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Lipopolysaccharide Domains Modulate Urovirulence

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Uropathogenic *Escherichia coli* (UPEC) accounts for 80 to 90% of urinary tract infections (UTI), and the increasing rate of antibiotic resistance among UPEC isolates reinforces the need for vaccines to prevent UTIs and recurrent infections. Previous studies have shown that UPEC isolate NU14 suppresses proinflammatory NF-κB-dependent cytokines (D. J. Klumpp, A. C. Weiser, S. Sengupta, S. G. Forrestal, R. A. Batler, and A. J. Schaeffer, Infect Immun 69:6689–6695, 2001, http://dx.doi.org/10.1128/IAI.69.11.6689-6695.2001; B. K. Billips, A. J. Schaeffer, and D. J. Klumpp, Infect Immun 76:3891–3900, 2008, http://dx.doi.org/10.1128/IAI.00069-08). However, modification of lipopolysaccharide (LPS) structure by deleting the O-antigen ligase gene (*waaL*) enhanced proinflammatory cytokine secretion. Vaccination with the Δ*waaL* mutant diminished NU14 reservoirs and protected against subsequent infections. Therefore, we hypothesized that LPS structural determinants shape immune responses. We evaluated the contribution of LPS domains to urovirulence corresponding to the inner core (*waaP*, *waaY*, and *rfaQ*), outer core (*rfaG*), and O-antigen (*waaL*, *wzzE*, and *wzyE*). Deletion of *waaP*, *waaY*, and *rfaG* attenuated adherence to urothelial cells *in vitro*. In a murine UTI model, the Δ*rfaG* mutant had the most severe defect in colonization. The mutation of *rfaG*, *waaL*, *wzzE*, and *wzyE* resulted in an inability to form reservoirs in mouse bladders. Infection with the LPS mutant panel resulted in various levels of urinary myeloperoxidase. Since the Δ*waaL* mutant promoted Th_1-associated adaptive responses in previous studies (B. K. Billips, R. E. Yaggie, J. P. Cashy, A. J. Schaeffer, and D. J. Klumpp, J Infect Dis 200:263–272, 2009, http://dx.doi.org/10.1086/599839), we assessed NU14 for Th_2-associated cytokines. We found NU14 infection stimulated TLR4-dependent bladder interleukin-33 (IL-33) production. Inoculation with *rfaG*, *waaL*, *wzzE*, and *wzyE* mutants showed decreased IL-33 production. We quantified antigen-specific antibodies after infection and found significantly increased IgE and IgG1 in Δ*waaP* mutant-infected mice. Our studies show LPS structural constituents mediate multiple aspects of the UPEC life cycle, including the ability to acutely colonize bladders, form reservoirs, and evoke innate and adaptive immune responses.

Urinary tract infection (UTI) is the second most common infection that leads to physician visits (1). Nearly half of all women will have a UTI in their lifetime, and 25% will have recurrent infections (2–4), suggesting that the remaining women suffering an initial UTI are immunized against subsequent infections. Uropathogenic *Escherichia coli* (UPEC) isolates account for 80 to 90% of infections and are treated with antibiotics (4, 5). However, in recent years there has been an increase in antibiotic resistance (6–8). The increasing rate of antibiotic resistance among UPEC isolates reinforces the urgent need for a UTI vaccine.

Previous studies have shown that recurrent UTI can occur as a consequence of reinfection from persistent bacteria or reinitiation of infection from a fecal source (9). Fecal flora can serve as a UPEC reservoir for subsequent recolonization of the bladder (10). Additionally, previous studies have shown UPEC isolates invade epithelial urothelial cells and are able to proliferate intracellularly upon infection (11–13). These intracellular bacterial communities (IBCs) are bacterial reservoirs protected from phagocytosis, neutralizing antibodies, and antibiotics and may be responsible for recurrence (12, 14–16). UPEC IBCs can survive in the bladder as intracellular reservoirs, leaving the host latently infected (14, 17). Accordingly, follow-up studies in UTI patients have shown that approximately one-third to two-thirds of recurrent UTIs are caused by the same UPEC strain (15, 16, 18).

UPEC strain NU14 is a B2 group cystitis isolate that is considered an archetypal cystitis strain due to the presence of UPEC-associated virulence factors, and it has been used in many studies to characterize UPEC pathogenesis (11, 12, 19–21). UPEC strains evade immune recognition by utilizing the Toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) (8). Previous studies have shown NU14 suppresses proinflammatory NF-κB-dependent cytokine secretion compared to infection with the K-12 strain in urothelial cultures and in a murine UTI model (12, 19, 22). However, deletion of O-antigen ligase (*waaL*), which modifies the LPS structure, enhanced NF-κB-dependent cytokine secretion and showed increased neutrophil recruitment (19, 23), suggesting that structural components of UPEC LPS modulate immune recognition and host response.

Despite its clinical importance, the adaptive immune response to UPEC infection is not well characterized. Previous studies have shown adoptive transfer of T cells or serum from UPEC-infected mice partially protects naive mice from infection; however, there was no indication of T helper cell skewing toward a Th_1 or Th_2 response (2). Similarly, there are no previous studies on UPEC-specific antibody isotypes that might inform T cell responses to UPEC infection. However, inoculation with a Δ*waaL* O-antigen-deficient NU14 mutant enhanced NF-κB-dependent cytokine secretion, protected against NU14 challenge, and partially eradi-

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cated NU14 reservoirs (23), suggesting O-antigen modulates cell-mediated responses that target IBCs. Therefore, we hypothesized NU14 infection results in a humoral-associated cytokine production for a more antibody-mediated response. Here, we performed a systematic interrogation of LPS structure to identify LPS domains modulating immune responses. We deleted individual LPS biosynthetic genes in the outer core, the inner core, and O-antigen. We used cell culture and murine UTI models and found that LPS components modulate the UPEC life cycle and host responses.

