

Uropathogenic *Escherichia coli* remodels LPS to evade Caspase-1 dependent pyroptosis to establish intracellular bacterial communities

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Research Article

Keywords:

Posted Date: July 31st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10514633/v1>

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Additional Declarations: The authors declare no competing interests.

Abstract

Uropathogenic *Escherichia coli* (UPEC), a major urinary tract infection (UTI) pathogen, is the currently known bacterium capable of extensively replicate in host cytosol. UPEC rapidly and clonally replicates to approximately 10^5 bacteria, establishing biofilm-like intracellular bacterial communities (IBCs) in superficial bladder epithelial cell (BEC) cytosol. Caspase-4/11-dependent noncanonical inflammasome is the major cytosolic immune surveillance monitoring gram-negative bacteria, *via* lipopolysaccharide (LPS) detection. However, how UPEC evades recognition by Caspase-4/11 to replicate extensively in the BEC cytosol is unknown. In this study, transcriptional profiling of UPEC during the IBC formation process found that the expression of *lpxT*, which encodes a phosphotransferase that adds an additional phosphate group at the 1-position of LPS lipid A, was upregulated. We showed that lack of this modification not only reduced initial IBC formation, but also reduced the reinfection of UPEC to form subsequent rounds of IBCs in acute infection, and also reduced the incidence to cause recurrent UTIs in mice. We confirmed that LpxT modifies LPS in UPEC, and that the expression of *lpxT* is highly induced in UPEC-infected mouse bladders, resulting in an increase in the percentage of 1-pyrophosphate lipid A. We showed that phosphate modification of lipid A reduces the interaction between LPS and Caspase-4 in BECs, and diminishes Caspase-4/11 activation both in BECs and in mouse bladders, leading to reduced host cell pyroptosis. Caspase-11 knockout mouse experiments confirmed that UPEC increases LPS lipid A modification by LpxT to reduce Caspase-11 activation in mouse bladder, thereby facilitating its IBC formation and virulence.

Introduction

Uropathogenic *Escherichia coli* (UPEC) is the primary causative agent of urinary tract infections (UTIs), which are among the most common infections in humans¹. Nearly 11 million UTI cases are reported each year, resulting in approximately \$5 billion in direct and indirect socioeconomic costs². All-age death counts caused by UPEC are 120 thousand (96.4 thousand – 154 thousand) per year, and the age-standardised mortality is 1.6 (1.3-2.0)³. Approximately 12% of men and 40% of women experience symptomatic UTIs during their lifetime⁴. After the initial infection, recurrence occurs in 30%-50% of patients within 6 months, 68% of which are caused by the original strain, despite the use of appropriate antibiotic therapy⁵⁻⁸. UTIs, which have the symptoms of urinary urgency, frequency and dysuria, accompanied by frequent recurrence, cause long-lasting pain and discomfort in patients⁹.

UPEC are thought to reside in the colon and cause acute disease on gaining access to the urinary bladder *via* an ascending route¹⁰. UTIs progress from planktonic bacteria in urine by invasion of bladder epithelial cells (BECs) by incorporating into fusiform vesicles¹¹. UPEC may then escape into the cytosol via lipase-mediated disruption of the fusiform vesicle membrane¹², where it rapidly replicates to establish biofilm-like intracellular bacterial communities (IBCs), a critical bottleneck in UPEC pathogenesis¹¹. The formation of each IBC starts with a single bacterium that escapes the fusiform vesicle to the host cytosol and undergoes clonal expansion to approximately 10^5 bacteria within a few

hours¹³. IBC formation is transient and terminates in a dispersal stage, during which the bacteria escape infected host cells and spread to neighboring naïve superficial BECs, where the IBC cycle is repeated¹⁴. IBC formation is essential for UPEC pathogenesis, as UPEC that is unable to form IBCs is cleared and cannot induce acute infection¹⁵. Additionally, IBC formation is also crucial for recurrent UTIs, as UPEC strains that are able to invade BECs but defective in IBC formation do not cause recurrent UTIs^{15,16}.

The cytosolic pathogen-sensing system is primarily mediated by inflammasomes that detect bacterial component contamination in the host cell cytosol; and which is critical for host defense against intracellular pathogens¹⁷. Two types of inflammasomes have been described: the canonical and noncanonical inflammasomes. In response to a diverse range of signals, including bacterial flagellin, bacterial virulence-associated effectors, double-stranded DNA, and single-stranded RNA, canonical inflammasomes activate Caspase-1¹⁸. An alternative Caspase-1-independent inflammasome, termed the noncanonical inflammasome, primarily mediates inflammatory responses to gram-negative bacteria¹⁹. The noncanonical inflammasome is formed by Caspase-11 in mice (an ortholog in humans, Caspase-4)²⁰. These Caspases are activated upon the binding of lipopolysaccharide (LPS), a major outer membrane component of gram-negative bacteria²¹. Active Caspase-4/11 cleaves gasdermin-D (GSDMD) to form active N-GSDMD, a latent pore-forming protein that makes holes in cell membranes, thereby causing pyroptosis²¹, and leading to interleukin (IL)-1 β and IL-1 α maturation and release^{19,22}. The destruction of cytosolic replication niches and the release of inflammatory cytokines are the underlying mechanisms of the inflammasome-mediated inflammatory response to intracellular bacterial infection²³.

Inflammasomes detect UPEC in the BEC cytosol and activate pyroptosis, which can result in the exfoliation of superficial BECs, thereby eliminating infected cells that harbor IBCs^{24,25}. Deletion of UPEC two component system (TCS) CpxRA, upregulates the expression of α -hemolysin (*hlyA*), resulting in increased activation of inflammasomes, and decreased IBC formation in UPEC-infected mouse bladders²⁵. Caspase-11 activation can be induced by even small amounts of bacterial LPS in the host cytosol²⁰. IBC formation begins with UPEC, which escapes into the cytosol to clonally replicate to a density of 10⁵ bacteria, and is not membrane bound. These indicate that the ability of Caspase-11 to monitor the amount of LPS in the host cytosol decreases as IBC formation progresses. However, how UPEC evades recognition by Caspase-11 to form IBCs is unknown.

LPS, which is composed of lipid A, a core oligosaccharide and an O-antigen polysaccharide²⁶, is a major constituent of the outer membrane of gram-negative bacteria. LPS is essential for bacterial survival and resistance to harsh environments, and bacteria have evolved diverse mechanisms to modify LPS to resist harsh environments, including immune system surveillance²⁷. LPS serves as a pathogen-associated molecular pattern (PAMP) and is recognized by both Toll-like receptor 4 (TLR4) and Caspase-4/11, which defend against pathogenic bacteria^{28,29}. However, pathogens utilize various mechanisms to evade immune system recognition of LPS, including LPS modification, thereby promoting the

pathogenicity of the organisms. LPS modifications, especially those that remove phosphates or alter the acylation of lipid A, can reduce recognition by TLR4^{30,31}. To our knowledge, only the deacylation of lipid A can reduce recognition by Caspase-11, and this effect has been observed in *Francisella novicida* and *Yersinia pestis*^{32,33}. However, whether other forms of lipid A modifications affect the interaction between LPS and Caspase-4/11 is unknown.

In this study, to further investigate whether and how UPEC evades Caspase-11 recognition, we performed transcriptional profiling of UPEC during the IBC formation process to identify potential genes that contribute to virulence using mouse models of UTI. The expression of *lpxT*, which encodes a phosphotransferase that adds an additional phosphate group at the 1-position of LPS lipid A, was upregulated during IBC formation and contributed to UPEC virulence. The lack of *lpxT* not only decreased the first round of IBC formation, but also reduced the reinfection of UPEC to form subsequent rounds of IBCs in acute infection, and also reduced the incidence to cause recurrent UTIs in mice. We confirmed that LpxT modifies LPS in UPEC, and that the expression of this gene is highly induced in UPEC-infected mouse bladders, resulting in an increase in the percentage of 1-pyrophosphate lipid A. We showed that phosphate modification of lipid A reduces the interaction between LPS and Caspase-4 *in vitro*, and further results demonstrated that this modification reduces Caspase-4/11 activation both in BECs and in mouse bladder, leading to reduced release of the inflammatory cytokines IL-1 α and IL-1 β , and host cell pyroptosis. Caspase-11 knockout mouse experiments showed that by diminishing Caspase-11 activity, LpxT contributes to UPEC virulence and IBC formation. In this study, we showed that by increasing the modification of the phosphate group of lipid A, UPEC escapes Caspase-11 detection, thus enabling its substantial replication to form IBCs. Furthermore, we identified a new form of LPS modification by phosphorylation that allows for the evasion of Caspase-11 recognition, in addition to the previously known deacylation of LPS lipid A.

Results

***lpxT* is essential for UPEC virulence and has upregulated expression during IBC formation**

To gain insight into the molecular mechanisms utilized by UPEC to evade Caspase-11 recognition during IBC formation, UPEC CFT073 was transurethrally administered to C3H/HeN mice, which is an *in vivo* model of UPEC infection¹⁴; the transcriptional profiles of intracellular bacteria scraped from bladders of C3H/HeN mice were determined using RNA sequencing (RNA-seq) at 6 h p.i. At this time point, the first round of IBC formation peaked in mouse UTI model¹⁴. Compared with those of the CFT073 inoculum (CFT073 grown in Luria–Bertani (LB) medium), the expression of 242 genes was upregulated, and that of 20 genes was downregulated (Dataset S1 and S2). The clustering results of gene ontology (GO) analysis revealed that the genes with upregulated expression included type I fimbria and secreted autotransporter toxins (Fig S1A), in line with previous studies showing that type I fimbria contribute to IBC formation at 6 h p.i.^{34–36}. The genes with downregulated expression included those related to the cation efflux system (Fig S1B), indicating that intracellular UPEC sequesters cations during infection; this result is consistent

with the findings of a previous study³⁷. Notably, the expression of *lpxT*, a lipid A modification gene, was significantly upregulated according to the transcriptomic results (13.06-fold). Quantitative reverse transcription PCR (qRT–PCR) results confirmed the upregulation of *lpxT* expression at 6 h p.i. (4.06-fold), and further revealed upregulated expression at 16 and 24 h p.i. (4.28- and 4.83-fold) (Fig. 1A), indicating that the expression of *lpxT* increased in the first and subsequent rounds of IBC formation. To verify the qRT–PCR results, we constructed a transcriptional fusion plasmid, *lpxT*-prompter-GFP, containing the *lpxT* promoter linked to a GFP reporter gene and transformed this plasmid into wild-type (WT) strain. GFP expression in WT-infected superficial BECs and in LB medium was determined using confocal microscopy. The results showed that GFP expression was detectable in WT-infected superficial BECs at 6, 16 and 24 h p.i., whereas no fluorescence was detected in bacteria grown in LB medium (Fig. 1B), thus confirming that the *lpxT* gene had upregulated in UPEC-infected superficial BECs of the mouse bladder during IBC formation.

