

RESEARCH ARTICLE

Protective vascular coagulation in response to bacterial infection of the kidney is regulated by bacterial lipid A and host CD147

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One sentence summary: Kidney response to infection is influenced by both host and pathogen. The structure of bacterial LPS effects the kinetics of protective coagulation, while infected epithelia releases CD147, activating tissue factor.

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ABSTRACT

Bacterial infection of the kidney leads to a rapid cascade of host protective responses, many of which are still poorly understood. We have previously shown that following kidney infection with uropathogenic *Escherichia coli* (UPEC), vascular coagulation is quickly initiated in local perivascular capillaries that protects the host from progressing from a local infection to systemic sepsis. The signaling mechanisms behind this response have not however been described. In this study, we use a number of *in vitro* and *in vivo* techniques, including intravital microscopy, to identify two previously unrecognized components influencing this protective coagulation response. The acylation state of the Lipid A of UPEC lipopolysaccharide (LPS) is shown to alter the kinetics of local coagulation onset *in vivo*. We also identify epithelial CD147 as a potential host factor influencing infection-mediated coagulation. CD147 is expressed by renal proximal epithelial cells infected with UPEC, contingent to bacterial expression of the α -hemolysin toxin. The epithelial CD147 subsequently can activate tissue factor on endothelial cells, a primary step in the coagulation cascade. This study emphasizes the rapid, multifaceted response of the kidney tissue to bacterial infection and the interplay between host and pathogen during the early hours of renal infection.

Keywords: coagulation; UTI; UPEC; kidney; tissue microbiology; CD147; lipid A; *Escherichia coli*

INTRODUCTION

Bacterial infection of the kidney leads to an immediate and complex 'dialogue' between host and pathogen that is influ-

enced by the bacterial repertoire of virulence factors, the host immune response and the microenvironment of the tissue. Understanding the sometimes-subtle interplay between these

Table 1. Bacterial strains, plasmids and oligonucleotides.

Strain	Genotype	Reference
CFT073	O6:K2:H1	(Mobley et al. 1990; Mansson et al. 2007)
LT002	CFT073 <i>hlyA::km^r</i>	(Mansson et al. 2007)
LT004	CFT073 <i>cobS::</i> Φ ($\Phi_{\text{LTetO-1-gfp}^+}$), <i>cm^r</i>	(Mansson et al. 2007)
LT005	LT004 <i>hlyA::km^r</i>	(Mansson et al. 2007)
LT004 Δ <i>msbB</i>	LT004 <i>msbB::km^r</i>	This study
Plasmids		
<i>pKD4</i>	Kanamycin resistance cassette source	(Datsenko and Wanner 2000)
<i>pKD46</i>	Lambda red recombinase	(Datsenko and Wanner 2000)
Oligonucleotides		
MsbBKO_F1	ATAATAGCGAATACATTCCTGAGTTTGATAAA TCCTTTTCGTGTAGGCTGGAGCTGCTTC	This study
MsbBKO_R2	AATTTTAATATCCAGGTGATTTGTTCT GGTCGCGGACCAACATATGAATATCCTCCTTAG	This study

factors is crucial to understanding the complete picture of kidney infection *in vivo*. We previously developed a ‘Tissue Microbiology’ *in vivo* model of uropathogenic *Escherichia coli* (UPEC) infection to study this interaction (Mansson et al. 2007; Melican et al. 2008; Melican et al. 2011). Combining the spatial and temporal control of microinfusion with multiphoton intravital microscopy, this model allowed us to study UPEC infection in ‘real-time’ within living kidneys. Visualizing the ongoing infection process, with subcellular resolution, revealed a remarkable complexity. Differential expression of bacterial virulence factors was shown to alter the course of infection in terms of bacterial adhesion and bacterial lifestyles (Mansson et al. 2007; Melican et al. 2008; Melican et al. 2011). We also demonstrated the effect of these bacterial virulence factors in influencing the kinetics of the host inflammatory response (Mansson et al. 2007; Melican et al. 2008; Melican et al. 2011). A major finding from this previous work was that UPEC infection of kidney tubules induces a rapid vascular coagulation in local peritubular capillaries (Melican et al. 2008). This coagulation leads to local tissue hypoxia and vascular isolation of the infection site, preventing a progression to sepsis and facilitating bacterial clearance (Melican et al. 2008).

The next step has been to find the signaling mechanisms that initiate this infection-mediated vascular coagulation. A key point is that at the onset of this coagulation, bacteria are still located at the epithelial barrier and not in direct contact with the blood vessels. The importance of understanding the complex associations between bacterial infection and coagulation has been highlighted in recent reviews (Davis, Miller-Dorey and Jenne 2016; Peetermans et al. 2016). Coagulation in response to bacterial sepsis and blood-borne bacteria has been comprehensively studied (McDonald et al. 2017). Sepsis can be considered, however, a ‘late stage’ of infection, the result of a local infection that the host has failed to control. Our previous work showed that if coagulation can be initiated before bacteria came into direct contact with the blood vessels, sepsis might be avoidable (Melican et al. 2008). This indicates that there is some form of cell-cell communication from the infected epithelia, initiating the protective coagulation response, prior to the onset of sepsis. An important aspect of clinical treatment of sepsis is the use of anticoagulants. We have shown that anticoagulation may actually enhance the progression from local to systemic infection by disrupting this important protective response (Melican et al. 2008). It is becoming more apparent through both basic research and clinical trials that there is a need to be able to

functionally uncouple essential physiological hemostasis and infection-mediated coagulation so that we can pharmacologically target only the latter when necessary (Davis, Miller-Dorey and Jenne 2016). It may also be that by understanding the mechanism of this protective response that we may be able to harness or enhance this as an immunotherapeutic alternative to antibiotic treatment of early stage infections.

Deciphering the mechanisms initiating infection-mediated coagulation during the early hours of bacterial infection is the focus of this work. Our hypothesis is that signaling events occurring at the bacterial-epithelial interface initiates tissue signaling which leads to a coagulation response in the local vasculature. To address this, we analyze both host and bacterial elements of epithelial infection that affect the kinetics of coagulation *in vitro* and *in vivo*.

