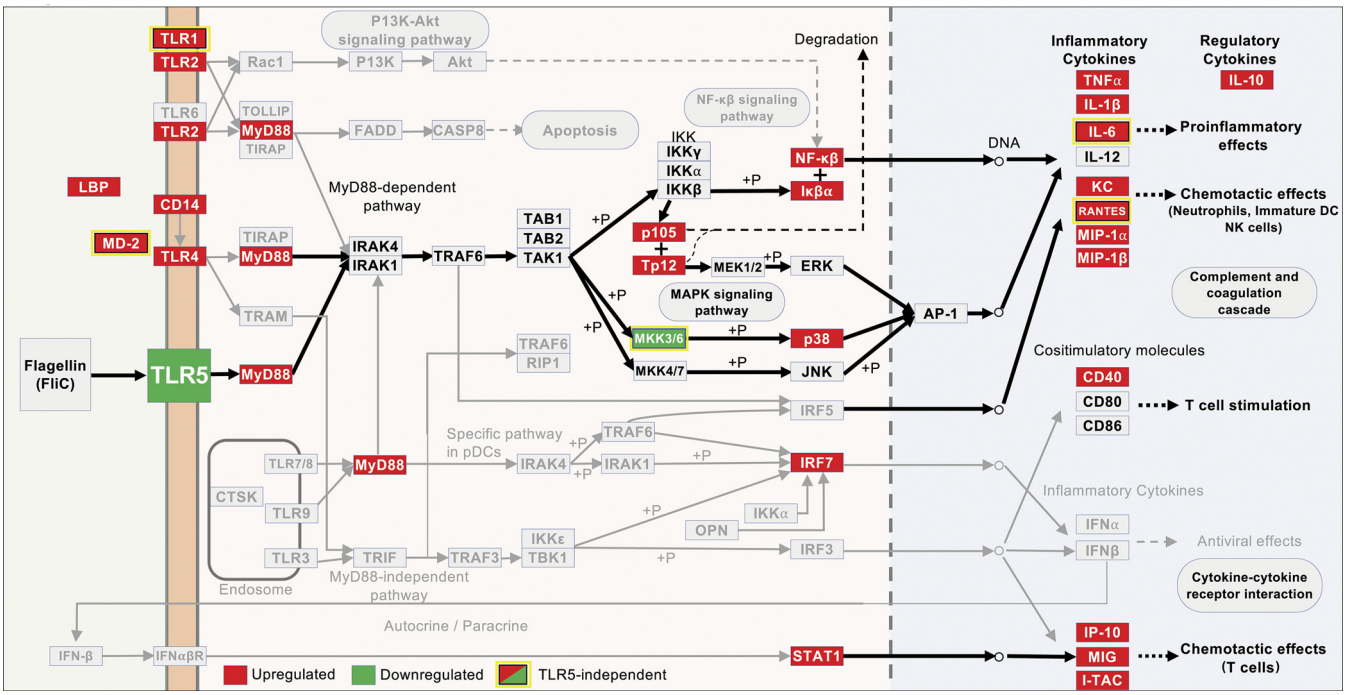


FIG 6 Cellular context of TLR5 engagement by UPEC FliC in the bladder leading to early IL-10 induction. Gene transcriptional responses analyzed using innateDB and overlaid on KEGG pathway 4620 Toll-like receptor signaling. Color key: green, downregulated; red, upregulated; yellow box, TLR5-dependent; other diagram components are per KEGG definitions. The illustration highlights possible signaling transduction mechanisms (center) that are engaged by FliC, leading to rapid IL-10 synthesis in the bladder. IL-10 does not form part of the canonical KEGG pathway 4620 but is included as a notional product of TLR5 engagement based on the findings of this study.

phenotypes related to flagella) and *in vitro* models of UTI used in this study, we suggest rapid IL-10 induction in the bladder response to FliC forms part of a TLR5-dependent program within a complex innate host defense strategy initiated to combat UPEC.