LpxT modifies the 1-position of lipid A to form the 1-pyrophosphate lipid A of LPS in *E. coli* W3110³⁸. We speculated that LpxT modification of lipid A may contribute to UPEC virulence by preventing Caspase-11 recognition. First, we investigated whether deletion of *lpxT* affects UPEC colonization in C3H/HeN mice during acute infection¹⁴. We transurethrally infected C3H/HeN mice with WT, Δ *lpxT* (*lpxT* deletion strain) or Δ *lpxT*+ (*lpxT* deletion complementation), and assessed bacterial colonization at 24 h p.i. The bacterial titers in mouse bladders, as determined by the enumeration of colony-forming units (CFUs), of Δ *lpxT*-infected was 0.98 log-fold lower than those of WT-infected (Fig. 1C). Δ *lpxT*+ infection reversed the decrease in bacterial colonization of Δ *lpxT*-infected to a level similar to that of WT (Fig. 1C). Furthermore, the Δ *lpxT* strain grew as well as the WT strain in LB and RPMI 1640 media, thereby ruling out a growth defect as the cause of UPEC colonization *in vivo* (Fig S2A and S2B). These results indicate that a lack of *lpxT* decreases UPEC colonization in mouse bladders during acute infection.

Invasion into superficial BECs is a prerequisite for subsequent cytosolic escape and replication³⁹. The effect of *lpxT* on invasion was then investigated by transurethrally infecting C3H/HeN mice with WT, Δ *lpxT* or Δ *lpxT* + and quantifying bacterial titers in mouse bladders at 2 h p.i. At this time point, most intracellular UPEC participated in invasion⁴⁰. The bacterial titers in the bladders of mice infected with Δ *lpxT* or WT were similar at 2 h p.i. (Fig S2C), indicating that LpxT has no effect on invasion into superficial BECs of mouse bladders. This result was confirmed using a UPEC host cell infection model. UPEC can invade human BEC 5637 cells (5637 cells), but cannot replicate appreciably once inside the cytosol to form IBCs⁴¹. There was no difference in the bacterial load of 5637 cells infected with Δ *lpxT* at 2 h p.i., but the bacterial load decreased to 32.38% and 32.09% at 6 and 8 h p.i., respectively, when UPEC escaped fusiform vesicles and resided in the BEC cytosol³⁷, compared to that of WT (Fig S2D). These results indicate that LpxT is not required for UPEC invasion into 5637 cells, but is required for its survival in the host cytosol. These results suggest that LpxT is required for UPEC virulence during acute infection and is not required for invasion, indicating that LpxT may contribute to UPEC virulence by affecting bacterial replication in superficial BECs.

LpxT promotes IBC formation

Cytosolic UPEC replicates to form IBCs³⁴. To investigate whether the expression of *lpxT* contributes to UPEC replication in the cytosol to form IBCs, C3H/HeN mice were transurethrally infected with WT, $\Delta lpxT$, or $\Delta lpxT+$, and the number of IBCs per bladder was determined at 6 h p.i., which is the time point at which most UPEC formed the first round of IBCs during acute infection. The number of IBCs per bladder, as determined by confocal microscopy of whole bladders, was 77.3% lower in $\Delta lpxT$ -infected than in WT-infected mice, and was restored in $\Delta lpxT+$ -infected mice, whereas the IBC size did not significantly differ between WT and $\Delta lpxT$ (Fig. 1D, S2E).

The number of bacteria in the bladders of the mice was determined to confirm the results of the IBC enumeration experiment at 6 h p.i. The bacterial titer was also 1.39-log-fold lower in the bladders of the $\Delta lpxT$ -infected mice than in those of the WT-infected mice, whereas it was restored to the WT level in the bladders of $\Delta lpxT+$ -infected mice (Fig. 1E). The reduced percentage of bacterial titers in the bladders of $\Delta lpxT$ -infected mice was comparable to the reduced percentage of IBC numbers in the bladders of $\Delta lpxT$ -infected mice (Fig. 1D, 1E), thus confirming that the size of IBC was not affected by the deletion of *lpxT*. These results suggest that *lpxT* promotes UPEC replication to form IBCs in the cytosol.

A reduction in IBC formation and bacterial titers caused by the deletion of *lpxT* was also detected at 24 h p.i., indicating subsequent rounds of IBC formation were also inhibited by *lpxT* expression (Fig. 1C and 1F). Therefore, the expression of *lpxT* contributes to UPEC virulence by promoting UPEC replication to form IBCs during acute infection.

Next, we used a mouse chronic cystitis model which is the indicative of recurrent UTIs, to confirm the lack of *lpxT* reduces recurrent UTIs. Infection of C3H/HeN mice resulted in a bimodal distribution of bacterial titers at 28 d p.i., reflecting mice that developed chronic cystitis and those mice that resolved infection or formed quiescent intracellular reservoirs (QIRs)⁴². At 28 d p.i., the presence of high-titer persistent bacteriuria ($> 10^4$ CFUs/mL) during infection and bladder bacterial burdens $> 10^4$ CFUs at death is defined as chronic cystitis. The ability of $\Delta lpxT$ to cause chronic cystitis was attenuated compared with that of WT (Fig. 1G), whereas was restored in $\Delta lpxT+$ -infected mice (Fig. 1G). These results suggest that the development of recurrent UTIs requires *lpxT* expression.

The increase in *lpxT* expression promotes the formation of lipid A 1-pyrophosphate in UPEC

LpxT is responsible for the formation of lipid A 1-pyrophosphate by the addition of a phosphate group at the 1-position on lipid A in *E. coli* W3110³⁸. We next investigated whether an increase in *lpxT* expression increases lipid A 1-pyrophosphate formation in UPEC CFT073. We first confirmed that LpxT is required for the formation of lipid A 1-pyrophosphate in UPEC CFT073 by thin-layer chromatography (TLC) analysis of ³²P-labeled lipid A species isolated from WT, $\Delta lpxT$, and *lpxT+* strains grown *in vitro* overnight. According to the TLC results, about 35.83% of the lipid A isolated from the WT exhibited 1-

pyrophosphate modification, and lipid A from the $\Delta lpxT$ lacked 1-pyrophosphate modification (Fig. 2A), indicating that LpxT is required for 1-pyrophosphate modification on lipid A in UPEC CFT073.

Next, we investigated whether overexpressing *lpxT* increases the effect of 1-pyrophosphate modification on lipid A. The TLC results showed that lipid A isolated from the WT exhibited 35.83% 1-pyrophosphate modification and that from the *lpxT*⁺ exhibited 57.98% 1-pyrophosphate modification. Compared with the WT, the amount of 1-pyrophosphate lipid A of *lpxT*⁺ increased 1.46-fold (Fig. 2A), indicating that the upregulation of *lpxT* expression results in increased modification of the phosphate group at position 1 of LPS lipid A. Compared with that of WT, the expression of *lpxT* in the *lpxT*⁺ increased 10.38-fold (Fig S2F). The expression level of *lpxT* in the bladders of WT-infected mice increased 4.06-fold compared with *in vitro* (Fig. 1A). These results suggest that UPEC upregulated expression of *lpxT* to promote the formation of lipid A 1-pyrophosphate in mouse bladders, with modification increasing potentially between 35.83% and 57.98%.

LpxT inhibits Caspase-4/11-dependent pyroptosis triggered by UPEC infection

We next investigated whether LpxT-dependent lipid A modification facilitates UPEC evasion of Caspase-11 recognition. We first investigated whether LpxT inhibits cell death upon UPEC infection. 5637 cells were infected with WT, $\Delta lpxT$, $\Delta lpxT$ + or *lpxT*⁺, and the release of cytoplasmic lactate dehydrogenase (LDH) was measured at 4 h p.i. The results showed higher LDH release in 5637 cells infected with $\Delta lpxT$, and lower LDH release in 5637 cells infected with *lpxT*⁺ than in cells infected with WT (Fig. 2B). Morphological analysis, which uses trypan blue to distinguish live (excluding blue) or dead cells (blue), was performed by confocal microscopy to confirm the above results. It was shown that compared with the WT-infected, the $\Delta lpxT$ -infected induced more, and *lpxT*⁺-infected induced less 5637 cell death (Fig S3A). These results indicated that LpxT inhibits 5637 cell death after UPEC infection.

Treatment of 5637 cells with an inhibitor specific for Caspase-4 (Ac-LEVD-CHO) prior to infection reduced LDH production in $\Delta lpxT$, $\Delta lpxT$ +, and *lpxT*⁺ to levels similar to those in the WT (Fig. 2C). Furthermore, LDH production was reduced to similar levels in Caspase-4-silenced 5637 cells (nearly 100% reduction) infected with either strain (Fig S3B and S3C). These results suggest that a lack of *lpxT* triggers Caspase-4-dependent pyroptosis.

BEC pyroptosis can lead to the exfoliation of superficial BECs, which facilitates the clearance of intracellular bacteria from the bladder^{24,25}. We next tested the ability of LpxT to influence superficial BEC exfoliation. By determining the abundance of binucleated superficial BECs on the luminal surface of the bladder using DAPI staining of whole-mount bladder tissue⁴³, and analyzing the relative expression level of UpIII, which is one of the main surface antigens present on the superficial BECs of the urothelium and is a marker for terminal differentiation⁴⁴, the effect of *lpxT* on the exfoliation response was assessed. As expected, we observed a reduction in the abundance of binucleated superficial BECs (Fig. 2D and 2E),

and reduced UpIII levels (Fig. 2F and 2G) infected with $\Delta lpxT$ compared with WT at 6 and 24 h p.i., indicating that the lack of *lpxT* increased the exfoliation of superficial BECs. These results suggest that LpxT inhibits superficial BEC exfoliation *in vivo*; this result does not contradict the finding that LpxT inhibits pyroptosis in cell infection.

Expression of *lpxT* reduces the interaction between LPS and Caspase-4

The lipid A moiety of LPS binds directly to the CARD domain of Caspase-4/11²⁰. We investigated the effect of LpxT on the interaction between LPS and Caspase-4 in 5637 cells. Using confocal microscopy, we examined the percentage of total LPS on WT, $\Delta lpxT$, $\Delta lpxT+$, and *lpxT+* that colocalized with Caspase-4 at 4 h p.i. The results showed that the colocalization of $\Delta lpxT$ -LPS with Caspase-4 was significantly higher than that of WT-LPS and $\Delta lpxT+$ -LPS in 5637 cells, and the colocalization of *lpxT+*-LPS with Caspase-4 was significantly lower than that of WT-LPS and $\Delta lpxT+$ -LPS (Fig. 2H). These results indicate that *lpxT* reduces the interaction between LPS on UPEC and Caspase-4 in cell infection. qRT-PCR results showed that the expression of Caspase-4 and Caspase-11 were at the similar level in WT- and $\Delta lpxT$ -infected 5637 cells or mouse bladders (Fig S4A), indicating that LpxT modification did not affect the expression of Caspase-4 and Caspase-11.