METHODS

Bacterial strains

Bacterial strains, plasmids and oligonucleotides are presented in Table 1. LT004 Δ *msbB*, was created using the one step allelic knockout method (Datsenko and Wanner 2000), a kanamycin resistance cassette was inserted into the *msbB* gene of LT004 between nt 281,464–281,858. No significant alterations in growth rates, capsule morphology or expression of α -hemolysin were observed. Bacteria were grown in Luria broth aerated over night at 37°C. On the day of infection, bacteria were diluted 1:50 or 1:100 and grown to OD_{600nm} 0.5, corresponding to $\approx 10^8$ CFU/ml.

Human cells

RPTEC/TERT1 (ATCC® CRL-4031) cells were plated at 2×10^6 cells per well and grown for 11 days at 37°C, 5% CO₂, 95% humidity in complete DMEM-F12 media containing DMEM-F12 (ATCC, Sweden cat #30–2006), 5pM T3 (#T6397), 10 ng/ml recombinant human EGF (#PMG8041, Life Technologies), 3.5 μ g/ml ascorbic acid (#A4403), 5 μ g/ml human transferrin (#T1147), 5 μ g/ml bovine insulin (#I6634), 25 ng/ml prostaglandin E1 (#P8908), 25 ng/ml hydrocortisone (#H0888), 8.65 ng/ml sodium selenite (#S5261) and 100 μ g/ml G418 (#G418-RO) (Sigma, Sweden). Monolayer formation, was evaluated using a mouse-anti human-ZO1 antibody (Invitrogen, Sweden cat #339 100), Cy3- donkey anti-mouse IgG (Jackson ImmunoResearch Europe cat#715–1650-150) Phalloidin-Alexa Fluor633 (Life Technologies, Sweden, cat

#A22284) and Hoechst 33 258 pentahydrate (bis-benzimide) (Life Technologies, Sweden cat # H1398). LIVE/DEAD® Viability/Cytotoxicity kit (Invitrogen, Sweden cat #L3224) for eukaryotic cells was used according to the manufacturer's instructions.

HMEC-1 ATCC® CRL-3243™ was grown in MCDB 131 medium (#10 372 019, Invitrogen, Sweden) supplemented with 10% of FBS (Lot # 111M3397 Sigma, Sweden), 10 mM GlutaMAX (#35 050 061, Gibco), 10 ng/ml recombinant mouse epidermal growth factor (mEGF # PMG804, Life Technologies) and 1 µg/ml of hydrocortisone (# H0888 Sigma) at 37°C and 5% CO₂.

Epithelial cell infection

Cells were infected at an MOI of 1:20 or stimulated with 5 µg/ml lipopolysaccharide (LPS). LPS was either prepared as previously described (Backhed et al. 2001) or Ultrapure K-12 LPS (Invitrogen, France, cat #tlr1-peklps). Where indicated, bacteria or media were pre-treated with Polymyxin B (PMB) (Sigma-Aldrich cat #P4932), 8U/ml, for 30 min at 8°C.

Endothelial cell stimulation

HMEC-1 cells were plated at 8 × 10⁵ cells per well. Cells were stimulated for 4 h at 37°C with aliquots of supernatant collected from the 'Epithelial cell infection' described above, (diluted 1:5), LPS (5 µg/ml) or PMB (8 U/ml) in complete RPTEC/TERT1 medium (see above). The complete DMEM-F12 medium did not activate HMEC-1 cytokine/chemokine secretion. Cell supernatants were collected, centrifuged and stored at -80°C.

Cytokine and chemokine determinations

ELISA kits for IL-6 (#550 799), IL-8 (#555 244 BD Bioscience), EMMPRIN (#DY972) and DPPVI/CD26 (#DY1180 R&D Systems, Minneapolis, MN) were used according to the manufacturer's instructions. IL-6 (#558 276), IL-8 (#558 277), IL-1β (#558 279) and TNF-α (#560 112) were analyzed simultaneously using the Cytometric Bead Array (CBA) Human Master Buffer Kit (#558 264 BD Biosciences, Sweden), using a FACS Canto II flow cytometer operated with the FACSDiva software. Data were analyzed with FCAP Array software, following the manufacturer's tutorial.

Tissue factor determination

Cell lysates were prepared by lysis using 150 µl 15mM octyl-β-D-glycopyranoside (#O8001 Sigma, Sweden) for 15 min at 37°C or by 3 repeated cycles of freeze-thaws in 150 µl of buffer of 50 mM Tris-HCl, 100 mM NaCl, 0.1% Triton X-100, pH 7.4. Tissue factor (TF) was extracted for 18 h at 4°C -8°C. TF-procoagulant activity was measured using either the chromogenic assay ACTICHROME® TF kit (Sekisui Diagnostics, # 846) or TF Human Chromogenic Activity Assay Kit (ABCAM, #ab108906) according to the manufacturer's instructions.

Animals

Studies were performed in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee (Indianapolis, Indiana, USA). Male Munich-Wistar rats (255 ± 22 g body weight) (Harlan, USA), with free access to chow and water, were used. Rats were anesthetized with 130–150 mg/kg thiobutabarbital (Inactin) (#T133 Sigma, St. Louis, MO and Sigma, Sweden). Animals underwent tracheostomy, cannulation

of a femoral artery, femoral vein and jugular vein. Infection by tubular microperfusion was performed as previously described (Mansson et al. 2007). Fresh LT004 Δ*msbB* was cultivated and concentrated to 10⁹ CFU/ml in PBS⁺ (PBS with 1 mM CaCl₂ and 2 mM MgCl₂) containing 1 mg/ml Fast Green FCF (Fisher, Fair Lawn, NJ) and 0.2 mg/ml cascade blue-conjugated 10 kDa dextran (#D1976 Molecular Probes, Eugene, OR). The left kidney was exposed via a subcostal flank incision and supported in a kidney cup. The bacterial suspension was infused over 10 min into the lumen of superficial proximal tubules using a micromanipulator (Leitz or World Precision Instruments, UK) and mercury leveling bulb. Bacteria or PBS were infused at an average rate of 54 nl/min.