In aligning this study with prior studies, several links between IL-10 induction and flagella are of note. For example, flagellum components of *Salmonella* and *Yersinia* have been shown to modify IL-10 production. *Salmonella* flagella trigger IL-10 secretion in splenocytes (60), monocytes (23, 61), and serum (62), but the type of host response may depend on the nature of antigen presentation (63). Flagella of *Yersinia* have been shown to induce IL-10 in macrophages (24). Interestingly, however, as part of a *Paracoccidioides* vaccine construct, *Salmonella* FliC inhibited IL-10 production in the lungs of mice (64). The effects of flagella on synthesis of cytokines such as IL-6 have

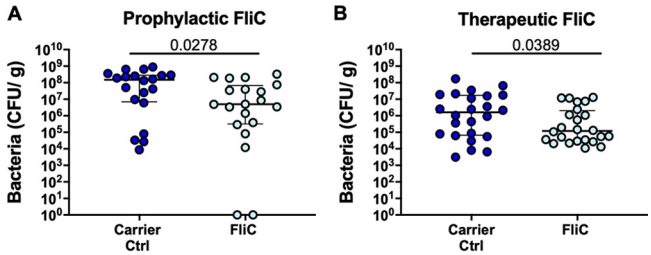


FIG 7 Control of UPEC UTI by FliC treatment. (A) Prophylactic FliC was administered to the bladders of mice 2 h prior to infectious challenge with UPEC. (B) Therapeutic FliC was administered to the bladders of mice 24 h after infectious challenge with UPEC. Bacterial loads were determined at 24 h (A) and 48 h (B) after infectious challenge. Both prophylactic and therapeutic FliC treatment significantly reduced the numbers of UPEC recovered from the bladders of mice treated with FliC compared to control mice that received carrier alone. Data for urine and kidneys are provided in Fig. S7. Data shown represent pooled data from 2 to 3 independent experiments, each comprising 8 to 10 mice per group (total $n = 20$ to 30 per group). *, $P < 0.05$ by Mann-Whitney U test (data did not satisfy Gaussian distribution or normality tests).

been associated with TLR5 (44, 45). In prior studies, we demonstrated the source of IL-10 in UPEC-infected human urothelial cell-monocyte cocultures is monocytes (not epithelial cells) (8, 10); however, by providing insight into the role of TLR5 in FliC-driven IL-10 responses in UPEC UTI *in vivo*, the current study provides a new understanding of the mechanism underlying this rapid bladder defense response triggered by UPEC. We suggest this is relevant to UPEC UTI in humans, because some individuals harbor a stop codon within the TLR5 open reading frame that is predicted to ablate host responses to flagella (65), and a TLR5 C1174T single-nucleotide polymorphism has been associated with recurrent UTI in adult women (66).

We used enriched *E. coli* flagellum protein preparations to initially study IL-10 responses in human cell cultures exposed to liquid- or soft-agar-grown *E. coli* harboring *flhDC* to drive hyperflagellated *E. coli* or *E. coli* deficient in *fliC*. The combinations of challenge conditions tested, and analysis of different UPEC strains in addition to MC4100, indicate that IL-10 responses to *E. coli* flagella are not limited to CFT073. Differences in environmental, growth, and stress conditions, or cross talk mechanisms, might affect flagellar expression differently in distinct *E. coli* strains (67); however, our analysis of UTI89 and EC958 shows a consistent role for FliC in IL-10 induction in the models tested here. MC4100 may be considered irrelevant to UPEC UTI, but inclusion of non-UPEC *E. coli* shows that the effects of *E. coli* flagella on IL-10 are not limited to UPEC. These findings are consistent with previous observations that different flagellum H types (H1, H4, and H7) can induce IL-10 secretion, although H4 flagella was identified as the most potent flagellin type able to induce this cytokine (68). Finally, our data are consistent with the well-established paradigm that TLR5 recognizes FliC monomers, not flagellar filaments, and flagellin-mediated stimulation of cytokine synthesis (including IL-10) occurs in the absence of assembled flagellar filaments (69).