In addition to the LPS on the bacterial surface, guanylate-binding protein 1 (GBP1) promoted LPS releasing from the bacteria into the host cell cytosol, also can activate Caspase-4-dependent pyroptosis⁴⁵. We next investigated whether the ability of LpxT to inhibit Caspase-4-dependent pyroptosis is mediated by GBP1. We generated GBP1-silenced 5637 cells (63.6% reduction, Fig S4B) and infected GBP1-silenced and control siRNA-treated 5637 cells with WT, $\Delta lpxT$, $\Delta lpxT+$, or *lpxT+*, and measured the release of LDH at 4 h p.i. The results showed that silencing GBP1 did not affect the release of LDH induced by $\Delta lpxT$ or *lpxT+* infection compared to that induced by WT or $\Delta lpxT+$ infection (Fig S4C), indicating that LpxT-mediated inhibition of Caspase-4-dependent pyroptosis is not mediated by GBP1. Combining with the above colocalization results between Caspase-4 and UPEC in BECs, it is highly likely that LpxT reduces the direct interaction between Caspase-4 and LPS which is on the bacterial surface.

LpxT diminishes Caspase-4/11 activity upon UPEC infection

Pyroptosis is triggered by the activation of Caspase-4/11, leading to the cleavage of GSDMD into active N-GSDMD²¹. To investigate whether LpxT affects Caspase-4 activation, 5637 cells were infected with WT, $\Delta lpxT$, $\Delta lpxT+$, or *lpxT+*, and full-length GSDMD and N-GSDMD were detected at 4 h p.i. The amount of N-GSDMD in 5637 cells increased 1.43-fold or decreased 54% following infection with $\Delta lpxT$ or *lpxT+* compared with that with WT (Fig. 3A). We next investigated whether LpxT affects Caspase-11 activation *in vivo*. Mice were transurethrally infected with WT, $\Delta lpxT$ or $\Delta lpxT+$, and the amount of full-length GSDMD and N-GSDMD in the mouse bladder was evaluated by western blotting at 6 and 24 h p.i. The results showed that the amount of N-GSDMD in the mouse bladder increased approximately 1.60-fold and 1.74-fold, when infected with $\Delta lpxT$ compared with that of WT at 6 and 24 h p.i., respectively (Fig. 3B and 3C).

Caspase-4/11 activation also led to release of the inflammatory cytokines IL-1 α and IL-1 β ^{20,22}. The lack of *lpxT* significantly increased, and overexpression of *lpxT* decreased IL-1 α and IL-1 β release in 5637 cells (Fig. 3D and 3E), but had no effect on the release of IL-1 α or IL-1 β in 5637 cells treated with a Caspase-4 inhibitor (Fig. 3D and 3E) or in Caspase-4-silenced 5637 cells (Fig S4D and S4E), indicating that LpxT reduced the release of IL-1 α and IL-1 β through inhibition of Caspase-4. To confirm the effect of LpxT on the release of IL-1 α and IL-1 β induced by Caspase-11 *in vivo*, Caspase-11 knockout (Caspase-11^{-/-}) mice and wild-type mice were transurethrally infected with WT or Δ *lpxT*, and the release of IL-1 α and IL-1 β in the urine was measured at 6 and 24 h p.i. The results showed that the lack of *lpxT* increased the release of IL-1 α and IL-1 β in the urine of wild-type mice (Fig. 3F, 3G, 3H, and 3I), but had no effect on that of Caspase-11^{-/-} mice (Fig. 3F, 3G, 3H, and 3I); this result is consistent with the cell infection data.

In addition to triggering pyroptosis through Caspase-4/11, LPS also directly activates the TLR4 pathway, which is the major innate immune response used to monitor extracellular bacteria⁴⁶. In UPEC infection, LPS-induced TLR4 pathway activation results in the release of IL-6⁴⁶. We thus determined whether deletion of *lpxT* affects the release of IL-6. 5637 cells were infected with WT, Δ *lpxT* or Δ *lpxT*+, and the release of IL-6 was determined by ELISA at 4 h p.i. The results showed that the release of IL-6 was not significantly different between WT and Δ *lpxT* (Fig S4F). This result indicated that LpxT may not affect activation of the LPS-TLR4 pathway.

Collectively, these results indicate that by reducing the direct interaction between UPEC LPS and Caspase-4, LpxT diminishes Caspase-4/11 activation, resulting in reduced pyroptosis triggered by UPEC infection and therefore increased IBC formation.

Caspase-11 knockout restores the virulence and IBC formation of Δ *lpxT*

We next confirmed the interactions between Caspase-11-dependent pyroptosis and UPEC virulence in Caspase-11^{-/-} mice. We transurethrally infected wild-type and Caspase-11^{-/-} mice with WT and Δ *lpxT*, and assessed the bacterial titers 24 h p.i. The results showed that the bacterial titers in the bladders of Δ *lpxT*-infected were lower than those of WT-infected in wild-type mice, whereas the bacterial titers of Δ *lpxT*-infected were restored to levels similar to those of WT-infected in Caspase-11^{-/-} mice (Fig. 4A). This result indicated that LpxT-mediated inhibition of Caspase-11-dependent pyroptosis is required for acute infection caused by UPEC.

Next, we confirmed that LpxT contributes to IBC formation by reducing Caspase-11 activation in Caspase-11^{-/-} mice. We transurethrally infected wild-type and Caspase-11^{-/-} mice with WT and Δ *lpxT*, and assessed the number of IBCs and bacterial titers at 6 h p.i. The number of IBCs and bacterial titers were significantly lower in the bladders of Δ *lpxT*-infected wild-type mice than in those of WT-infected wild-type mice. However, the number of IBCs and bacterial titers were restored in the bladders of Δ *lpxT*-infected Caspase-11^{-/-} mice to levels similar to those in the bladders of the WT-infected Caspase-11^{-/-} mice (Fig. 4B and 4C). We also investigated whether LpxT-mediated diminishing of Caspase-11 activation was essential for subsequent rounds of IBC formation by infecting wild-type and Caspase-11^{-/-}

Δ mice with WT or Δ lpxT and assessed the number of IBCs at 24 h p.i. The number of IBCs was significantly lower in the bladders of Δ lpxT-infected wild-type mice than in those of WT-infected wild-type mice, whereas it was restored in the bladders of Δ lpxT-infected Caspase-11^{-/-} mice to levels similar to those in WT-infected Caspase-11^{-/-} mice (Fig. 4D). These results suggested that LpxT-mediated reduction of Caspase-11 activation is required for UPEC formation of IBCs, both the initial and subsequent rounds, in mouse bladders during acute infection.

Discussion

Caspase-4/11-dependent noncanonical inflammasome is the major cytosolic immune surveillance monitoring invading gram-negative bacteria, *via* the detection of bacterial common outer membrane component, lipid A of LPS. Many gram-negative bacterial pathogens replicate in vesicles for refuge to avoid this immune surveillance. Some bacteria deacylate lipid A of LPS in the position of 2' or 3', to reduce the interaction of LPS and Caspase-4/11 in some degree to evade Caspase-4/11 detection³³. But this modification only allows bacteria to modest replication (such as in the case of *Francisella novicida* which can replicate to about 100-folds³³) or achieve basic cytosolic survival (such as in the case of *Yersinia pestis*³²). UPEC is the only currently known pathogenic bacteria which can extensively replicate in cytosol of host cells. It can rapidly replicate from one bacterium to approximately 10⁵ bacteria within six hours, establishing IBCs in the cytosol of superficial BECs. The mechanisms by which UPEC avoids recognition by Caspase-4/11, enabling their extensive and rapid replication, remain unknown. In this study, we found that UPEC phosphorylates the 1-position of lipid A, which is a new form modification in a new position of lipid A to reduce the interaction between LPS and Caspase-4/11, different from the known deacylation modification, to allow UPEC massive replication in the BEC cytosol. It implies that the phosphorylation modification that we discovered in this study is a more efficient mechanism for evading Caspase-4/11 recognition, as it supports rapid and massive bacterial replication in host cytosol.

Acute UPEC infection can develop into recurrent infection even with appropriate antibiotic treatments, which makes UTI an increasingly common public health problem⁴⁷. There is a surge in demand for new therapeutics to combat this disease. In the community, presumably, only a few microorganisms enter the bladder, and these microorganisms are quickly eliminated by a robust host response if they are left in the harsh bladder lumen⁴⁸. By invading BECs and forming IBCs, UPEC rapidly increases in number to high levels. Once high bacterial titers are achieved, UPEC bursts into the lumen, and this sudden flood of microorganisms increases the chances of bacteria invading neighboring naïve superficial BECs to start the cycle anew to form subsequent rounds of IBCs¹⁴. During acute infection, UPEC undergoes at least two rounds of IBC formation, and acute infection terminates with exfoliation of the infected BECs. Exfoliation accompanied by IBC progression leads to the formation of bacterial QIRs in the underlying transitional cells that seed recurrent UTIs⁴⁹. Hence, IBC formation represents a bottleneck and a particular characteristic of UPEC infection, making infection difficult to cure. In this study, we found that by phosphorylating the lipid A in the position of 1, UPEC evades the cytosolic innate immune surveillance of Caspase-11, resulting in increased IBC formation. Our results also demonstrated that the lack of this

modification not only reduced initial IBC formation, but also reduced the reinfection of UPEC to form subsequent rounds of IBCs, and the incidence of recurrent UTIs. These results suggest that the modification encoded by *lpxT* is a prominent target for the development of new therapeutics to combat recurrent UPEC infection.

The CARD domain of Caspase-4/11 directly binds to the lipid A moiety and triggers the oligomerization and catalytic activation of Caspase-4/11²⁰. However, the structural basis of lipid A/LPS recognition by Caspase-4/11 has not yet been defined⁵⁰. Our colocalization result showed that overexpression of *lpxT* decreased the direct interaction between LPS and Caspase-4, from 21.8% to 10.7%. It is indicated that phosphorylation of lipid A by LpxT, which increases the negative charge of LPS, can hinder the interaction between Caspase-4 and LPS. The lipid A molecule can mainly be modified by altering its acyl chains and by adding a positively or negatively charged group to its sugar backbone. Deacylation of lipid A can reduce the detection by Caspase-11 in *F. novicida* and *Y. pestis*^{32,33}. Substitution of the phosphate group of lipid A with a positively charged appendage (phosphoethanolamine) in EHEC O157:H7 may correlate with Caspase-4/11 activation⁵⁰. In the present study, the addition of more phosphate residues reduced the interaction between LPS and Caspase-4. Thus, the change in charge of LPS is likely the reason that Caspase-4/11 can alter the ability to recognize LPS.