Multiphoton microscopy

All multiphoton imaging was performed using the set-up previously optimized and described (Mansson et al. 2007; Melican et al. 2008). Images were collected using a Bio-Rad MRC 1024 confocal/2-photon system (Bio-Rad, Hercules, CA) attached to a Nikon Diaphot inverted microscope (Fryer Co, Huntley, IL) with a Nikon 20x water-immersion objective. Images were collected using an excitation wavelength of 810 nm and neutral density filters set to 25%–40%. Fluorescent probes were injected as a single bolus via a jugular vein access line. Tetramethylrhodamine-conjugated 500 kDa dextran (~2.5 mg/400 µl 0.9% saline, Molecular Probes, Eugene, OR) and Hoechst 33 342 (~600 µg/0.4 ml of 0.9% saline, Molecular Probes, Eugene, OR). Line scan measurements were performed as previously described (Kleinfeld et al. 1998), and as illustrated in Fig. 3. For line scanning, a capillary adjacent to the infected tubule, which lay parallel to scan head direction, was required. Typically, only the single most appropriate capillary per infection site (*n* = 4) was selected for scanning. Images and data-volumes were processed using MetaMorph Image Processing Software (Universal Imaging-Molecular Devices, PA, USA), and ImageJ (U. S. National Institutes of Health, MD, <http://rsb.info.nih.gov/ij/>). Final figures were prepared with Adobe Photoshop (Adobe, CA, USA).

Proteome Array

The human XL Cytokine array kit (Proteome Profiler™ array) (R&D Systems, # ARY022B) was used according to manufacturer's protocols. The chemiluminescence was measured using a imageQuant LAS 4000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Spot size and pixel intensity were determined using ImageJ software Fiji (NIH, Bethesda, MD, USA).

EMMPRIN and CD26 stimulation

Endothelial cells were stimulated with homo-dimer human recombinant EMMPRIN (Abcam, Sweden #AB155636) or human CD26/DPP4 recombinant protein (Sino Biological #10 688-HNCH-10) for 4 h before TF activation determination at the noted concentrations.

Statistical analysis

Data are shown as mean ± standard deviation (SD). Statistics were determined using ANOVA with Tukeys correction for multiple comparisons. Independent samples were analyzed using an unpaired t-test with Welches correction. The proteome array was analyzed with an uncorrected multiple t-test. Significance is considered = *P* ≤ 0.05 and annotated by * unless otherwise

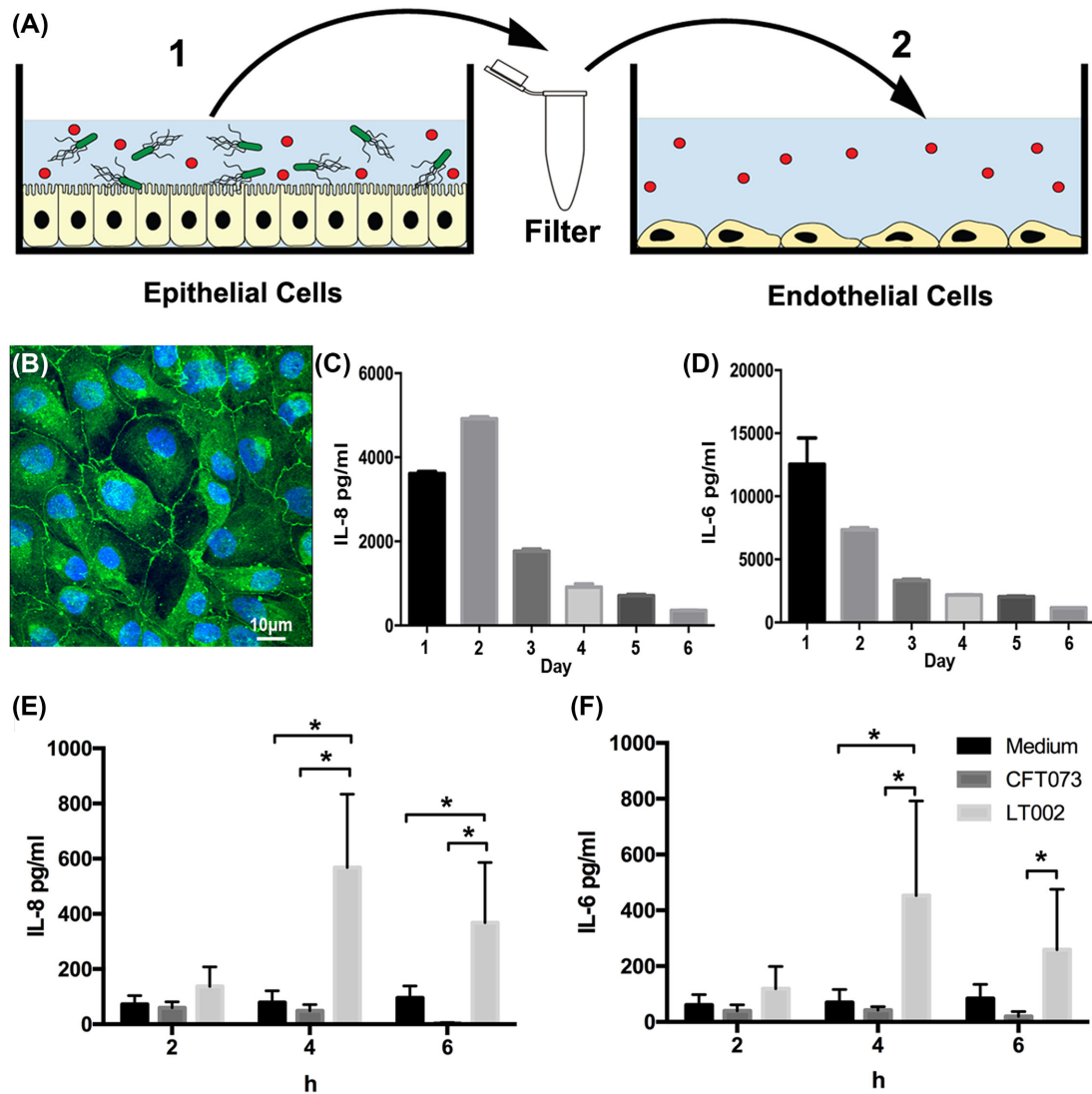


Figure 1. Epithelial cell response to UPEC infection. (A) Schematic of the *in vitro* infection model system. (B) Z0-1 staining (green) showing tight junction formation in RPTEC/TERT1 cells at cultivation day 10. Cell nuclei are seen in blue. (C) ELISA measurements of basal IL-8 and (D) IL-6 expression in RPTEC/TERT1 cells on days 1 to 6 of cultivation, $n = 3$. (E) IL-8 and (F) IL-6 expression in renal RPTEC/TERT1 epithelial cells infected with an MOI 1:20 of CFT073 (dark grey) or LT002 (light grey) for 2, 4 and 6 h. Uninfected cell culture medium (black) was used as the comparative control. Expression was measured using the cytometric bead array (CBA), $n = 5$. All bars represent mean \pm SD. Significance was tested with a 2-way ANOVA with Tukey's post-test for multiple comparisons.

stated. All data were analyzed using the GraphPad InStat version 6.0 (GraphPad software, San Diego, CA, USA).

