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O-antigen of uropathogenic *Escherichia coli* is required for induction of neutrophil extracellular traps

Wei-Hung Lin [a], Shew-Meei Sheu [b], Ching-Fang Wu [c], Wen-Chun Huang [d,e], Li-Jin Hsu [f], Kuan-Chieh Yu [f], Hui-Ching Cheng [e], Cheng-Yen Kao [g], Jiunn-Jong Wu [h], Ming-Cheng Wang [a,i,**], Ching-Hao Teng [d,e,*]

[a] *Division of Nephrology, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan*
[b] *Department of Medical Research, Ditmanson Medical Foundation Chia-Yi Christian Hospital, Chia-Yi City, Taiwan*
[c] *Division of Nephrology, Department of Internal Medicine, E-Da Cancer Hospital, Kaohsiung, Taiwan*
[d] *Institute of Molecular Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan*
[e] *Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan*
[f] *Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan, Taiwan*
[g] *Institute of Microbiology and Immunology, School of Life Science, National Yang-Ming University, Taipei, Taiwan*
[h] *Department of Medical Laboratory Science and Biotechnology, Asia University, Taichung, Taiwan*
[i] *Institute of Clinical Pharmacy and Pharmaceutical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan*

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ABSTRACT

Background: Urinary tract infections (UTIs) are prevalent bacterial infection, with uropathogenic *Escherichia coli* (UPEC) as the primary causative agent. The outer membrane of UPEC contains a lipopolysaccharide (LPS), which plays crucial roles in the host's immune response to infection. Neutrophils use neutrophil extracellular traps (NETs) are mechanism by which neutrophils defend against bacterial infections. However, the exact mechanism by which a bacterial LPS induces NET formation is not well understood. Therefore, the objective of this study is to identify the possible mechanism of LPS-mediated NETs and dissect the LPS domains of UPEC that predominantly modulate NET formation and NET-mediated killing.

Methods: To investigate the mechanism of bacterial LPS-induced NET formation, we constructed UPEC CFT073 mutants that had *rfaD*, *rfaL* and the *wzzE* deleted with individual LPS biosynthetic genes including the inner core synthase, O-antigen ligase and O-antigen polymerase, respectively. Subsequently, we evaluated the NET/reactive oxygen species (ROS)/IL-1β induction abilities and assessed the activation of toll-like receptor 4 (TLR4)/JNK signaling by CFT073 and its mutants.

Results: The results showed that the O-antigen of CFT073 LPS is essential for inducing NET formation through TLR4/JNK/NOX pathways. Inhibition of either pathway significantly decreased the production of ROS, induction of NETs, and secretion of IL-1β.

Conclusion: Our results demonstrate that CFT073 LPS is essential for inducing ROS-dependent NETs and IL-1β secretion from neutrophils. This study also provides evidence for the crucial roles of O-antigen in the immune response to UPEC infection, as well as its potential as a therapeutic target for the treatment of UTIs.

1. Introduction

Uropathogenic *Escherichia coli* (*E. coli*) (UPEC) is the most common causative pathogens of urinary tract infections (UTIs), which account for about three quarters of ambulatory care visits.[1,2] UTIs usually start as bladder infections and, in some cases, ascend to the kidneys, causing cystitis, mild to severe pyelonephritis, severe sepsis, septic shock, and even mortality.[3] During the course of UTIs, invading UPEC activates the

* Corresponding author. Division of Nephrology, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan.
** Corresponding author. Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan, Taiwan.
E-mail addresses: wangmc@mail.ncku.edu.tw (M.-C. Wang), chteng@mail.ncku.edu.tw (C.-H. Teng).

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host immune system, leading to inflammation that manifests as cystitis and pyelonephritis. Various UPEC components are recognized by the innate immune system's pattern recognition receptors, triggering the production of pro-inflammatory cytokines and the recruitment of immune cells, which together contribute to urinary tract inflammation.[4] Among these components, lipopolysaccharide (LPS) is one of the most well-known bacterial components capable of initiating immune responses during infections.[5] LPS is present on the outer membrane of most Gram-negative bacteria and participates in host-pathogen interactions.[5] During the course of UTIs, the LPS of UPEC can be recognized by toll-like receptor 4 (TLR4) on uroepithelial cells. It induces the secretion of proinflammatory cytokines to recruit neutrophils, which are the most rapid and robust responders to UTIs. Neutrophils are one of the major defense cells of the innate immune system. They effectively eliminate invading microorganisms through multiple antimicrobial strategies, including the release of neutrophil extracellular traps (NETs).[6] The NETs released by neutrophils have been shown to participate pathogen elimination in UTIs.[7] In addition to triggering neutrophil recruitment, LPS triggers the release of NETs (NETosis).[8] During LPS-induced NETosis, LPS can activate NADPH oxidase (NOX) in neutrophils, leading to the production of reactive oxygen species (ROS).[9] These ROS disrupt the cytoplasmic granules and nuclear envelope, releasing myeloperoxidase (MPO) and neutrophil elastase (NE) from the granules and allowing these enzymes to access the nucleus. NE then degrades histones, and together with MPO, promotes chromatin decondensation. Finally, cell membrane rupture causes neutrophil lysis and the release of NETs, which are composed of chromatin and granule enzymes.[10] The NET components can effectively trap and kill bacteria. Further characterization of the mechanisms by which the LPS of UPEC induces the release of neutrophil NETs would facilitate the understanding of the pathogenesis of UPEC-induced UTIs.

Although the LPS of UPEC is known to contribute to the induction of NETs during UTIs, which LPS structural domains needed to be expressed in the bacteria to induce NET formation remained unclear. The structure of LPS is composed of three distinct domains: (1) hydrophobic lipid A, which anchors the LPS molecules to the outer membrane; (2) the core oligosaccharide, which links to the lipidA; and (3) The O-antigen, an extended O-specific polysaccharide chain attached to the core oligosaccharide, is exposed on the surface of *E. coli*. Its antigenic properties make it a key target for serotyping, which is why it is referred to as the O-antigen. The O-antigen polysaccharide chains consist of multiple repeating oligosaccharide subunits.[11] The core oligosaccharide can be further divided into the inner and outer core regions. The inner core connects lipid A and the outer core connects the O-antigen component. The biosynthesis of LPS is a multistep process mediated by distinct enzymes. Bacteria with mutations in the enzyme-encoding genes express truncated LPS components. The rfa gene clusters (also called waa) encode the enzymes required for the core oligosaccharide biosynthesis of LPS (Fig. 1).[12,13] Among the biosynthetic genes, *rfaD* encodes ADP-L-*glycero*-D-*manno*-heptose-6-epimerase, which converts ADP-D-D-heptose to ADP-L-D-heptose to provide the substrate of the inner core.[14] The *rfaD* mutant of *E. coli* expresses a truncated LPS that lacks part of the inner core, the whole outer core, and the O-antigen components.[15] The rfaL gene encodes a ligase that mediates the bonding between the O-antigenic polysaccharide and the lipid A core acceptor.[15] The deletion of rfaL results in the expression of LPS molecules that lack O-antigen. In addition, the *wzzE* gene encodes an enzyme that regulates the length of the O-antigen polysaccharide chain.[15,16] A mutation of *wzzE* in *E. coli* alters the length of the O-antigen chains of LPS.[17] The aforementioned mutations that cause the expression of distinct forms of LPS defects in bacteria can be used to determine which structural components of LPS are required for UPEC to induce NETs.

In the present study, we evaluated the roles of LPS structural domains in NET formation, IL-1β secretion and ROS production during the interaction between UPEC and neutrophils.