Separate from flagella, other factors in UPEC are likely to contribute to IL-10 responses in the bladder. Our findings based on acellular flagellum stimulation assays and experiments using WT *E. coli* and *fliC*-deficient strains in cell cultures show levels of IL-10 above the baseline *in vitro* even under conditions where FliC was absent. The main cell types used in the coculture model in this study act in synergy in response to UPEC to promote IL-10 synthesis (10), which is a phenotype not discernible from monocultures (12, 70). Other bacterial factors associated with IL-10 induction are lipopolysaccharide and type III secretion system proteins (71), the latter of which is not relevant to UPEC but is shed from some *E. coli* strains (enteropathogenic *E. coli* and enterohemorrhagic *E. coli*) under some conditions (72). We were careful to remove endotoxin from the treatments used in this study, and the use of pure FliC shows that this factor of UPEC significantly contributes to IL-10 bladder induction. However, it is likely that FliC (and flagella more broadly) is not the sole PAMP of UPEC that triggers IL-10 production in host cells. Other PAMPS of different bacteria may also induce IL-10; for example, peptidoglycan-embedded lipopeptides and cell wall glycopolymers of *Staphylococcus* induce IL-10 in monocytes and macrophages (22). Chlamydial major outer membrane protein triggers the production of IL-10 in macrophages (73). LPS-induced IL-10 production through TLR4 is well described (74–77). Thus, it is likely that additional UPEC factors induce IL-10; however, the current findings are consistent with several prior observations of flagella from *Salmonella* (23, 61) and *Yersinia* (24), which are reported to induce IL-10 in monocytes and macrophages, respectively.

In addition to the effects of UPEC FliC on IL-10 induction, this study defines a multifaceted innate immune response that is engaged in the bladder immediately upon detection of FliC. RNA sequencing identified many factors that have been associated with the host response to flagella in other experimental systems (provided in Table S1 in the supplemental material), illustrating a large degree of consistency in the overall response of the bladder to FliC than other systems. Some factors, such as the genes for serum amyloid A (e.g., *saa1*), that were strongly induced by FliC in this study have been linked to flagellar function previously (78) and may be critical to host defense against UTI (79). In addition, the many novel factors identified to be induced after exposure to FliC in this study, such as multiple predicted genes and genes

encoding solute carriers and receptors, have no known links to flagella and will require future investigation into their potential roles in UTI. The transcriptomic data of this study expand our insight into the extent to which the innate immune system is engaged by FliC in the bladder. Several of the responses occurring in the mouse bladder in response to FliC can also be interpreted alongside the responses of human uroepithelial cell-monocyte cocultures to discern numerous consistent responses, such as those for IL-10, IL-1, and IL-6. The complex interplay between IL-10 and the regulation of inflammation in the context of other cytokines, such as IL-6, is reviewed elsewhere (77). Topology analysis identified IL-17 as strongly induced in the mouse bladder response to FliC, consistent with the elevated levels of IL-17 observed in the human cell coculture model of bladder; that IL-17 plays a role in innate defense to UPEC UTI in mice (80); and the findings of the current study implicate FliC in this response. It is likely that several of the cytokines identified as induced by FliC in this study contribute to control of UPEC; for example, other than IL-17, previous studies have shown roles for IL-1β (81), IL-6 (82), and G-CSF (83) in modulating host resistance to UPEC UTI. Taken together, these findings support the idea that IL-10 responses to FliC occur concurrently with a diverse repertoire of antimicrobial products and innate immune mediators that are produced as a result of sensing not only flagellum proteins but also other UPEC cell components.