RESULTS

Epithelial cell inflammatory responses to UPEC infection

To study the communication between infected renal proximal tubule epithelial cells and vascular endothelial cells we used a controlled 2-step *in vitro* cell culture model (Fig. 1A). In an effort to faithfully replicate the *in vivo* microenvironment of the kidney, we sought an epithelial cell line that closely replicated the morphological and functional characteristics of renal proximal tubules. We used RPTEC/TERT1 (ATCC® CRL-4031), a human renal epithelial cell line that has been shown to reproduce renal proximal tubular characteristics in both 2D and 3D culture (Wieser et al. 2008; Simon-Friedt et al. 2015; Secker et al. 2018).

We optimized growth conditions, with extended (10 day) culturing, high-density seeding and frequent media changes, which resulted in a confluent monolayer that displayed tight junction formation (Fig. 1B), dome formation indicative of fluid transport and a reduced basal cytokine secretion over time (Fig. 1C, D). Tubular infection was modeled by infecting RPTEC/TERT1 with the prototypic UPEC strain CFT073 (Table 1) at an MOI of 1:20. As the response of RPTEC/TERT1 to bacterial infection had not been previously reported, we started by evaluating the epithelial inflammatory profile of these cells following UPEC infection. Supernatants from infected RPTEC/TERT1 were collected at 2, 4 and 6 h post infection and the epithelial inflammatory profile evaluated using a cytometric bead array (CBA). CFT073 infection led to no increase in epithelial IL-8 or IL-6 (Fig. 1E, F). We have shown previously that expression of the UPEC protein toxin α -hemolysin affects the kinetics of infection-mediated coagulation *in vivo* (Mansson et al. 2007). To analyze the epithelial inflammatory response in the absence of α -hemolysin, we infected

RPTEC/TERT1 with LT002, an isogenic mutant of CFT073 that lacks expression of the α -hemolysin toxin (Δ hly) (Table 1). Following LT002 infection, epithelial IL-8 (Fig. 1E) and IL-6 (Fig. 1E) production was increased at 4 and 6 h. The α -hemolysin toxin expressed by UPEC has a concentration-dependent bi-phasic action either modulating cell signaling at sub-lytic concentrations or as a cytotoxic pore-forming toxin at higher concentrations (Uhlen et al. 2000; Dhakal and Mulvey 2012). We evaluated the cytotoxic action of α -hemolysin on RPTEC/TERT1 using a live dead assay (Supplementary Fig. S1), which showed that infection with the α -hemolysin expressing CFT073 strain caused increased epithelial cell death/detachment after 6 h of infection when compared to the LT002 (Δ hly) strain.

Together this data indicated that the RPTEC/TERT1 cell line could respond to UPEC infection, and that bacterial expression of the toxin HlyA influenced the renal epithelial inflammatory response, a phenomenon that has been previously described on bladder epithelial cells (Dhakal and Mulvey 2012; Hilbert et al. 2012; Ristow and Welch 2016).

Endothelial cell activation following epithelial cell infection

We then investigated how endothelial cells may respond to the multitude of factors produced by infected renal epithelia. Working from our previous *in vivo* data showing initiation of infection-mediated coagulation at 4 h (Melican et al. 2008), we focused on signaling components produced up to 4 h post infection. Supernatant from a 4 h epithelial infection (step 1, Fig. 1A) was collected, filtered (0.2 μ m) and then used to stimulate a human microvascular endothelial cell line (HMEC-1) for 4 h (Step 2, Fig. 1A).

HMEC-1 cells stimulated for 4 h with supernatant from CFT073 infected epithelial cells showed an increased production of IL-8, IL-6 and IL-1 β , and a moderate increase of TNF- α (Fig. 2A-D). Endothelial stimulation with supernatant from LT002 (Δ hly) infected epithelial cells showed a similar pattern, with significant up-regulation of IL-8, IL-6, and an increase of TNF- α and IL-1 β (Fig. 2A-D). The similarity of the endothelial response to the supernatants from the different epithelial infections (Fig. 1E) demonstrated that epithelial production of IL-8 and IL-6 are not decisive factors in this cell-cell signaling and that other factors must be involved.

We were also interested in the ability of the epithelial factors to induce coagulation. Tissue factor (TF) is a central regulator of the coagulation cascade. We therefore measured the activation of endothelial TF following stimulation with infected epithelial supernatants. Stimulation of endothelial cells with supernatant from epithelial cells infected with both CFT073 and LT002 led to significant endothelial TF activation (Fig. 2E). This data supports our hypothesis that factors originating from the infected epithelium can mediate endothelial activation and the initiation of coagulation.

Role of LPS Lipid A in endothelial activation

We then investigated what factors may be mediating TF activation. A major component of the filtered supernatants is the bacterial endotoxin LPS. As bacteria grow, they shed significant amounts of LPS into their environment (Mattsbj-Baltzer et al. 1991). Control experiments with purified LPS showed that LPS alone could stimulate endothelial production of IL-8, IL-6 and IL-1 β (Supplementary Fig. 2A-D) and also induced significant TF activation (Supplementary Fig. 2E).