MATERIALS AND METHODS

Bacterial strains and culture. Targeted NU14 LPS genes were deleted by homologous recombination using lambda red recombination (20). PCR primers were developed for the flanking 5′ and 3′ ends of the gene-coding region of each gene targeted for deletion with pKD3 as a template for the chloramphenicol cassette. The following the primers were used (uppercase letters refer to the gene sequence found on NU14, and lowercase letters refer to the chloramphenicol cassette sequence): *waaP* Fwd, 5′-GTGGATTAAAATAGTGGGCACTCATATTTCTCTCCGGAAAgtgtaggctggagctgcttc-3′; *waaP* Rev, 5′-TTATACAGTCTGCCAGAGAAAGCGGCGGACATCATAACGGcatatgaatatcctccttag-3′; *waaY* Fwd, 5′-AGCTAATAAATCCATGTTGGTTCCGTTTTGACTGTGTGGTgtgtaggctggagctgcttc-3′; *waaY* Rev, 5′-TTCATTAATTAAGTACAAGCTTAAGAAATAAATTACTCTTcatatgaatatcctccttag-3′; *rfaQ* Fwd, 5′-TTCATTAATTAAGTACAAGCTTAAGAAATAAATTACTCTTcatatgaatatcctccttag-3′; *rfaQ* Rev, 5′-CTTCACCTTAATCGGATAATCTCAACAAAAAGAGTTACTTcatatgaatatcctccttag-3′; *rfaG* Fwd, 5′-TTCATTAATTAAGTACAAGCTTAAGAAATAAATTACTCTTcatatgaatatcctcctag-3′; *rfaG* Rev, 5′-TCGATAAATTACTGCCCTCCTCCACGACAGGTACGTCGTTcatatgaatatcctccttag-3′; *wzzE* Fwd, 5′-GTAGAAATCGTGGTGGCAGCCCCAATTTAACCAAATAAATgtgtaggctggagctgcttc-3′; *wzzE* Rev, 5′-TTTCACATCGATTCTCTTCGAATAAGCGGCGAGCGCCTTTcatatgaatatcctccttag-3′; *wzyE* Fwd, 5′-GCTGGCAGCGGGCGTTGGCGATTGCCGCCGGGGAGGTCGCgtgtaggctggagctgcttc-3′; *wzyE* Rev, 5′-GCAGCGTATAGGTCGGTGCCGTGGTGTTGTTATTCATTGTcatatgaatatcctccttag-3′.

The PCR product was purified and electroporated into an NU14/pKD46 lambda red plasmid and plated on LB agar plates containing chloramphenicol at 30 μg/ml. Colonies selected were verified by sequencing for the insertion of the cassette. Bacterial cultures were grown at 37°C in LB under static conditions to promote the expression of type 1 pili (24). The mutation of LPS biosynthetic genes did not alter type 1 pilus expression as determined by hemagglutination assay (data not shown). To quantify antigen-specific antibody production, we transformed each of the mutants with an ovalbumin (OVA)-coding plasmid (pnir15.OVA) as described previously (2, 25). The expression of OVA by the bacterial strains was confirmed via Western blot analysis after 48 h of growth at 37°C under static and anaerobic conditions (data not shown) in LB medium with 100 μg/ml ampicillin added (25).

Bacterial growth curves. Starter bacterial cultures were grown in LB with appropriate antibiotics under static conditions to promote the expression of type 1 pili (24). To assess the role of LPS structure on bacterial growth and detergent susceptibility, bacterial cultures were diluted to an optimal density (OD) of 0.5 and grown at 37°C shaken in LB alone, LB with 1 μM EDTA (26), or LB with 1% sodium dodecyl sulfate (SDS) (27). The OD at 650 nm (OD_{650}) was determined at 1-h intervals for 4 h.

Cell culture. PD07i human bladder urothelial cells were maintained in EpiLife medium supplemented with human keratinocyte growth supplement (Invitrogen) and penicillin-streptomycin (Cellutions) at 37°C in a 5% CO_2 atmosphere (28–30).

Animals. Ten- to 12-week-old female C57BL/6 mice and $TLR4^{-/-}$ (B6.B10ScN-Tlr4lps-del/JthJ) mice were purchased from JAX and maintained at the Center for Comparative Medicine. All animals were used and maintained under Northwestern IACUC-approved protocols.

LPS purification. Purified LPS from NU14 and the LPS mutant panel (LPS purification kit; Boca Scientific) was treated with DNase, RNase, and proteinase K and repurified as described previously (31). LPS was quantified by Purpald assay, and 5 μg of each LPS was subsequently run on an SDS-PAGE gel and silver stained to visualize differently glycosylated LPS species (32, 33).

Invasion assays. PD07i cells were inoculated with NU14 or the panel of LPS mutants for 2 h, at which point they were treated with 100 μg/ml gentamicin for 30 min (11). Total CFU and gentamicin-protected (intracellular) CFU were quantified by plating onto LB agar plates.

Murine UTI. Ten- to 12-week-old female C57BL/6 mice were anesthetized by isoflurane and then instilled with a 10-μl volume containing 2×10^8 CFU from a stock solution of 10^{10} CFU/ml of bacteria in phosphate-buffered saline (PBS) via transurethral catheterization (4, 19, 20). Colonization was determined at 24 h postinoculation (hpi) by harvesting and homogenizing the bladders and then plating serial dilutions on eosin-methylene blue (EMB) agar with selective antibiotics (4). Latent colonization was determined at 2 weeks postinoculation by harvesting and homogenizing the bladders and then plating serial dilutions on EMB agar with selective antibiotics (4). The limit of detection for bladder colonization is 10 CFU/bladder.

Urinary neutrophil myeloperoxidase and bladder IL-33 ELISA. Ten- to 12-week-old female C57BL/6 mice were anesthetized by isoflurane and then instilled via transurethral catheter with a 10-μl volume of PBS or 2×10^8 CFU of each bacterial strain in PBS. At 6 hpi urine was collected and frozen until urinary myeloperoxidase (MPO) was quantified by enzyme-linked immunosorbent assay (ELISA) (Hycult Biotech) as described by the manufacturer (19). The limit of detection for the MPO ELISA is 1.6 ng/ml. To quantify interleukin-33 (IL-33) production, at 24 hpi bladders were harvested and homogenized with a Tissue-Tearor (Research Products International) in tissue extraction reagent (Invitrogen), and bladder IL-33 was assayed by ELISA (R&D).

IL-33 staining. Bladders were harvested at 24 hpi, fixed in 4% paraformaldehyde, and embedded in paraffin. The bladder sections were deparaffinized and antigen was retrieved using target retrieval buffer (S1699; Dako) in a decloaking chamber. The sections were then blocked with Fc receptor blocker and background buster (Innovex) to avoid mouse-on-mouse background staining. The sections were treated with mouse IL-33 monoclonal antibody (AF3626; R&D Systems) and exposed to goat anti-mouse (AP124-K; EMD Millipore) as a secondary antibody. For development we used rabbit anti-goat conjugated to alkaline phosphatase (A4062; Sigma) and a Vulcan fast red chromogen kit 2 (Biocare Medical FR805) as described by the manufacturer.

Antigen-specific antibody isotype ELISA. We quantified antigen-specific antibody production from NU14 and the LPS mutant panel by using the murine reinfection assay (2, 4). Briefly, we inoculated female C57BL/6 mice with either NU14 or the different LPS mutants (all strains contained the OVA plasmid) with 2×10^8 CFU bacteria via transurethral catheterization (day 0). Control mice were instilled with 10 μl of PBS. At 14 days postinoculation we reinoculated the mice by instilling OVA-expressing NU14 or the respective OVA-expressing LPS mutant with 2×10^8 CFU bacteria via transurethral catheterization. Control mice were instilled with 10 μl of PBS. We then challenged the mice 14 days after the second inoculation (on day 28) with wild-type (WT) NU14. At 24 h after NU14 challenge, serum was collected for antigen-specific antibody isotype ELISA. High-binding ELISA plates (Fisher Scientific) were coated with 0.1 mg/ml ovalbumin (Calbiochem) overnight at 4°C. Wells were washed with PBS with 0.5% Tween 20, and plates were incubated overnight with the collected mouse serum. The wells were washed and then incubated for 1 h with the respective anti-mouse antibody IgE, IgG2[c], or IgG1 (BD Biosciences). The wells were then incubated with streptavidin-horseradish peroxidase (R&D Systems). The wells were washed and incubated with substrate solution (R&D Systems) and visualized as indicated by the manufacturer's instructions.