The function of *lpxT* was first characterized in *E. coli* W3110³⁸. LpxT reportedly increased resistance to deoxycholate (a major component of bile) and enhanced *E. coli* colonization in the gut^{38,51}. Analysis of the 2,136 publicly available complete genomes of *E. coli* showed that *lpxT* is conserved in all the genomes (Fig S5). *lpxT* is also present in *Salmonella* species⁵², and in the same genomic position as *E. coli* (Fig S6), indicating that this gene was present in the common ancestor of *E. coli* and *Salmonella*, and has been retained to date. In this study, we found that the expression of *lpxT* can be highly induced in UPEC infected BECs. Overexpression of *lpxT* results in increased phosphate modification of lipid A to prevent the recognition of UPEC by Caspase-11, thereby promoting IBC formation in the BEC cytosol. UPEC could have gained the ability to overexpress *lpxT* in the host cytosol during the coevolution of bacteria and host cells. Some intestinal pathogenic *E. coli*, such as the *E. coli* derivative *Shigella*, also invade intestinal epithelial cells and survive in the host cytosol. Determining whether *lpxT* is also overexpressed in *Shigella* when in the host cytosol and helps bacteria evade attack by Caspase-11, in addition to its currently known mechanism⁵³, requires further study. *Salmonella* suppresses Caspase-11 dependent-pyroptosis in macrophages⁵⁴, whether *Salmonella* also upregulates *lpxT* to evade Caspase-11 recognition for its replication need further study.

UPEC replicates massively in BEC cytosol to cause UTI. Hence, UPEC must diminish the detection and activation of inflammasomes for its successful infection. The UPEC TCS CpxRA downregulates the expression of *hlyA*, resulting in decreased activation of inflammasomes, and increased IBC formation in UPEC-infected mouse bladders²⁵. UPEC virulence factor Toll/IL-1 receptor-containing (TIR-containing) protein C (TcpC) attenuates the activation of the NLRP3 inflammasome by binding both NLRP3 and Caspase-1, and thereby preventing activation of Caspase-1⁵⁵. Our results showed that UPEC uses LpxT

to modify LPS lipid A, which is the major target of Caspase-11, to evade detection by Caspase-11, thereby facilitating IBC formation. Combined with the function of CpxRA and TcpC, these findings suggest that UPEC diminishes both the Caspase-1-mediated canonical inflammasome and the Caspase-11-mediated noncanonical inflammasome to avoid the cytosolic innate immune surveillance and support UPEC substantially replicates to form IBC in the BEC cytosol.

Methods

Animal Experiments

All animal studies were conducted according to protocols approved by the Institutional Animal Care Committee of Nankai University (Tianjin, China) and performed under protocol no. IACUC 2020030501. Six- to eight-week-old female C3H/HeN and C57/BL6 mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). *Caspase-11* knockout (*caspase-11*^{-/-}) C57/BL6 mice were generated using CRISPR-Cas9 at the Nankai University Laboratory Animal Center. All infections were performed in a biosafety level two facility and under the approved protocols.

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table S1. The UPEC strain CFT073 was used as the WT strain. The Δ *lpxT* mutant strain was generated using the λ -Red recombination method. The complemented Δ *lpxT*⁺ strain was generated by ligating *lpxT* and its own promoter into the genomic DNA between *glmS* and *pstS* of the Δ *lpxT* strain. For the overexpression of *lpxT*, CFT073 bacteria were transformed with a pACYC184 plasmid harboring the *lpxT* gene under its promoter between the XbaI I and BamH I sites. For fluorescence microscopy, CFT073 bacteria were transformed with a pETDuet plasmid expressing GFP under control of the T7 promoter. For the biosensor assay, CFT073 was transformed with a pETDuet plasmid harboring the *gfp* gene under control of the *lpxT* promoter. The plasmids used in this study are described in Table S2.

Bacterial strains were generally cultured in LB media under aerobic conditions at 37°C. The media were supplemented with antibiotics when required to the following final concentrations: ampicillin, 100 µg/mL; chloramphenicol, 25 µg/mL; and kanamycin, 50 µg/mL.

Cell culture and infections

The bladder epithelial cell line 5637 (ATCC HTB-9) was seeded in 24-well plates at a density of 2×10^5 cells/well, cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), and incubated at 37°C in 5% CO₂. Cells were infected with the indicated strains (WT, Δ *lpxT*, or Δ *lpxT*⁺) at a multiplicity of infection (MOI) of 10 for 1, 3, and 4 h, followed by an additional 1, 3, and 4 h of incubation in medium containing 100 µg/mL gentamicin to kill extracellular bacteria. After 1 h, the plate was

washed three times with PBS. The cells in each well were lysed with 200 μ L of 0.1% Triton X-100. Intracellular bacteria were counted by plating serial dilutions onto LB agar plates. A *caspase-4* knockdown of 5637 cells was generated using lentiviruses expressing shRNA constructs (Table S3).

Mouse infections and IBC enumeration

C3H/HeN mice were transurethrally inoculated with 10^7 CFUs of WT, Δ *lpxT*, or Δ *lpxT* + in 50 μ L of PBS after being anesthetized with an intraperitoneal injection of 3% chloral hydrate (Sangon Biotech; A600288). At predetermined timepoints, the mice were sacrificed, and the bladders were harvested aseptically. To quantify CFUs, the bladders were washed in PBS for three times, and then homogenized, serially diluted, and plated to quantify CFUs. For IBC enumeration, C3H/HeN mice were inoculated with WT, Δ *lpxT*, or Δ *lpxT*+ expressing GFP. After 6 or 24 h p.i., the bladders were cut open, fixed with 4% paraformaldehyde for 30 min and stained with 1 mg/mL wheat germ agglutinin (WGA; Invitrogen; W32464) for 5 min. Then, the bladders were washed with PBS for three times and stained with DAPI (Abcam, ab104139) for 5 min. The IBCs were visualized with a Zeiss LSM800 confocal microscope (Carl Zeiss). The number of IBCs in the whole bladder of each mouse from top to bottom and from left to right was counted via confocal microscopy¹². For chronic infection, 10^8 CFUs WT, Δ *lpxT*, or Δ *lpxT*+ suspended in PBS were inoculated into lightly anesthetized C3H/HeN mice. Infection proceeded for 28 days, the bladders were removed and homogenized in sterile PBS. Homogenates were plated for bacterial enumeration.

Transcriptomics by RNA sequencing (RNA-seq)

Total RNA was isolated from the bacteria containing BECs that was scraped from the mouse bladders using TRIzol (Invitrogen, 15596026). RNA quality and quantity were assessed using a NanoDrop spectrophotometer (Thermo) after each step. A MICROBEnrich Kit (Thermo Fisher Scientific, AM1901) was used to deplete host cell RNA. A MICROBExpress Bacterial mRNA Enrichment Kit (Thermo Fisher Scientific, AM1905) was used to remove bacterial 23S and 16S rRNA. Illumina library preparation, sequencing, and data analysis were conducted by Novogene. RNA-seq was performed on triplicate samples. RNA-seq data were mapped to the genome of CFT073 (AE014075.1). Normalized gene locus expression levels were calculated as RPKM. The genes showing an RPKM of ≥ 10 were chosen for subsequent statistical analysis. Differentially expressed gene analysis was performed using the edgeR package with the default parameters. Genes exhibiting *p* values < 0.05 and a threefold or greater difference in RPKM between two conditions were defined as differentially expressed⁵⁶. The raw sequence read files are available in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA1118349.

RNA extraction and qRT-PCR

The infected cells and bladders containing intracellular UPEC were harvested, RNA was extracted from bacteria, infected cells, or mouse bladders with an RNeasy Mini Kit (Qiagen; 74104), and DNase digestion was performed on a column using an RNase-free DNase set (Qiagen; 79254) to remove DNA

contamination. The integrity and purity of the RNA were checked by gel electrophoresis, and the absorbance was measured using a Nanodrop 2000 (Thermo). The ratio of A260 to A280 was between 1.9 and 2.1 for all RNA samples. For qRT–PCR analysis, total RNA was reverse transcribed with a StarScript III RT Master Mix reagent kit to synthesize cDNA. cDNA was used as a template and mixed with a SYBR Green PCR Master Mix kit supplemented with forward and reverse primers (Table S4). qRT–PCR was performed on an ABI 7500 thermocycler. The level of *lpxT* was normalized to that of *gyrA*. Fold changes in transcript levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Student's t test was used to analyze differences between groups.

³²P-labeled Lipid A extraction and TLC analysis

Cultures from overnight growth were diluted to an OD₆₀₀ of ~ 0.05 in 5 mL of M9 minimal medium, and 2.5 µCi/ml ³²Pi was added. Lipid A was prepared as described previously⁵⁷, with slight modifications. Bacteria were harvested at an OD₆₀₀ of ~ 0.8 and washed with 5 mL of PBS. After centrifugation, the pellet was resuspended in 3 mL of a single-phase Bligh/Dyer mixture consisting of chloroform/methanol/water (1:2:0.8)⁵⁸. After mixing and incubating for 1 h at room temperature, the insoluble residue was recovered by centrifugation. The residue was subjected to hydrolysis at 100°C for 30 min in 12.5 mM sodium acetate buffer, pH = 4.5. The ³²P-labeled lipid A species were recovered by two-phase Bligh/Dyer extraction and dried using a nitrogen dryer. The ³²P-labeled sample was dissolved in 50 µL of 4:1 chloroform-methanol (v/v). The mixture was vortexed and sonicated in a bath to completely dissolve the lipid material. Five microlitres of sample was added to a scintillation vial containing 5 mL of scintillation cocktail. Count in a scintillation counter and calculate total counts/min of sample. Using a microcapillary pipette, the sample was spotted onto a silica gel 60 TLC plate at 10,000 counts/min/spot and allowed to dry (> 15 min). The plate was developed in the solvent chloroform:methanol:water:glacial acetic acid (65:25:4:1, v/v). The plate was dried and exposed to a PhosphorImager Screen for 1 h to visualize the resolved ³²P-lipid A species.

LDH cytotoxicity analysis

5637 cells were cultured in 96-well plates until 80% confluence was reached. The Caspase-4 inhibitor Ac-LEVD-CHO (MedChemExpress; HY-136727) was added to the medium at 40 µM for 2 h before UPEC infection. The cells were infected with the indicated strains at an MOI of 10. After 2 h, the culture media were replaced with media supplemented with 100 µg/mL gentamicin for an additional 2 h to kill extracellular bacteria, the supernatants were collected, and the amounts of LDH were measured spectrophotometrically using a CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega; G1780). Cytotoxicity (%) was calculated by comparing the LDH in culture supernatants to the total cellular LDH (the amount of LDH released upon cell lysis with lysis buffer) in the presence of DMSO (vehicle control) or inhibitor.