Several lines of evidence relating to flagellin and TLR5 have been established using studies of *Salmonella enterica* serotype Typhimurium flagella, probably reflecting in part its abundant peritrichous expression and commercial availability. Most signaling in response to flagellin occurs through TLR5 (44), which relays sensing to cell response networks that drive production of cytokines and chemokines. TLR5 signaling can vary depending on experimental conditions, such as specific tissue or cell location and the type of pathogen (44, 84). Our results show that IL-10 induction in the bladder as part of early defense against UPEC UTI requires TLR5. TLR5-dependent IL-10 secretion has also been described as part of the response to a flagellin fusion protein studied to prevent allergy (85); our findings are consistent with this observation. In the context of UTI, a previous study of mice treated with flagellin by transurethral inoculation showed upregulation of KC (CXCL1), MIP2 (CXCL2), MCP-1 (CCL2), IL-6, and TNF-α in the bladder (86). That study did not investigate *il10*; however, the findings of the current study support the view that TLR5 recognition of flagellin is an important element of the innate immune response to UPEC during the early stages of UTI in mice. It is interesting that Andersen-Nissen et al. (86) found that TLR5-deficient mice are able to control UTI initially with a defect in resistance apparent only after 2 to 5 days postinoculation. Our findings show extensive responses to UPEC FliC within just 2 h; it seems likely this early response (including *il10*) is critical to shape an effective host response that requires additional time to develop and effect restriction of UPEC in the bladder, detectable one or more days later. Flagellin also activates renal collecting duct cells via TLR5, which enables upregulation of CXCL1 and CXCL2 to provide renal host defense against pyelonephritis (87). Additionally, this study describes a novel group of 591 genes that exhibited altered expression (mostly downregulated) in response to FliC independent of TLR5. Identification of this group prompted a search for candidates associated with NLRC4/NAIP activation and IL-1β signaling, because NLRC4 is one of the key inflammasome sensors that responds to bacteria (88); most notably, flagellin from *Salmonella* triggers the pathway following cytosolic recognition of the bacterial ligand, as discussed elsewhere (89). It is NAIP5 and NAIP6, rather than NLRC4, that recognize flagellin (90). Activation of this pathway can lead to NLRC4-mediated pyroptosis and other antimicrobial responses, including shedding of infected epithelial cells and release of prostaglandins and leukotrienes. Among the genes identified as significantly upregulated via TLR5-independent mechanisms following exposure to FliC were those encoding caspase-7 and Gasdermin-D; recently, both of these factors were identified as key substrates downstream of the NLRC4/NAIP5 inflammasome required for resistance to *Legionella* infection (91). Thus, it would be interesting to investigate the role of NLRC4/NAIP5 and associated factors, such as caspase-7 and Gasdermin-D, in resistance

to UPEC, particularly in the context of TL5-independent driven responses to flagellin in the host response.

We observed significant downregulation of *tlr5* in WT mice treated with FliC, which is consistent with a previous study that showed treatment with various bacterial ligands downregulated TLR5 expression (92). Other studies have shown responses to flagella in the absence of functional TLR5 signaling (41, 44). TLR11 also forms part of the defense response of the bladder to UPEC in experimental infection in mice (93); we excluded TLR11 from this study because of its absence from the human receptor repertoire and because it has been demonstrated that TLR11 is not a sensor for FliC (52). Further studies are needed to characterize the signaling mechanisms underlying UPEC FliC-mediated and TLR5-dependent IL-10 production. Examples of candidates that would be useful to investigate in characterizing these signaling mechanisms are shown in the TLR signaling KEGG pathway used to interpret these data, which we illustrated with IL-10 highlighted as a notional product of TLR5 engagement (at the time of writing, IL-10 is not included in KEGG pathway 4620). For example, significant upregulation of *myd88*, *nfkb1*, and *nfkb2* suggests these contribute to rapid production of IL-10 through MyD88-dependent mechanisms with quick activation of NF-κB and mitogen-activated protein kinase (MAPK). Other differentially expressed genes, such as those related to macrophage and neutrophil inflammation (e.g., *ccl20–mip-3α* and *ngp*), stress a convergence between canonical TLR signaling and early cellular defense responses to UPEC; others, such as *mrgpra2b* (expressed by neutrophils and mast cells, suggested to have important roles in the innate immune system [94]), *mir-351*, and several predicted genes that were the most strongly upregulated independent of TLR5 (but which have largely uncharacterized functions), underscore the gaps in knowledge of how innate immune responses to UPEC develop and how these might affect the pathogenesis of UTI. Other limitations of this work are the concentrations of FliC used, which are difficult to relate to natural infection; however, similar assay conditions are reported in many published studies on FliC (that have used microgram amounts of less pure FliC); this enables comparison between studies of similar nature.