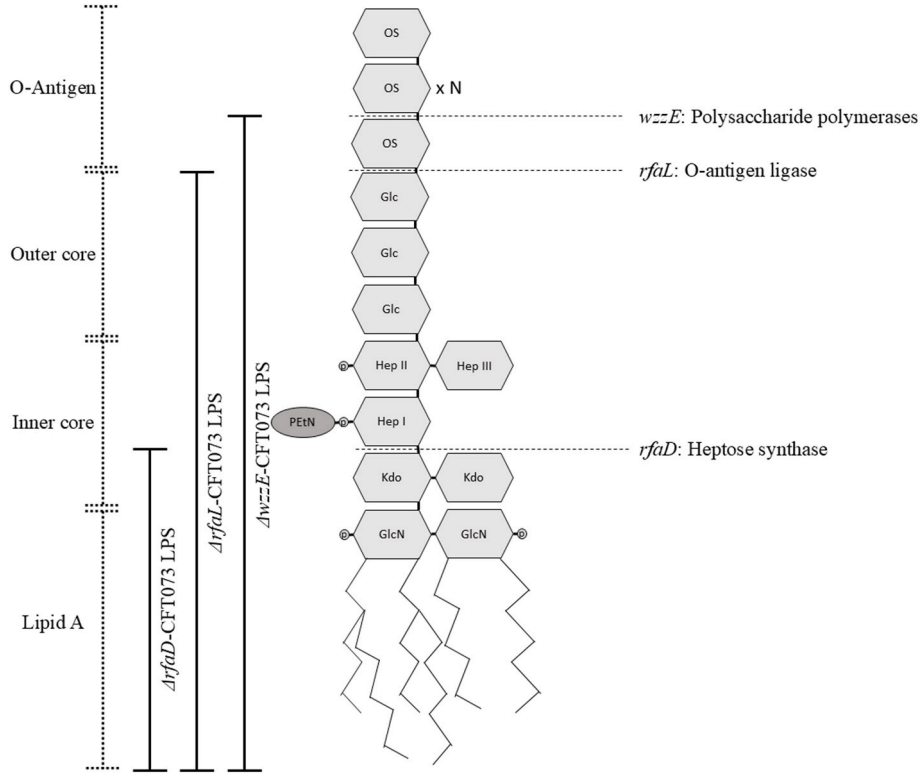

Fig. 1. LPS structures in *E. coli* strains with mutations in *rfaD*, *rfaL*, and *wzzE*. OS, oligosaccharide; Glc, glucose; Hep, L-*glycero*-D-*manno*-heptose; Kdo, 2-keto-3-deoxyoctonoic acid; GlcN, glucosamine; PEtN, phosphoethanolamine.

2. Materials and Methods

2.1. Construction of mutant and complementary strains

In this study, *rfaD*, *rfaL*, *wzzE* mutants were constructed using a previously described PCR-based gene inactivation method (Table 1).[18–21] The PCR products, including a kanamycin (Km)-resistance cassette for the *rfaD* and *wzzE* mutants and chloramphenicol (*Cm*)-resistance cassette for the *rfaL* mutant, were amplified using specific primers (Table 2). These PCR products were separately electroporated into the UPEC CFT073 strain with pKD46 plasmid to generate individual gene mutants. The mutant strains were selected on Luria Bertani (LB) plates containing Km or *Cm*. The mutation was confirmed by the detection of the fragment change of target gene with PCR amplification. To construct recombinant plasmids pCL1920-*rfaD*, and pCL1920-*rfaL*, the DNA fragments encoding *rfaD* and *rfaL*, respectively, were amplified from the UPEC CFT073 genome by PCR with the primers (Table 2). The amplified fragment was cloned into the digested plasmid vector pCL1920. The two recombinant plasmids were used to generate the complementary strains of *rfaD* and *rfaL* mutants, respectively.

2.2. Bacteria and culture condition

The bacterial strains and plasmids used in this study are shown in Table 1. The bacteria were cultured in 5 ml of LB with the appropriate antibiotics in test tubes, incubated at 37 °C with shaking at 200 rpm for 18 h (overnight), unless otherwise specified. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml), spectinomycin (100 μg/ml), Km (50 μg/ml), and *Cm* (15 μg/ml).[22]

To verify whether an *E. coli* strain harbors the expected antibiotic resistance, such as that of newly constructed mutant strains (Table 1), the following procedure was performed. The strain was cultured in LB broth without antibiotics overnight. Then, the culture was spread onto LB agar plates containing the relevant antibiotics to which the strain was expected to be resistant. Colony formation on the antibiotic-supplemented agar indicated that the strain exhibited the anticipated antibiotic resistance.

Table 1
E. coli strains and plasmids used in this study.

Strain or plasmid	Relevant information	AR marker*	Reference
CFT073	A clinical *E. coli* isolates associated with acute pyelonephritis	-	39
Δ*rfaD*-CFT073	CFT073 with a *rfaD* deletion	Km	This study
Δ*rfaD*-CFT073/ pCL-*rfaD*	Δ*rfaD*-CFT073 harboring the plasmid pCL-*rfaD*	Km, Sp	This study
Δ*rfaL*-CFT073	CFT073 with a *rfaL* deletion	*Cm*	This study
Δ*rfaL*-CFT073/ pCL-*rfaL*	Δ*rfaL*-CFT073 harboring the plasmid pCL-*rfaL*	*Cm*, Sp	This study
Δ*wzzE*-CFT073	CFT073 with a *wzzE* deletion	Km	This study
Plasmid			
pKD46	λ Red recombinase expression plasmid	Ap	16
pKD3	Template plasmid for FRT-flanked *cat* cassette	*Cm*	16
pCL1920	Low copy-number plasmid vector	Sp	40
pCL-*rfaD*	pCL1920 harboring a His-tagged *rfaD* gene that is under control of the *lac* promoter	Sp	This study
pCL-*rfaL*	pCL1920 harboring a His-tagged *rfaL* gene that is under control of the *lac* promoter	Sp	This study

AR markers*, antimicrobial resistance markers commonly used in laboratories; Ap, ampicillin; Sp, spectinomycin; Km, kanamycin; *Cm*, chloramphenicol.

2.3. LPS isolation

The protocol for isolating LPS followed that described in a previous study.[23] The purified LPS was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 10 % gel and visualized by silver staining.

2.4. Primary human neutrophils

The study design was approved by the Institutional Review Board at National Cheng Kung University Hospital (B-ER-108-228). Informed consent was obtained for the use of whole blood samples. All methods were performed in accordance with the relevant guidelines and regulations. Neutrophils were isolated from the whole blood of healthy volunteers using the EasySep Direct Human Neutrophil Isolation Kit (#19666, STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions.

2.5. Quantification of NET formation induced by bacterial infection

Neutrophils (5×10^5/well) suspended in 1X phosphate-buffered saline (PBS) (#10010023, Gibco, Grand Island, NY, USA) containing 2 % fetal bovine serum (Cat#16000044, Gibco, Grand Island, NY, USA) were seeded in a 4-well chamber slide pre-coated with poly-L-lysine (Thermo Fisher Scientific, Cleveland, OH, USA) and allowed to adhere for 4 h at 37 °C in a humidified CO_2 incubator. After 4 h of incubation, neutrophils were infected with 2.5×10^6 (multiplicity of infection, MOI = 5) of UPEC CFT073 or its mutants suspended in phenol-red-free RPMI medium (#32404014, Gibco, Grand Island, NY, USA) with 1 nM SYTOX Green (#S7020, Gibco, Grand Island, NY, USA) for 1 h at 37 °C. After 1 h of infection, the cells were stained with 5 μg/ml Hoechst 33,342 (Invitrogen, Waltham, MA, USA) and fluorescence images were captured by using a Leica DFC7000T camera with 10 × objective lens. To evaluate the intensity of bacteria induced NET production, SYTOX green images were split into an RGB stack and the percentage of green fluorescence was analyzed using the software ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.6. Immunofluorescence

Following infection, cells were fixed with 4 % paraformaldehyde for 10 min at room temperature and subsequently washed with PBS. The cells were then incubated with rabbit anti-NE (1:100, cat. #481001, Millipore, MA, USA) and mouse *anti*-MPO (1:100, cat. #0400–0002, Bio-Rad, CA, USA) primary antibodies in PBS for 18 h at 4 °C. After 18 h of incubation, the slides were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-rabbit (1:500) and Alexa Fluor 647-conjugated anti-mouse (1:500) secondary antibodies for 1 h at room temperature in the dark. Following secondary antibody incubation, slides were washed with PBS, mounted, and sealed with coverslips. Fluorescent images were acquired using a confocal microscope (LSM-900, Zeiss, Jena, Germany).