To study the extent of the role played by LPS in endothelial activation in our model, we used the antibiotic Polymyxin B (PMB), which binds to the lipid A portion of LPS, antagonizing LPS- TLR4 signaling (Backhed et al. 2003). Control experiments confirmed that PMB treatment (8 U/ml) of purified LPS reduced endothelial IL-8, IL-6 and IL-1 β production as well as TF activation (Supplementary Fig. 2A-E). PMB treatment of the supernatant from epithelial cells infected with both CFT073 and LT002 prior to endothelial cell stimulation (Step 2, Fig. 1A), resulted in a reduction in endothelial IL-8 and IL-6 production (Fig. 2A,B, +PMB). Endothelial TF activation was significantly reduced following PMB treatment of the supernatant from CFT073 and LT002 infected epithelial cells (Fig. 2E, +PMB).

The reduction in both cytokine signaling (Fig. 2A-D) and TF activation (Fig. 2E) was, however, not complete abrogation. PMB treated supernatant from both CFT073 and LT002 still stimulated a significant IL-8 and IL-6 response when compared to its non-infected controls (Fig. 2A-B, +PMB). PMB treated epithelial supernatants also showed an increase in TF activation (Fig. 2E, +PMB). These results indicate that lipid A mediated LPS signaling is an important element in our *in vitro* model, and blocking it reduces, but does not eliminate, inflammation and TF activation.

LPS lipid A mediated signaling affects coagulation kinetics *in vivo*

Testing the relevance of this lipid A mediated LPS signaling *in vivo* is more challenging. PMB is nephrotoxic and cannot be used in animal studies (Falagas and Kasiakou 2006). To overcome this, we used an isogenic *msbB* mutant of the GFP expressing CFT073 strain LT004, LT004 Δ *msbB* (Table 1), which produces a penta-acylated lipid A that is poorly recognized by the TLR4 signalling complex (Somerville et al. 1996; Backhed et al. 2003). Epithelial infection *in vitro* with LT004 Δ *msbB* for 4 h showed lower IL-8 (Fig. 2F) and TF activation (Fig. 2G) in stimulated endothelial cells, as compared to its CFT073 counterpart (Fig. 2A, 2E). In agreement with the PMB results, neither of these reductions were complete abrogation (Fig. 2F, G), with LT004 Δ *msbB* epithelial infection still demonstrating a significant effect on endothelial TF activation. This confirmed the reduced inflammatory profile of LT004 Δ *msbB* and a reduced, though not eliminated, ability of this strain to induce TF activation *in vitro*.

To test the role of lipid A in infection-mediated coagulation *in vivo* LT004 Δ *msbB* was introduced into our renal infection model (Melican et al. 2008). LT004 Δ *msbB* was microinfused (10⁵ CFU in 40 μ l over 10 min) into a single proximal tubule of an exposed kidney in a live rat and the progress of infection followed by intravital multiphoton microscopy. The LT004 Δ *msbB* strain (green in Fig. 3A) adhered to and colonized the renal epithelium (Fig. 3A, 2 h – 8 h). Within 4–5 h, the bacteria filled the tubular lumen in the majority of infected tubules (Fig. 3A, 4–5.5 h). Bacterial adherence and colonization patterns were comparable to that previously described for the LT004 (Mansson et al. 2007; Melican et al. 2008). The first signs of vascular perturbation and coagulation (assessed by the red plasma marker in Fig. 3A) were seen approximately 6.5 h after infection (Fig. 3A, 6.5 h arrow). This represented a delay compared to the 4–5 h time frame seen in LT004 infection (Mansson et al. 2007; Melican et al. 2008). To measure the velocity of red blood cells (RBC) in the peritubular capillaries, line scanning (Kleinfeld et al. 1998) was performed (Fig. 3B-D). A peritubular capillary adjacent to the infected tubule was scanned along its central axis at 2ms per line for 4 s (Fig. 3B dotted line). Individual RBC velocity was determined by calculating