ELISA analysis

5637 cells were seeded in 96-well plates at a density of 1×10^4 cells/well for 24 h. The cells were infected with WT, $\Delta lpxT$, or $\Delta lpxT +$ at an MOI of 10. After 2 h, the culture media were replaced with media supplemented with 100 $\mu\text{g/mL}$ gentamicin for an additional 2 h to kill extracellular bacteria; then, the supernatants were collected. Concentrations of IL-1 β and IL-1 α in the collected supernatants were determined by an ELISA development kit (R&D Systems; DY201-05, DY200-05). After being anesthetized, 6- to 8-week-old female C3H/HeN mice were infected *via* intraurethral catheterization with 50 μL of the bacterial suspension ($\sim 1 \times 10^7$ CFU). At 6 and 24 h p.i., urine was collected, and the concentrations of IL-1 β and IL-1 α in the urine were quantified by an ELISA development kit (R&D Systems; DY401-05, DY400-05) according to the manufacturer's protocol.

Western blotting

The cells and bladders were harvested and then lysed in RIPA lysis buffer supplemented with protease inhibitors (Roche; 4693116001). The samples were boiled in $4 \times$ SDS loading buffer. The proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked in TBS with 0.05% Tween 20 (TBST) containing 5% milk at room temperature for 1 h and probed with anti-mouse uroplakin III (Abcam; ab231576), anti-human gasdermin D (Cell Signaling Technology; 39754S), anti-mouse gasdermin D (Abcam; ab219800) or anti-cleaved gasdermin D (Cell Signaling Technology; 36425S) monoclonal primary antibodies overnight. After being washed 3 times with TBST, the membranes were incubated with the secondary antibodies HRP-conjugated rabbit anti-mouse IgG (1:10000; Abcam; ab6728) or goat anti-rabbit IgG (1:10000; Abcam; ab205718) at room temperature for 1 h. The proteins were visualized with enhanced chemiluminescence (ECL) western blotting detection reagents (Thermo Fisher Scientific; 34580) using the ChemiDoc™ MP imaging system (Bio-Rad). The protein band densities were analyzed using ImageJ 1.50 (NIH). β -actin or GAPDH was used as the loading control.

Immunofluorescence staining and microscopy

5637 cells were cultured on 6-well plates containing coverslips and subjected to bacterial infection. After infection, the cells were washed, fixed for 20 min with 4% paraformaldehyde in PBS, permeabilized for 20 min in 0.1% Triton X-100 in PBS, and blocked with 5% BSA for 1 h. Then, the cells were stained with the primary antibody anti-Caspase-4 (1:100; Cell Signaling Technology; 4450S), anti-*E. coli* LPS (1:100; Abcam; ab35654) diluted in PBS containing 1% BSA overnight at 4°C, and incubated with fluorescence-conjugated goat anti-rabbit IgG Alexa Fluor® 488 (1:200; Abcam; ab150077) or goat anti-mouse IgG Alexa Fluor® 594 (1:200; Abcam; ab150116). The nuclei were mounted in antifade reagent with DAPI for 5 min and imaged with a Zeiss LSM800 confocal microscope. The confocal images shown are representative of three independent experiments.

RNA interference

5637 cells were seeded in 96-well plates. The following day, the cells were transfected with 10 pmol of siRNA (Thermo; HSS104021) using Lipofectamine RNAiMAX (Invitrogen; 13778100) according to the manufacturer's instructions⁵⁹. The non-targeting negative control (Thermo; 12935300) was used as a

control. The degree of GBP1 knockdown was assessed by western blotting with the indicated antibody (Proteintech; 15303-1-AP), and the cells were used for experiments 48 h after the introduction of siRNAs (Table S3). Cells were infected with WT, $\Delta lpxT$, or $\Delta lpxT +$ at an MOI of 10. Then, the supernatants were collected, and the amount of LDH was measured.

Phylogenetic analysis

Phylogenetic analysis was performed on 2,136 complete *E. coli* genomes that were downloaded from the NCBI database. Single-copy core orthologous genes were determined using OrthoFinder⁶⁰, and recombination was detected using ClonalFrameM⁶¹. Conserved core genes in these genomes were identified and used for tree construction. A maximum-likelihood phylogeny with 1000 bootstrap replicates was generated using IQ-TREE⁶² under the generalized time reversible (GTR) model and visualized using iTOL⁶³.

Quantification and statistical analysis

All experiments were repeated three times independently. For all *in vitro* experiments, quantitative data are shown as the mean $\pm$ standard deviation (SD) ($n = 3$). The bacterial titers were measured at 2, 6, and 24 h p.i., and the quantitative IBC enumeration data are shown as the mean $\pm$ SD for a number ($n = 9$ or 15) of animals (indicated in each figure) pooled from three experiments, where each experiment employed at least 3 mice. Statistical analyses were performed using GraphPad Prism version 9.0.1 (GraphPad Software, La Jolla, CA, USA). The results were analyzed with an unpaired Student's *t* test and two-way ANOVA. For *in vivo* results, the Mann–Whitney U test was used according to the test requirements. Differences for which $p < 0.05$ were considered statistically significant.

Declarations

Competing Interest Statement:

The authors declare no competing interest.

Author Contributions:

L.W., L.F. and Y.P. designed research; X.L., S.Z., L.J., Q. W., H. S., C.J., J. M., Q. Z., X. Z., P. D., J. Z., J. Q., Y. L. and J.Q. performed research; S. S., B.L. and L.F. provided technical support and insights; X.L., S. Z. and Y.P. analyzed the data; and L.W., L.F. and Y.P. wrote the paper.

Acknowledgments

This work was funded by National Natural Science Foundation of China Grant, 32130003, 31820103002, and 32370194 (to L.W.), 32070133, 32470111 (to L.F.), 32400153 (to Y.P.), 32170110 (to L.J.), Shenzhen

References

1. Dielubanza EJ, Schaeffer AJ (2011) Urinary tract infections in women. *Med Clin North Am* 95:27–41. 10.1016/j.mcna.2010.08.023
2. Foxman B (2014) Urinary tract infection syndromes: occurrence, recurrence, bacteriology, risk factors, and disease burden. *Infect Dis Clin North Am* 28:1–13. 10.1016/j.idc.2013.09.003
3. Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the Global Burden of Disease Study 2019 (2022) *Lancet* 400, 2221–2248. 10.1016/s0140-6736(22)02185-7
4. Nielubowicz GR, Mobley HL (2010) Host-pathogen interactions in urinary tract infection. *Nat Rev Urol* 7:430–441. 10.1038/nrurol.2010.101
5. Brauner A, Jacobson SH, Kuhn I (1992) Urinary *Escherichia coli* causing recurrent infections—a prospective follow-up of biochemical phenotypes. *Clin Nephrol* 38:318–323
6. Russo TA, Stapleton A, Wenderoth S, Hooton TM, Stamm WE (1995) Chromosomal restriction fragment length polymorphism analysis of *Escherichia coli* strains causing recurrent urinary tract infections in young women. *J Infect Dis* 172:440–445
7. Bruxvoort KJ, Bider-Canfield Z, Casey JA, Qian L, Pressman A, Liang AS, Robinson S, Jacobsen SJ, Tartof SY (2019) Outpatient urinary tract infections in an era of virtual health care: trends from 2008 to 2017. *Clin Infect Dis*. 10.1093/cid/ciz764
8. Ambite I, Butler D, Wan MLY, Rosenblad T, Tran TH, Chao SM, Svanborg C (2021) Molecular determinants of disease severity in urinary tract infection. *Nat Rev Urol* 18:468–486. 10.1038/s41585-021-00477-x
9. Scott VC, Haake DA, Churchill BM, Justice SS, Kim JH (2015) Intracellular Bacterial Communities: A Potential Etiology for Chronic Lower Urinary Tract Symptoms. *Urology* 86, 425–431. 10.1016/j.urology.2015.04.002
10. Worby CJ, Olson BS, Dodson KW, Earl AM, Hultgren SJ (2022) Establishing the role of the gut microbiota in susceptibility to recurrent urinary tract infections. *J Clin Invest* 132. 10.1172/jci158497
11. Klein RD, Hultgren SJ (2020) Urinary tract infections: microbial pathogenesis, host-pathogen interactions and new treatment strategies. *Nat Rev Microbiol* 18:211–226. 10.1038/s41579-020-0324-0
12. Pang Y, Cheng Z, Zhang S, Li S, Li X, Li X, Zhang X, Li X, Feng Y, Cui H et al (2022) Bladder epithelial cell phosphate transporter inhibition protects mice against uropathogenic *Escherichia coli* infection. *Cell Rep* 39:110698. 10.1016/j.celrep.2022.110698
13. Schwartz DJ, Chen SL, Hultgren SJ, Seed PC (2011) Population dynamics and niche distribution of uropathogenic *Escherichia coli* during acute and chronic urinary tract infection. *Infect Immun* 79:4250–4259. 10.1128/IAI.05339-11

14. Justice SS, Hung C, Theriot JA, Fletcher DA, Anderson GG, Footer MJ, Hultgren SJ (2004) Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. *Proc Natl Acad Sci USA* 101:1333–1338. 10.1073/pnas.0308125100
15. Hunstad DA, Justice SS (2010) Intracellular lifestyles and immune evasion strategies of uropathogenic *Escherichia coli*. *Annu Rev Microbiol* 64:203–221. 10.1146/annurev.micro.112408.134258
16. Chen SL, Hung CS, Pinkner JS, Walker JN, Cusumano CK, Li Z, Bouckaert J, Gordon JI, Hultgren SJ (2009) Positive selection identifies an in vivo role for FimH during urinary tract infection in addition to mannose binding. *Proc Natl Acad Sci USA* 106:22439–22444. 10.1073/pnas.0902179106
17. Wu C, Lu W, Zhang Y, Zhang G, Shi X, Hisada Y, Grover SP, Zhang X, Li L, Xiang B et al (2019) Inflammasome Activation Triggers Blood Clotting and Host Death through Pyroptosis. *Immunity* 50:1401–1411e1404. 10.1016/j.immuni.2019.04.003
18. Latz E, Xiao TS, Stutz A (2013) Activation and regulation of the inflammasomes. *Nat Rev Immunol* 13:397–411. 10.1038/nri3452
19. Ta A, Vanaja SK (2021) Inflammasome activation and evasion by bacterial pathogens. *Curr Opin Immunol* 68:125–133. 10.1016/j.coi.2020.11.006
20. Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P, Hu L, Shao F (2014) Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* 514:187–192. 10.1038/nature13683
21. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, Zhuang Y, Cai T, Wang F, Shao F (2015) Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* 526:660–665. 10.1038/nature15514
22. Wiggins KA, Parry AJ, Cassidy LD, Humphry M, Webster SJ, Goodall JC, Narita M, Clarke MCH (2019) IL-1 α cleavage by inflammatory caspases of the noncanonical inflammasome controls the senescence-associated secretory phenotype. *Aging Cell* 18:e12946. 10.1111/ace1.12946
23. Demirel I, Persson A, Brauner A, Särndahl E, Kruse R, Persson K (2018) Activation of the NLRP3 Inflammasome Pathway by Uropathogenic *Escherichia coli* Is Virulence Factor-Dependent and Influences Colonization of Bladder Epithelial Cells. *Front Cell Infect Microbiol* 8:81. 10.3389/fcimb.2018.00081
24. Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, Hultgren SJ (1998) Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. *Science* 282:1494–1497. 10.1126/science.282.5393.1494
25. Nagamatsu K, Hannan TJ, Guest RL, Kostakioti M, Hadjifrangiskou M, Binkley J, Dodson K, Raivio TL, Hultgren SJ (2015) Dysregulation of *Escherichia coli* α -hemolysin expression alters the course of acute and persistent urinary tract infection. *Proc Natl Acad Sci USA* 112:E871–E880. 10.1073/pnas.1500374112
26. Wang X, Quinn PJ (2010) Lipopolysaccharide: Biosynthetic pathway and structure modification. *Prog Lipid Res* 49:97–107. 10.1016/j.plipres.2009.06.002