2.7. Cell line

The HL-60 cell line originally derived from a 36-year-old Caucasian patient with acute promyelocytic leukemia can be induced to differentiate in vitro to morphologically mature cells including banded and segmented neutrophils.[24] The HL-60 cells obtained from the American Type Culture Collection were stimulated with 1.3 % dimethyl sulfoxide (Merck, Darmstadt, Germany) for 5 d to differentiate them into a neutrophil-like state in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) and 1 % penicillin-streptomycin (GenedireX, Las Vegas, NV, USA) at 37 °C in 5 % CO_2.

Table 2
Primers used in this study.

Primer name	Sequence (5′-3′)
ND-*rfaD*-F	TCCAATCCCCTCTACCAATG
ND-*rfaD*-R	TCGGACGTTCTGATGAAAGC
pCL1920-*rfaD*-*Hin*dIII-F	CGCCAAGCTTCAGGAAGAGTAGAGAATCGC
rfaD-His tag-SalI-R	TAGAGTCGACTTAGTGATGGTGATGGTGATGTGCGTCACGATTCAGCCAG
NK-*rfaL*-F	AAATAACCCATCCTCGTAGCATAGGTTGAAATTATATGATAGGAATATCCTCCTTAGTTC
NK-*rfaL*-R	TTTATTTCACATATTCATTACTTATCTAATAAACATTGTGTGTAGGCTGGAGCTGCTTCG
pCL1920-*rfaL*-*Hin*dIII-F	CGCCAAGCTTATGCGATATGTATGCGGTTC
pCL1920-*rfaL*-His tag-*Bam*HI-R	CCGGGGATCCTTAGTGATGGTGATGGTGATGCTTATCTAATAAACATTGGTCCG
NK-*wzzE*-F	CCATCCTGCCATACATCATG
NK-*wzzE*-R	CGCACCCATAACAGTGCATC

2.8. Western blot analysis

After LPS treatment, equal amounts of whole cell lysates were prepared as previously described.[25] The target proteins were detected using the following antibodies: *anti*-JNK (#9258, Cell Signaling Technology, Beverly, MA, USA), *anti*-p-JNK (#9255, Cell Signaling Technology, Beverly, MA, USA), and *anti*-GAPDH (#GTX100118, GeneTex, Irvine, CA, USA). For Ponceau S staining, a polyvinylidene fluoride (PVDF) membrane was washed with TBS-T, stained with 0.1 % Ponceau S/5 % acetic acid solution for 10min at room temperature, and rinsed with distilled water to remove the background.

2.9. Determination of reactive oxygen species

Neutrophils (1×10^5) were plated into a 96-well white plate for 4 h as described above and infected with 5×10^5 (MOI = 5) of WT-CFT073 or its mutants for 30 min. The neutrophils were further washed with 1XPBS and treated with 12.5 mg/ml luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). Chemiluminescence was continuously detected every 5 min for 1 h at 37 °C using a microplate reader (Synergy HTX, BioTek, Winooski, VT, USA).

2.10. IL-1β determination

The amount of IL-1β in the infected supernatant was analyzed by human IL-1β/IL-1F2 DuoSet ELISA (DY201–05, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.11. NET killing ability

Neutrophils (1×10^6) were seeded into a 24-well plate and activated with 100 nM Phorbol-12-myristate 13-acetate (PMA) at 37 °C for 4 h. After activation, 100 μl of cytochalasin D (50 μg/ml) was added with or without DNase for 15 min of incubation at 37 °C. The neutrophils were further infected with 10^5 (MOI = 0.1) of CFT073 WT or its mutants for 3 h. The supernatant was collected, serially diluted, and inoculated on an LB agar plate at 37 °C for 10 h. Bacterial killing dependent on NETosis was calculated as done in a previous report.[26] The NET killing ability (%) was determined as (colony-forming units, CFUs, in presence of DNase – CFUs in absence of DNase)/CFUs in presence of DNase × 100 %.[27]

2.12. Statistical analysis

At least three independent experiments were conducted, and the results are presented as mean ± standard deviation (SD). Data normality was assessed using the Shapiro-Wilk test in GraphPad software, confirming that all data followed a normal distribution. Statistical analyses were conducted using one-way ANOVA or Student's t-test in GraphPad. Statistical significance is presented as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ or denoted by different letters ($p < 0.05$).[28,29]

3. Results

3.1. UPEC induces NET formation

NET formation is a critical immune response against *E. coli* infections. We investigated whether *E. coli* strains involved in different type of infections induce different levels of NET formation. The NET-inducing abilities of the uropathogenic *E. coli* (UPEC) strain, CFT073, the neonatal meningitis pathogenic *E. coli* (NMEC) strain, RS218, and the non-pathogenic strain MG1655[30,31] were evaluated after incubation with primary human polymorphonuclear neutrophils (PMNs) by using a plasma membrane-impermeable DNA-binding dye, SYTOX Green to lable NET structures. As shown in Fig. 2A and B, CFT073 and RS218 showed significant NET formation, whereas MG1655 did not induce apparent NETs. In addition, CFT073 induced higher levels of NETs than those induced by RS218. These finding suggest that pathogenic *E. coli* strains may induced higher levels of NETs than those induced by non-pathogenic strains, and that pathogenic strains involved in different infections may induce different levels of NETs. Given that the UPEC strain induced the highest levels of NETs, we further investigated how this strain induces NETs.

3.2. Expression of O-antigen is essential for the induction of NETs in UPEC

To assess the impact of UPEC LPS structures on NET induction, the deletion mutants of *wzzE*, *rfaD*, and *rfaL* in CFT073 were constructed; they are denoted as Δ*wzzE*-CFT073, Δ*rfaD*-CFT073, and Δ*rfaL*-CFT073, respectively (Table 1). The NET-inducing abilities of these mutants were assessed using SYTOX Green staining and compared with that of the wild-type strain. As shown in Fig. 3A and B, Δ*rfaD*-CFT073 and Δ*rfaL*-CFT073 triggered significantly lower levels of NET formation than did WT-CFT073, and Δ*wzzE*-CFT073 induced a NET production level similar to that induced by WT-CFT073. Notably, transcomplementation of the *rfaD* or *rfaL* genes restored the NET-inducing ability of their respective mutants. In addition, the NET-inducing abilities of the strains were verified by quantifying neutrophil elastase (NE) and myeloperoxidase (MPO) associated with NET structures induced by each bacterial strain. Consistent with the SYTOX Green results, deletion of *rfaD* and *rfaL* significantly reduced CFT073's capacity to induce NET formation. (Fig. S1). Since only the *wzzE* mutant expressed O-antigen (Fig. 1), these findings strongly suggest that the expression of the O-antigen domain of LPS is essential for NET induction.

As the results of DNA labeling with SYTOX Green were consistent with the quantification of NET-associated NE and MPO, we used SYTOX Green staining to assess NET formation levels in subsequent experiments.