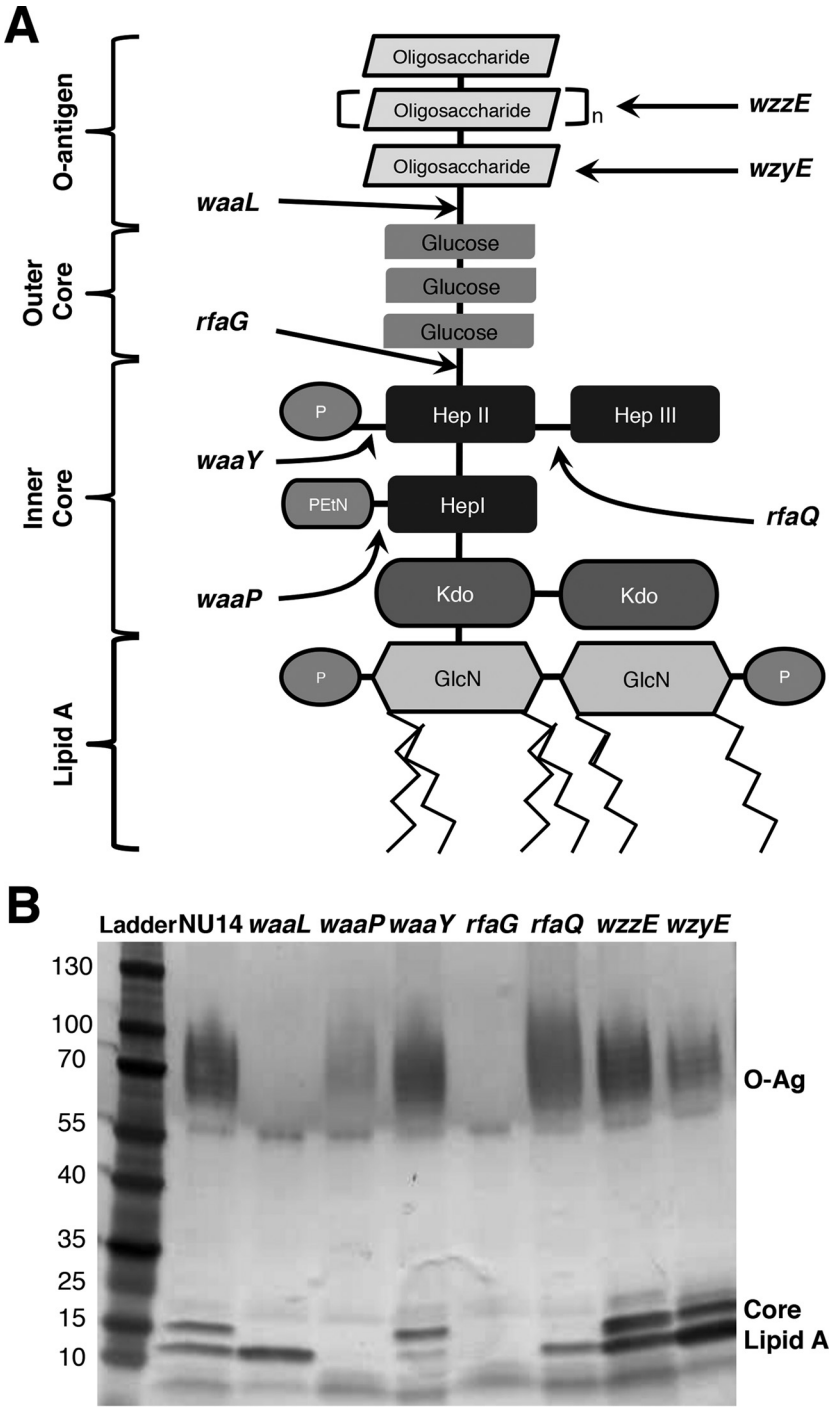

FIG 1 Deletion of LPS biosynthetic genes modulates LPS structure. (A) Structure of LPS, with mutants depicted with arrows. Mutation of the inner core (*waaP*, *waaY*, and *rfaQ*), outer core (*rfaG*), and O-antigen (O-Ag) (*wzzE*, *wzyE*, and *waaL*). (B) Silver-stained SDS-PAGE of purified LPS of NU14, *waaL*, *waaP*, *waaY*, *rfaG*, *rfaQ*, *wzzE*, and *wzyE* strains. Lower-molecular-weight species pertain to the core-lipid A region; higher-molecular-weight species pertain to O-antigen.

Statistical analysis. Results were analyzed by one-way analysis of variance followed by Dunnett's multiple-comparison test or Tukey's multiple-comparison test with the use of Prism software, version 6 (GraphPad, Inc.). Differences were considered statistically significant at a P value of <0.05.

RESULTS

Targeted deletion of LPS biosynthetic genes alters LPS structure. To evaluate the contribution of LPS domains to uropathogenic virulence, we deleted individual biosynthetic genes in each section of the LPS structure, the inner core (*waaP*, *waaY*, and *rfaQ*), outer core (*rfaG*), and O-antigen (*waaL*, *wzzE*, and *wzyE*), as depicted in Fig. 1A. LPS structure is assembled such that the lipid A-Kdo_2 serves as the acceptor for the core and lipidoligosaccharide chains (34). Sequential glycosyl transfer assembles both the core oligosaccharide and lipidoligosaccharide chains from nucleotide sugar precursors; the deletion of one gene can disrupt that sequential

TABLE 1 Targeted LPS biosynthetic genes and functions

Gene	Function
waaP	Adds phosphoryl substituent to Hep I
waaY	Phosphorylates Hep II
rfaQ	Transferase for branch Hep III residue
rfaG	Glucosyltransferase
waaL	Ligates O-antigen
wzzE	Determines O-antigen chain length
wzyE	Glycosidic linkages of O-antigen

assembly and results in modification of the core and O-antigen (34). Table 1 lists the function of each gene targeted for deletion. To visualize whether the deletion of these genes resulted in changes to the LPS structure, we purified LPS from the panel of NU14 mutants and performed SDS-PAGE (31, 33). Deletion of individual LPS biosynthetic genes resulted in alteration of the LPS structure (Fig. 1B). More specifically, the inner and outer core mutants, as well as the Δ*waaL* mutant, which ligates the O-antigen on the outer core, have changes in the lower-molecular-weight species consistent with changes to the core-lipid A region (35). Conversely, there is no change in the lower-molecular-weight species (core and lipid A regions) from the Δ*wzzE* and Δ*wzyE* O-antigen mutants; instead, these mutants exhibit a slight modification of the higher-molecular-weight species which corresponds to O-antigen. Mutant *rfaG* shows the most significant changes in the LPS structure with loss of bands in the core-lipid A region and in the O-antigen region, while the Δ*waaL* mutant appears to have no O-antigen, shown by the lack of higher-molecular-weight species, as predicted. Thus, targeted deletion of biosynthetic genes results in defects in a specific domain of the LPS structure.