27. Samantha A, Vrielink A (2020) Lipid A Phosphoethanolamine Transferase: Regulation, Structure and Immune Response. *J Mol Biol* 432:5184–5196. 10.1016/j.jmb.2020.04.022
28. Simpson BW, Trent MS (2019) Pushing the envelope: LPS modifications and their consequences. *Nat Rev Microbiol* 17:403–416. 10.1038/s41579-019-0201-x
29. Santos JC, Dick MS, Lagrange B, Degrandi D, Pfeffer K, Yamamoto M, Meunier E, Pelczar P, Henry T, Broz P (2018) LPS targets host guanylate-binding proteins to the bacterial outer membrane for non-canonical inflammasome activation. *The EMBO journal* 37. 10.15252/embj.201798089
30. Kong Q, Six DA, Liu Q, Gu L, Roland KL, Raetz CR, Curtiss R 3rd (2011) Palmitoylation state impacts induction of innate and acquired immunity by the *Salmonella enterica* serovar typhimurium msbB mutant. *Infect Immun* 79:5027–5038. 10.1128/iai.05524-11
31. Kong Q, Six DA, Liu Q, Gu L, Wang S, Alamuri P, Raetz CR, Curtiss R 3rd (2012) Phosphate groups of lipid A are essential for *Salmonella enterica* serovar Typhimurium virulence and affect innate and adaptive immunity. *Infect Immun* 80:3215–3224. 10.1128/iai.00123-12
32. Rebeil R, Ernst RK, Gowen BB, Miller SI, Hinnebusch BJ (2004) Variation in lipid A structure in the pathogenic yersiniae. *Mol Microbiol* 52:1363–1373. 10.1111/j.1365-2958.2004.04059.x
33. Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA (2013) Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science* 341:1250–1253. 10.1126/science.1240988
34. Smith YC, Rasmussen SB, Grande KK, Conran RM, O'Brien AD (2008) Hemolysin of uropathogenic *Escherichia coli* evokes extensive shedding of the uroepithelium and hemorrhage in bladder tissue within the first 24 hours after intraurethral inoculation of mice. *Infect Immun* 76:2978–2990. 10.1128/iai.00075-08
35. Guyer DM, Radulovic S, Jones FE, Mobley HL (2002) Sat, the secreted autotransporter toxin of uropathogenic *Escherichia coli*, is a vacuolating cytotoxin for bladder and kidney epithelial cells. *Infect Immun* 70:4539–4546
36. Snyder JA, Haugen BJ, Buckles EL, Lockett CV, Johnson DE, Donnenberg MS, Welch RA, Mobley HLT (2004) Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. *Infect Immun* 72:6373–6381. 10.1128/IAI.72.11.6373-6381.2004
37. Miao Y, Li G, Zhang X, Xu H, Abraham SN (2015) A TRP Channel Senses Lysosome Neutralization by Pathogens to Trigger Their Expulsion. *Cell* 161:1306–1319. 10.1016/j.cell.2015.05.009
38. Touzé T, Tran AX, Hankins JV, Mengin-Lecreulx D, Trent MS (2008) Periplasmic phosphorylation of lipid A is linked to the synthesis of undecaprenyl phosphate. *Mol Microbiol* 67:264–277. 10.1111/j.1365-2958.2007.06044.x
39. Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ (2003) Intracellular bacterial biofilm-like pods in urinary tract infections. *Science* 301:105–107. 10.1126/science.1084550
40. Song J, Bishop BL, Li G, Duncan MJ, Abraham SN (2007) TLR4-initiated and cAMP-mediated abrogation of bacterial invasion of the bladder. *Cell Host Microbe* 1:287–298. 10.1016/j.chom.2007.05.007

41. Eto DS, Sundsbak JL, Mulvey MA (2006) Actin-gated intracellular growth and resurgence of uropathogenic *Escherichia coli*. *Cell Microbiol* 8:704–717. 10.1111/j.1462-5822.2006.00691.x
42. Hannan TJ, Mysorekar IU, Hung CS, Isaacson-Schmid ML, Hultgren SJ (2010) Early severe inflammatory responses to uropathogenic *E. coli* predispose to chronic and recurrent urinary tract infection. *PLoS Pathog* 6:29–30. 10.1371/journal.ppat.1001042. e1001042
43. Blango MG, Ott EM, Erman A, Veranic P, Mulvey MA (2014) Forced resurgence and targeting of intracellular uropathogenic *Escherichia coli* reservoirs. *PLoS ONE* 9:e93327. 10.1371/journal.pone.0093327
44. Wu XR, Sun TT (1993) Molecular cloning of a 47 kDa tissue-specific and differentiation-dependent urothelial cell surface glycoprotein. *J Cell Sci* 106(Pt 1):31–43. 10.1242/jcs.106.1.31
45. Wandel MP, Kim BH, Park ES, Boyle KB, Nayak K, Lagrange B, Herod A, Henry T, Zilbauer M, Rohde J et al (2020) Guanylate-binding proteins convert cytosolic bacteria into caspase-4 signaling platforms. *Nat Immunol* 21:880–891. 10.1038/s41590-020-0697-2
46. Schilling JD, Mulvey MA, Vincent CD, Lorenz RG, Hultgren SJ (2001) Bacterial invasion augments epithelial cytokine responses to *Escherichia coli* through a lipopolysaccharide-dependent mechanism. *J Immunol* 166:1148–1155
47. Hooton TM (2012) Uncomplicated urinary tract infection. *N Engl J Med* 366:1028–1037. 10.1056/NEJMcp1104429
48. Anderson GG, Martin SM, Hultgren SJ (2004) Host subversion by formation of intracellular bacterial communities in the urinary tract. *Microbes Infect* 6:1094–1101. 10.1016/j.micinf.2004.05.023
49. Mysorekar IU, Hultgren SJ (2006) Mechanisms of uropathogenic *Escherichia coli* persistence and eradication from the urinary tract. *Proc Natl Acad Sci U S A* 103:14170–14175. 10.1073/pnas.0602136103
50. Zamyatina A, Heine H (2020) Lipopolysaccharide Recognition in the Crossroads of TLR4 and Caspase-4/11 Mediated Inflammatory Pathways. *Front Immunol* 11:585146. 10.3389/fimmu.2020.585146
51. Tian X, Manat G, Gasiorowski E, Auger R, Hicham S, Mengin-Lecreulx D, Boneca IG, Touzé T (2021) LpxT-Dependent Phosphorylation of Lipid A in *Escherichia coli* Increases Resistance to Deoxycholate and Enhances Gut Colonization. *Front Microbiol* 12:676596. 10.3389/fmicb.2021.676596
52. Herrera CM, Hankins JV, Trent MS (2010) Activation of PmrA inhibits LpxT-dependent phosphorylation of lipid A promoting resistance to antimicrobial peptides. *Mol Microbiol* 76:1444–1460. 10.1111/j.1365-2958.2010.07150.x
53. Li Z, Liu W, Fu J, Cheng S, Xu Y, Wang Z, Liu X, Shi X, Liu Y, Qi X et al (2021) *Shigella* evades pyroptosis by arginine ADP-ribosylation of caspase-11. *Nature* 599:290–295. 10.1038/s41586-021-04020-1
54. Zhou L, Li Y, You J, Wu C, Zuo L, Chen Y, Kang L, Zhou Z, Huang R, Wu S (2024) *Salmonella* *spvC* gene suppresses macrophage/neutrophil antibacterial defense mediated by gasdermin D. *Inflamm Res*

73:19–33. 10.1007/s00011-023-01818-9

55. Waldhuber A, Puthia M, Wieser A, Cirl C, Dürr S, Neumann-Pfeifer S, Albrecht S, Römmeler F, Müller T, Zheng Y et al (2016) Uropathogenic *Escherichia coli* strain CFT073 disrupts NLRP3 inflammasome activation. *J Clin Invest* 126:2425–2436. 10.1172/jci81916
56. Liu Y, Liu B, Xu T, Wang Q, Li W, Wu J, Zheng X, Liu B, Liu R, Liu X et al (2021) A fructose/H(+) symporter controlled by a LacI-type regulator promotes survival of pandemic *Vibrio cholerae* in seawater. *Nat Commun* 12:4649. 10.1038/s41467-021-24971-3
57. Tran AX, Karbarz MJ, Wang X, Raetz CR, McGrath SC, Cotter RJ, Trent MS (2004) Periplasmic cleavage and modification of the 1-phosphate group of *Helicobacter pylori* lipid A. *J Biol Chem* 279:55780–55791. 10.1074/jbc.M406480200
58. Henderson JC, O'Brien JP, Brodbelt JS, Trent MS (2013) Isolation and chemical characterization of lipid A from gram-negative bacteria. *J Vis Exp* e50623. 10.3791/50623
59. Cheng Z, Zheng Y, Yang W, Sun H, Zhou F, Huang C, Zhang S, Song Y, Liang Q, Yang N et al (2023) Pathogenic bacteria exploit transferrin receptor transcytosis to penetrate the blood-brain barrier. *Proc Natl Acad Sci U S A* 120:e2307899120. 10.1073/pnas.2307899120
60. Liu B, Liu Y, Yang B, Wang Q, Liu X, Qin J, Zhao K, Li F, Feng X, Li L et al (2022) *Escherichia coli* O157:H7 senses microbiota-produced riboflavin to increase its virulence in the gut. *Proc Natl Acad Sci U S A* 119:e2212436119. 10.1073/pnas.2212436119
61. Didelot X, Wilson DJ (2015) ClonalFrameML: efficient inference of recombination in whole bacterial genomes. *PLoS Comput Biol* 11:e1004041. 10.1371/journal.pcbi.1004041
62. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ (2015) IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 32:268–274. 10.1093/molbev/msu300
63. Letunic I, Bork P (2021) Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res* 49:W293–w296. 10.1093/nar/gkab301