FliC has been topical in vaccine development for decades and forms part of several recently developed experimental vaccines, including as adjuvant comixed with vaccine antigens and as chimeric or fusion proteins, as reviewed elsewhere (95). For example, an FliC adjuvant has been tested in the context of influenza vaccines in human clinical trials (96). We explored the potential of FliC-driven innate immune responses of the bladder as an approach to infection control of UPEC distinct from vaccine-driven adaptive immunity to gain proof of principle that UPEC FliC is useful for prevention or control of UTI. Our observations of mice administered FliC prophylactically as well as therapeutically provide evidence that FliC is useful for new approaches to the treatment of UTI. In these experiments, higher recovery of UPEC from urine and kidneys of mice treated prophylactically (with carrier or FliC) than from mice treated therapeutically most likely reflects the different time periods used between infectious challenge and UPEC load measurement (i.e., 24 h for prophylactic model versus 48 h for therapeutic model); these differences in recovery of UPEC occurred regardless of the use of FliC; thus, we consider these a reflection of the model rather than effects of FliC. Several flagellum H antigen types have been investigated as part of polyvalent vaccine studies in rats for UPEC (97); however, the problem of flagellin variation and the related need to target multiple virulence factors is an important consideration (98). Finally, the nature of flagellin to shape both innate and adaptive arms of immunity has led to its use as an immunomodulatory antitumor agent (42). How FliC or the immune response to it might be incorporated into novel approaches to treat or prevent UTI remains unclear, but this study establishes that FliC can be used to increase the host's ability to control UPEC bladder infection. The mechanism of the protective effect observed in this study remains unknown, and addressing the potential role of factors, such as *il10*, would necessitate different models, such as double TLR5$^{-/-}$ and IL-10$^{-/-}$ mice, for example. However, we have no evidence that IL-10 is responsible for the protective effect, and given the many genes and cytokines that are altered in expression after FliC

inoculation, future work will need to examine the mechanism by which the observed protective effect from FliC is afforded. Another avenue for analysis could be the use of UPEC FliC as an adjuvant to promote the efficacy of experimental UPEC vaccines, as reported for other pathogens (99), or alternatively, as an immunomodulatory agent; such an approach was shown to activate TLR5 and induce the production of a host defense peptide, BD2, that may boost control of recurrent UPEC UTI (100).

MATERIALS AND METHODS

Cell lines and bacteria. Human 5637 uroepithelial (ATCC number HTB-9), U937 monocyte (ATCC number CRL-1593.2), and mouse J774A.1 (ATCC number TIB-7) cell lines were used. Cells were grown at 37°C with 5% CO_2 in complete RPMI (cRPMI) medium (RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin; Life Technologies, USA).

UPEC reference strains CFT073 (101), UTI89 (102), and EC958 (103) and various derivatives, as well as the commensal *E. coli* MC4100, were used (Table 1). UPEC derivatives included mutants with targeted deletions in *fliC*, namely, CFT073Δ*fliC* (57), EC958Δ*fliC* (68), and UTI89Δ*fliC* (this study) strains. Additionally, a multiple mutant, termed the CFT073Δ*4* strain, with combined deletions in four major chaperone-usher fimbriae operons (type 1, F1C, and two P fimbrial gene clusters) (113), was used along with its *fliC*-deficient derivative, the CFT073Δ*4* Δ*fliC* strain (57). *E. coli* MC4100 and UPEC strains carrying isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible p*flhDC* (master operon for flagellar biosynthesis) were used to study hyperflagellated states. Unless otherwise stated, bacteria were grown with agitation (200 rpm) at 37°C in lysogeny broth (LB) or on LB agar (1.5% and 0.25% as required) overnight with antibiotic selection (50 μg/ml kanamycin, 30 μg/ml chloramphenicol) and IPTG (20 mM) as indicated. For motility assays, overnight cultures were prepared in LB broth (with appropriate antibiotics where necessary), and 1 μl of phosphate-buffered saline (PBS) containing approximately 1×10^6 CFU was spotted onto the center of fresh 0.25% LB agar plates (in triplicate) that were supplemented with IPTG and kanamycin as necessary. The plates were incubated at 37°C for 9 h, and rates of motility were determined by measuring the diameter of growth over time. The data are shown as the mean diameter (in millimeters) of motility ± standard errors of the means (SEM) for at least 3 independent experiments.

Cell coculture and cytokine measurement. A coculture model of human 5637 uroepithelial cells and U937 monocytes was used for most *in vitro* assays, essentially as described previously (8). Briefly, 1×10^5 uroepithelial cells and 5×10^4 monocytes in cRPMI were seeded together into the wells of a 96-well plate. The cocultures were infected with 1.5×10^6 CFU of UPEC (multiplicity of infection [MOI], 10) and incubated at 37°C with 5% CO_2 for 5 h, a time point previously associated with IL-10 induction by UPEC *in vitro* (10). For cytokine measurements, supernatants were analyzed using ELISA specific for human IL-10 (number 88-7106-86; eBioscience, USA) or multiplex cytokine assays (Bio-Rad, USA). J774A.1 macrophages were used in parallel assays, as indicated. Cell coculture assays were performed at least three times in independent experiments.