3.3. Deletion of O-antigen reduced total ROS production and IL-1β secretion

Previous studies have shown that Gram-negative bacteria can trigger

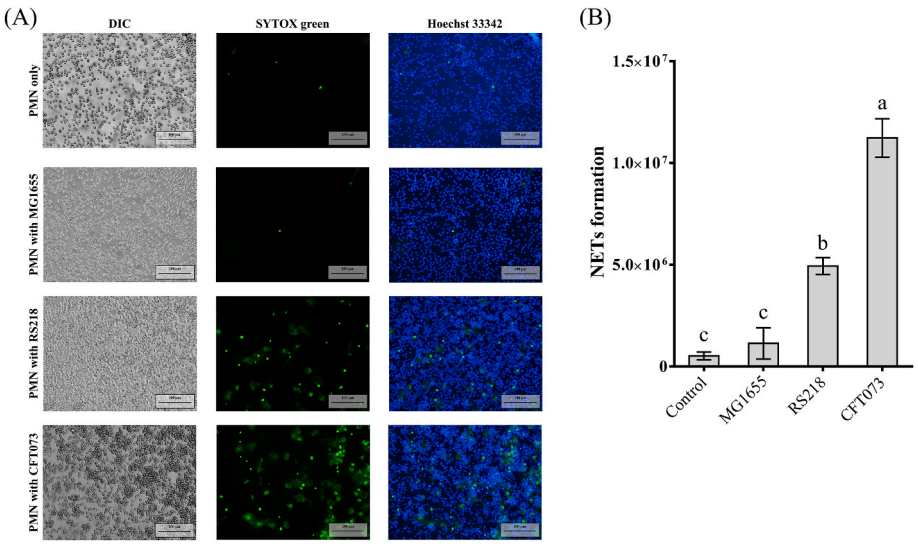

Fig. 2. Abilities of different *E. coli* strains to induce NET formation in primary human neutrophils. (A) Images of NET formation after co-incubation of CFT073, RS218, and MG1655 with primary human PMNs for 1 h. The released NETs were labeled with SYTOX green for visualization. (B) Quantitative data of the NET formation induced by *E. coli* strains. The experiments were conducted at least three times and the results are presented as the means ± SD. Letters above the bars indicate groups with statistically significant differences ($p < 0.05$, one-way ANOVA).

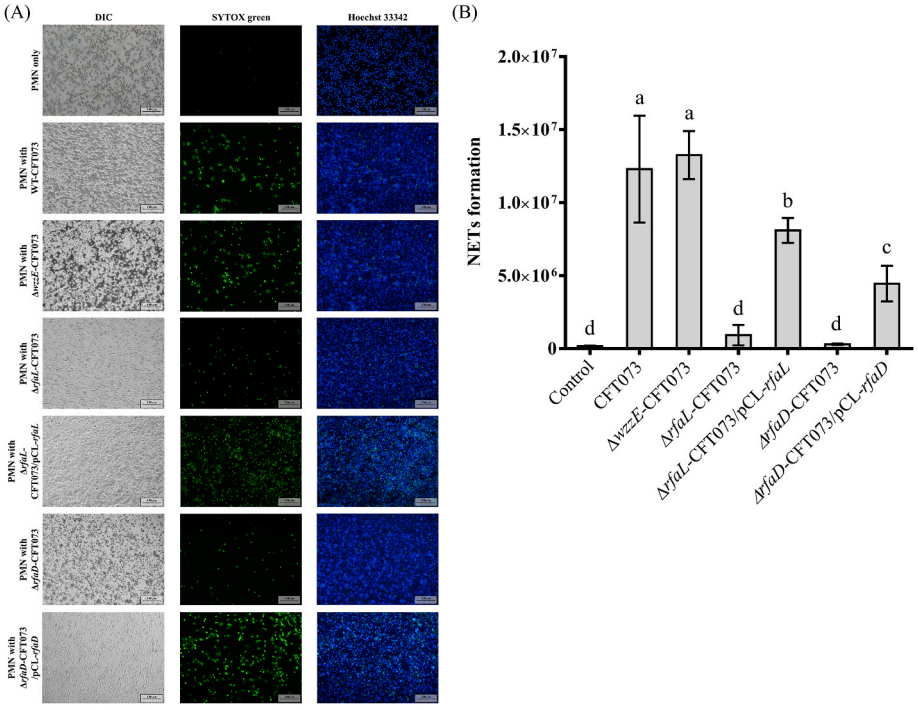

Fig. 3. Impacts of *ΔwzzE*, *ΔrfaL*, and *ΔrfaD* mutations on ability of CFT073 to induce NET formation. (A) Images of NET formation after co-incubation of indicated CFT073 strains with primary human PMNs for 1 h. The released NETs were labeled with SYTOX green for visualization. (B) Quantitative data of NET formation induced by CFT073 strains. The experiments were conducted at least three times and the results are presented as the means ± SD. Letters above the bars indicate groups with statistically significant differences ($p < 0.05$, one-way ANOVA).

NADPH oxidase (NOX)-dependent NETosis, wherein the activated NOX initiates ROS production, leading to the release of NETs from neutrophils.[9] In addition, the proinflammatory cytokine IL-1β is known to promote NET formation and can itself be induced by NET formation.[32] We assessed the capacities of WT-CFT073, *ΔwzzE*-CFT073, *ΔrfaD*-CFT073, and *ΔrfaL*-CFT073 to induce neutrophil ROS production and IL-1β secretion. After incubation with primary human PMNs, *ΔrfaD*-CFT073 and *ΔrfaL*-CFT073 exhibited significantly lower levels of ROS production and IL-1β secretion compared to those of WT-CFT073, whereas *ΔwzzE*-CFT073 induced ROS and IL-1β levels similar to those triggered by the wild-type strain (Fig. 4A and B). Transcomplementation of *rfaD* and *rfaL* restored the abilities of *ΔrfaD*-CFT073 and *ΔrfaL*-CFT073 to induce ROS and IL-1β production. These findings indicate that the O-antigen expression in CFT073 is required for triggering ROS and IL-1β production. Furthermore, inhibition of NOX activity by the NOX inhibitor DPI significantly reduced NET formation triggered by the CFT073 strains expressing O-antigen (Fig. 4C, D, and 4 E), demonstrating that O-antigen expression in CFT073 contributes to