Figures

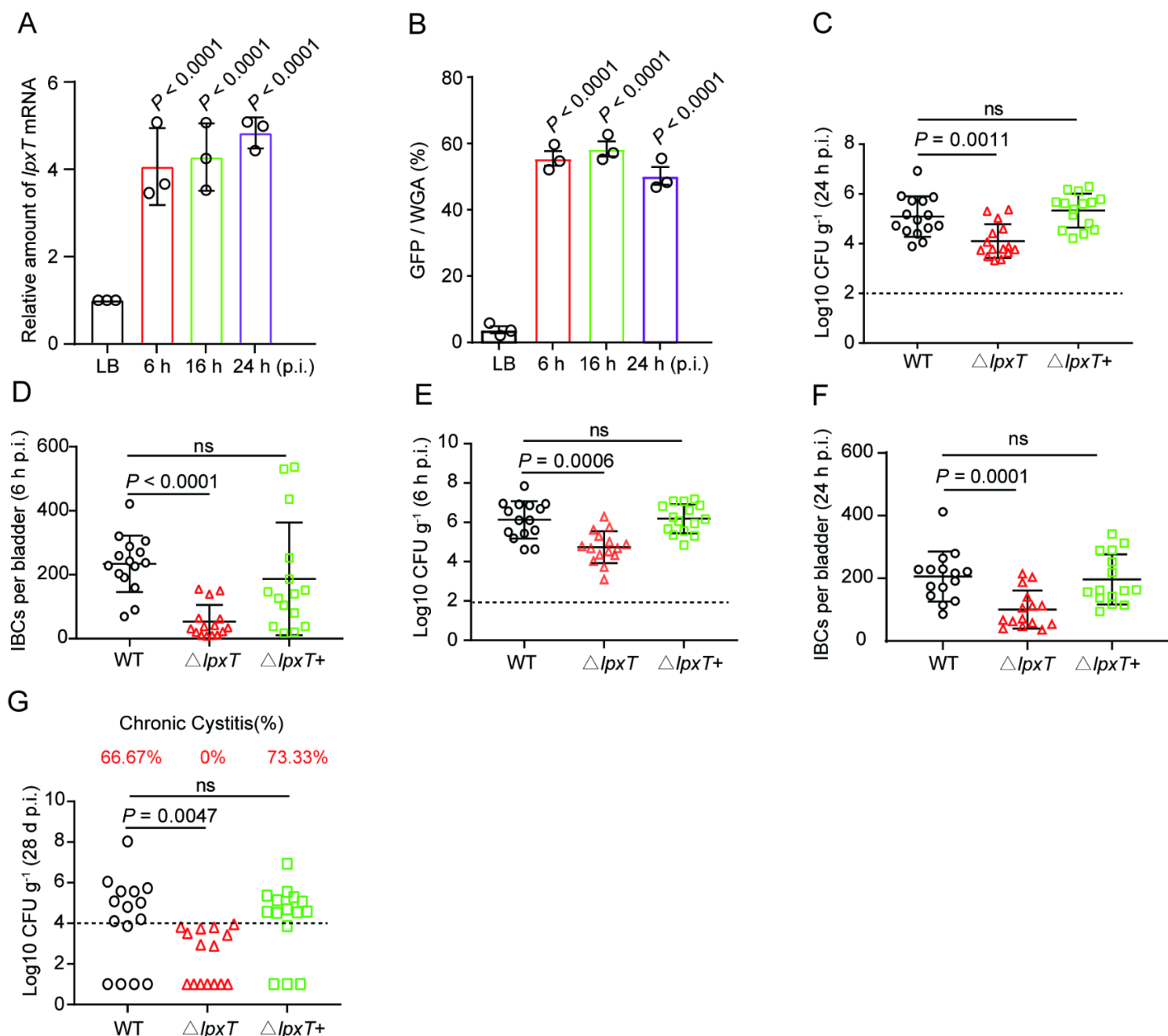


Figure 1

***lpxT* is essential for UPEC virulence and has upregulated expression during IBC formation.**

A. Fold change in the mRNA levels of *lpxT* in CFT073-infected mouse bladders compared to CFT073 inoculum at the indicated time points.

B. *lpxT* expression was calculated based on the percentage of the intensity of the GFP reporter gene under the control of the *lpxT* promoter to the intensity of WGA staining in CFT073-infected mouse bladders at the indicated time points and CFT073 cultured in LB medium.

C. Bacterial titers in the bladders of C3H/HeN mice transurethrally infected with WT, $\Delta lpxT$ or $\Delta lpxT+$ at 24 h p.i. The experiments were repeated three times, where each experiment employed five mice, and the

data were pooled ($n = 15$). The dotted line indicates the limit of detection.

D. IBC enumeration in C3H/HeN mouse bladders infected with WT, Δ/pxT , or $\Delta/pxT+$ at 6 h p.i. The experiments were repeated three times, where each experiment employed five mice, and the data were pooled ($n = 15$).

E. Bacterial titers in the bladders of C3H/HeN mice transurethrally infected with WT, Δ/pxT or $\Delta/pxT+$ at 6 h p.i. The experiments were repeated three times, where each experiment employed five mice, and the data were pooled ($n = 15$). The dotted line indicates the limit of detection.

F. IBC enumeration in C3H/HeN mouse bladders infected with WT, Δ/pxT , or $\Delta/pxT+$ at 24 h p.i. The experiments were repeated three times, where each experiment employed five mice, and the data were pooled ($n = 15$).

G. Bacterial titers in the bladders of C3H/HeN mice infected with WT, Δ/pxT or $\Delta/pxT+$ at 28 d p.i. The experiments were repeated three times, where each experiment employed five mice, and the data were pooled ($n = 15$).

Significance is indicated as the P value. ns, no significant difference.

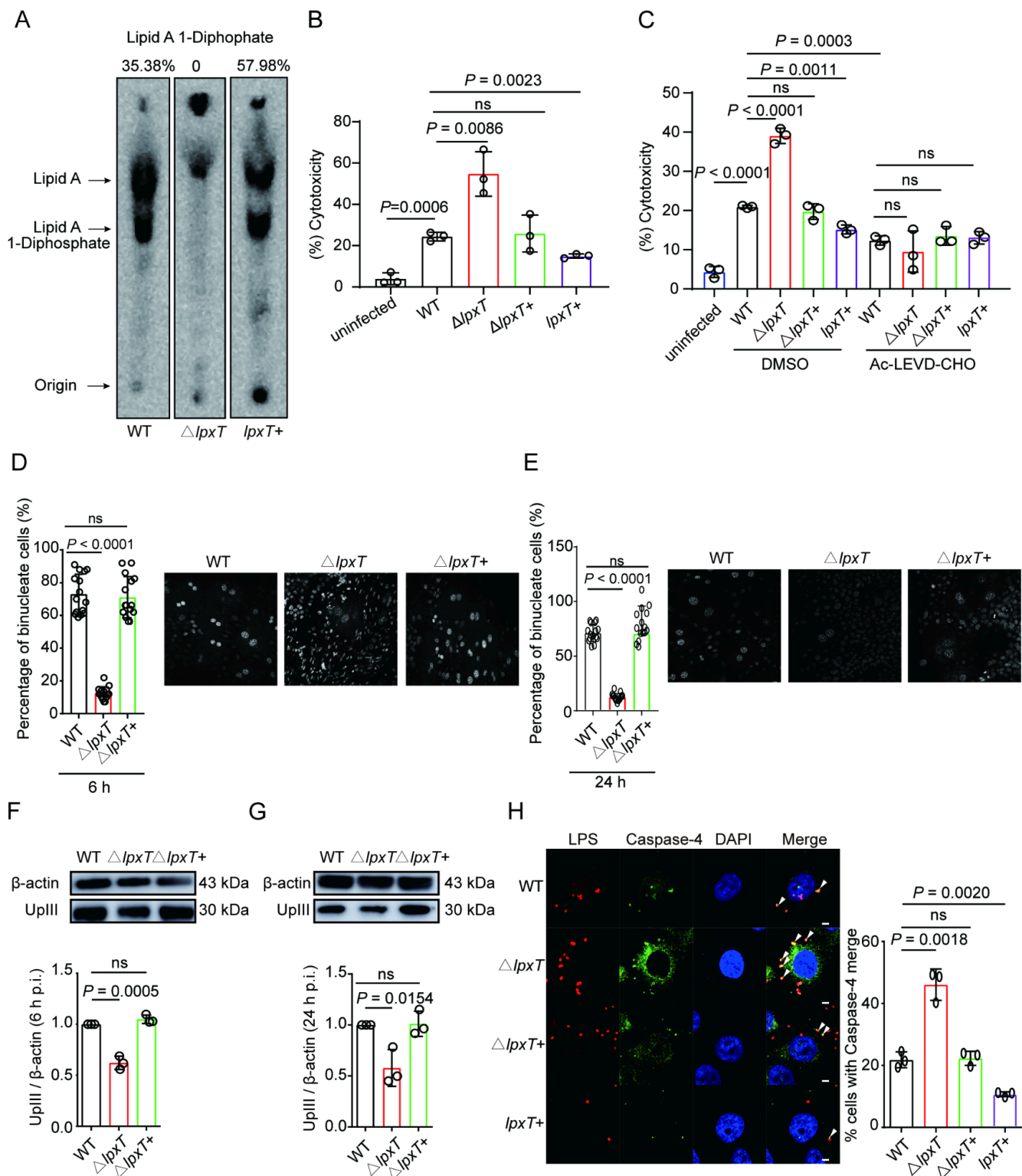


Figure 2

The increase in *lpxT* expression promotes the formation of lipid A 1-pyrophosphate in UPEC and inhibits Caspase-4/11-dependent pyroptosis.

A. ^{32} P-labeled lipid A species isolated from WT, $\Delta lpxT$, and $lpxT^+$ cultured in M9 minimal medium were separated by TLC and visualized by phosphorimaging analysis.

B. 5637 cells were infected with WT, $\Delta lpxT$, $\Delta lpxT^+$ or $lpxT^+$. Cell death at an MOI of 10 for 4 h p.i. was determined by measuring LDH release. Uninfected 5637 cells were used as a control.

C. 5637 cells were treated with 40 μ M Caspase-4 (Ac-LEVD-CHO) inhibitors for 2 h before infection with WT, $\Delta lpxT$, $\Delta lpxT^+$ or $lpxT^+$. Cell death at an MOI of 10 for 4 h p.i. was determined by measuring LDH release in the presence of DMSO (vehicle control), or Ac-LEVD-CHO. Uninfected 5637 cells were used as a control.

D and E. C3H/HeN mice were transurethrally infected with WT, $\Delta lpxT$ or $\Delta lpxT^+$, and the bladders were removed at 6 (D) and 24 h (E) p.i. Fixed whole-mounted bladders were stained with DAPI and imaged by confocal microscopy. Representative images showing the exfoliation of superficial BECs in mouse bladders (Right). Bar = 20 μ m. The percentage of superficial BECs was calculated from five random fields of view. The percentage relative to that in uninfected bladders was shown (Left). The experiments were repeated three times, where each experiment employed five mice, and the data were pooled (n = 15).