Preparation of flagellum-enriched *E. coli*. Broth cultures of *E. coli* were grown overnight (10 ml LB) at 37°C with shaking (200 rpm), harvested, and washed in PBS three times (8,000 × *g* for 10 min at 4°C). The cells were adjusted to 3×10^7 CFU/ml in cRPMI medium for use in cocultures. Initially, we tested whether *E. coli* grown to be flagellum enriched would induce more IL-10 than non-flagellum-enriched *E. coli*; for this, we used soft-agar cultures to promote swarming growth, which is associated with increased expression of flagellin (105). Soft-agar flagellum-enriched *E. coli* was prepared using LB agar plates (0.25% agar), onto the surface of which was spotted 10 μl containing 3×10^8 CFU (from overnight LB cultures), as previously described (106). The plates were incubated overnight (37°C), and subsequently areas of hyperflagellated *E. coli* were excised from the agar, resuspended in 500 μl PBS by pipetting, and centrifuged (1,000 × *g*, 5 min at 4°C) to pellet any residual agar. The supernatants containing the bacteria were then diluted in cRPMI for assay (1.5×10^6 CFU/ml; MOI, 10). Colony counts were performed to determine MOI. Results shown represent at least four independent experiments.

Preparation of acellular flagella, purification of FliC, and protein analysis. Protein preparations enriched for flagella were isolated from *E. coli* using a combination of mechanical shearing and ultracentrifugation, essentially as described elsewhere (107). Briefly, 500-ml cultures were grown with shaking (60 rpm) and washed in PBS, and flagella were sheared using a bead beater (57). The suspensions were centrifuged and the supernatants (with flagella) were filtered (0.45 μm). Bacteria-free flagella were pelleted (135,000 × *g*, 90 min, 4°C) and resuspended in 2 ml PBS for freezing at −20°C. Depolymerization of flagellar filaments into FliC monomers was achieved by heating (60°C, 10 min) prior to analysis, postpurification, or use in downstream assays. FliC was postpurified using fast protein liquid chromatography (FPLC) with an ÄKTA pure protein purification system and a Superdex 200 increase 10/300 GL column (GE Lifesciences) (57). Endotoxin was removed using high-capacity columns (88274; Pierce). Proteins were analyzed using a bicinchoninic acid protein assay kit (number 23227; Thermo Scientific Pierce, USA). Western blots (with anti-flagellum H-pool-E antibody; number 54394; Staten Serum Institut, Denmark) used anti-rabbit IgG horseradish peroxidase conjugate (number sc-2030; Santa Cruz Biotech, USA) and 3,3′-diaminobenzidine substrate. The UPEC FliC proteins prepared in this manner were pure and endotoxin free, as previously described (57).

Mouse experiments and treatment of bladder. Examination of the bladder response to flagella and FliC was undertaken using female C57BL/6J or B6.129S1-Tlr5^{tm1Flv}/J mice (The Jackson Laboratory, USA, and Animal Resources Centre, Canning Vale, WA) at 10 to 12 weeks of age. Mice were administered approximately 1.5×10^8 to 2.0×10^8 CFU UPEC or 30 μg FliC via the transurethral route in 50 μl PBS at

a slow infusion rate (5 μl s^{-1}). For the collection of tissues, mice were euthanized by isoflurane anesthesia overdose followed by cervical dislocation. Bladder tissue was collected at 2 h postinoculation, a time point associated with IL-10 responses in UPEC-infected mice (8). For ELISA, bladder was homogenized in a protease inhibitor cocktail (Roche, Castle Hill, NSW, Australia) and clarified at 12,000 $\times$ *g* for 20 min at 4°C. Supernatants were stored at −80°C until assay, which was performed using quintuplicate samples in a commercial IL-10 ELISA (Pierce Endogen, Scoresby, VIC, Australia). Independent experiments using groups of five were repeated at least twice.