LPS structure alters UPEC's susceptibility to detergents. To characterize the role of LPS structure in uropathogenesis, we assessed piliation of the NU14 LPS mutant panel by hemagglutination assay. We did not find any significant decrease in expression of type I pili after mutation of individual LPS biosynthetic genes with bacterial dilution to $\log_2(8)$ (data not shown). Further characterization of bacterial phenotypes showed that although piliation was unaffected, growth in rich medium was impacted by the deletion of O-antigen (Fig. 2A). We found that removal of the O-antigen structure by deleting either *rfaG* or *waaL* resulted in a significant decrease in bacterial growth in a rich medium. LPS is found on the outer surface of the outer membrane of *E. coli* and other Gram-negative bacteria; thus, it alters outer membrane functions (34, 36). EDTA has been shown to cause release of LPS and outer membrane proteins (26, 37). Δ*rfaG* and Δ*waaL* mutants showed a significant decrease in bacterial growth in LB medium containing 1 μM EDTA; however, these mutants showed a defect in bacterial growth in rich media, suggesting the decrease is not due to increased susceptibility to EDTA but instead due to overall growth defects (Fig. 2B). Resistance to SDS is a characteristic of outer membrane integrity (27, 38, 39). We sought to identify whether mutation of the LPS structure resulted in increased susceptibility to SDS by growing NU14 and the LPS mutant panel in LB containing 1% SDS. Δ*waaL*, Δ*waaP*, Δ*waaY*, Δ*rfaG*, Δ*rfaQ*, and Δ*wzzE* mutants showed a significant decrease in bacterial growth in LB media containing 1% SDS (Fig. 2C). These results suggest that mutation of LPS structure alters susceptibility to environmental stressors, potentially impacting survival in the urinary tract.

LPS structure modulates UPEC's ability to adhere to and invade PD07i cells. UPEC adherence to urothelial cells is essential for the initiation of infection and necessary for the eventual internalization of bacterial cells (11, 40–42). In order to characterize whether LPS domains played a role in the UPEC life cycle, including adherence and invasion of urothelial cells, we used a gentamicin protection assay. We found Δ*waaP*, Δ*waaY*, and Δ*rfaG* mutants had severe defects in the ability to adhere to bladder cells (Fig. 3A). Δ*waaP*, Δ*waaY*, Δ*rfaQ*, and Δ*rfaG* mutants were severely attenuated in their ability to invade bladder cells. Previous studies in our laboratory have revealed that the Δ*waaL* NU14 mutant showed a significant increase in invasion of PD07i urothelial cells (43). Consistent with those findings, here we found a significant increase in intracellular CFU (Fig. 3B); these data suggest that O-antigen plays a role in endocytic processing. Overall, NU14 Δ*rfaG* and Δ*waaP* mutants showed the most severe defects in both adherence and ability to invade urothelial cells *in vitro* (Fig. 3). These results suggest that LPS structure promotes the UPEC life cycle at the levels of adherence and invasion.

LPS structure modulates colonization in murine UTI model. We previously demonstrated that the Δ*waaL* mutant had attenuated colonization in murine UTI, suggesting that LPS structure plays a role in uropathogenicity. Indeed, inoculation with the panel of LPS mutants resulted in differential ability to acutely colonize bladders. The most striking colonization defect came from the Δ*rfaG* NU14 outer core mutant, where 25% of mice had sterile bladders at 24 hpi (Fig. 4A). Only inoculation with the Δ*wzzE* mutant failed to result in a significant decrease in bladder colonization compared to the level with WT NU14. Interestingly, deletion of *wzzE* also resulted in the least visible change in LPS structure compared to that of NU14 (Fig. 1B). The acute colonization phenotypes of the LPS mutant panel verify a role for the importance of specific LPS structural components in urovirulence.

To further characterize the role of LPS domains in later UPEC life cycle events, we quantified residual bladder colonization at 2 weeks postinoculation after stable reservoirs are established (44). Only inoculation with the inner core LPS mutants resulted in colonized bladders at 2 weeks postinoculation, indicative of formed reservoirs. Figure 4B shows inoculation with NU14 Δ*rfaG*, Δ*waaL* Δ*wzzE*, and Δ*wzyE* mutants resulted in sterile bladders at 2 weeks postinoculation. These findings suggest there is a role for the O-antigen structure in reservoir establishment or maintenance.

Mutation of the LPS domains modulates innate immune responses. We previously demonstrated infection with the NU14 Δ*waaL* mutant resulted in increased myeloperoxidase (MPO) levels in bladder homogenates compared to those of WT UPEC, suggesting enhanced neutrophil recruitment (19). To determine whether inoculation with the LPS panel of mutants resulted in altered activation of innate immune responses *in vivo*, we utilized a murine UTI model. We quantified functional differences in innate immune responses by assessing urinary MPO levels. While there was no significant change in urinary MPO compared to the level for NU14, we found significantly increased urinary MPO by mice inoculated with NU14, Δ*rfaQ*, Δ*waaL*, Δ*wzzE*, and Δ*wzyE* strains compared to PBS-instilled mice, suggesting LPS structure plays a role in innate immune responses (Fig. 5). There was no correlation between the ability to acutely colonize bladders and neutrophil recruitment (compare Fig. 4A and 5). Δ*waaP*, Δ*waaY*,