F and G. C3H/HeN mice were infected with WT, $\Delta lpxT$, or $\Delta lpxT^+$ for 6 (F) or 24 h (G). The homogenized bladder tissues were analyzed for the expression of UpIII by immunoblotting. The band intensity of UpIII was quantified and calculated in arbitrary units set to a value of 1 for WT-infected bladder samples by ImageJ.

H. Colocalization of Caspase-4 (green) with LPS on WT, $\Delta lpxT$, $\Delta lpxT^+$, and $lpxT^+$ (red) in infected 5637 cells at 4 h p.i. Representative images are shown (Right). Bar = 5 μ m. The bar graph shows the percentage of intracellular LPS that colocalized with Caspase-4 relative to total 5637 cells (Left).

Significance is indicated as the *P* value. ns, no significant difference.

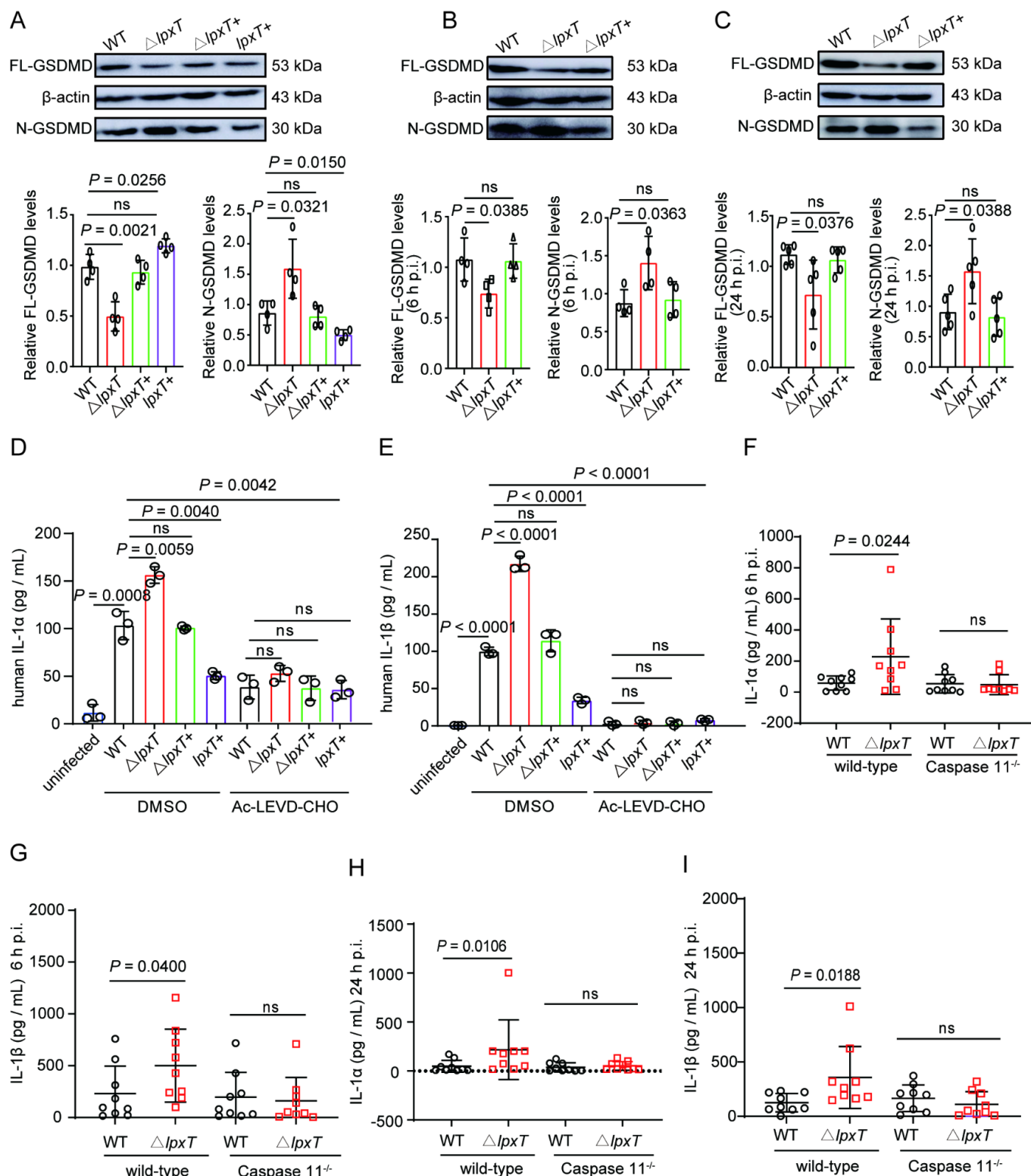


Figure 3

LpxT diminishes Caspase-4/11 activity upon UPEC infection.

A. The expression of full-length GSDMD and N-GSDMD in 5637 cells infected with WT, $\Delta lpxT$, $\Delta lpxT^+$ or $lpxT^+$ at 4 h p.i. The blots are representative of four independent experiments (Upper). The band intensity of full-length GSDMD and N-GSDMD were quantified by ImageJ (Bottom).

B and C. The expression of full-length GSDMD and N-GSDMD in mouse bladders infected with WT, $\Delta lpxT$ or $\Delta lpxT+$ at 6 (B) and 24 h (C) p.i. The blots are representative of four (B) or five (C) independent experiments (Upper). The band intensity of full-length GSDMD and N-GSDMD were quantified by ImageJ (Bottom).

D and E. 5637 cells were treated with DMSO or Ac-LEVD-CHO for 2 h before infection with WT, $\Delta lpxT$, $\Delta lpxT+$ or $lpxT+$. After 4 h, the amount of IL-1 α (D) and IL-1 β (E) in the culture medium was determined by ELISA.

F and G. Wild-type and Caspase-11^{-/-} C57/BL6 mice infected with WT or $\Delta lpxT$ were sacrificed at 6 h p.i. The amount of IL-1 α (F) and IL-1 β (G) in the urine was determined by ELISA. The experiments were repeated three times, where each experiment employed three mice, and the data were pooled (n = 9).

H and I. Wild-type and Caspase-11^{-/-} C57/BL6 mice infected with WT or $\Delta lpxT$ were sacrificed at 24 h p.i. The amount of IL-1 α (H) and IL-1 β (I) in the urine was determined by ELISA. The experiments were repeated three times, where each experiment employed three mice, and the data were pooled (n = 9). Significance is indicated as the *P* value. ns, no significant difference.

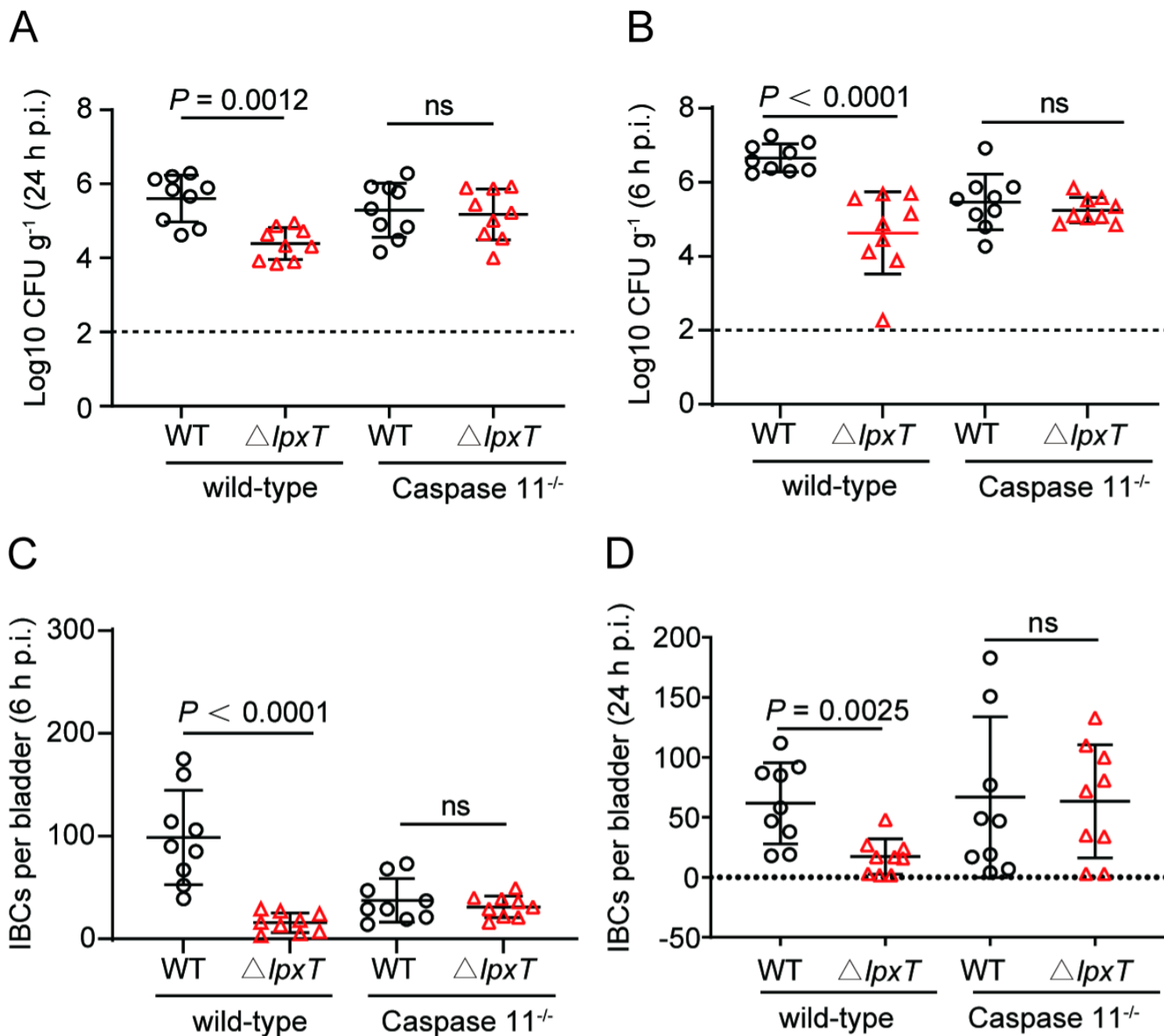


Figure 4

Caspase-11 knockout restores the virulence and IBC formation of $\Delta lpxT$.

A and B. Bacterial titers at 24 (A) and 6 h p.i. (B) in wild-type and Caspase-11^{-/-} C57/BL6 mouse bladders infected with WT or $\Delta lpxT$. The experiments were repeated three times, where each experiment employed three mice, and the data were pooled (n = 9). The dotted line indicates the limit of detection.

C and D. IBC enumeration at 6 (C) and 24 h p.i. (D) of wild-type and Caspase-11^{-/-} C57/BL6 mouse bladders infected with WT or $\Delta lpxT$. The experiments were repeated three times, where each experiment employed three mice, and the data were pooled (n = 9).

Significance is indicated as the *P* value. ns, no significant difference.

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