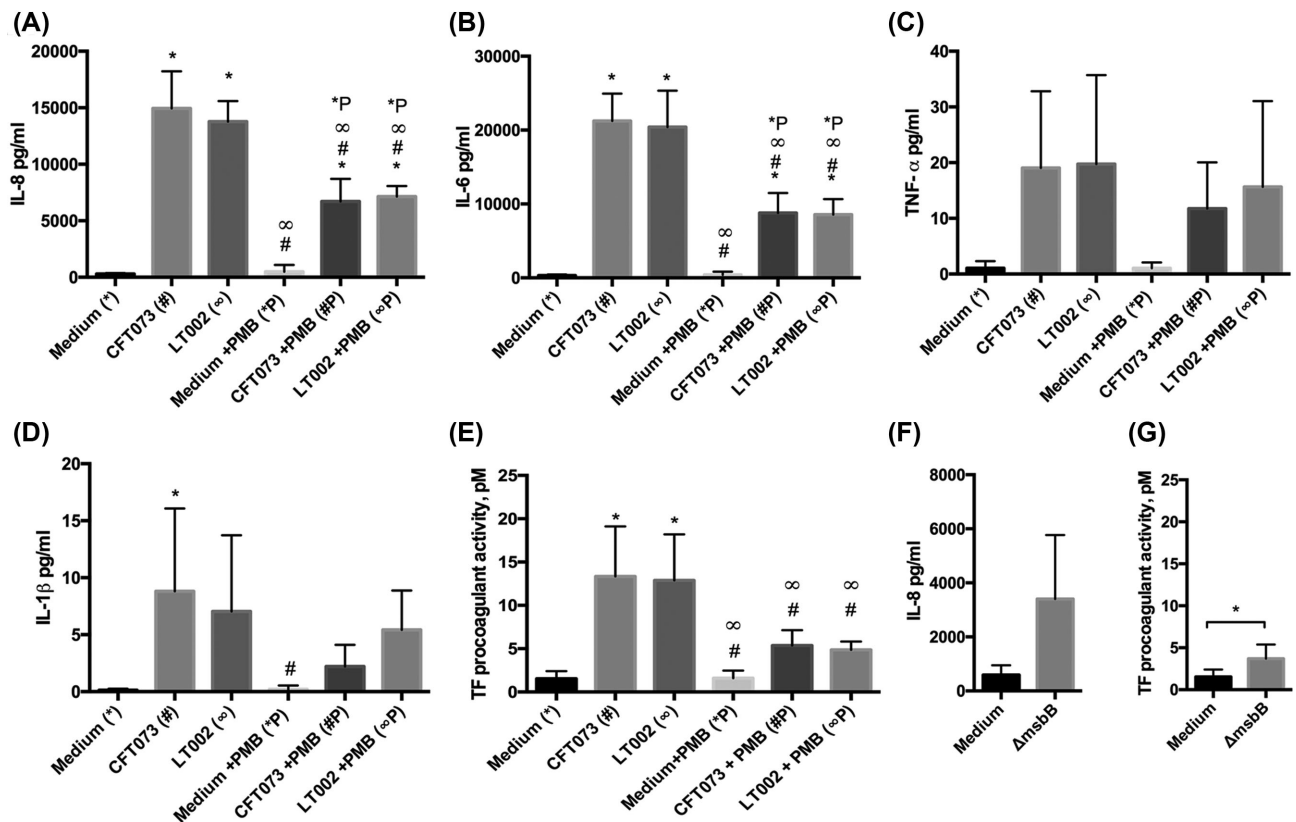


Figure 2. Activation of endothelial cells by supernatant from infected epithelia. (A) IL-8, (B) IL-6, (C) TNF- α and (D) IL-1 β expression measured by CBA from HMEC-1 endothelial cells stimulated for 4 h with supernatant from RPTEC/TERT1 epithelial cells infected for 4 h with either CFT073 or LT002 $n = 5$. Supernatant from epithelial cultures treated with uninfected cell culture medium was used as a comparative control. Epithelial supernatants were pre-treated with 8 U/ml PMB prior to endothelial stimulation (+PMB), $n = 5$. (E) TF pro-coagulant activity, pM, measured using chromogenic assays, in HMEC-1 cells treated with supernatant from infected RPTEC/TERT1 epithelial cells as described above, $n = 5$ (F) IL-8 expression measured by ELISA of HMEC-1 cells stimulated for 4 h with supernatant from RPTEC/TERT1 epithelial cells infected with LT004 $\Delta msbB$ for 4 h, $n = 3$ (G) TF pro-coagulant activity, pM, in HMEC-1 cells treated for 4 h with supernatant from RPTEC/TERT1 infected with LT004 $\Delta msbB$ for 4 h, $n = 5$. All bars represent mean \pm SD. In A-E statistical significance ($P < 0.05$) is shown by the use of symbols corresponding to the comparative experiment. i.e. significance to the medium control is shown with * and significance to the CFT073 culture is shown by #.

the distance it travelled through the vessel over time (Fig. 3C). Line scanning demonstrated a gradual slowing in RBC flow following LT004 $\Delta msbB$ infection (Fig. 3D). In wild-type LT004 infections the RBC rate has been shown to drop to $0 \mu\text{m}/\text{sec}$ by 4.5 h (Melican *et al.* 2008). This data confirmed a slowing of infection-mediated coagulation kinetics in response to the penta-acylated lipid A of LT004 $\Delta msbB$, but not complete abrogation of coagulation. This demonstrates a role for lipid A mediated signaling in modulating the kinetics of infection-mediated coagulation *in vivo*.

Host epithelial signaling factors induce endothelial tissue factor activation.

The acylation state of lipid A can now join α -hemolysin as bacterial factors affecting the kinetics of infection-mediated coagulation *in vivo*, however, neither of these bacterial factors act in isolation (Amori, Lau and Pittas 2007; Melican *et al.* 2008). Elimination of either of these factors is not enough to completely abrogate infection-mediated coagulation, but rather delay its onset. Our data also indicated that other factors may be involved in this response as the *msbB* mutation could not completely eliminate the TF activation response (Fig. 2G). Our hypothesis is that *in vivo* the expression of these bacterial virulence factors affects

the epithelial response to infection rather than acting directly on the endothelium as occurs in the *in vitro* models.

We therefore screened for candidate host derived molecules involved in infection-mediated coagulation by performing a proteome array study on supernatant from RPTEC/TERT1 cells infected with the wild-type CFT073 strain. The array covered 106 human cytokines (see methods). Following 4 h of epithelial infection we could detect signal for 15 proteins (Fig. 4A). Of these, ICAM-1, CD26 and CD147 were significantly up-regulated compared to non-infected controls. ICAM-1 up-regulation in UPEC infection has already been reported, and is believed to be involved in neutrophil recruitment (Mansson *et al.* 2007). CD26 (also known as DPP4) and CD147 (also known as EMMPRIN) represent new candidate proteins in epithelial UPEC infection.

To confirm the infection-induced production of CD26 and CD147 we performed ELISA on supernatant of RPTEC/TERT1 cells infected with CFT073 for 4 h. Infection induced significant levels of both CD26 and CD147 in epithelial supernatant (Fig. 4B,C). As we had seen that LPS played a role in infection kinetics, we tested whether LPS alone could stimulate CD26 and CD147. Stimulation of epithelial cells with purified LPS (5mg/ml) for 4 h did not induce release of either CD26 or CD147 into the supernatant (Fig. 4B, C), demonstrating a limited role for the Lipid A pathway in this response. To evaluate the role