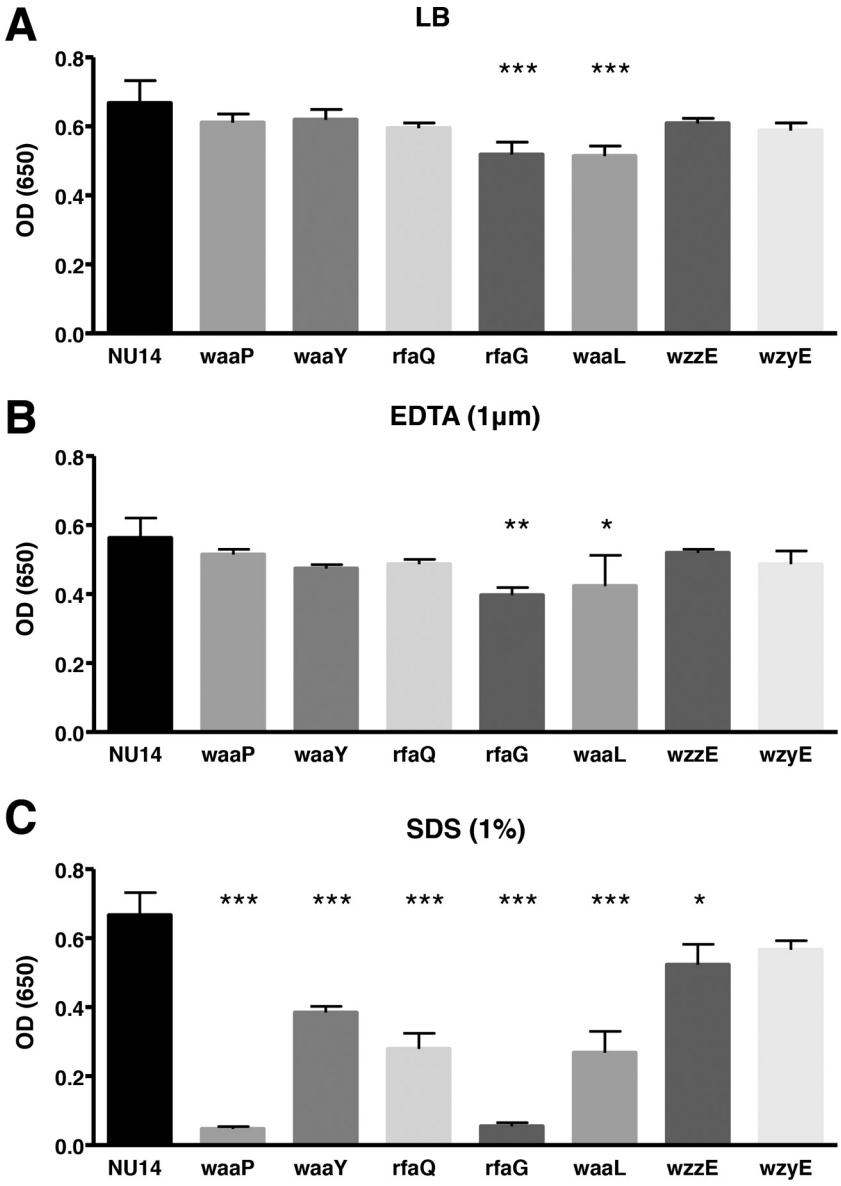

FIG 2 LPS structure modulates bacterial growth and outer membrane functions. (A) Bacterial growth of NU14, Δ*waaL*, Δ*waaP*, Δ*waaY*, Δ*rfaG*, Δ*rfaQ*, Δ*wzzE*, and Δ*wzyE* strains in LB medium. Δ*rfaG* and Δ*waaL* mutants showed a significant decrease in bacterial growth in LB medium. (B) Bacterial susceptibility to 1 μM EDTA. Δ*rfaG* and Δ*waaL* mutants showed a significant decrease in bacterial growth in LB medium containing 1 μM EDTA. (C) Bacterial susceptibility to 1% SDS. Δ*waaL*, Δ*waaP*, Δ*waaY*, Δ*rfaG*, Δ*rfaQ*, and Δ*wzzE* mutants showed a significant decrease in bacterial growth in LB medium containing 1% SDS. Data represent means ± standard errors of the means; this experiment was performed in duplicate and repeated three times. Statistical analysis was determined by one-way analysis of variance followed by Dunnett's multiple-comparison test; the asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate statistically significant differences between NU14 and the LPS mutants.

and Δ*rfaG* mutants failed to elicit a significant innate immune response. These results suggest the inner and outer core play a role in the activation of innate immune responses, as inoculation with the inner and outer core mutants fails to elicit a significant immune response.

UPEC infection stimulates TLR4-dependent IL-33 production. While it is less clear in humans, murine UTI models show recurrent UTI infections are the product of IBCs and reservoirs, presumably because the bacteria are protected from neutralizing antibodies, antibiotics, and phagocytosis (14). Therefore, we hypothesized that NU14 infection would induce a humoral adaptive immune response that would promote antibody production and thus would quantify Th_2-related cytokines IL-33 and IL-13 via ELISA in a murine UTI model. There was no significant increase in IL-13 bladder production at 24 hpi (data not shown). There was a significant increase in bladder IL-33 production at 12 hpi, with a peak in IL-33 concentration at 24 hpi (Fig. 6A). We next sought to assess the role of TLR4 in IL-33 production via ELISA, since UPEC infection results in activation of the TLR4 pathway (45). Indeed, $TLR4^{-/-}$ mice did not exhibit a significant increase in IL-33 production upon UPEC infection compared to WT C57BL/6 mice (Fig. 6B). We found prominent IL-33 staining in the urothelium

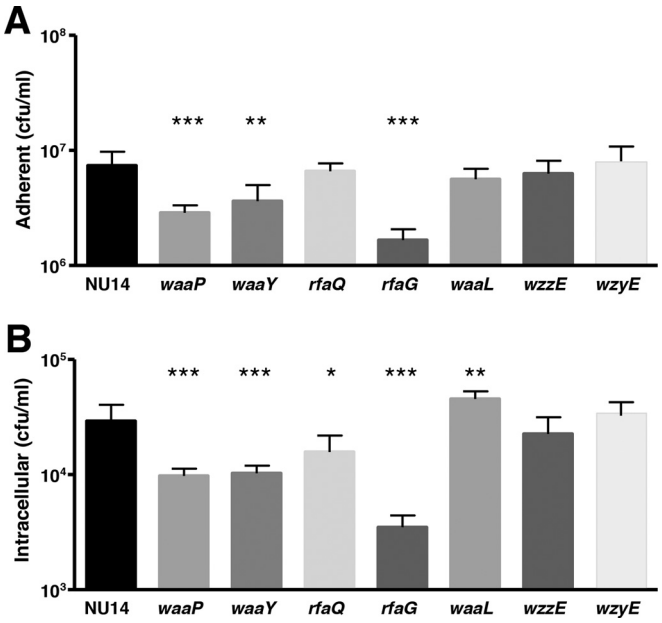

FIG 3 LPS structure modulates bacterial adherence and invasion. (A) Cell adherence to PD07i cells was determined at 2 hpi. Δ*waaP*, Δ*waaY*, and Δ*rfaG* mutants had severe defects in the ability to adhere to bladder cells. (B) Intracellular CFU were determined after gentamicin treatment. Δ*waaP*, Δ*waaY*, Δ*rfaQ*, and Δ*rfaG* mutants were severely attenuated in their ability to invade bladder cells, while the Δ*waaL* mutant had a significant increase in the number of intracellular CFU. Data represent means ± standard errors of the means; each experiment was performed three times in triplicate. Statistical analysis was determined by one-way analysis of variance followed by Dunnett's multiple-comparison test; the asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate statistically significant differences between NU14 and the LPS mutants.