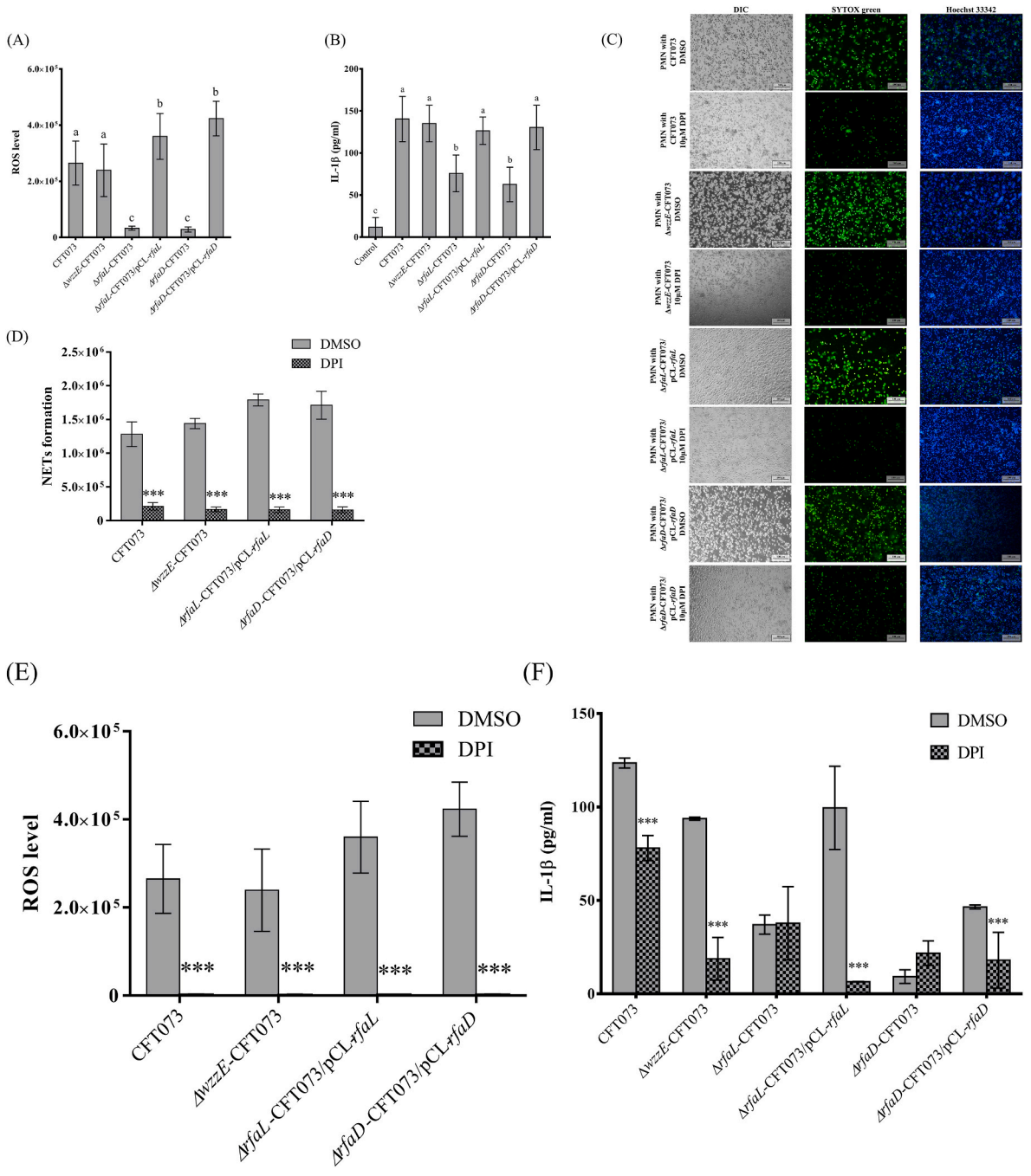

Fig. 4. Abilities of CFT073 mutants to induce ROS production and IL-1β secretion, and roles of ROS in CFT073-induced NET formation and IL-1β secretion. (A) ROS levels in human PMNs induced by indicated CFT073 strains. (B) Levels of IL-1β secretion in human PMNs induced by the indicated CFT073 strains. (C) Images of the NET formation of human PMNs induced by indicated CFT073 strains with and without blockade of ROS production by DPI. (D) Quantitative data of NET formation suppressed by DPI. (E) ROS levels of human PMNs induced by indicated CFT073 strains with and without blockade of ROS production by DPI. (F) The levels of IL-1β secretion in human PMNs induced by the indicated CFT073 strains with and without the blockade of ROS production by DPI. The experiments were conducted at least three times and the results are presented as the means ± SD. Letters above the bars indicate groups with statistically significant differences ($p < 0.05$, one-way ANOVA). ***$p < 0.001$ compared to its vehicle control group.

NET formation mainly through the activation of the NOX-ROS pathway. In addition, inhibiting NOX activity markedly reduced IL-1β production in neutrophils incubated with the CFT073 strains expressing O-antigen. However, inhibiting NOX activity had no significant impact on IL-1β production in the cells incubated with the strains without O-antigen expression (Fig. 4F). These findings suggest that the O-antigen-mediated IL-1β production in neutrophils is mainly through the NOX-ROS pathway.

3.4. Purified LPS from ΔrfaL-CFT073 shows decreased NET production

The above findings demonstrate that the O-antigen expression in CFT073 is required for the bacteria to induce NETs. We further investigated whether the O-antigen structure directly contributes to NET induction. The LPS of the wild-type CFT073 (CFT073 LPS) and the LPS of *ΔrfaL*-CFT073 (*ΔrfaL*-CFT073 LPS) were purified (Fig. 5A) and then subjected to NET induction analyses using primary human PMNs. As shown in Fig. 5B and C, *ΔrfaL*-CFT073 LPS triggered a significantly

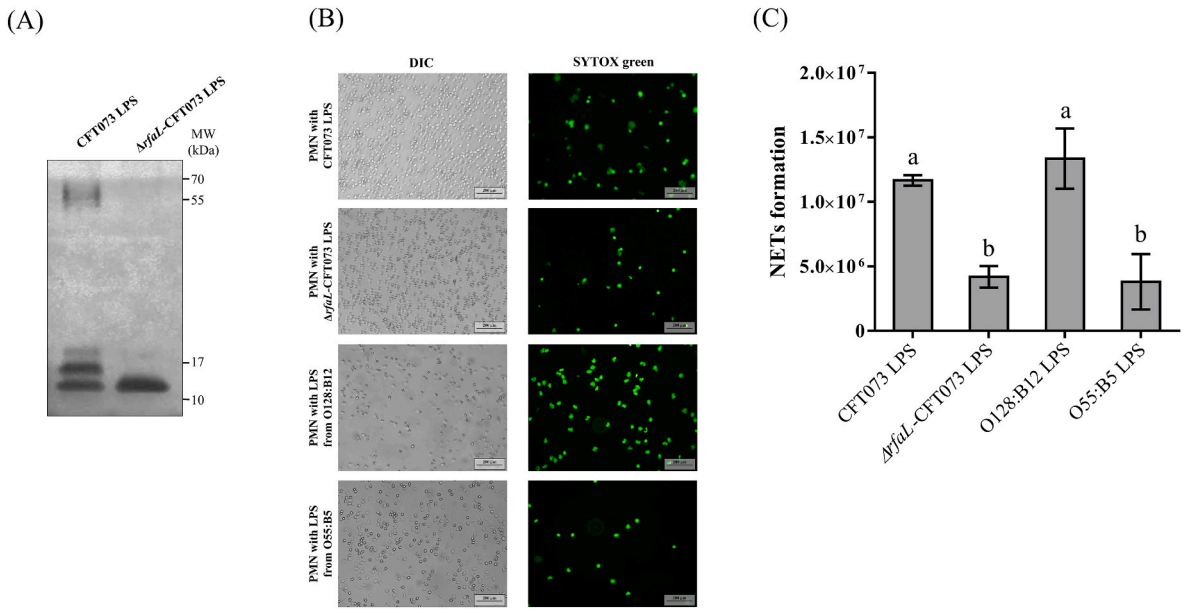

Fig. 5. LPS from CFT073 strains and their ability to induce NET formation. (A) LPS purified from CFT073 (CFT073 LPS) and Δ*rfaL*-CFT073 (Δ*rfaL*-CFT073 LPS) visualized by silver staining. (B) Images of NET formation of human PMNs induced by purified CFT073 LPS, Δ*rfaL*-CFT073 LPS, O128:B12 LPS and O55:B5 LPS. The experiments were conducted at least three times and the results are presented as the means ± SD. Letters above the bars indicate groups with statistically significant differences ($p < 0.05$, one-way ANOVA).

lower level of NET formation than that triggered by CFT073 LPS, suggesting that the O-antigen structure of the CFT073 LPS is physically involved in triggering NET formation during the bacterium-neutrophil interaction.

Since the O-antigen of CFT073 LPS belongs to the O6 serotype, we further evaluated the abilities of LPS with different O-antigen to induce NET formation under the same NET formation conditions. The commercially available LPS serotypes O128:B12 (O antigen type O128) and O55: B5 (O antigen type O55) were subjected to the assays. LPS O128:B12 induced NET formation similar to that triggered by CFT073 LPS, and LPS O55:B5 induced a level of NET formation similar to that triggered by the O-antigen truncated Δ*rfaL*-CFT073 LPS (Fig. 5B and C). These findings demonstrated that the O-antigen-mediated NET formation is O-antigen-type dependent.

3.5. LPS-induced NETs can be blocked by TLR4 and JNK inhibitors

It has been reported that the *E. coli* LPS O111:B4 (O antigen type O111) can trigger ROS-mediated NOX-dependent NETosis through the TLR4-dependent JNK signaling pathway.[9] We determined whether the