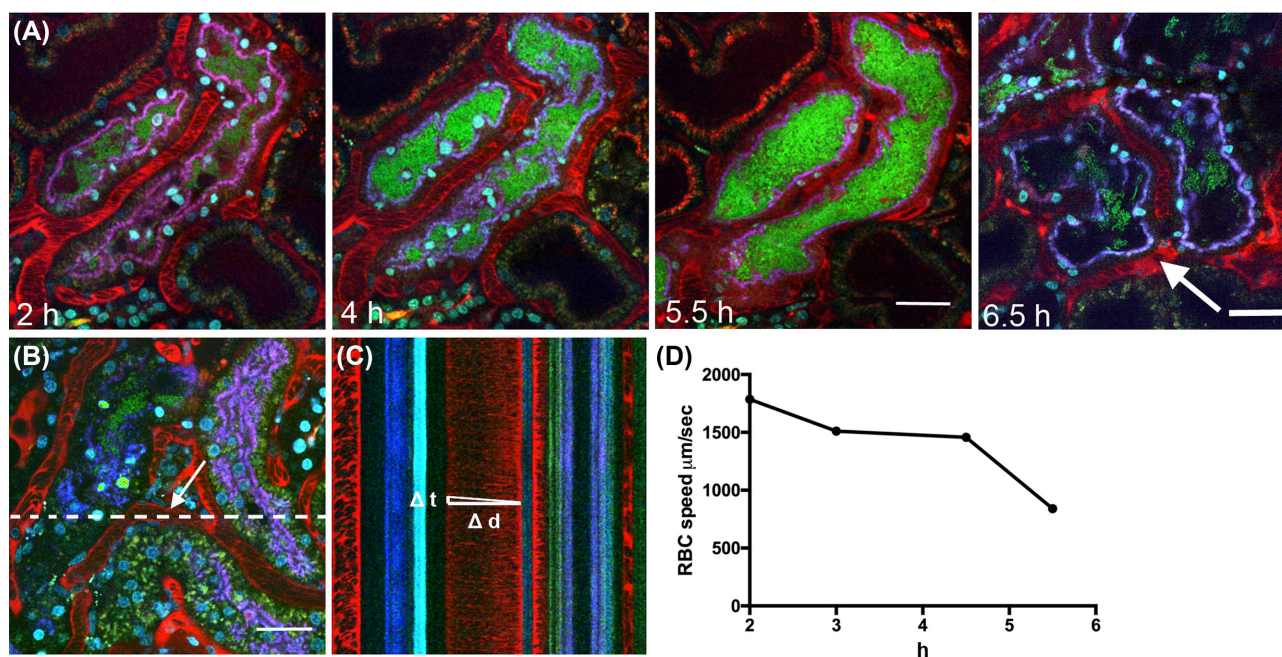


Figure 3. Penta-acylated Lipid A delays coagulation *in vivo*. Representative intravital microscopy showing the infection kinetics over time of (A) LT004 ΔmsbB microinjected into a single renal proximal tubule of a live rat ($n = 4$). Bacteria express GFP (green), blood plasma is visualized by i.v. delivered 500 kDa tetramethylrhodamine conjugated dextran (red) and cell nuclei with i.v. delivered Hoechst 33 342 (cyan). The infected tubule is outlined with a 10 kDa cascade blue conjugated dextran co-infused with the bacteria and selectively endocytosed by the proximal tubule cells (blue). Scale bars = 30 μm . (B) Reference image showing the location of the line scan (dashed line). The arrow shows the location of the vessel of interest shown in (C). (C) Part of the cross-section of the line scan from (B) showing the calculation of RBC velocity, calculated by determining the distance Δd a RBC has travelled over time Δt . (D) Representative RBC velocity in the selected vessel adjacent to an LT004 ΔmsbB infected tubule (vessel shown in B) over time.

of α -hemolysin in this signaling, we tested supernatant from epithelial cells infected with the α -hemolysin deficient LT002 strain. No CD26 or CD147 was released from epithelial cells infected with LT002 (Fig. 4B, C). This suggests that their release appears to be specific to infection with the wild-type CFT073 bacteria, the strain that shows the most rapid coagulatory response *in vivo* (Mansson et al. 2007).

To investigate whether CD26 and CD147 may play a direct role in infection-mediated coagulation caused by CFT073, we stimulated HMEC-1 endothelial cells with purified CD26 and CD147 and tested for TF activation. Concentrations of active human CD26 from 10–50 ng/ml showed no significant up-regulation of endothelial TF activation following 4 h of stimulation (Fig. 4E). CD147, however, revealed an increase in endothelial TF activation at 4 h at higher concentrations (50mg/ml) (Fig. 4E). This data implies that high local concentrations of CD147, expressed by CFT073 infected epithelia, could play a role in epithelial-endothelial signaling and the initiation of infection-mediated coagulation during renal infection. This suggests CD147 as a possible host-derived factor in infection-mediated coagulation.

DISCUSSION

This work highlights two new players in host-pathogen cross-talk that influences local infection-mediated coagulation: (1) the acylation state of bacterial LPS and (2) host derived CD147. The importance of infection-mediated coagulation has already been shown; it plays a protective role in renal UPEC infection, preventing the transition to urosepsis (Melican et al. 2008). Our present work adds new knowledge by showing that both bacterial viru-

lence determinants and host cell factors are involved in the activation of TF.

The role of Lipid A and effect of the *msbB* mutation on TLR4 mediated IL-8 production has been reported previously (Backhed et al. 2001) but the exclusivity of this interaction in UPEC urinary tract infection has been less clear (Hedlund et al. 1999; Backhed et al. 2003). Ambiguity in previous results using the common renal epithelial cell line A498 (Backhed et al. 2001; Demirel et al. 2015), led us to seek out an alternative *in vitro* model that more closely represented the morphology of the *in vivo* microenvironment. The RPTEC/TERT1 cell line originates from a healthy male donor and is immortalized by telomerase overexpression (Wieser et al. 2008). This line is being extensively used as a model for nephrotoxicity (Simon-Friedt et al. 2015). Our data demonstrated that this cell line did respond to UPEC infection. The lack of IL-6 and IL-8 signaling in response to the hemolysin expressing CFT073 strain suggests that hemolysin may influence the regulation of pro-inflammatory signaling, as has been well described in bladder epithelium (Dhokal and Mulvey 2012; Hilbert et al. 2012) and even for the non-related *Staphylococcus aureus* beta-hemolysin toxin (Tajima et al. 2009).

Antagonizing Lipid A mediated signaling *in vitro* with polymyxin B demonstrated a significant role for this pathway in the activation of the endothelial cell line. A drawback of our *in vitro* model is that the bacterial products come into direct contact with the endothelia, a situation we do not believe represents the *in vivo* situation. We see coagulation occurring *in vivo* when bacteria and seemingly their products are still maintained at the epithelial barrier (Melican et al. 2008; Melican et al. 2011). *In vivo* infection with the penta-acylated lipid A