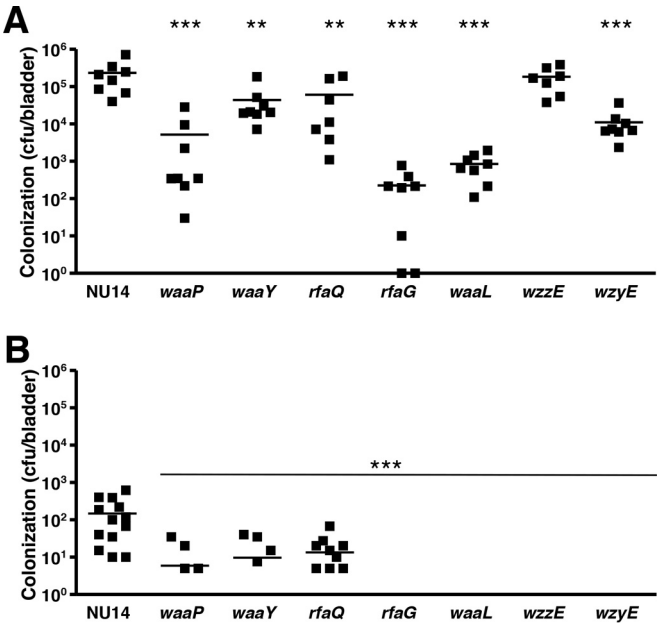

FIG 4 LPS structure modulates acute bladder colonization and bladder reservoirs. (A) Acute bladder colonization. Female C57BL/6 mice were instilled via transurethral catheterization with 2×10^8 CFU of NU14, Δ*waaL*, Δ*waaP*, Δ*waaY*, Δ*rfaG*, Δ*rfaQ*, Δ*wzzE*, and Δ*wzyE* strains. Δ*waaP*, Δ*waaY*, Δ*rfaQ*, Δ*rfaG*, Δ*waaL*, and Δ*wzy*E mutants were severely attenuated in their ability to colonize bladders at 24 hpi. (B) Colonization at 2 weeks postinfection likely to indicate reservoirs. Mice infected with Δ*rfaG*, Δ*waaL*, Δ*wzzE*, and Δ*wzyE* mutants had sterile bladders at 2 weeks postinoculation. Data represent means ± standard errors of the means; each experiment was performed at least twice. Statistical analysis was determined by one-way analysis of variance followed by Dunnett's multiple-comparison test; the asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate statistically significant differences between NU14 and the LPS mutants.

of WT mice after NU14 infection, suggesting bladder epithelial cells produce IL-33 in response to UPEC infection. Indeed, urothelial IL-33 immunostaining is largely absent from the TLR4$^{-/-}$ mice (Fig. 6C). Additionally we find that mutation of the LPS structure modulates bladder IL-33 production (Fig. 6D). Any mutation that altered O-antigen resulted in significantly decreased IL-33 levels compared to WT UPEC, suggesting a role for the O-antigen domain in modulating the innate adaptive immune interface.

LPS structure modulates UPEC-specific IgG1 and IgE production in murine UTI. Previous studies have shown UPEC infection activates adaptive immune responses and results in the generation of protective antibodies (2). However, the production of UPEC-specific antibodies and antibody isoforms has not been characterized. Here, we used a reinfection model to assess the production of Th_1- and/or Th_2-dependent antigen-specific antibodies and whether specific domains of the LPS structure skewed the antigen-specific antibody production toward Th_1- or Th_2-dependent antibodies (2, 4, 25). All bacterial strains were transformed with a plasmid encoding OVA to facilitate the detection of antigen-specific antibody production (2, 25). The bacterial strains transformed with the OVA-encoding plasmid expressed OVA under anaerobic conditions (data not shown), and as such all strains are likely to continue to express OVA while growing under the anaerobic conditions of the bladder (2, 25). To allow for induction of the adaptive immune response, mice were inoculated twice (with a 14-day interval) with either NU14-OVA or the OVA-expressing LPS mutant panel and then challenged 14 days after the second inoculation (day 28) (2, 4, 25). Serum from these animals was used to quantify OVA-specific antibody isoforms relative to saline-instilled mice. There were no significant increases in Th_1-associated antibody IgG2c after inoculation with any of the LPS mutants or WT UPEC compared to PBS-instilled mice (Fig. 7C). Mice inoculated with the Δ*waaP* and Δ*waaY* mutants had a significant increase in OVA-specific IgG1, a Th_2-associated antibody (Fig. 7A). Additionally, mice inoculated with the Δ*waaP* mutant also had significant OVA-specific IgE antibody production compared to mice instilled with PBS (Fig. 7B). These data suggest that while WT UPEC infection does not result in a significant increase in UPEC-specific antibody isoforms, the inner core domain of the LPS structure plays a role in adaptive immune responses, specifically Th_2-associated antibody production.

DISCUSSION

We sought to identify the impact of LPS structural elements in uropathogenesis. We find that although deletion of individual LPS biosynthetic genes in the outer core (*waaP*, *waaY*, and *rfaQ*), the inner core (*rfaG*), and O-antigen (*waaL*, *wzzE*, and *wzyE*) did not affect the expression of type I pili, deletion of *rfaG* and *waaL* impacted bacterial growth even in rich medium. We therefore examined the growth of these bacterial strains in EDTA to produce a

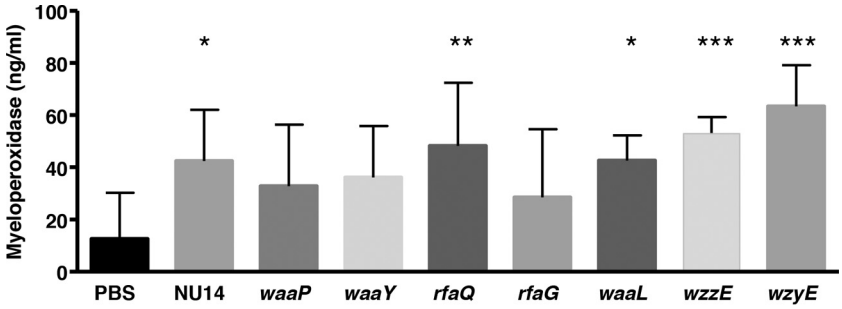

FIG 5 O-antigen suppresses urinary MPO. Female C57BL/6 mice were instilled via transurethral catheterization with PBS or 2×10^8 CFU of NU14, Δ*waaL*, Δ*waaP*, Δ*waaY*, Δ*rfaG*, Δ*rfaQ*, Δ*wzzE*, and Δ*wzyE* strains, and urine was collected at 6 hpi. Mice infected with NU14, Δ*rfaQ*, Δ*waaL*, Δ*wzzE*, and Δ*wzyE* strains had increased urinary MPO compared to PBS-instilled mice. There were no differences between NU14-infected mice and the LPS mutant-infected mice ($n = 8$). Data represent means ± standard errors of the means; each experiment was performed at least twice. Statistical analysis was determined by one-way analysis of variance followed by Dunnett's multiple-comparison test; the asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate statistically significant differences between results for PBS- and LPS mutant-treated mice.

chelating environment and again only saw growth defects in Δ*rfaG* and Δ*waaL* mutants. Alternatively, we find that only NU14 and Δ*wzyE* strains were unaffected by growth in medium with 1% SDS, confirming the importance of multiple LPS domains in maintaining outer membrane integrity (27, 39). Bacterial survival in the gastrointestinal tract may have a higher need for the selection of detergent-resistant fitness (38), and while the bladder may not constitute the same type of stresses, we find that multiple LPS domains have a role in maintaining the detergent-resistant capabilities found normally in *E. coli* strains.