RNA isolation, sequencing, and bioinformatics. For RNA isolation, bladder tissues were homogenized in TRIzol (Life Technologies, Mulgrave, VIC, Australia). RNase-free DNase-treated RNA that passed Bioanalyzer 2100 (Agilent) analysis was used for RNA sequencing. We performed mRNA sequencing on RNA from C57BL/6 and B6.129S1-Tlr5^{tm1Flv}/J mice (n = 3 to 5 per group) using the Illumina NextSeq 500 platform. Total RNA was subjected to 2 rounds of poly(A)$^+$ selection and converted to cDNA. We used TruSeq library generation kits (Illumina, San Diego, California). Library construction consisted of random fragmentation of the poly(A) mRNA, followed by cDNA production using random primers. The ends of the cDNA were repaired and A-tailed, and adaptors were ligated for indexing (with up to 12 different barcodes per lane) during the sequencing runs. The cDNA libraries were quantitated using qPCR in a Roche LightCycler 480 with the Kapa Biosystems kit (Kapa Biosystems, Woburn, Massachusetts) prior to cluster generation. Clusters were generated to yield approximately 725,000 to 825,000 clusters/mm^2. Cluster density and quality were determined during the run after the first base addition parameters were assessed. We ran paired-end 2$\times$ 75-bp sequencing runs to align the cDNA sequences to the reference genome. For data preprocessing and bioinformatics, STAR (version 2.5.3) was used to align the raw RNA sequencing fastq reads to the Gencode GRCm38 p4, release M11, mouse reference genome (108). HTSeq-count, version 0.9.1, was used to estimate transcript abundances (109). DESeq2 then was used to normalize and test for differential expression and regulation. Genes that met certain criteria (i.e., fold change of $\geq \pm 2.0$, q value of <0.05) were accepted as significantly altered in expression (110).

Control of UPEC in the bladder using FliC. To explore the potential for FliC and associated innate immune responses in the bladder to be used for infection control or disease prevention purposes, we examined UPEC numbers in the bladders of mice that were treated with 30 μg FliC (in 50 μl PBS) either prophylactically or therapeutically. In the prophylactic model, infectious challenge with UPEC occurred 2 h after administration of FliC, and UPEC titers were measured 24 h after infectious challenge. In the therapeutic model, mice received infectious challenge and, 24 h later, received FliC; 24 h later, UPEC titers were measured. The infectious challenge in both models was 1.5×10^8 to 2.0×10^8 CFU of UPEC CFT073 in 50 μl of PBS inoculated via the transurethral route. Control mice received 50 μl of carrier and were challenged in the same manner. The total bacterial loads in the bladders, urine samples, and kidneys of mice were assessed using standard colony count methods, as previously described elsewhere (8).

Ethics statement. This study was carried out in accordance with the national guidelines of the Australian National Health and Medical Research Council. The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and the Animal Ethics Committee of Griffith University reviewed and approved all animal experimentation protocols used in this study (permits: University of Alabama at Birmingham animal protocol IACUC-10089 and Griffith approval MSC/01/18/AEC).

Statistics. Statistical significance was set at a P value of ≤ 0.05. Welch's independent samples t test was used to compare IL-10 levels in ELISAs, and analysis of variance (ANOVA) was performed with Tukey's *post hoc* comparison for multiple-target Bio-Plex assay. Mann-Whitney U test was used to evaluate mouse bladder titer data. Statistical testing of RNA sequencing data was undertaken using DESeq2 and included significance criteria of a fold change of $\geq \pm 2.0$ and q value of <0.05, as described elsewhere (110). Other statistical analyses were performed using GraphPad Prism v8.0 and SPSS Statistical Package v22.

Data availability. Raw and processed data were deposited in Gene Expression Omnibus (GEO; accession no. GSE132294).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00545-19.

FIG S1, TIF file, 0.5 MB.
FIG S2, PDF file, 1.5 MB.
FIG S3, TIF file, 0.6 MB.
FIG S4, TIF file, 0.1 MB.
FIG S5, PDF file, 1.3 MB.
FIG S6, PDF file, 0.4 MB.
FIG S7, PDF file, 0.1 MB.
TABLE S1, DOCX file, 0.2 MB.
DATA SET S1, XLSX file, 10.7 MB.
DATA SET S2, XLSX file, 0.4 MB.

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