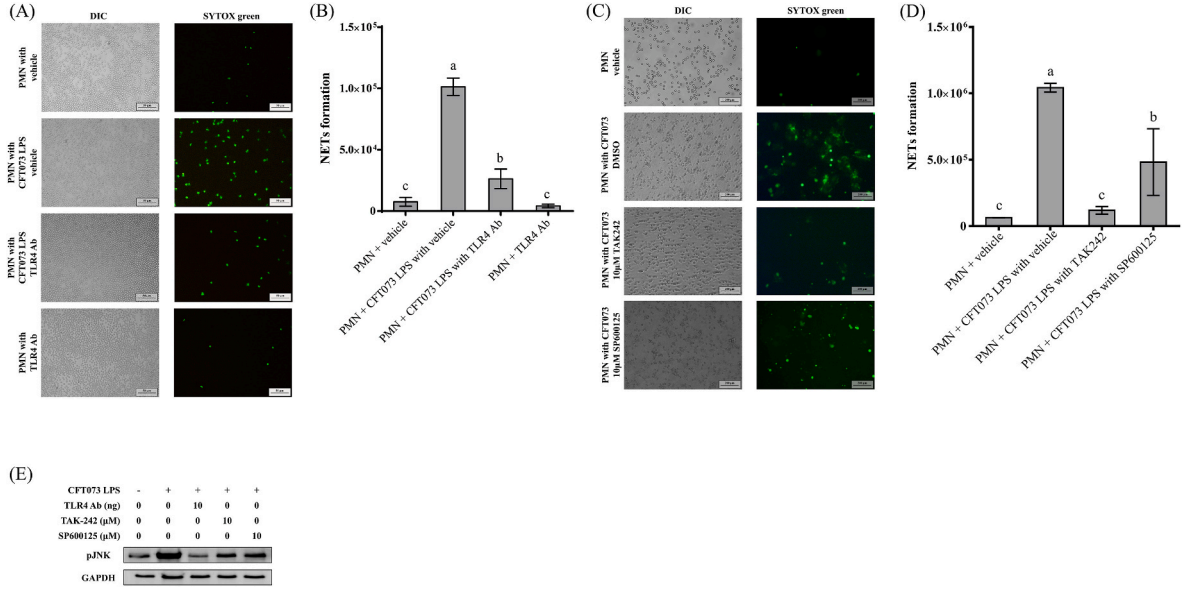

Fig. 6. Roles of TLR4/JNK pathway in CFT073-induced NET formation in neutrophils. (A) Images of NET formation by human PMNs induced by CFT073 LPS with and without blocking of TLR4 signaling with *anti*-TLR4 antibodies. (B) Quantitative data of NET formation inhibited by *anti*-TLR4 antibodies. (C) Images of NET formation by human PMNs induced by CFT073 LPS with and without treatment of the TLR4 inhibitors, TAK-242 and JNK inhibitors, SP600125. (D) The quantitative data of the NET formation decreased by TLR4/JNK inhibitors. (E) The levels of phosphorylated JNK induced by CFT073 LPS with and without inhibition of TLR4 and JNK signaling in human PMNs. The experiments were conducted at least three times and the results are presented as the means ± SD. Letters above the bars indicate groups with statistically significant differences ($p < 0.05$, one-way ANOVA).

CFT073 LPS triggers NET formation through the same signaling pathway. Blocking the activation of TLR4 (Fig. 6A and B) and JNK signaling (Fig. 6C and D) in primary human PMNs significantly decreased the NETosis induced by the purified LPS of CFT073. Additionally, blocking TLR4 signaling inhibited CFT073 induced JNK signaling (Fig. 6E). This observation demonstrates that the CFT073 LPS triggers NETosis through the TLR4/JNK pathway and suggests that the TLR4/JNK pathway may be commonly involved in LPS-induced NETosis.

Accordingly, we further investigated whether the O-antigens of *E. coli* LPS contribute to the activation of the TLR4/JNK pathway. *ΔrfaL*-CFT073 LPS induced lower levels of JNK signaling than those induced by the LPS purified from CFT073 in both primary human PMNs (Fig. 7A) and the neutrophil cell line HL-60 (Fig. 7B). In addition, LPS O55:B5 exhibited a significantly lower ability to induce the activation of JNK signaling than that of LPS O128:B12 in both primary human neutrophils and HL-60 (Fig. 7C and D). These findings demonstrate that the O-antigen structure of LPS contributes to triggering TLR4/JNK signaling and thus inducing NETosis and indicate that the O-antigen type determines the activation level of the TLR4/JNK signaling.

3.6. *O-antigen is required for bacterial resistance to NET-mediated killing*

To investigate whether the LPS structure affects UPEC sensitivity to NET-mediated killing, we incubated WT-CFT073, its LPS-related mutants, and the complemented strains with primary human PMNs pretreated with PMA to stimulate NET formation, with and without DNase treatment. After 3 h of incubation, live bacterial counts were measured, and the levels of NET-mediated killing for each strain was determined (see Materials and Methods). The results showed no significant difference in NET-mediated killing between WT-CFT073 and ΔwzzE-CFT073 (Fig. 8A). However, NET-mediated killing of *ΔrfaD*-CFT073 and *ΔrfaL*-CFT073 was significantly lower than that of WT-CFT073 and ΔwzzE-CFT073. Complementation of *ΔrfaD*-CFT073 and *ΔrfaL*-CFT073 with *rfaD* and *rfaL* genes, respectively, restored the level of NET-mediated killing to those of CFT073 and *ΔwzzE*-CFT073. In this experimental setup, the observed lower levels of NET-mediated killing of *ΔrfaD*-CFT073 and *ΔrfaL*-CFT073 may have two possible explanations. One possibility is that *ΔrfaD*-CFT073 and *ΔrfaL*-CFT073 are inherently more resistant to NET-mediated killing than WT-CFT073. Alternatively, since these mutants are less effective at inducing NET formation, they may stimulate lower levels of NET formation when interacting with PMNs, resulting in a reduced level of NET-mediated killing compared to the wild-type strain.

To identify the correct explanation, we assessed the survival rates of these mutants compared to WT-CFT073 under equal NET levels. Equal amounts of WT-CFT073 and either *ΔrfaD*-CFT073 or *ΔrfaL*-CFT073 were co-incubated in PMA-pretreated primary human PMNs. After 3 h of incubation, we measured the survival rate of each bacterial strain. As shown in Fig. 8B and C, the survival rates of ΔrfaD-CFT073 and ΔrfaL-CFT073 were significantly lower than that of CFT073, indicating that these mutants are more susceptible to NET-mediated killing. Overall, these results suggest that while the O-antigen of LPS is crucial for NET induction, it also plays a key role in bacterial resistance to NET-mediated killing.

4. Discussion

This study confirms the essential role of the O-antigen in LPS in inducing NETosis during neutrophil-UPEC interaction. The O-antigen structure is required for initiating the TLR4-mediated activation of the JNK pathway, which consequently leads to ROS-mediated NOX-dependent NETosis. Moreover, this O-antigen-mediated pathway activation induces the expression of IL-1β, a known facilitator of NET formation.[4,32] Given that neutrophils are the early responders in UTIs caused by UPEC,[4] these findings significantly contribute to our comprehension of UPEC pathogenesis.

Our findings reveal the contribution of the O-antigen of UPEC to the induction of TLR4-dependent NETosis. While the conserved lipid A region of LPS is conventionally recognized as the primary domain