We find LPS structure impacts the ability of UPEC to invade urothelial cells *in vitro* and *in vivo*, as we saw variations in acute and long-term colonization among mutants. More specifically, we find genes that alter O-antigen structure (*rfaG*, *waaL*, *wzzE*, and *wzyE*) have variable effects on acute colonization (Δ*wzzE* mutant infects at WT levels). However, all O-antigen mutants had sterile bladders at 2 weeks postinoculation, suggesting that O-antigen plays an additional role in establishment and maintenance of reservoirs. Consistent with these results, other studies have shown that removal of O-antigen from pyelonephritis isolate CFT073 and extraintestinal *E. coli* isolate CP9 also resulted in attenuated acute colonization of murine bladders (46, 47). Previous studies have shown the expression of type I pili is required for efficient adherence and invasion of urothelial cells (42); however, here we show that O-antigen is also able to modulate adherence and invasion independent of type I pilus expression. Previous studies have shown a fraction of IBCs are able to reach a latent state of infection within urothelial cells known as quiescent intracellular reservoirs (QIR), which can remain latent in a host and result in reinfection (17, 44, 48–50). Interestingly, we find that the NU14 Δ*wzzE* mutant colonizes at wild-type levels acutely but is unable to colonize bladders long term; this could be due to the ability of the Δ*wzzE* strain to form IBCs but inability to form QIR, and as such we speculate that O-antigen plays a critical role in the UPEC intracellular life cycle.

TLR4 signaling has been well established in modulating susceptibility to UPEC infections. C3H/HeJ mice lack functional TLR4 signaling, and as such they are hyporesponsive to LPS and are unable to clear UPEC infections due to defects in neutrophil recruitment and function (51–53). Here we find that altering the LPS structure by deleting individual biosynthetic genes alters neutrophil recruitment, as there are various levels of urinary MPO, such that core mutants *waaP*, *waaY*, and *rfaG* fail to induce significant neutrophil recruitment characteristic of wild-type NU14. We do not find a correlation between urinary MPO collected at 6 h postinfection and bladder colonization at 24 h postinoculation. However, there may be differences in bladder colonization at this earlier time point. We find that inner and outer core mutants that do not modify the Hep III residue fail to substantially induce neutrophil recruitment, suggesting that the LPS core is important for adequate neutrophil recruitment.

We assessed production of the Th_2 initiator cytokine IL-33 and found a significant increase in bladder IL-33 at 12 h postinoculation in a TLR4-dependent manner. IL-33 has been well established in helping skew adaptive immune responses toward a Th_2 response (54). Consistent with our hypothesis, we find that inoculation with the Δ*waaL* LPS mutant does not result in the significant production of IL-33. Additionally, we find that there is a role for O-antigen in the innate/adaptive immune interface, as inoculation with any O-antigen mutant resulted in low IL-33 production. Recently it has been shown that IL-33 also plays a role in neutrophil recruitment in a sepsis model of infection (55). We did not find a correlation between IL-33 and neutrophil recruitment, but future studies will further explore the role of TLR4-dependent IL-33 production and neutrophil recruitment in UPEC pathogenesis.

We previously demonstrated that infection with NU14 results in suppression of NF-κB activation and downstream NF-κB-dependent cytokine production (12, 19). While pyelonephritis isolate CFT073 suppresses NF-κB activation by secreting TcpC toxin, which contains a Toll/IL-1 receptor domain that binds to MyD88, other UPEC isolates, including NU14, lack *tcpC* (20, 56, 57). Alternatively, many UPEC strains express and secrete the pore-forming toxin α-hemolysin (HlyA), which suppresses NF-κB activation and proinflammatory cytokine secretion in an Akt-dependent manner (58, 59). More recently, it has been shown that IL-33 reduces NF-κB activation and NF-κB-dependent cytokine production (60). Thus, our findings suggest that the secretion of IL-33 in response to NU14 infection is an additional potential mechanism for UPEC-mediated NF-κB suppression. IL-33 has been characterized as promoting a Th_2 response (61, 62). We previously demonstrated that UPEC infection activates adaptive immune responses and results in the generation of protective antibodies (2). However, there was no previous characterization of the