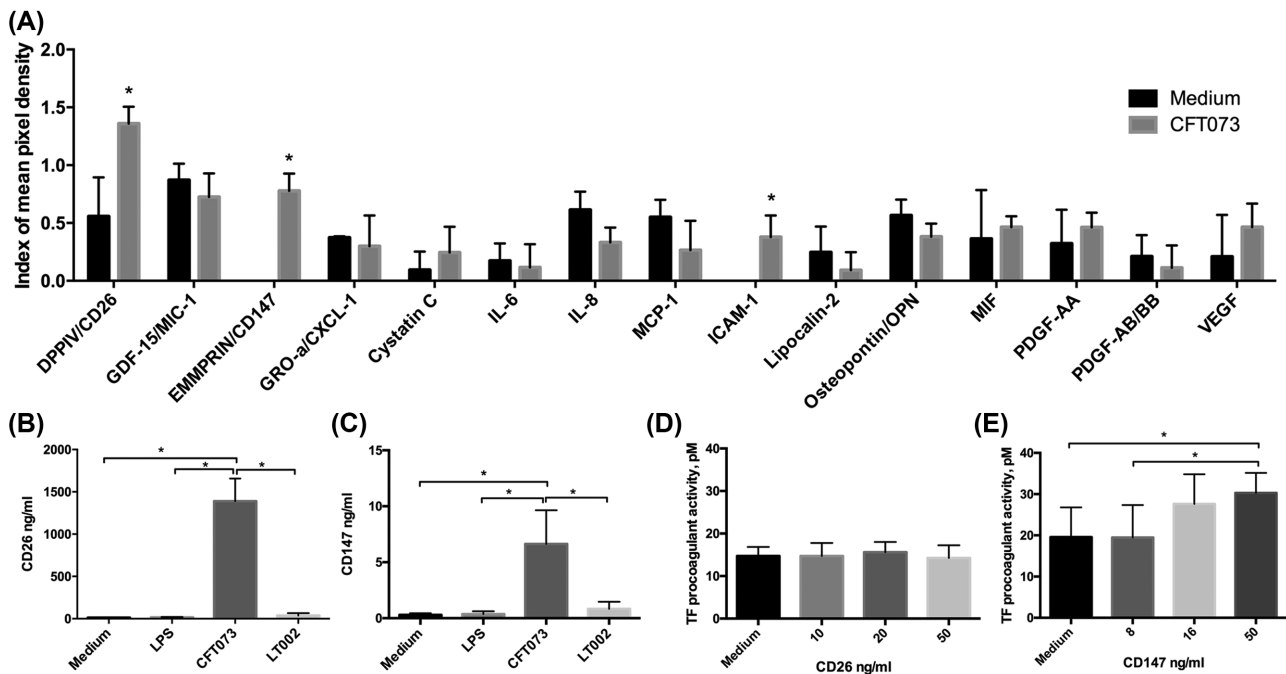


Figure 4. Epithelial signaling factors induce endothelial TF activation. (A) Proteome array of supernatant of epithelial cells infected with CFT073 for 4 h. Supernatant from uninfected cells was used as the comparative control. Bars represent index of mean pixel density from the nitrocellulose membranes, $n = 3$. (B) CD26 and (C) CD147 expression in the supernatant of RPTEC/TERT1 cells stimulated for 4 h with cell culture medium, 5 mg/ml LPS or infected at an MOI 1:20 with CFT073 or LT002. Measurements were performed by ELISA, $n = 5$. (D) TF pro-coagulant activity, pM, measured using chromogenic assays in endothelial cells treated with the noted concentrations of (D) CD26, or (E) CD147, $n = 3$. All bars represent mean \pm SD.

UPEC strain demonstrated slower kinetics, though not elimination, of coagulation as compared with the WT strain. While a strain may have a less immunogenic LPS, it can still express α -hemolysin, and, as each of these factors appears to play a role in the onset of coagulation, the result is a nuanced change in host response kinetics, not an on-or-off switch. LPS plays an enormous role in the pathogenesis of many gram-negative infections, our data suggests a role for LPS Lipid A triggered cell-cell communication as a factor in renal coagulation following tubule infection.

Our data also introduces CD147 as the first host epithelial factor to be directly implicated in infection-mediated coagulation. CD147, also known as EMMPRIN and Basigin (Grass and Toole 2015), is perhaps best recognized for its role in cancer, where it acts to regulate the expression of VEGF and matrix metalloproteinase, contributing to malignancies (Grass and Toole 2015). Targeting of CD147 has been suggested as a novel cancer treatment strategy (Hao et al. 2010). In terms of bacterial infection, CD147 has been implicated as an adhesion factor for *Neisseria meningitidis* (Bernard et al. 2014) and *Listeria monocytogenes* (Till et al. 2008). We show here that CD147 may play a role in infectious cell signaling and the initiation of local coagulation during renal infection. In support of our findings, CD147 has been shown to be released from epithelial cells in vesicles and to alter the angiogenic state of endothelial cells (Millimaggi et al. 2007). We show that epithelial cells infected with the α -hemolysin expressing CFT073 strain secrete CD147. CD147 may be an effector explaining the enhanced *in vivo* kinetics of infection-mediated coagulation for the wild-type UPEC vs the α -hemolysin deficient LT005 strain (Mansson et al. 2007). Further investigation of the role of CD147 during renal infection *in vivo* is of huge interest and the focus of ongoing work. Human CD147 however

exhibits only 65% homology with the rodent protein (Watanabe et al. 2010), making *in vivo* experimentation challenging at this point.

While CD26, also known as dipeptidyl peptidase-4 (DPP4), did not show a direct action on TF in our study, its high expression during epithelial infection is also of interest. The DPP4 inhibitor sitagliptin, an oral glucose-lowering agent, has been shown to inhibit platelet aggregation (Gupta et al. 2012). Clinical trials with sitagliptin also demonstrated an increased risk for urinary tract infections (Amori, Lau and Pittas 2007). This suggests that CD26 may play a role in infection-mediated coagulation, just not via TF activation as measured here.

Our work has revealed roles for bacterial LPS lipid A and host CD147 in the modulation of infection-mediated coagulation kinetics. The picture that is emerging highlights the complexity, cross talk and dynamics of renal infection. Understanding this cross-talk will be important to evaluate the possible detrimental influence that seemingly unrelated treatments such as anticoagulation, cancer treatment (anti-CD147) and diabetes treatment (anti-CD26) may have on infection progression in UTI. A single molecular mechanism cannot control the multifaceted tissue response to bacterial infection; evolution has ensured that multiple pathways are involved, ensuring that fail-safes are in place to prevent bacterial spread and life-threatening sepsis. Learning to harness and enhance this host response is an attractive possibility in the search for new treatments in the post-antibiotic era.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSPD](https://www.femsdpd.com) online.

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Conflict of interest. None declared.

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