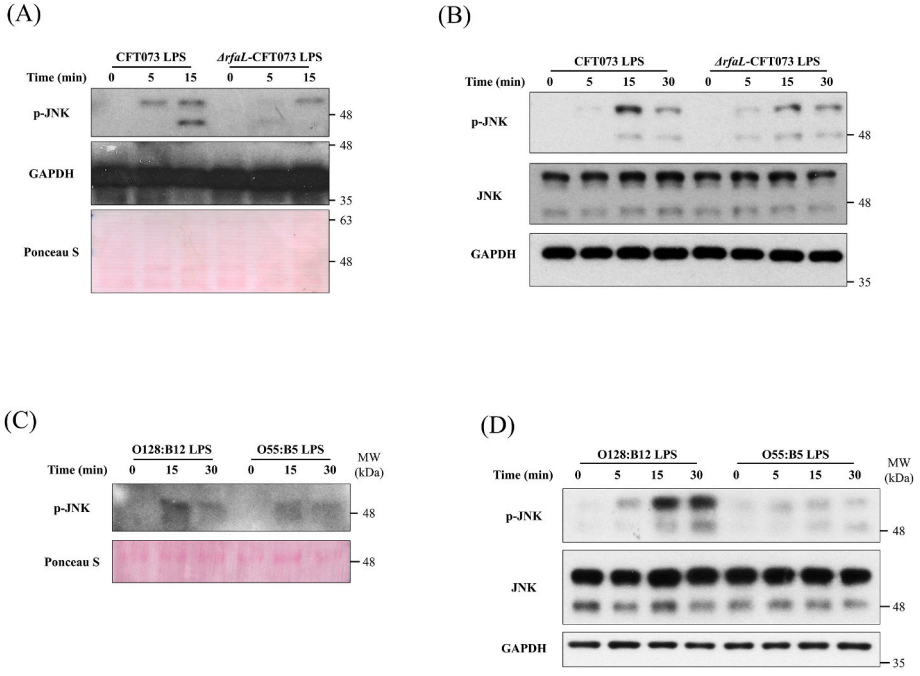

Fig. 7. JNK signaling in neutrophils induced by CFT073 LPS, Δ*rfaL*-CFT073 LPS, O128:B12 L PS, and O55:B5 LPS. (A) Levels of phosphorylated JNK in human PMNs induced by CFT073 LPS and Δ*rfaL*-CFT073 LPS. (B) Levels of phosphorylated JNK in neutrophil cell line HL-60 induced by CFT073 LPS and Δ*rfaL*-CFT073 LPS. (D) Levels of phosphorylated JNK in human PMNs induced by O128:B12 LPS and O55:B5 LPS. (E) Levels of phosphorylated JNK in neutrophil cell line HL-60 induced by O128:B12 LPS and O55:B5 LPS. Total cellular protein samples were subjected to Western blot analysis using an antibody recognizing total JNK protein or its phosphorylated form (p-JNK). GAPDH immunoblots and Ponceau S staining of the PVDF membrane were used as the loading controls.

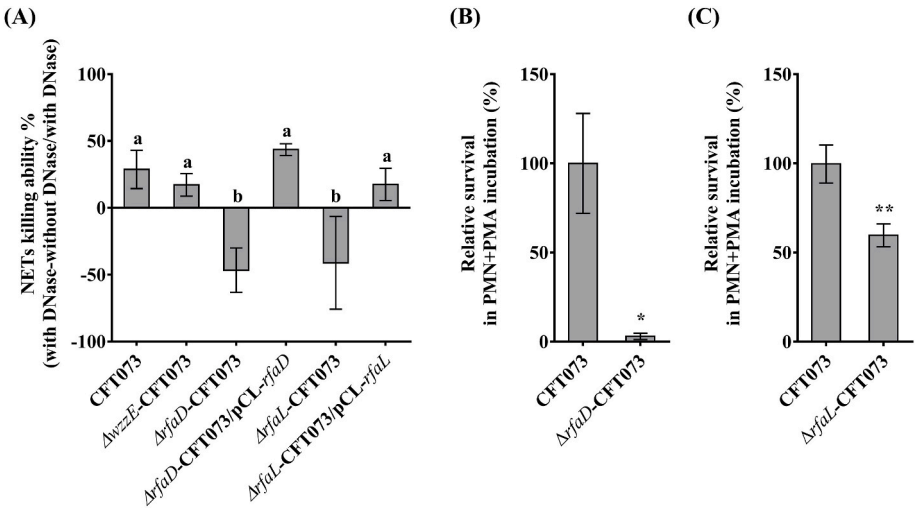

Fig. 8. Δ*rfaD*-CFT073 and Δ*rfaL*-CFT073 were more sensitive to NET-mediated killing. (A) NET-mediated killing of WT-CFT073, Δ*wzzE*-CFT073, Δ*rfaD*-CFT073, Δ*rfaL*-CFT073, and the complemented strains. The indicated strains were independently incubated with PMA-pretreated human PMNs with or without DNase for 3 h. Surviving bacteria in the supernatant were collected, serially diluted, and inoculated on an LB agar plate with specific antibiotics to count CFUs. The percentage of NET killing ability was calculated as (CFUs with DNase - CFUs without DNase/CFU with DNase) × 100 %. The data are presented as the mean ± SD of at least three independent experiments. Letters marked above the bars indicate groups with statistically significant differences ($p < 0.05$, one-way ANOVA) (B) The relative survival of Δ*rfaD*-CFT073 compared to that of WT-CFT073 under a similar level NET induction in a co-incubation setup. (C) The relative survival of Δ*rfaL*-CFT073 compared to that of WT-CFT073 under a similar level NET induction in a co-incubation setup. For (B) and (C), Equal numbers of the indicated two strains were mixed and co-cultured with PMA-pretreated human PMNs for 3 h. Then, the numbers of the surviving bacteria were determined. The survival rates of each strains were calculated by the ratio of the surviving bacterial counts to those of the original inoculum. The results are presented as the relative survival rates of WT-CFT073. The data are presented as the mean ± SD of at least three independent experiments. *, $P < 0.05$; **, $p < 0.01$ (Student's *t*-test).

responsible for TLR4 interaction on innate immune cells, our study highlights the essential role of O-antigen in TLR4-dependent NETosis induction. Specifically, only LPS with a specific type of O-antigen significantly induces NETosis (Fig. 5C), suggesting that the O-antigen may exert either a positive or negative regulatory effect on TLR4-dependent NETosis during bacterium-neutrophil interactions. That is, the O-antigen of an LPS capable of inducing NETosis may play a positive role in the TLR4-dependent pathway, leading to NETosis, or alternatively, the O-antigen of an LPS incapable of inducing NETosis may act negatively by impeding TLR4-dependent signaling. Our findings that CFT073 LPS can significantly induce NETosis and O-antigen truncation significantly decreases its ability to induce NETosis (Fig. 3A and B) strongly imply that O-antigen is more likely to function as a positive regulator in TLR4-dependent NETosis induction.

This study demonstrates the crucial role of O-antigen in inducing the production of IL-1β during UPEC-induced NETosis. IL-1β appears to act as an amplifier in NETosis during infections. As a cytokine, IL-1β is essential for recruiting circulating neutrophils to infection sites and has the ability to directly induce NETosis in neutrophils.[4,32] Furthermore, the process of NETosis leads to the production of IL-1β,[33,34] creating a feedback loop that further attracts more circulating neutrophils to the infection site, thereby amplifying the source of NETosis. Therefore, as a key component in inducing IL-1β production, O-antigen is not only crucial for NETosis induction but also for amplifying the NETosis reaction during infections.

Our results indicate that the deletion of the O-antigen enables UPEC to evade NET induction, potentially helping the pathogen escape NET-mediated killing during UPEC-neutrophil interactions in vitro. However, previous studies have shown that UPEC strains lacking the O-antigen exhibit significantly reduced virulence in a mouse model of UTIs.[30,35] This seemingly contradictory observation can be attributed to the additional roles of the O-antigen in UPEC virulence. For example, the O-antigen structure aids UPEC in resisting phagocytosis by immune cells and complement-mediated killing.[36,37] Therefore, although the loss of O-antigen may allow UPEC to avoid NET induction, its overall impact on UPEC is a reduction in their virulence during infections.

It has been proposed that NETs is a double-edged sword innate immunity.[38] The formation of NETs provide protection against invading pathogens, while it has been noted that NETs may leads to tissue or organ damage due to the NET-associated compound that non-specifically attack both the invading pathogens and host tissues. Accordingly, a proper balance of the bacteria-killing effects and the detrimental effects of NETosis is necessary. Given that the O-antigen is an important NETosis inducer of *E. coli*, it is also a potential target to modulate the NETosis during *E. coli* infections. For example, blocking the O-antigen on the surface of the pathogen may mitigate the over-induced detrimental NETosis effect on host tissues.

5. Conclusion

Neutrophils play a pivotal role in the early innate immune response to *E. coli*-induced UTIs, utilizing NETosis as a key strategy to counter invading pathogens. The O-antigen of UPEC, involved in both NETosis induction and the amplification of the IL-1β-mediated NETosis reaction, is a promising target for innovative strategies against *E. coli*-induced UTIs. Further exploration is warranted to elucidate how distinct O-antigen types influence the outcomes of NETosis.

CRediT authorship contribution statement

Wei-Hung Lin: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Shew-Meei Sheu:** Writing – review & editing, Methodology, Conceptualization. **Ching-Fang Wu:** Funding acquisition, Conceptualization. **Wen-Chun Huang:** Writing – original draft, Methodology, Investigation. **Li-Jin Hsu:** Writing – review & editing, Methodology, Investigation, Data curation. **Kuan-Chieh Yu:** Methodology, Investigation. **Hui-Ching Cheng:** Methodology, Investigation. **Cheng-Yen Kao:** Conceptualization. **Jiunn-Jong Wu:** Conceptualization. **Ming-Cheng Wang:** Conceptualization. **Ching-Hao Teng:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization.