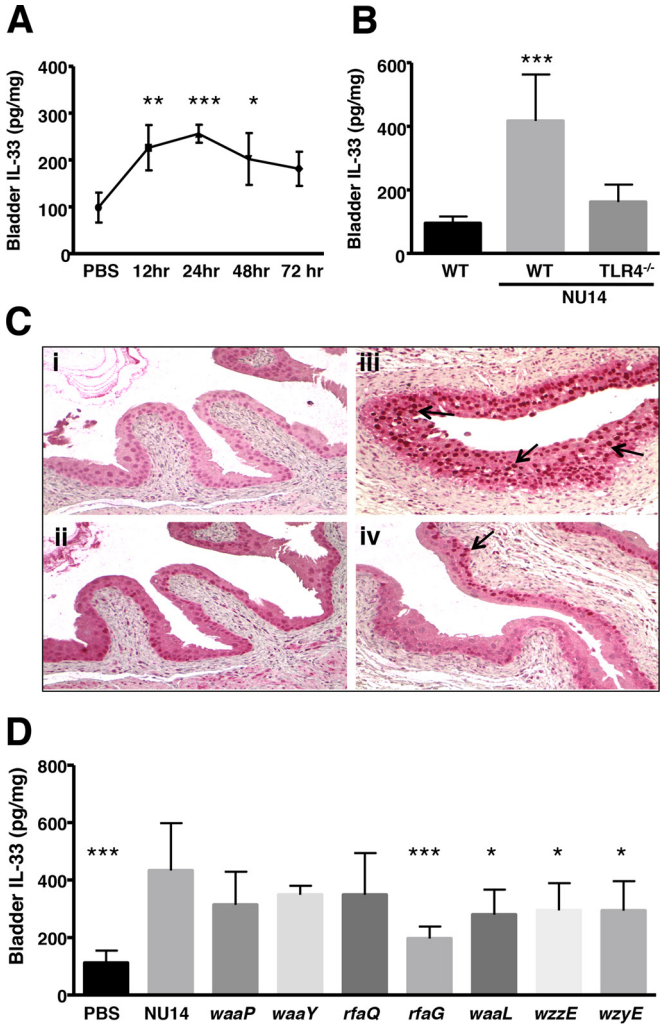

FIG 6 UPEC induces IL-33 secretion in a TLR4-dependent manner from bladder urothelial cells. (A) Female C57BL/6 mice were instilled via transurethral catheterization with PBS or 2×10^8 CFU of NU14, and bladders were harvested at 12, 24, 48, and 72 h postinoculation ($n = 5$). IL-33 production was increased at 12 hpi, peaked at 24 hpi, and decreased by 72 hpi. Statistical analysis was determined by one-way analysis of variance followed by Dunnett's multiple-comparison test; the asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate statistically significant differences between PBS- and NU14-instilled mice. (B) Female C57BL/6 and TLR4$^{-/-}$ mice were instilled via transurethral catheterization with PBS or 2×10^8 CFU of NU14, and bladders were harvested at 24 hpi. TLR4$^{-/-}$ mice infected with NU14 did not result in significant IL-33 production. Statistical analysis was determined by one-way analysis of variance followed by Tukey's multiple-comparison test; the asterisks (**, $P < 0.01$; ***, $P < 0.001$) indicate statistically significant differences between WT C57B6 mice instilled with PBS, WT C57B6 mice inoculated with NU14, and TLR4$^{-/-}$ NU14-inoculated mice ($n = 5$). (C) Bladder IL-33 staining. (i) Negative control of C57BL/6 bladder section stained for IL-33, with all antibodies carried out except IL-33. (ii) PBS-instilled C57BL/6 bladder section stained for IL-33 shows minimal staining. (iii) NU14-infected C57BL/6 bladder shows increased IL-33 staining in the urothelium (arrows). (iv) NU14-infected TLR4$^{-/-}$ mouse bladder shows minimal IL-33 staining. (D) O-antigen induces bladder IL-33 production. Female C57BL/6 mice were instilled via transurethral catheterization with PBS or 2×10^8 CFU of NU14, Δ*waaL*, Δ*waaP*, Δ*waaY*, Δ*rfaG*, Δ*rfaQ*, Δ*wzzE*, Δ*wzyE* strains, and bladders were harvested at 24 hpi. Mice infected with Δ*rfaG*, Δ*waaL*, Δ*wzzE*, and Δ*wzyE* mutants showed no significant induction in IL-33 production compared to NU14-infected mice. Statistical analysis was determined by one-way analysis of variance followed by Dunnett's multiple-comparison test; the asterisks (*, $P < 0.05$; **, $P < 0.01$) indicate statistically significant differences between WT NU14 and the LPS mutants.

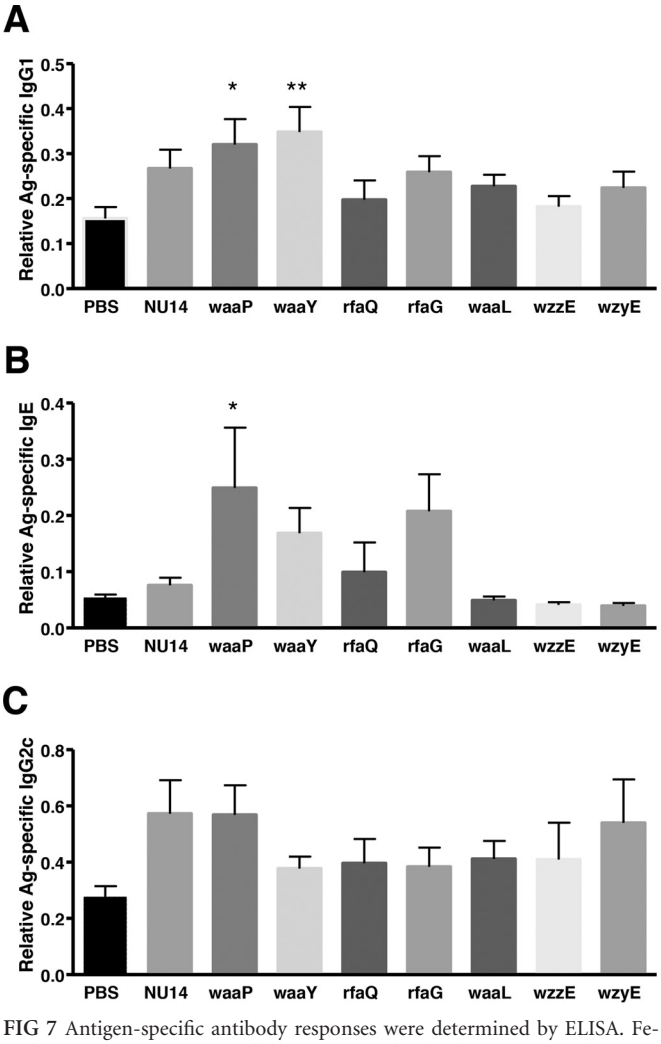

FIG 7 Antigen-specific antibody responses were determined by ELISA. Female C57BL/6 mice were instilled via transurethral catheterization with PBS or 2×10^8 CFU of NU14-OVA, Δ*waaL*-OVA, Δ*waaP*-OVA, Δ*waaY*-OVA, Δ*rfaG*-OVA, Δ*rfaQ*-OVA, Δ*wzzE*-OVA, and Δ*wzyE*-OVA strains on day 1 and again on day 14. The mice were challenged with NU14 on day 28, and serum was collected 24 h after UPEC challenge. (A) Mice infected with Δ*waaP* and Δ*waaY* mutants showed significant OVA-specific IgG1 production compared to PBS-instilled mice ($n = 15$). There were no significant increases compared to NU14. (B) Mice infected with the Δ*waaP* mutant showed significant OVA-specific IgE production compared to PBS-instilled mice ($n = 5$). There were no significant increases compared to NU14. (C) No significant differences in OVA-specific IgG2c between groups ($n = 15$). Data represent means ± standard errors of the means; each experiment was performed at least twice. Statistical analysis was determined by one-way analysis of variance followed by Dunnett's multiple-comparison test; the asterisks (*, $P < 0.05$; **, $P < 0.01$) indicate statistically significant differences between PBS and LPS mutant treatment.

production of UPEC-specific antibody isoforms. Here, we find that although UPEC isolate NU14 induces Th_2-associated IL-33, inoculation with NU14 does not result in a significant production of UPEC-specific Th_2-associated antibody IgG1 or IgE. However, the *waaP* inner core mutant results in a significant increase in UPEC-specific Th_2-associated antibodies IgG1 and IgE. These results implicate the inner core in inhibiting humoral immune responses.

These studies demonstrate that LPS domains modulate UPEC

life cycle and host responses. In addition to impacts on invasion and reservoirs, we find that LPS structure differentially modulates the innate immune response, the innate/adaptive immune interface, and the adaptive immune response. These findings can inform the development of optimized live attenuated vaccine candidates for UTI. For example, the NU14 Δ*wzyE* mutant has significantly increased urinary MPO and decreased acute colonization and is unable to chronically infect murine bladders. Additionally, the Δ*wzyE* mutant produces the highest levels of Th_1-associated antibody IgG2c, suggesting it promotes a cell-mediated adaptive response (similar to the Δ*waaL* mutant), while inoculation with the Δ*waaP* mutant promotes a humoral adaptive immune response. Thus, the deletion of LPS genes *waaL*, *waaP*, and *wzyE* should result in a strain that is deficient in acute and chronic colonization but able to activate of both cell-mediated and humoral adaptive immune responses, and this would be ideal for a live-attenuated vaccine candidate. Future studies will be aimed at combining the mutations to generate an optimized vaccine candidate.

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