Data statement

The data supporting the findings of this study are available within the article. Raw data and additional details regarding the datasets can be obtained upon request from the corresponding author.

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Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jmii.2024.12.007.

References

1. Foxman B. The epidemiology of urinary tract infection. *Nat Rev Urol*. 2010;7:653–660.
2. Lin WH, Wang MC, Liu PY, et al. Escherichia coli urinary tract infections: host age-related differences in bacterial virulence factors and antimicrobial susceptibility. *J Microbiol Immunol Infect*. 2022;55:249–256.
3. Wagenlehner FME, Bjerklund Johansen TE, Cai T, et al. Epidemiology, definition and treatment of complicated urinary tract infections. *Nat Rev Urol*. 2020;17:586–600.
4. Kuhn HW, Hreha TN, Hunstad DA. Immune defenses in the urinary tract. *Trends Immunol*. 2023;44:701–711.
5. Whitfield C, Trent MS. Biosynthesis and export of bacterial lipopolysaccharides. *Annu Rev Biochem*. 2014;83:99–128.
6. Pruchniak MP, Arazna M, Demkow U. Life of neutrophil: from stem cell to neutrophil extracellular trap. *Respir Physiol Neurobiol*. 2013;187:68–73.
7. Yu Y, Kwon K, Tsitrin T, et al. Characterization of early-phase neutrophil extracellular traps in urinary tract infections. *PLoS Pathog*. 2017;13, e1006151.
8. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science*. 2004;303:1532–1535.
9. Khan MA, Farahvash A, Douda DN, et al. JNK activation turns on LPS- and gram-negative bacteria-induced NADPH oxidase-dependent suicidal NETosis. *Sci Rep*. 2017;7:3409.
10. Huang J, Hong W, Wan M, Zheng L. Molecular mechanisms and therapeutic target of NETosis in diseases. *MedComm*. 2020;3:e162.
11. Bertani B, Ruiz N. Function and biogenesis of lipopolysaccharides. *EcoSal Plus*. 2018;8.
12. Amor K, Heinrichs DE, Frirdich E, Ziebell K, Johnson RP, Whitfield C. Distribution of core oligosaccharide types in lipopolysaccharides from Escherichia coli. *Infect Immun*. 2000;68:1116–1124.
13. Kuo CJ, Chen JW, Chiu HC, et al. Mutation of the enterohemorrhagic Escherichia coli core LPS biosynthesis enzyme RfaD confers hypersusceptibility to host intestinal innate immunity in vivo. *Front Cell Infect Microbiol*. 2016;6:82.
14. Wang J, Ma W, Wang Z, Li Y, Wang X. Construction and characterization of an Escherichia coli mutant producing Kdo_2-lipid A. *Mar Drugs*. 2014;12:1495–1511.
15. Aguiniga LM, Yaggie RE, Schaeffer AJ, Klumpp DJ. Lipopolysaccharide domains modulate urovirulence. *Infect Immun*. 2016;84:3131–3140.
16. Leo V, Tran E, Morona R. Polysaccharide co-polymerase WzzB/WzzE chimeras reveal transmembrane 2 region of WzzB is important for interaction with WzyB. *J Bacteriol*. 2020;203:e00598–20.
17. Phan MD, Peters KM, Sarkar S, et al. The serum resistome of a globally disseminated multidrug resistant uropathogenic Escherichia coli clone. *PLoS Genet*. 2013;9, e1003834.
18. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A*. 2000;97:6640–6645.
19. Hsu PC, Chen CS, Wang S, Hashimoto M, Huang WC, Teng CH. Identification of MltG as a prc protease substrate whose dysregulation contributes to the conditional growth defect of prc-deficient Escherichia coli. *Front Microbiol*. 2020;11:2000.
20. Huang WC, Lin CY, Hashimoto M, et al. The role of the bacterial protease Prc in the uropathogenesis of extraintestinal pathogenic Escherichia coli. *J Biomed Sci*. 2020;27:14.
21. Baba T, Ara T, Hasegawa M, et al. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol*. 2006;2:8–2006.
22. Huang WC, Wong MY, Wang SH, et al. The ferric citrate uptake system encoded in a novel bla CTX-M-3- and bla TEM-1-harboring conjugative plasmid contributes to the virulence of Escherichia coli. *Front Microbiol*. 2021;12, 667782.
23. Kariyawasam S, Wilkie BN, Hunter DB, Gyles CL. Systemic and mucosal antibody responses to selected cell surface antigens of avian pathogenic Escherichia coli in experimentally infected chickens. *Avian Dis*. 2002;46:668–678.
24. Collins SJ. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood*. 1987;70:1233–1244.
25. Tsai CW, Lai FJ, Sheu HM, et al. WWOX suppresses autophagy for inducing apoptosis in methotrexate-treated human squamous cell carcinoma. *Cell Death Dis*. 2013;4:e792.
26. Ermert D, Zychlinsky A, Urban C. Fungal and bacterial killing by neutrophils. *Methods Mol Biol*. 2009;470:293–312.
27. Xie Y, Parthasarathy G, Di Cello F, Teng CH, Paul-Satyaseela M, Kim KS. Transcriptome of Escherichia coli K1 bound to human brain microvascular endothelial cells. *Biochem Biophys Res Commun*. 2008;365:201–206.
28. Scaria J, Warnick LD, Kaneene JB, May K, Teng CH, Chang YF. Comparison of phenotypic and genotypic antimicrobial profiles in Escherichia coli and Salmonella enterica from the same dairy cattle farms. *Mol Cell Probes*. 2010;24:325–345.
29. Janvilisri T, Scaria J, Teng CH, et al. Temporal differential proteomes of Clostridium difficile in the pig ileal-ligated loop model. *PLoS One*. 2012;7, e45608.
30. Sarkar S, Ulett GC, Totsika M, Phan MD, Schembri MA. Role of capsule and O antigen in the virulence of uropathogenic Escherichia coli. *PLoS One*. 2014;9, e94786.
31. Brzuszkiewicz E, Brüggemann H, Liesegang H, et al. How to become a uropathogen: comparative genomic analysis of extraintestinal pathogenic Escherichia coli strains. *Proc Natl Acad Sci U S A*. 2006;103:12879–12884.
32. Keshari RS, Jyoti A, Dubey M, et al. Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. *PLoS One*. 2012;7, e48111.
33. Demirel I, Persson A, Brauner A, Sarndahl E, Kruse R, Persson K. Activation of NLRP3 by uropathogenic Escherichia coli is associated with IL-1 beta release and regulation of antimicrobial properties in human neutrophils. *Sci Rep*. 2020;10, 21837.
34. Papagoras C, Chrysanthopoulou A, Mitsios A, et al. IL-17A expressed on neutrophil extracellular traps promotes mesenchymal stem cell differentiation toward bone-forming cells in ankylosing spondylitis. *Eur J Immunol*. 2021;51:930–942.
35. Billips BK, Schaeffer AJ, Klumpp DJ. Molecular basis of uropathogenic Escherichia coli evasion of the innate immune response in the bladder. *Infect Immun*. 2008;76:3891–3900.
36. Burns SM, Hull SI. Comparison of loss of serum resistance by defined lipopolysaccharide mutants and an acapsular mutant of uropathogenic Escherichia coli O75:K5. *Infect Immun*. 1998;66:4244–4253.
37. Burns SM, Hull SI. Loss of resistance to ingestion and phagocytic killing by O(-) and K(-) mutants of a uropathogenic Escherichia coli O75:K5 strain. *Infect Immun*. 1999;67:3757–3762.
38. Phey Liana KM, Hafy Zen, Andriyani Liberty Iche, Umar Tungki Pratama. Neutrophil extracellular traps and its correlation with several pathological conditions: prosperities and deleterious implications. *Mol Cell Biomed Sci*. 2022;6:1–11.